HEALTH AND FUNCTION OF GASTROINTESTINAL TRACT AS INFLUENCED BY DIETARY IMMUNOMODULATORY COMPONENTS IN POULTRY

by

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Dedicated to GOD, and my family

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NOMENCLATURE

ACTH	Adrenocorticotropic hormone
AID	Apparent ileal digestibility
AME	Apparent metabolizable energy
BW	Body weight
Ca	Calcium
CAT	Catalase
CP	Crude protein
CC	Coccidia Challenge
CD	Crypt depth
Cr	Chromium
COS	Chitosan Oligosaccharide
CP	Crude protein
d	Day
DADS	Diallyldisulphide
DATS	Diallyltrisulphide
DNA	Deoxyribonucleic acid
DM	Dry matter
DEX	Dexamethasone
FI	Feed intake
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Goblet cell
GE	Gross energy
G:F	Gain to feed ratio
GIT	Gastrointestinal tract
GPx	Glutathione peroxidase
IDE	Ileal digestible energy

IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IL	Interleukin
LPS	Lipopolysaccharide
Lys	Lysine
ME	Metabolizable energy
MHC	Major histocompatibility complex
MUC2	Mucin 2
AMEn	Nitrogen corrected apparent metabolizable energy
NE	Necrotic enteritis
NO	Nitric oxide
NRC	National Research Council
NSP	Non-starch polysaccharides
PAMP	Pathogen associated molecular pattern
P	Phosphorus
PP	Phytate phosphorus
PC	Positive control
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SBP	Soybean Bioactive Pepetide
SOD	Superoxide dismutase
TGF-β	Transforming growth factor beta
TH	T-helper
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
VH	Villus height
ZO-1	Zonula occludens

ABSTRACT

Experiments were conducted to investigate the efficacy of soybean bioactive peptide and chitosan oligosaccharide on a stress and disease challenge model. For the first study, responses of broiler chickens (Cobb 500) to graded concentrations of dietary soybean bioactive peptide (SBP) (0, 1, 2, 3, 4, 5 g of SBP/kg of diet) and coccidia challenge (CC) were investigated. In Experiment 1, 384 male broiler chicks were used to investigate the effect of graded dietary concentrations of SBP in a randomized complete block design. There were linear effects ($P < 0.05$) of graded concentrations of SBP on BW, BW gain, gain:feed, ileal villus height and jejunal crypt depth at d 22 post hatch. There were linear effects ($P < 0.01$) of graded SBP concentrations on ileal dry matter and energy digestibility**.** Experiment 2 was conducted to investigate the immune-protective properties of SBP during a coccidia challenge. Dietary SBP ameliorated the CC-induced effect ($P = 0.01$) on gain:feed. Coccidia challenge reduced ($P < 0.05$) jejunal villi height whereas dietary SBP supplementation increased ($P < 0.05$) jejunal villi height. Dietary SBP attenuated the CC-induced effects ($P < 0.05$) on the expression of plasma interleukin-1β, transforming growth factor-β, claudin-1 and occludin genes.

The second study examined the response of broiler chickens (Cobb 500) to graded dietary concentrations of dietary chitosan oligosaccharide (COS) (0.0, 0.5, 1.0, 1.5, 2.0, and 2.5 g of COS/kg of diet) and coccidia challenge (CC). Experiment 1 was conducted to investigate the effect of graded dietary concentration of COS in a randomized complete block design. There were linear effects ($P < 0.05$) of graded COS concentration on body weight (BW), BW gain, feed intake, jejunal villus height and villus height to crypt depth ratio at d 22 post hatch. Between 0.50 and 1.18 g COS/kg diet was optimum for BW gain, jejunal villus height, and villus height to crypt depth ratio. There were linear increases ($P < 0.05$) in ileal dry matter and energy digestibility.

Experiment 2 was conducted to investigate the immune-protective properties of COS on broiler chickens during CC. Coccidia challenge reduced ($P < 0.05$) and dietary COS increased ($P < 0.05$) BW gain, and feed intake. Dietary COS mitigated ($P < 0.05$) the CC-induced effects ($P < 0.05$) on gain:feed. Coccidia challenge reduced ($P < 0.05$) jejunal villus height whereas dietary COS supplementation increased ($P < 0.05$) jejunal villus height. Dietary COS supplementation attenuated the CC-induced effects ($P < 0.05$) on the expression of plasma interleukin-6 and 10, claudin-1 and occludin genes.

Next, a study was conducted to investigate the effect of dietary chitosan oligosaccharide (COS) on growth performance, nutrient digestibility, jejunal morphology, gene expression, and plasma antioxidant enzymes in male broiler chickens under experimentally induced stress via in-feed dexamethasone (DEX). The results showed that dietary COS also decreased ($P < 0.05$) DEX-induced effects (interaction; $P < 0.05$) on BW, BW gain, and gain:feed. Dietary COS supplementation attenuated the DEX effects (interaction; $P < 0.05$) on villus height, crypt depth, villus height to crypt depth ratio, and ileal digestibility of DM and energy. The DEX-induced effect of relative mRNA expression of jejunal mucosa IL-6, IL-10, and claudin-1 was reduced by dietary COS supplementation (interaction; $P < 0.05$). Responses (interaction; $P < 0.05$) in the activity of plasma superoxide dismutase, catalase, and glutathione peroxidase to COS and DEX were similar to that observed with the relative mRNA expression. Chitosan oligosaccharide supplementation increased ($P < 0.05$) the mRNA expression of IL-8 and occludin.

In the final experiment, the objectives of this study were to determine the effect of dietary supplementation of garlic-derived diallyl disulfide (DADS) and diallyl trisulfide (DATS) on growth performance, gut morphology, and cecal microflora in broiler chickens. Dietary concentrations of garlic-derived DADS + DATS between 0 and 75 mg/kg had no effect ($P > 0.05$)

on growth performance and gut morphological responses examined. There was a quadratic effect ($P < 0.05$) of DADS + DATS on $\log_{10}$ number of copies of cecal *Escherichia coli* at d 8 post hatching. However, *Lactobacillus spp* $\log_{10}$ number of counts were not affected by dietary supplementation of garlic derived DADS + DATS.

In summary, the overall results from these studies shows that dietary supplementation of SBP or COS may be a potential to ameliorate stress or disease challenge in broiler chickens.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

The world's economy in terms of food consumption patterns have moved towards animal products because of the increasing country-specific purchasing power and world population growth (Adedokun and Olojede, 2019). Studies have indicated that the increase in animal protein consumption is mostly linked to the poultry industry (Windhorst, 2006; Thornton, 2010), which is attributed to a high global consumption of poultry meat. Relative to the aforementioned increases, the maintenance of health and well-being of poultry birds is faced with an huge challenges.

For the past 50 years, antibiotic growth promoters and anticoccidia drugs have been used in poultry production to improve the growth performance and protect the birds from enteric challenges. The extensive use of these drugs could result in the development of antibiotic-resistant human pathogenic bacteria, hence, antibiotics have come under intense scrutiny. (Phillips, 1999; Ratcliff, 2000). As a result of the aforementioned issue the United States have prohibited the use of of antibiotics to prevent disease or promote growth.

The removal of antibiotics as growth promoters from poultry diets has led to problems in animal performance and rise in the incidence of certain poultry diseases such as (sub clinical) necrotic enteritis and coccidiosis (Huyghebaert et al., 2011). However, it is pertinent to explore new solutions for anti-infective therapy to maintain and promote the gut health for the improvement in the performance of birds. One promising approach that has received a great level of attention is the use of of immunomodulatory feed additives than can boost the host natural defence mechanism.

The gastro-intestinal tract is a vital organ of the digestive system which mediates uptake of nutrients (Yegani and Korver, 2008). Therefore, a healthy gut is important for optimum

performance of the birds. If gut health and its functions are impaired then digestion and absorption of the nutrients are affected and thus growth and development of the birds will be compromised (Sugiharto, 2016).

Nutritional strategies to mitigate gastrointestinal dysfunction have been extensively studied and include dietary supplementation of probiotics or prebiotics (Lowry et al., 2005), phytochemicals (Wlodarska et al., 2016), organic acids (Du et al., 2016), feed enzymes and egg yolk antibodies (Witcombe and Smith, 2014; Martinez-Cummer, 2015). There is a dearth of literature on dietary supplementation of soybean bioactive peptides (SBP) and chitosan oligosaccharides (COS) in broiler chickens diets to mitigate gastrointestinal dysfunction. Soybean bioactive peptides and COS have been shown to have antimicrobial, antioxidant, and immunomodulatory properties (Yang et al., 2009; He et al., 2013; Niu et al., 2013; Xiao et al., 2013; Swiatkiewicz et al., 2015). Therefore, supplementation COS and SBP to diets of broiler chickens diet may be a nutritional option to alleviate gastrointestinal stressors.

The objective of studies presented in this dissertation include; first, to determine the effect of SBP in broiler diets to support growth performance, digestive functions, intestinal morphology, and immune organs. The second objective was to evaluate the immune-protective properties of SBP in broiler chickens diet during coccidia challenge. The third objective was to determine an optimum dietary concentration of COS in broiler diets to support growth performance, digestive functions, intestinal morphology, and immune organs. The fourth objective was to evaluate the immune-protective properties of COS on broiler chickens following coccidia challenge. Lastly, the fifth objective was to investigate the effect of dietary COS supplementation on anti-oxidative function and immune response induced by in-feed DEX supplementation.

1.2 Poultry Gastrointestinal Tract

Poultry has specialized anatomical features, such as crop and a gizzard, that are unique to avian species (Figure 1-1). Although the digestive tract of poultry is simple, it is typical to avian species as it reflects some of the evolutionary adaptations to reduce body weight to enhance flight (Michael, 1998). Birds pick the feed with the beak and swallow it as whole due to lack of teeth. Salivary gland in the mouth secretes saliva, which moistens the feed for easy swallow, and starts the digestion process by providing enzymes such as amylase (Jacquie, 2013). The crop is an enlarged portion in the esophagus, where food is stored. Thereafter, the feed present in the crop empties into the proventriculus, where the enzymatic digestion primarily begins. Mucosal glands in the proventriculus secrete hydrochloric acid and pepsinogen to initiate protein digestion. Hydrochloric acid secretion decreases the pH of digesta in both proventriculus and gizzard. The gizzard is unique to birds, it is commonly referred to as mechanical stomach which grinds and mixes the bird's feed. (Svihus, 2011; Jacquie, 2013).

The digesta enters into the small intestine, which comprises of the duodenum, jejunum and ileum, from the gizzard. The duodenum is formed as a loop surrounding the pancreas, which secretes digestive enzymes. The pancreas also secretes bicarbonate which neutralizes the acidic pH of the digesta from the proventriculus. Liver secretes bile, which passes into the duodenum through the gall bladder, plays a key role in the digestion of lipids and the absorption of fat-soluble vitamins. The jejunum and ileum are the major sites of nutrient digestion and absorption due to the secretion of digestive enzymes and the presence of villi and microvilli (Jacquie, 2013; Michael, 1998).

From the ileum, the digesta passes directly to the colon via the ileo-ceco-colonic junction. Anti-peristaltic movements from the direction of the cloaca are responsible for the filling of the caeca, which in the chicken consist of two blind pouches. Retro-peristalsis pushes the contents

against a meshwork of villi at the ileo-ceco-colonic junction, which functions as a filter allowing only fine particles to enter the ceca. Cecal content is excreted separately and has a soft consistency and a characterized color. The main function of the caeca is to absorb remaining water and salts and to carry out the fermentation of indigestible plant material by the abundant microflora. During fermentation, lactic acid, volatile fatty acids and other compounds like ammonia, amines, phenols and indoles are produced in the ceca (Svihus et al., 2013). The digesta enters the very short colon where the final reabsorption of water occurs and undigested feed mixes with uric acid in the cloaca before excretion through the vent (Jacquie, 2013).

1.3 Gut Development in Poultry

Gastro-intestinal tract development in poultry begins as soon as the egg is fertilized until it is hatched. By day 3 incubation, the gut originates from the endoderm, which also develops into the epithelial lining of the mucous glands, whereas the muscular wall and connective tissue evolves from the mesoderm which is partitioned into foregut, midgut, and hindgut (Gilbert, 1997). Most organs such as liver and pancreas are derived from gut tube (Romanoff, 1960). uring day 6, the gut will consist of duodenal loop, small intestine, and cecum by day 6 post incubation. The walls of the yolk sac and GI tract are continuous, and the midgut is connected to the yolk duct (Freeman and Vince, 1974). The cloaca and bursa are derived from the hindgut. As the incubation period progresses, the embryonic small intestine weight increases to a much greater than body weight. During the last 3 days of incubation the ratio of body weight to small intestinal weight increases (Uni et al., 2003).

It is pertinent to note that suitable conditions are strictly needed to support the gut development. The chicks require appropriate timing and form of nutrient available to grow, because initial growth is critical for intestinal development. Activity of the GIT and digestive

organs are stimulated by early access to nutrients (Noy and Sklan, 2001). Especially during the first week post-hatch period, development of the GIT is an important aspect of growth (Sell et al., 1991). Vivid changes occur within the first 24 h post hatching. The immature small intestine of the newly hatched chick undergoes morphological changes in the 2-week post-hatch period (Geyra et al., 2001). During the post-hatch period, the small intestine continues to increase in weight more rapidly than the body mass. Enzymatic and absorptive activities also increase during this period (Uni et al., 1999). Earlier feeding in the post-hatch period is not only able to promote the growth of entire organism, but also stimulate growth of the GIT (Bigot et al., 2003).

1.4 Gut Health in Poultry

Optimal nutrient absorption requires a healthy gut for efficient conversion of feed into its basic components. Digestion, absorption of nutrients, performance, and welfare will be affected if gut health is compromised. Birds with a well-functioning intestinal tract are generally characterized as being healthy. A well-functioning intestinal tract is characteristized by a well balanced microbial population. Stressful conditions such as change of feed, and transportation, pathogenic infections, high temperature and humidity, could lead to an of poultry gastro-intestinal tract (Jin et al., 1997). Poor intestinal health caused by pathogens as a result of gut damage, will depress efficiency in nutrient utilization. Intestinal infection with no subclinncal signs of lesions such as in necrotic enteritis (NE) are more destructive than acute infections. Therefore, a healthy gut largely depends on the the way nutrients are distributed and utilized for general development, and immune system maturation (Kelly and Conway, 2001).

1.5 Factors Affecting Gut Health in Poultry

Potentially harmful microorganisms, nutrients, and non-nutrients could be present in some of the materials ingested by poultry. However, most of these materials are processed in the digestive tract of poultry, which makes it a major site of exposure to pathogens. Gastrointestinal tract functions by allowing nutrients to cross the intestinal barrier while selectively preventing the passage of harmful substances across the intestinal wall into the body of poultry (Korver, 2006). Some factors associated with diet, disease agents, management practices and the environment, could result in impairment of growth rate, feed efficiency and the balance among the components of the chicken gut (Hughes, 2005).

1.5.1 Diet

1.5.1.1. Non-starch Polysaccharides

The major group of anti-nutritional compounds present in various feed ingredients is the non-starch polysaccharides (NSP). It is present in most of the cereals used in poultry diets (Iji, 1999). Resistance to digestive enzymes and creating a viscous environment are the major attributes of NSPs, which results in excretion of sticky droppings (Choct and Annison, 1992). In the small intestine, NSP increases digesta viscosity (Waldenstedt et al., 2000). High concentrations of NSP are found in some cereal grains such as barley, wheat, rye, and oats, and when added to poultry diets in high amount leads to reduced feed efficiency, growth rate, increased digesta viscosity, decreased digesta passage rate, digestive enzymatic activities and nutrient digestibility, of birds (Bedford and Schulze, 1998).

1.5.1.2. Physical Texture and Form of Feed

Physiological characteristics of the intestinal tract may be affected by the physical form of cereal components of feed fed to poultry (Engberg et al., 2004). Mortality may be associated feeding finely ground feed and this could lead to necrotic enteritis as compared with coarsely ground feed. Finely ground wheat fed to poultry increased mortality by 28.9%, whereas observed that the use of coarsely ground wheat diet resulted in a mortality of 18.1% (Branton et al., 1987). Coccidiosis and necrotic enteritis were associated with Mortalities. Development of *Eimeria tenella* in the ceca was associated with feeding of whole wheat to broiler chickens and challenging them with coccidia (Gabriel et al., 2003). Birds fed whole-wheat had a lower weight gain as compared with ground wheat-fed broilers. There was no effect of feeding whole wheat on the performance of broiler chickens before before and after coccidia challenge (Banfield et al., 2002). Birds fed whole-wheat-fed had significant increases in the activity and size of the pancreas and gizzard. Response to gizzard size increase could be as a result of the need to do more grinding to process the whole grain before digestion in the GI tract. Supplementation of whole wheat had no effect on the control of coccidia infections as described by previous studies (Banfield et al., 1999; Banfield and Forbes, 2001).

1.5.2 Infectious Agents

Nutrients are absorbed into the body system from the environment through the gastrointestinal tract, which also provides various protective mechanisms against external invasion. Understanding the manner of causation of enteric disease is complex, as it involves the combinations of several entities (Reynolds, 2003). The main function of the GI tract is for digestion and absorption of nutrients for growth, maintenance and reproduction. Alteration of these biological processes can result in GI dysfunction (Dekich, 1998).

1.5.2.1. Bacterial Infections

Pathogenic bacteria invasion of the gastrointestinal tract could result in reduced weight gain and feed efficiency. Intestinal challenges by pathogenic bacterial such as *Clostridium perfringens* will result in high mortality of poultry birds (Porter, 1998). One of the most severe in poultry intestine is the lesions caused by *Eimeria* infection also known as coccidiosis (Long et al., 1974).

1.5.2.2. Parasites

Internal parasites that commonly affects the commercial poultry are the protozoans that causes diseases. These parasites must spend part of their developmental stages outside the body of the host or they cannot continue to exist. Parasitic diseases such as coccidiosis ususally occurs in birds that are exposed to confinement rearing and high-density. In the past most these parasitic diseases rely on several microorganisms as, most of which have been practically excluded (McDougald, 2003).

1.5.2.3. Toxins

Feed-borne toxins can cause enteric challenge in poultry. The most common example of toxins in poultry feed are mycotoxins (Dekich, 1998). Poultry feed contaminated with mycotoxins would cause a huge economic loss to the poultry industry due to reduced performance (Sklan et al., 2003). Trichothecene mycotoxicoses have detrimental effects on the GI tract which results in major economic losses in the poultry industry (Schiefer and Beasley, 1989). Girgis et al., (2008) reported that following *Eimeria* challenge in broiler chickens, mycotoxins decreased the percentage helper and cytotoxic T-cells in the jejunal mucosa of broiler chickens. Concurrent expose of the small intestine to deoxyvalenol and *Salmonella* resulted in increased expression of

pro-inflammatory cytokines (TNF-α and IL-6) (Grenier et al., 2016). Other factors in combination with mycotoxins can exacerbate outbreaks of diseases.

1.6 Biomarkers for Monitoring Intestinal Health in Poultry

Intestinal health is critical for poultry, particularly for efficient absorption of nutrients. The development of biomarkers of gastrointestinal functionality is crucial in order to understand the events that affect the intestinal barrier function of the gastrointestinal tract in poultry. These would provide techniques for preventions against gastro-intestinal dysfunction.

1.6.1 Biomarkers in the Intestinal Wall

Crypt is an area where stem cells divide into transit amplifying cells, and further differentiate in to mature epithelial cell populations. The villus is a specialized structure with repetitive formation protruding into the lumen for increasing absorptive surface area. Epithelial cells migrate up the villus, to finally enter apoptosis (Fouquet et al., 2004) and exfoliate from the villus tip (Zhang et al., 2015). Differentiated cells near the villus tips are most important for nutrient absorption during migration. Epithelial cell death is usually caused by some enteric pathogens such as coccidia. Intestinal health problems are usually associated with increased loss of villous epithelial cells (Zhang et al., 2015). Decreased epithelial cell population is partly compensated by increased proliferation, resulting in increased crypt depth. Measuring the villus height, crypt depth and the villus/crypt ratio is currently the major criteria for assessing intestinal health status in monogastric animals. The above-mentioned entities are commonly measured in the small intestine to evaluate gut barrier function in poultry studies assessin the efficacy of feed ingredients and feed additives (Teirlynck et al., 2009). Broiler chickens at 23 days of age have a

villus height of approximately 1400 at the duodenum whereas the crypt depth is about 190 (de Verdal et al., 2010).

1.6.2 Biomarkers in blood and in liver

Inflammation could be induced when there is increased number of bacterial translocation insulting the gastrointestinal epithelium which could also come in contact with the liver (Ilan, 2012). Secretion of proteins by the liver cells is altered during acute phase response. Three different experimental models have been developed in order to induce the expression of acute phase protein indicating gastrointestinal dysfunction, but none has been effective (Gilani et al., 2017). Gastrointestinal tract could serve as a pipeline for pathogens to reach the liver. In order to assess intestinal permeability in broilers and turkeys, bacterial counts in the liver have been used as biomarker (Tellez et al., 2014). Coccidia challenge models in poultry have resulted in the leakage of epithelial junctional complexes that protects the intestinal barrier, resulting in easy access of pathogenic derived molecule that will compromise the GI tract (Chen et al., 2015).

1.7 Physiology of Stress in Poultry

Homeostasis is the maintenance of stable internal environment by the birds despite environmental insults. Stress occurs when there is deviation from the normal physiological function of the body. During stress conditions, body resources such as major nutrients are redirected at the expense of other normal body physiological function such as growth and reproduction (Beck, 1991). Birds become fatigued under the long-term stress condition. This results in starvation and susceptibility to infectious diseases (Dohms, 1990). Therefore, it is pertinent to have a management program to minimize the negative effects of stress on the performance and health of birds.

Acute stressor stimulates the activation of the sympathetic nervous system which triggers the first hormonal response to release catecholamines, most importantly adrenaline and noradrenaline (Romero and Butler, 2007). These hormones triggers the channeling of energy resources to muscular tissue and increases the heart rate and blood pressure to prepare the body for necessary response. By redirecting away from the stress factor in order to minimize further responses (Moberg and Mench, 2000). Moving forward, a cascade of hormonal responses ending with the release of glucocorticoids then follows, which is corticosterone in birds. Acute stress triggers the activation of the sympathetic nervous system, which occurs for a short period of time, whereas long lasting effects are induced as result of glucocorticoid release. Increased expression of glucocorticoids signifies stress response in broiler chickens which could eventually result in impaired immune function (O'Mahony et al., 2009) and deteriorated growth (Satterlee et al., 2000).

There is strong evidence that glucocorticoids inhibit gastrointestinal function. Administration of in-feed dexamethasone is followed by decreased jejunal villi height resulting in reduced intestinal absorptive area, and increased permeability of intestine (Chang et al., 2015; Vicu˜na et al., 2015). The change in intestinal permeability allows passage of pathogenic orgnanisms through the intestinal barrier into the blood stream.

1.8 Avian Immune System

1.8.1 Innate and Adaptive Immune Systems

Poultry birds are constantly exposed to pathogenic organisms during their life cycle, but the avian immune system helps protect the birds from pathogenic infection by minimizing their negative effects on host cells. Subsequent responses of poultry to previously encountered pathogens are more robust and efficient which results in the development of immunological memory. The innate and adaptive immune system coordinates and sustains the complex

immunological memory. Physical and chemical barriers of the skin, mucosal tissue, and soluble proteins are the major components of the innate immune system. Macrophages, dendritic cells, natural killer cells, and heterophils are the main cells of the innate immune system. These cells highly depends on recognition of pathogenic features such as mannose and glucan found in bacterial cell walls.

Antigen presenting cells such as macrophages and dendritic cells, presents antigens to the adaptive immune system, and thereby linking the innate and adaptive immunity. M1 macrophages also known as classically activated macrophages, clear pathogens through phagocytosis, nitrogen species (Nitrous oxide), and secretion of antimicrobial peptides and enzymes. Interleukin-1β (IL-1β) and interleukin-6 (IL-6) are secreted by M1 macrophages (Murray and Wynn, 2011). Anti-inflammatory cytokines such as interleukin-10 (IL-10) are involved in tissue repair and they are secreted by M2 macrophages (Murray and Wynn, 2011). Acute-phase response are initiated when pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α, are secreted by macrophages into the blood stream (Klasing, 1988). Liver cells produce acute-phase proteins and are secreted into circulation after been stimulated by pro-inflammatory cytokines. Adaptive immune system is divided into two; humoral and cell-mediated immunity. These are mediated by B and T lymphocytes. The naïve T cells proliferates and mature into activatedT cell, these cells needs to have an antigen presented to it by MHC class I molecule, the origin of T cells in poultry is in the thymus, whereas B cells originates from the bursa of fabricius. The B cell has two major functions; it activates internal signals when bound to its specific antigen, it also serves to bring antigen inside the cell where it is degraded and displayed by the appropriate MHC molecules.

1.9 Common Enteric Diseases in Poultry

Commensal microbial species are usually present in the GIT to maintain the overall health of poultry. There is a balance among the commensal microflora within the lumen of the intestine and the mucosa when the birds are in a healthy state. (Allen and Torres, 2008). Dysbiosis can occur when there is disruption in the balance of commensal microorganisms. This results in the proliferation of microbial population beyond the threshold the can tolerate, and this lead to emergence of diseases.

1.9.1 Coccidiosis

Its an enteric disease in poultry caused by intracellular protozoan parasites belonging to the genus *Eimeria* (McDougald and Reid, 1991). Currently in the poultry industry, it is one of the most devastating disease which results in huge economic loss (Williams, 2005). *Eimeria* invades the intestinal linning and distrupts the enterocyte layer of the gastrointestinal tract of poultry. Examples of different species that affects poultry includes; *Eimeria acervulina*, *Eimeria tenella, Eimeria mitis*, *Eimeria praecox*, *Eimeria maxima*, *Eimeria necatrix*, and *Eimeria brunetti* (McDougald, 2003). Each of the aforementioned species infects different sections in the GI tract, such as duodenum, jejunum, ileum and caecum. These *Eimeria* species multiply rapidly within a short period of time resulting in the birds becoming highly susceptible to coccidia infection (McDougald and Reid, 1991). Broiler chickens are usually affected during the growing phase of their lifecycle.

1.9.1.1. Lifecycle

The life cycle *Eimeria* parasites occurs both externally and internally within the host. A schematic lifecycle of *Eimeria* is shown in Figure 1-2. The cycle starts by shedding the

unsporulated oocysts into the environment in the feces of infected animals. The memebrane of the oocysts are very thick,it helps them to survive outside the hosts. Oocyst becomes infective under a favorable environmental conditions such as warmth, oxygen, and moisture (Allen and Fetterer, 2002). Thereafter the host ingests the oocyst, and the thick walled membrane is broken to release the activated oocyst. The sporocysts that excystate during this process each form two sporozoites and subsequently these are able to infect the intestine (McDougald, 1998). Schizonts develop in the intestinal tissue and undergo at least two generations of asexual reproduction. Micro- and macrogametes are formed after the asexual reproduction. These gametes may fuse producing zygotes. The zygote matures into an oocyst and is subsequently excreted in the feces after which a new lifecycle is ready to begin. The life cycles of the different poultry *Eimeria* species are largely similar, though differences occur in length of pre-patent period, number of schizogony cycles, localization within the gut and in the amount and duration of oocyst shedding (Rose et al., 1996).

1.9.1.2. Symptoms and Lesions

Infected birds will have ruffled feathers and a breakdown in intestinal function. One of first observable symptom is blood in the excreta. The degree of pathogenicity largely depends on the specie of *Eimeria*, number of oocyst and the age of the birds. Reduction in feed and water intake are seen coupled with high vulnerability to other diseases in poultry (Idris et al, 1997). In the gastrointestinal tract, *Eimeria* damage the intestinal mucosa, result in atrophied villi, and reduced intestinal absorptive area (Assis et al., 2010). Transit time of digesta is increased resulting in decreased viscosity, and high permeability of plasma proteins, are also observed during coccidia infection (Williams, 2005).

1.9.1.3. Immune Response to *Eimeria* Infection

Coccidia infection is caused by different species of *Eimeria* that have slightly different life cycles. They also challenge the immune system of broiler chickens differently, however there are some similarities in their mode of infection. Shortly after invasion of *Eimeria* in the small intestine, they activate the specific and nonspecific (phagocytes, chemokines and complement system) immune responses which are linked to cellular and humoral immunity. The gut associated lymphoid tissues coordinates most of the immune responses during *Eimeria* challenge. They are mainly involved in the processing and presentation of antigens to lymphocytes, production of antibodies, and the activation of T-cells. Vervelde et al. (1996) reported that macrophages and T cells mostly observed through histological staining following *Eimeria* infection. Recent studies also indicated that a number of cytokines are produced locally during coccidia infection for enhancing protective immunity against *Eimeria* (Lillehoj et al., 2004; Dalloul and Lillehoj, 2005).

Various cytokines and T helper cells (TH) coordinates cell-mediated immune responses to *Eimeria* infection. The classical and alternative M1 and M2 macrophages are also involved in the regulation of pro and anti-inflammatory responses during coccidia challenge. M1 macrophages enhances the secretion proinflammatory cytokines such as IL-6, TNF-α, and IL-1β, whereas M2 macrophages secretes IL-10, Interferon-γ, and TGF-β, which functions to minimize tissue, damage by suppressing the excessive secretion pro-inflammatory cytokines. A well-balanced M1/M2 cytokine response is key to successful immunity to *Eimeria*.

1.9.1.4. Control Strategies

Administration of anticoccidial medication in poultry feed is the most commonly used method to control coccidiosis in commercial poultry. Currently, there is an enormous reduction in

the use of in-feed anticoccidial drugs due to the increasing consumer concern regarding antibiotic resistance, which necessitates an alternate method for controlling coccidiosis.

1.9.2 Necrotic Enteritis

Clinical Necrotic enteritis (NE) is defined as bacteria disease in poultry that affects the enteric region of chicks within their first few weeks life, caused by toxin production from *Clostridium perfringens.* This bacteria has two strains, type A and type C which can induce NE in poultry. Necrotic enteritis is considered the most clinically dramatic bacterial enteric disease of poultry. Losses due to NE are estimated to cost the commercial poultry industry about $2 billion annually (McDevitt et al., 2006).

1.9.2.1. Pathogenesis

Clostridium perfringens is usually present in the GI tract of poultry without resulting in an insult, but overabundance of this bacteria results in NE (McDevitt et al., 2006). It is also sometimes exacerbated by co-infection with *Eimeria*. Generally, the disease occurs in broilers during the growing phase of their life cycle (Long, 1973). Mucosal damage by activated sporocysts, reduction in intestinal pH, and increased transit time allow the establishment of *Clostridium perfringens*.

Increased inclusion of animal byproducts in diets predispose birds to the disease. Anything that promotes excessive microbial growth or slows the rate of passage in the small intestine could promote the occurrence of NE. In many cases, concurrent coccidiosis, most importantly *Eimeria maxima*, and *E acervulina* is associated with outbreaks in commercial poultry production.

1.9.2.2. Symptoms and Lesions

Usually the main sign of necrotic enteritis in a commercial broiler production is a sudden increase in mortality. However, birds with ruffled feathers and diarrhea are also present. Lesions are primarily found in the small intestine (jejunum/ileum), but most occur in the jejunum, likely due to a favorable pH or lowered activity of digestive enzymes. The mucosa is usually covered with a tan to yellow pseudomembrane often referred to as a "Turkish towel" in appearance and may extend throughout the small intestine or be localized. The membrane is composed of degenerated cells and Gram-positive bacteria (Long et al., 1974).

1.10 Nutritional Immunomodulators Supporting Intestinal Health

1.10.1 Probiotics

Probiotics are live microbial organisms used as feed supplements for poultry, to protect them from gastrointestinal infections and improves gut health (Dahiya et al., 2006). Probiotics improves gut health through several strategies such as; (1) competitive exclusion, (2) enhancing gut barrier function (3) reduction of enterotoxins (4) enhancing host immunity. Several gram-positive bacteria such as *Bacillus, Lactobacillus, Enterococcus and Bifidobacteria* are commonly used to prepare probiotics culture for initial colonization of the GI tract in poultry. Supplementation of probiotic in broiler feed improves growth performance, feed efficiency and intestinal health by reducing intestinal pH, and intestinal bacteria composition (Ghasemi et al., 2014). Hanson et al. (1994) observed that supplementation of *Lactobacillu*s in broiler chickens diets increased cellularity of Peyer's patches in the ileum indicating a stimulation of mucosal immune system. Birds fed diets supplemented with *Bacillus*-based direct fed microbials had lower lesion scores than birds given the non-supplemented diets following an *Eimeria* challenge (Lee et

al., 2010). This indicates that probiotics could also play a major role against coccidia infection by mitigating the impact of parasitic infection on chickens (Giannenas et al., 2012).

1.10.2 Prebiotics

They have been defined as non-digestible feed components that beneficially affect the host health because of their fermentable properties that stimulate bacteria growth in the ileum and cecum (Gibson and Roberfroid, 1995). The most common examples of prebiotics used in poultry research includes mannanoligosaccharides, fructooligosaccharides, and galactooligosaccharides. These compounds are not digestible by the birds but serves as substrate for commensal bacteria such as *Lactobacillus* and *Bifidobacteria* (Jozefiak et al., 2008). These prebiotic components have the ability to either directly bind the pathogens and increase the osmotic values or indirectly have effects through metabolites that are generated by the intestinal microflora during metabolism of prebiotics. When prebiotics are ingested by the birds, they influence the microbiota composition, resulting in improved gut health (Park et al., 2016).

Administration of fructooligosaccharides at the dose of 4g / kg of diet had a positive effect on intestinal morphology and microbiota (Xue et al., 2003). Thus, the use of prebiotics is used as alternatives to antibiotics due to their ability to alter the intestinal microbiota and immune system to reduce colonization of pathogens.

1.10.3 Essential Oils

They are mixtures of fragrant and volatile aromatic compounds that originates from plants. Essential oils possess characteristics odor, and are soluble in organic solvents. They are sensitive to heat and light, so they should be stored in cool and dry places. The most commonly used essential oils are thymol, cinnamaldehyde, carvacrol and eugenol. Their modes of action lie in

their interference with the enzymatic system of the bacteria and the modulation of immune responses and inflammation. Some studies (Pirgozliev et al., 2015; Peng et al., 2016) showed that essential oils are promising alternatives to growth promoter antibiotics (e.g., avilamycin). Essential oils play key role as a preventive and curative role in necrotic enteritis in broiler chickens (Jerzsele et al., 2012). Rezaei–Moghadam et al. (2012) reported that supplementation of turmeric increases serum antioxidant levels and immune status of the birds. Essential oils also contain compounds with strong anti-inflammatory properties, mainly terpenoids and flavonoids, which suppress the metabolism of inflammatory prostaglandins (Krishan and Narang, 2014). There is strong evidence that essential oils can improve production performance and promote better immunity and health.

1.10.4 Organic Acids

Organic acids are weak acids that are dissociated partially. These acids have different properties, some could be used as supplements in drinking water or as additives in feed. Common examples of organic acids used includes; acetic acid, propionic acid and formic acid. The major potential of organic acid in poultry is to decrease pathogenic bacteria population by diffusing through the bacteria membrane by disrupting enzymatic reactions (Cherrington et al., 1991). Several studies (Hassan et al., 2010; Nava et al., 2009) have showed that organic acids addition to broiler chickens feed improves gut health, nutrient digestibility, and the stimulation of immune response. Addition of formic acid to broiler chickens diet reduced coliform count as compared to the control (Paul et al., 2007). Similarly, dietary supplementation of organic acids improved nutrient digestibility (Hernandez et al.,2006; Garcia et al., 2007). Improvement in antibody titres against Newcastle disease in broiler chickens was observed through dietary addition of organic acids (Houshmand et al., 2012), this indicates that organic acid could enhance natural immunity in broiler chickens. From the above mentioned studies, organic acid could be used as an alternative

to reduce pathogenic bacteria in the gastrointestinal tract in order to improve the gut health (Nava et al., 2009).

1.10.5 Bioactive Peptides

The awareness on the influence of diet on health has resulted in the high demand for functional foods and nutraceuticals. This is attributed to the specific functional groups and their molecular derivatives released during metabolism of functional foods and nutraceuticals. Dietary proteins have also been known to offer health benefits *in vivo* and *in vitro*, along with their ability to supply calories and amino acids. Bioactive peptides, which are specific protein fragments, have a positive influence on body functions and may impact gut health in poultry. These peptides are are derived from food protein hydrolysates that induce beneficial biological functionalities. They are produced by microbial fermentation, enzyme digestion or proteolysis by enzymes *in vitro*, and can perform physiological activities in the major body systems (Korhonen and Pihlanto, 2006). Yang et al. (2009) postulated that bioactive peptides could have beneficial functionalities including antioxidative, antimicrobial, and immunomodulatory effects. Bioactive peptides have been produced from a wide range of foods including animal sources such as milk (Gill et al., 2000), egg, cheese, beef (Jang and Lee, 2005), pork (Jang et al., 2008), sea- food (Kim and Wijesekara, 2010), fish (Kim and Wijesekara, 2010; Yang et al., 2009), chicken and plant sources such as rice, corn, soy and soy products (Chiang et al., 2006, *Brassica carinata* (Pedroche et al., 2007).

Biological activities of some bioactive peptides have been established to have prospects of being incorporated as ingredients in functional foods and nutraceuticals and where they may assist in the control and prevention of diseasesin poultry due to their antimicrobial roles as well as enhancement of the immune system. However, in order to firmly establish their therapeutic potency further insightful research of immunomodulatory peptides *in vivo* are needed.

1.10.6 Chitosan Oligosaccharide

The second most abundant natural polysaccharide on earth after cellulose is chitin (Muzzarelli). Chitin is found in exoskeletons of arthropods, molluscs, insects, mushrooms and cell walls of some microorgnaisms (Shahidi, 1999). Chitosan is the deacetylated derivative of chitin, and previous works revealed that chitosan plays a role in immune responses for animal (Villiers et al., 2009). The molecular chain of chitosan has free amino group, this provides a higher chemical and biochemical reactivity than chitin (Ogawa et al., 2004). The structures of cellulose, chitin and chitosan are shown in Figure 1-3.

Chitosan oligosaccharide (COS) has received recent attention as feed additive in animal nutrition. It is a non-toxic polyglucosamine found in nature (arthropods and crustaceans). Chitosan is derived from chitin through a deacetylation process, further hydrolysis will result in COS (Singla and Chawla, 2001. Chitosan oligosaccharides are produced by chitosan depolymerization using physical or acid hydrolysis, and enzymatic degradation (Lodhi et al., 2014) as shown in Figure 1-4. Chitosan oligosaccharide contain reactive functional amino and hydroxyl groups and unlike chitin, have antimicrobial (Holappa et al., 2006), anti-inflammatory (Ma et al., 2011), anti-oxidative (Yen et al., 2008), and immunostimulatory (Zaharoff et al., 2007) properties. Previous studies in monogastric animals have demonstrated that COS given orally in feed is biosafe (Hirano et al., 1990). A mice study concluded that maximum tolerated dose of COS was higher than 10 g/kg (Qin et al., 2006).

Summary

Based on the informations presented in the literature, it is clear that enteric health is extremely important to the overall performance of poultry. Therefore, strategies to maintain optimal gastrointestinal tract conditions could enhance poultry production. Due to changes in

consumer preferences, the poultry industry continues to strive to satisfy the desires of the consumer while ensuring economically profitable production and management practices.

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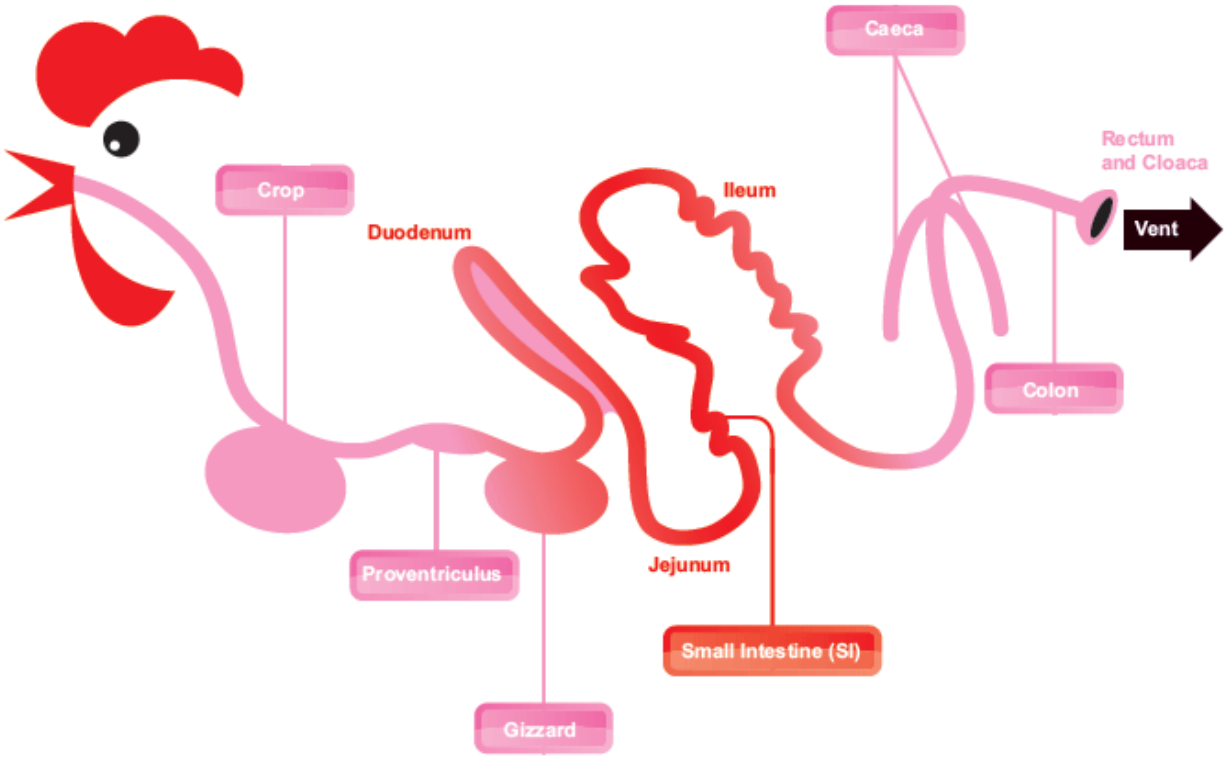

Figure 1.1 Digestive tract of poultry. Modified from Ross Tech Note – Gut Health in Poultry. (2013)

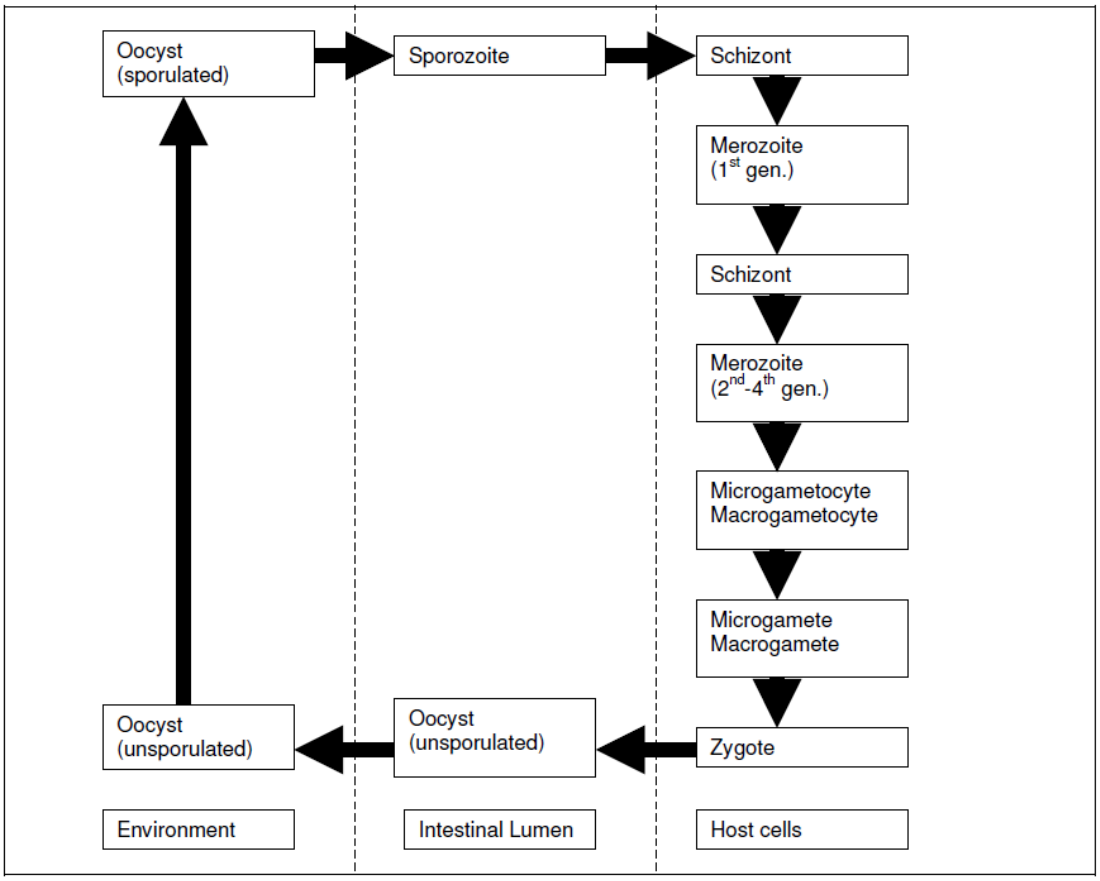

Figure 1.2 Schematic lifecycle of *Eimeria* parasites

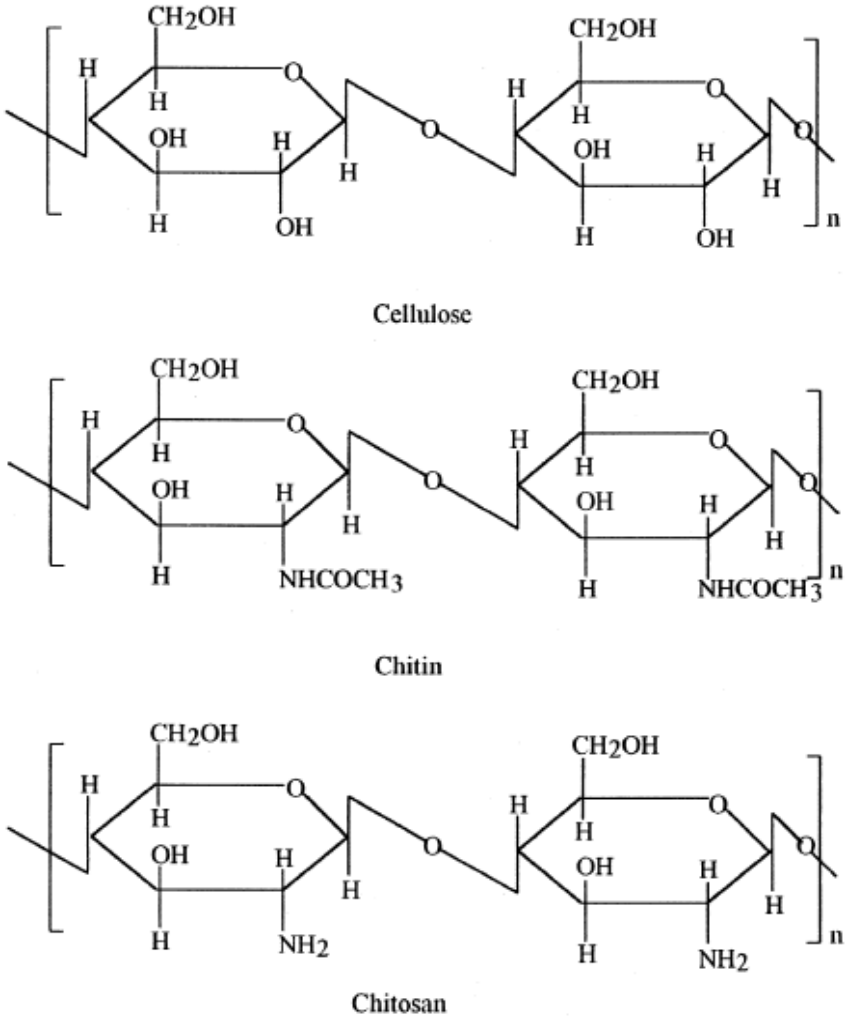

Figure 1.3 Structure of cellulose, chitin and chitosan

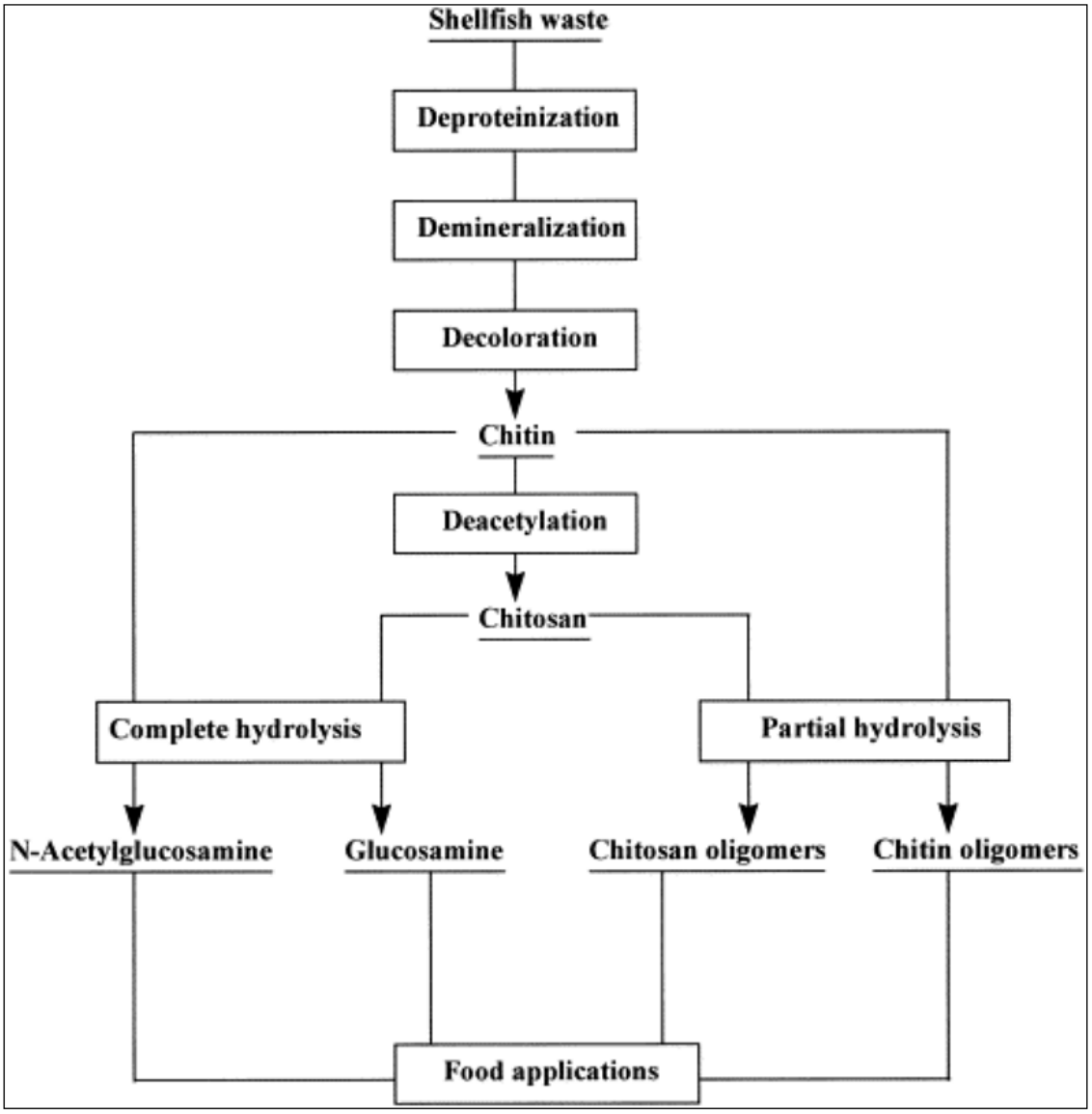

Figure 1.4 Flow chart showing preparation of chitin, chitosan and their products after hydrolysis, adapted from Shahidi et al. (1999).

CHAPTER 2. RESPONSE OF BROILER CHICKENS TO DIETARY SOYBEAN BIOACTIVE PEPTIDE AND COCCIDIA CHALLENGE

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2.1 Abstract

Growth performance, nutrient digestibility, jejunal morphology, plasma cytokine and gene expression responses of broiler chickens (Cobb 500) to graded concentrations of dietary soybean bioactive peptide (SBP) and coccidia challenge (CC) were investigated in 2 experiments. In Experiment 1, 384 male broiler chicks were used to investigate the effect of graded dietary concentrations of SBP on growth performance, nutrient digestibility, and intestinal morphology in in a randomized complete block design with 8 replicate cages and 6 diets. Corn-soybean meal-based diet was supplemented with 0, 1, 2, 3, 4, 5 g of SBP/kg of diet. There were linear effects ($P < 0.05$) of graded concentrations of SBP on BW, BW gain, gain:feed, ileal villus height and jejunal crypt depth at d 22 post hatch. There were linear effects ($P < 0.01$) of graded SBP concentrations on ileal DM and energy digestibility**.** Experiment 2 was conducted to investigate the immune-protective properties of SBP on broiler chickens during a coccidia challenge. A total of 256 male broiler chicks were randomly assigned to 8 replicate cages in a 2 × 2 factorial arrangement of treatments with 2 SBP levels (0 or 4 g/kg of diet) and with or without CC. On d 14 post hatch, birds in the challenged group received 20×, which is twice the recommended coccidia vaccine dose of 25 doses/kg BW, whereas the nonchallenged group received physiological saline. Dietary SBP ameliorated the CC-induced effect ($P = 0.01$) on gain:feed. Coccidia challenge reduced ($P < 0.05$) jejunal villi height whereas dietary SBP supplementation increased ($P < 0.05$) jejunal villi height. Dietary SBP attenuated the CC-induced effects ($P < 0.05$) on the expression of plasma

interleukin-1β, transforming growth factor-β, claudin-1 and occludin genes. In conclusion, dietary SBP improved growth performance, nutrient digestibility, and jejunal morphology. In addition, dietary SBP reduced the impact of coccidia challenge, and may be beneficial in the feed of broiler chickens for alleviation of health-related effects of coccidia infection.

Key words: Broiler chickens, coccidia challenge, intestinal health, soybean bioactive peptide

2.2 Introduction

Coccidiosis is one of the most common and economically important parasitic diseases in poultry production, costing producers more than $800 million each year (Swaggerty et al., 2011). In poultry, the causative agents of coccidiosis are apicomplexan protozoan parasites of the genus *Eimeria*. These parasites invade the intestinal epithelium in a site-specific manner, causing inflammation and necrosis of the gut mucosal barrier, resulting in reduced feed efficiency, and decreased weight gain (Vermeulen, 2001).

For the past five decades, the most common prevention and control measures for coccidiosis were limited to the use of in-feed anticoccidial drugs (such as Amprolium, Decoquinate, and Diclazuril) and vaccinations (De Gussem, 2007; Sharman et al., 2010). However, the long-term use of these drugs has led to the development of resistance against anticoccidial agents (Cox et al., 2010). Additionally, the implementation of the U.S. Veterinary Feed Directive, seeking to cease long-term applications of in-feed antimicrobials as growth promotants, has put into question the use of anticoccidials from the year 2012 onward (Wallace et al., 2010). Unfortunately, this ban has led to a decline in animal health, resulting in reduced growth performance (Castanon, 2007). This has generated significant interest in development of feed additives in diets fed to livestock in an attempt to improve animal health without negatively affecting performance in poultry production.

Among the numerous feed additives currently being investigated, bioactive peptides have piqued the interest of human and animal nutritionists. Bioactive peptides are food protein hydrolysates produced by microbial fermentation, enzymatic digestion (or in vitro enzymatic proteolysis), and alkali or acid hydrolysis. These bioactive peptides have antioxidative, antimicrobial, and immunomodulatory activities (Hartmann and Meisel, 2007; Yang et al., 2009). Bioactive peptides interact with microbial host invaders, suppressing or stimulating certain immune responses such as innate immune modulation (Hancock and Sahl, 2006). Few studies have evaluated the potential of soybean bioactive peptide (SBP) in broiler diets (Feng et al., 2007; Karimzadeh et al., 2016). Currently, low molecular weight peptides and free amino acids are used as high-protein feed ingredients in animal diets (Folador et al., 2006; Nechienzia et al., 2010). Several studies have shown that soybean peptides possess different bioactivities (Fico et al., 2000; Lucas et al., 2001; Ranich et al., 2001) and nutritional function and therefore, might be useful as a functional ingredient to promote the health status of broiler chickens.

To our knowledge, there is no information on the use of SBP as an immune-protective ingredient against coccidia challenge in broiler diets. To fill this research gap, we hypothesized that the dietary supplementation of SBP will not enhance growth performance or digestive function, and will not improve immune functions in broiler chickens. The objectives of the current study are to: 1) determine the effect of SBP in broiler diets to support growth performance, digestive functions, intestinal morphology, and immune organs from d 1 to 22 post hatching; 2) evaluate the immune-protective properties of SBP in broiler chickens diet during coccidia challenge.

2.3 Materials and Methods

The Purdue University Animal Care and Use Committee (West Lafayette, IN) approved the experiment protocol.

2.3.1 Experiment 1

2.3.1.1. Birds, Diets, and Experimental Design

A total of 384 one-day-old male broiler chicks (Cobb 500), maintained in electrically heated battery cages (Alternative Design Manufacturing and Supply Inc.) in an environmentally controlled room until d 22 post hatch were used. Basal diet was formulated to contain standard broiler starter diet, based on corn-soybean meal in mash form. Basal diet was supplemented with 0, 1, 2, 3, 4, or 5 g/kg of a commercial SBP (Fortide, Chengdu Mytech Biotech Co. Ltd., Chengdu, Sichuan, China; Table 2-1) in powdered form. Chromic oxide was added as an indigestible marker. All diets were formulated to meet the NRC (1994) nutrient requirements of broiler chickens. On d 1 post hatching, all birds were individually weighed and allotted to 6 dietary treatments based on BW in a randomized complete block design with 8 replicate cages and 8 birds per cage.

2.3.1.2. Growth Performance and Nutrient Utilization

Individual BW of birds and feed intake were determined at d 15, and 22 post hatch. Final BW, fed intake and feed efficiency for the weigh periods were measured for each period. On d 22 post hatch ileal digesta was collected from the distal two-thirds of ileum excised from each bird, and contents gently flushed with distilled, deionized water and pooled within cage (Kluth et al., 2005). Excreta samples from d 19 to 21 were collected from trays under each cage and care was taken to minimize contamination with feed and feathers. Ileal digesta and excreta samples were stored at – 20°C before further analyses. Pooled ileal and excreta samples were dried in a forced-air oven for 1 week at 56 ^{0}C. Diet, dried ileal digesta and excreta were finely ground using an electric coffee grinder and a mill grinder (Retsch ZM 100, GmbH & Co. K.C., Haan, Germany) respectively. Diet, ileal digesta, and excreta were analyzed for dry matter (DM), gross energy (GE), and nitrogen content. The concentration of DM in diets, ileal digesta, and excreta samples were

measured by drying at 105°C for 24 h in a forced-air oven (The precision Scientific Co., IL; method 934.01; AOAC, 2006). Gross energy was determined using an isoperibol bomb calorimeter (Parr 6200, Parr Instruments, Moline, IL) standardized with benzoic acid. Nitrogen was determined using a Leco analyzer (TruMac N, Leco Corp., St. Joseph, MO) standardized with EDTA (method 990.03, AOAC International 2006). Chromic oxide was used as an indigestible marker, and diet, digesta, and excreta chromium concentrations were determined according to the procedures of (Fenton and Fenton, 1979). Apparent ileal digestibility (AID) of DM, nitrogen, and energy were calculated using the index method as described by (Olukosi et al., 2007)

2.3.1.3. Intestinal Morphological Analysis

The proximal ileum and mid jejunum from the median weight bird in each cage was collected at d 22 post hatch. Excised intestinal segments were flushed in ice-cold 10% phosphate-buffered saline (VWR International, Radnor, PA), stapled to a cardboard background, and fixed in 10% buffered formalin (VWR International, Radnor, PA) for approximately 30 days. Samples were subsequently dehydrated with ethanol (VWR International, Radnor, PA), cleared with Sub-X® (Polysciences, Inc., Warrington, PA) and placed in paraffin (Polyfin paraffin, Sigma Polysciences, St. Louis, MO). Segments of the ileum and jejunum were sliced (5 μm) and subjected to hematoxylin and eosin (H&E) stain, by the Purdue Histology and Phenotyping Laboratory (Purdue University, West Lafayette, IN USA). Villus height and crypt depth were measured from 4 complete, vertically oriented villi per slide and villus height to crypt depth ratio was calculated. Villus length is defined as the length from the villus tip to the valley between each villi while crypt depth is defined as the length between the crypt opening and base. All measurements were performed under a National binocular light microscope (National Optical and Scientific Instruments, Inc., Schertz, TX).

2.3.1.4. Organ weight

Immediately following euthanasia on d 22 post hatch, the median weight bird in each cage was selected, and the liver, pancreas and spleen were promptly removed and weighed. The weights of liver, pancreas and spleen were expressed relative to kg live BW.

2.3.1.5. Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) with the fixed effect of treatment and the random effect of block. The following statistical model was used in the analysis: $Yij = \mu + W_i + B_j + \varepsilon_{ij}$ where Y is the response criterion; μ is the overall mean; W_i is the effect of ith treatment (i = 1,2,3,…,6); B_j is the effect of jth block (k = 1, 2, 3,… 8); and ε_{ij} is the error term. If there was an effect of treatment, single-degree-of-freedom linear and quadratic contrasts were conducted. Significant difference was defined as $P \leq 0.05$. Experimental unit was the cage.

2.3.2 Experiment 2

2.3.2.1. Bird Husbandry, and Dietary Treatments

Total of three hundred and twenty male broiler chickens (Cobb 500) were used in this study. All chicks were housed in electrically heated battery cages (Alternative Design Manufacturing and Supply Inc.) until d 21 post hatch. Battery cage temperature was maintained at 37 ± 1°C for the 1st week and gradually decreased to 27°C in the 3rd week. The lighting schedule was 22L:2D throughout the experiment. Feed and water were provided *ad libitum*. Chicks were inspected daily for any health problems, and mortality was recorded as it occurred. Standard broiler starter diets in mash form were fed from d 0 to 21 post hatch. All birds were provided with a corn-soybean meal basal diet (free of antibiotics growth promoters and coccidiostats) formulated to

meet or exceed all the NRC (1994) requirements for broiler chickens. Dietary treatments were formulated by supplementing a basal diet with two levels of soybean bioactive peptide (SBP) (0 or 4 g/kg) as shown in Table 2-1.

2.3.2.2. Experimental Design and Procedure.

On d 0 post hatch, 320 birds were individually tagged and weighed. Birds were assigned to 2 experimental diets with 2 levels of soybean bioactive peptide (SBP) at 0 or 4 g/kg based on results obtained in experiment 1. There were 16 replicate cages of 0 g SBP /kg and 16 replicate cages of 4 g SBP /kg diet with 10 birds per cage. On d 13 post hatch, all birds were weighed, and feed was weighed back. All birds were re-randomized within each of 0 or 4 g SBP/kg diet to equalize the average body weight of the diets before coccidia challenge. In each of the 0 or 4 g SBP/kg diet, 64 birds in 8 replicate cages of 8 birds per cage were orally gavaged with coccidia vaccine on d 14 post hatch for the challenged group (as shown in Figure 2-1). Also, in each of the 0 or 4 g SBP/kg diet, 64 birds in 8 replicate cages of 8 birds per cage were orally gavaged with physiological saline for the non-challenged group. This resulted in a 2 × 2 factorial arrangement of treatments with 2 levels of SBP at 0 or 4 g/kg and 2 coccidia challenge status (challenged or non-challenged) with 8 replicate cages of 8 birds per cage.

2.3.2.3. Coccidia Infection and Sampling

At d 14 post hatch, average BW of birds were 414 g. The manufacturer's recommendation of one dose of the coccidia vaccine (Coccivac®-B-52, Merck Animal Health) for 1-day-old birds weighing approximately 40 g was estimated to be 25 doses/kg BW. Thus, we calculated the approximate recommended dose for birds at 414 g to be 10 doses (10×). However, in order to challenge the birds, we doubled the recommended dose to 20 doses (20×). Therefore, birds in the

challenged group were orally gavaged with 1 mL of coccidia vaccine containing 20× while birds in the non-challenged group were gavaged with 1 mL of physiological saline. According to the manufacturer, the vaccine is a live oocyst, isolated from chickens, and prepared from anticoccidia-sensitive strains of *E. acervulina, E. maxima, E. maxima MF, E. mivati, and E. tenella.* On d 21 post hatch, birds and feeders were weighed to evaluate growth performance. Average feed intake and BW gain were corrected for mortality. In addition, all birds were euthanized (CO_2 asphyxiation), but 1 bird/cage (median weight bird) was euthanized (CO_2 asphyxiation) separately to prevent additional stressor for blood collection, tissue sampling, and mucosal scrapping from the jejunum. Ileal digesta samples were collected from the remaining birds and pooled within cage for the determination of ileal digestibility of nutrients. Chemical analysis of diets, and ileal digesta, and the measurements of jejunal morphology were similar to that previously described in experiment 1.

2.3.2.4. Total RNA Extraction and Reverse Transcription

The mid jejunum from 1 bird/cage were removed and flushed with ice-cold PBS (VWR International, Radnor, PA), cut in half (longitudinally) exposing the lumen, and mucosal contents were scraped with a metal spatula. Mucosal contents were immediately placed in 2 mL of Trizol reagent (Invitrogen, Grand Island, NY) and stored at -80°C until RNA isolation. Total RNA was extracted from the tissues using Trizol reagent (Invitrogen) following the manufacturer's protocol. RNA concentrations were determined by NanoDrop 1000 (Thermo Scientific), and RNA integrity was verified by 1% agarose gel electrophoresis. To eliminate contaminating DNA, extracted RNA was purified with DNA-*free* DNase Treatment and Removal Kit (Ambion). Afterwards, 2 mg of total RNA from each sample were reverse transcribed into cDNA using the MMLV reverse

transcription system (Promega), and cDNA was then diluted 1:10 with nuclease-free water (Ambion) and stored at -20°C until use.

2.3.2.5. Quantitative Real-time PCR Analysis

Real-time PCR was performed with Bio-Rad iCycler with the Faststart SYBR green-based mix (Life Technologies). PCR programs for all genes were designed as: 10 min at 95°C; 40 cycles of 95°C for 30 s, primer-specific annealing temperature for 30s, and 72°C for 30s; followed by melting curve analysis. The primer sequences used in this study are listed in Table 2-2. Primer specificity and efficiency (90 to 100%) were verified. Samples were analyzed in duplicate, and a difference lesser than or equal to 5% was acceptable. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with normalization against the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as described in a previous study (Tan et al., 2014).

2.3.2.6. Plasma Collection and Analyses

One bird closest to the median group weight for each cage was selected, and blood was collected via cardiac puncture into tubes containing EDTA. Tubes were placed on ice, centrifuged for 20 min at 1,250 × *g* and 4°C to separate plasma, and stored at -80°C until analysis. Plasma concentrations of pro-inflammatory cytokines and antibody were analyzed by a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) using a sandwich enzyme linked immunosorbent assay (ELISA) with chicken specific interleukin-6, interleukin-10, tumor necrosis factor-alpha, and immunoglobulin-A ELISA quantitation kits (MyBiosource, Inc., San Diego, CA), respectively. According to the instructions of the manufacturer (intra assay CV < 8%, inter assay CV < 10%), absorbance was measured at 450 nm. The concentrations of interleukin-6, interleukin-

10, tumor necrosis factor-alpha, and immunoglobulin-A were determined using standard curves constructed from the standards run on the plate.

2.3.2.7. Statistical Analysis

Post-coccidia challenge data were analyzed using the MIXED procedure of SAS as a 2 × 2 factorial consisting of 2 levels of SBP (0 or 4 g/kg) and 2 levels of coccidia challenge (unchallenged or challenged) and evaluated for main effects and interactions. Statistical significance was declared at a probability of $P \leq 0.05$ and cage was the experimental unit.

2.4 Results and Discussion

2.4.1 Experiment 1

Enzymatic hydrolysis is commonly used in food industry for the removal of irritants, detoxification, and enhancement of nutrient quality (Sathe et al., 2005). This process cleaves protein structure to oligopeptides, smaller peptides and free amino acids. These peptides have different length, and different amino acid residue compositions are produced, and can exhibit a variety of functional properties. Recently, immunomodulatory peptides have been isolated from several hydrolyzed food proteins. It was found that larger fractions of peptides found in whey and soybean could have immunomodulatory effects. These fractions have a size of over 1000 kDa and were composed of aggregates, which are formed during the hydrolysis process (Kiewiet et al., 2018). Soybean bioactive peptide (Fortide, Chengdu Mytech Biotech Co. Ltd., Chengdu, Sichuan, China) used in this study was derived by enzymatic hydrolysis and it is in powdered form. The Fortide product consists of 45% CP with about 5-6 peptide and a molecular weight ranging from 650-1000 Da.

Growth performance of broiler chickens fed graded concentrations of SBP from d 1 to 22 post hatch are presented in Table 2-3. Final BW, BW gain, and gain to feed ratio increased linearly ($P < 0.05$) with increasing concentrations of SBP in the diet at d 15 and 22 post hatch. Relative to broiler chickens fed 0 g SBP/kg of diet, there were 4.9, 8.1, 11.1, 10.7, and 13.6 % increases at d 15 post hatch and 3.4, 4.1, 5.4, 7.2, and 9.4% increases at d 22 post hatch on BW gain for broiler chickens fed 1, 2, 3, 4, and 5 g SBP/kg of diet, respectively. Wang, (2005) also observed improved final BW, BW gain, and gain to feed ratio at d 21 post-hatch by supplementing enzyme-hydrolyzed soybean meal in broiler diets. The observed improvement in growth performance could be explained by increased intestinal enzyme activities due to bioactive peptide supplementation as reported by (Feng et al., 2007 and Karimzadeh et al., 2016). The enhanced growth promotion might also be due to improvement in small intestinal morphology and nutrient digestibility observed in the current experiment.

Intestinal morphological measurements of broiler chickens fed dietary concentrations of SBP are presented in Table 2-4. Villus height to crypt depth ratio in the jejunum and villus height in the ileum were linearly increased ($P < 0.05$) with increasing concentrations of SBP in the jejunum and ileum respectively. Crypt depth linearly decreased ($P < 0.05$) with increasing SBP levels in the jejunum. Karimzadeh et al. (2016) observed similar response with the inclusion of canola bioactive peptide in broiler diet. Abdollahi et al. (2017) also showed improved villus height and crypth depth in the duodenum with SBP inclusion in broiler diet. The increase in villus height is paralleled by an increase in digestive, absorptive functions and expression of brush border enzymes (Caspary, 1992). Longer villi are correlated with improved absorptive capacity, addition of SBP in diets offers a comparative advantage in improving the gut health status of the birds. The crypt is associated with villus renewal as a production site of stem cells, and deeper crypts indicate

more rapid tissue turnover (Yason et al., 1987). Villus height to crypt depth ratio is considered a useful criterion for estimating the digestive capacity of the small intestine. A high villus height to crypt depth ratio suggests well-differentiated intestinal mucosa with high digestive and absorptive capability (Montagne et al., 2003). In this sense, the increased villus height to crypt depth ratio observed in jejunum of birds fed SBP could enhance digestive capacity. The mechanism by which SBP improves intestinal morphology is not well understood, although it was speculated by Karimzadeh et al. (2016) that small peptides increase the number and size of villus in the small intestine when compared with other intact proteins.

Relative liver, pancreas, and spleen weights linearly increased ($P < 0.05$) with increasing concentration of SBP in broiler diets (Table 2-4). The liver, spleen, and pancreas are important organs of poultry, and the development status of immune organs directly impacts the immune function (Fan et al., 2013). However, SBP inclusion increased the relative weight of the spleen of broiler chickens compared with control diet. It indicated that SBP could resist the effect of immunosuppression on immune organ development. Spleen weight is assumed to be directly correlated with the proliferation of immune cells within this secondary lymphoid tissue since avians lack lymph nodes (Elmore, 2006).

Increased dietary concentration of SBP linearly increased ($P < 0.05$) ileal digestibility of dry matter and energy (Table2-4). Canola and soybean bioactive peptide supplementation improved nutrient retention and digestibility in broiler chickens (Karimzadeh et al., 2016; Abdollahi et al., 2017). Improved nutrient retention in broiler chickens fed diets supplemented with SBP might be due to modulation of gut environment, improvement of beneficial intestinal microbial balance, improved small intestinal morphology or stimulation of the mucosal immune system (Jin et al., 2008; Tang et al., 2009; Ohh et al., 2010). Apparent metabolizable energy (AME)

and nitrogen-corrected apparent metabolizable energy (AMEn) showed a quadratic ($P < 0.05$) response with increasing concentration of SBP in broiler diet. Dietary inclusion rate of SBP higher than 4 g/kg did not show any further improvements in the ileal digestibility of dry matter and energy. However, further studies are required to identify the exact mechanism of action of SBP that underlies these observations.

2.4.2 Experiment 2

Intestinal health has recently been the subject of intense studies in poultry production (Gadde et al., 2017). When gut health is compromised, digestion and nutrient absorption are affected which, in turn, can have a detrimental effect on gain to feed and BW gain, leading to a greater susceptibility to disease and economic loss. Of the intestinal diseases in poultry, coccidiosis may be most economically important, because it has been shown to impair weight gain and gain to feed ratio (Lee et al., 2010). In the current experiment, we developed a consistent model that would create an acute or subclinical form of coccidiosis, without causing severe mortality. In coccidia challenge trials conducted by our lab (unpublished), the concentration of the coccidia vaccine was determined based on the BW of birds relative to the manufacturer's recommendation. In order to induce acute or subclinical forms of coccidiosis, we doubled the manufacturer's recommendation based on the BW. The aforementioned procedure has been consistent with previous broiler chicken trials conducted in our laboratory where BW gain was reduced between 25 and 35% with coccidia challenge.

Historically, the severity of experimental *Eimeria* infection in chickens have been assessed by decreased BW gain, and gain to feed ratio (Lee et al., 2010). These aforementioned parameters reflect host immunity status in avian coccidiosis (Lillehoj et al., 2007). Mortality in this experiment was below 5% thus, it was not a cause for concern during the trial. Data on growth performance

are presented in Table 2-5. During the post-challenge period of d 14 to 21 post hatch, dietary SBP supplementation attenuated the coccidia challenge induced effect ($P = 0.01$) on gain to feed ratio. Coccidia challenge reduced ($P < 0.05$) BW, BW gain and feed intake, while SBP supplementation increased ($P < 0.05$) BW and BW gain. In the current study, birds in the control group fed 0 g/kg SBP and challenged with coccidia vaccine had a 29% decrease in weight gain as compared with the non-challenged group fed 0 g/kg SBP. Whereas the birds fed 4 g/kg SBP and challenged with coccidia vaccine had a 13% decrease in BW gain as compared with the non-challenged birds fed 4 g/kg SBP. This translates to 55% improvement in weight gain due to SBP supplementation during coccidia challenge. The reduction in BW, BW gain, and feed intake due to the coccidia challenge were not surprising because coccidia infection are known to cause disruption to the intestinal mucosa, resulting in nutrient malabsorption, and reduced performance (Dalloul and Lillehoj, 2005). Furthermore, parasitic infection elicit a nutrient-demanding immune response, which may have also contributed to the impaired growth. This result indicates that the coccidia vaccine challenge had a considerable impact on BW, BW gain, feed intake and gain to feed ratio, but dietary supplementation of SBP shows immune-protective properties and can enhance host defenses against *Eimeria* challenge in broiler chickens. Immunomodulatory peptides are relatively nonspecific in their action and this may account for why the exact mechanism of action are largely unknown. However, it could be speculated that an underlying mechanism may be specific peptide-related regulation of gastrointestinal morphology, motility and function.

In the current study, coccidia challenge induced effect of crypt depth, and goblet cells was reduced by dietary SBP supplementation ($P < 0.05$) (Table 2-5). In the group of birds fed diet supplemented with 0 g/kg SBP, coccidia challenge decreased villus height from 840 to 485 µm, whereas in birds fed diet supplemented with 4 g/kg SBP, coccidia challenge decreased villus height

only from 1067 to 815 µm thus, resulting in a SBP × coccidia challenge interaction ($P < 0.05$). Coccidia parasites are known to cause substantial intestinal damage as they damage enterocytes during progression of their life cycle (Allen and Fetterer, 2002). Decreased villi height could result in poor nutrient absorption and lower performance (Xu et al., 2003). Birds in the challenge group had decreased villi height and villus height to crypt depth ratio, suggesting that coccidia challenge damaged the gut. Supplementation of SBP to birds in the challenged group reversed the coccidia challenge-induced decrease in villi height and villi height to crypt depth ratio, and these result are consistent with findings of previous studies (Cox et al., 2010; Morales-L´opez et al; 2009). The results indicates that SBP supplementation is effective in mitigating performance decline associated with coccidiosis, possibly by improving gut integrity and increasing villi height and villi height to crypt depth ratio. Goblet cells are known as special epithelial cells that are involved in intestinal mucus production (Osho et al., 2017). In the jejunum, SBP supplementation reduced the number of goblet cells in challenged birds by 29% compared with non-challenged birds.

Soybean bioactive peptide supplementation improved ($P < 0.01$) the digestibility of DM, energy and ileal digestible energy in birds challenged with coccidia vaccine (Table 2-5). Nutrient malabsorption during coccidiosis is largely attributed to inflammation, villi atrophy, and impaired activity of digestive enzymes at the site of infection (Adams et al., 1996). Amerah and Ravindran (2015) observed a reduction in ileal digestible energy when broiler chickens are subjected to mixed coccidia challenge. In the current study, we observed a 5.3%, 6%, and 6% unit improvement on ileal digestibility of nutrients and energy of birds fed SBP under coccidia challenge condition. Thus, it could be surmised that coccidia challenge decreased the digestibility of DM, energy, and ileal digestible energy.

The gene expression of cytokines in the jejunum are presented in Table 2-6. There were interaction effects ($P < 0.05$) between coccidia challenge and SBP supplementation on expression of jejunal interleukin **(**IL)-1β, tumor necrosis factor-alpha (TNF-α), and transforming growth factor-beta (TGF-β). The effect ($P < 0.05$) of challenge on the mRNA expression of IL-6 and IL-10 was observed (Table 2-6). Among birds in the coccidia challenged group, cytokine expression levels including IL1β, IL-6 IL- 8, IL-10, and TGF-β were lower in birds fed 4 g/kg SBP compared with birds fed 0 g/kg SBP. Previous studies demonstrated that several pro-inflammatory cytokines or chemokine such as IL-1β, IL-8, and IL-6 were produced in response to experimental *Eimeria* infections (Hong et al., 2006; Lee et al., 2011). Coccidia challenge stimulated increased production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) in the current study. Coccidia challenge remarkably increased the mRNA expression levels of IIL-1β, IL-6, IL-8, and TNF-α) that are involved in jejunal inflammation. Inflammation is an essential part of the innate immune system and involved in the recruitment of phagocytic cells to phagocytize and destroy infectious agents, clearing of cellular debris from the site of damage, and secretion of chemokines that attract other cell types to produce new tissue (Khatri et al., 2005).

Interleukin-1β is a representative powerful pro-inflammatory cytokine mainly produced by stimulated macrophages, monocytes and other cells (Laurent et al., 2001). Generally, IL-1β is not expressed in healthy cells or tissue but acts as a mediator of inflammation in mammals and avian (Fasina et al., 2008). The primary function of IL-8 is to recruit and activate neutrophils in response to infection (Wigley and Kaiser, 2003). During coccidia infection, survival followed by the growth performance of chickens becomes a priority. Therefore, jejunal inflammation plays an important role in the survival of broiler chickens through processing and clearing of the pathogen with subsequent tissue repair. These pro-inflammation cytokines ((IL-1β, IL-6, IL-8, and TNF-α)

are directly or indirectly related with inflammation and have been used as markers to detect inflammation. Therefore, decreased expression levels of pro-inflammatory cytokines in coccidia challenged group indicate that either host immune response was improved, or the inflammation was mediated by SBP. Coccidia challenge induced over expression of IL-10 to abate IL-6 production by macrophages, which may be a mechanism to evade parasite killing.

Dietary SBP supplementation reduced the coccidia-induced effect ($P < 0.05$) on the expression of Claudin-1 and Occludin (Table 2-6). Coccidia challenge reduced ($P < 0.05$) the expression of zonula occludens-1 (ZO-1), zonula occludens-2 (ZO-2), and mucin-2 (MUC-2). Maintaining tight junctions of intestinal epithelial cells is important to optimal health, regulating major immune functions, increasing absorption rate of nutrients, maintaining homeostasis, and protecting against invading pathogens (Rajput et al., 2013). In contrast to pro-inflammatory cytokines, coccidia challenge reduced expression levels of claudin-1, occludin, ZO-1, and ZO-2 in jejunum compared to birds in the non-challenged groups. However, the expression levels in birds given 4 g/kg SBP showed about 3- to 4 times as much as coccidia challenged birds fed the control diet. This increased expression of the tight junction proteins translates to improved barrier function in intestine, especially during invasion of pathogenic microorganisms. Visser et al. (2012), showed that casein hydrolysate intake decreased the epithelial permeability compared to a diet with sole amino acids. The underlying mechanisms of how the immune effects induced by bioactive peptides came about, could be that peptides can directly stimulate Toll-like receptors (TLRs), this activation leads to the production of TNF-α, IL-8, IL-10, and IL-6.

Dietary SBP supplementation attenuated the coccidia challenge induced effect ($P < 0.01$) on the expression of plasma IL-6, IL-10, and TNF-α (Table 2-6). Previous studies have demonstrated that several pro- and anti-inflammatory cytokines were produced in chickens due to

experimental *Eimeria* infections (Lillehoj and Choi, 1998; Min et al., 2001). The IL-6 is a necessary component of resistance to primary infections (Smith and Hayday, 1998). In the current study plasma level of IL-6, and TNF-α increased by 73 and 97% respectively due to coccidia challenge, which mediates the inflammatory immune response, suggesting that coccidia challenge could impair immune function. On the other hand, addition of SBP to the diets of the challenged birds resulted in a smaller increase (51 and 70%) of IL-6 and TNF-α production respectively, indicating that SBP improved the immune response of chickens, probably due to immune stimulating properties. There was an interaction effect ($P < 0.05$) between coccidia challenge and SBP supplementation on plasma immunoglobulin A (IgA) (Table 2-6). The result implies that coccidia challenge increased the expression of IgA, suggesting that coccidia challenge impaired the activity of intestinal mucosal immunity. However, adding SBP reduced the expression of plasma IgA during coccidia challenge. Bioactive peptide intake can increase IgA, as shown for a common carp egg peptide (Chalamaiah et al., 2015). In the current experiment, we observed a decrease in IgA secretion as a result of SBP supplementation, the reason could be because SBP has been fed for 13 days prior to coccidia challenge, which might have protected the intestinal mucosa. In addition, soybean peptides contain antibodies that enhance the immune function of animal, and can improve the level of animal health (Hou et al., 2017).

The principles of coccidiosis vaccination involves the provision of a mild mixed *Eimeria* species challenge within the first d of chick life to build immunity against exposure to field strains later in life (Chapman, 2014). Vaccination at this age increases the risk of enteric disturbances, including coccidiosis, because it requires up to 10 days to develop an acquired immune response (Kogut and Klasing, 2009). Enteric disturbances at this stage of chick's life may cause early reduction in growth and increase in chick's susceptibility to secondary infections (Chapman, 2014).

The current study highlights the potential therapeutic benefits of SBP and support a role for SBP in protecting the gastrointestinal tract from colonization with entero-pathogens and from microbe-induced barrier defects.

In conclusion, our data suggest dietary SBP improved growth performance, nutrient digestibility, and jejunal morphology. In addition, SBP alleviated the negative effects of the coccidia challenge and may be a promising and beneficial 'anticoccidial'. To our knowledge, this is the first study to evaluate the effects of dietary SBP on disease status and immune related gene expression during coccidia challenge in poultry, thus providing a new evidence for the potential use of SBP for the amelioration of coccidia infection.

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Table 2.1 Ingredients and nutrient composition of experimental diets used in Exp. 1 and 2, g/kg as-fed basis.

Ingredients	
Corn	537.1
Soybean meal (47% CP)	326.2
Corn DDGS	30.0
Soybean oil	35.0
Monocalcium phosphate[1]	17.0
Limestone (38% Ca)	15.5
Salt	4.0
Vitamin-mineral premix[2]	3.0
DL-Methionine	3.5
L-Lysine.HCl	3.0
Threonine	0.7
Chromic oxide premix[3]	25
SBP premix[4,5,6]	0
Total	1,000
Calculated Nutrients & Energy content	
CP, g/kg	220
ME, kcal/kg	3060
Ca, g/kg	9.6
P, g/kg	7.3
Non-phytate P, g/kg	4.7
Ca:Tp	1.3

[1] Contained 16% Ca, 21% P.

[2] Supplied the following per kilogram of diet: vitamin A, 5,484 IU; vitamin D_3, 2,643 IU; vitamin E, 11 IU; menadione sodium bisulfite, 4.38 mg; riboflavin, 5.49 mg; D-pantothenic acid, 11 mg; niacin, 44.1 mg; choline chloride, 771 mg; vitamin B_{12}, 13.2 μg; biotin, 55.2 μg; thiamine mononitrate, 2.2 mg; folic acid, 990 μg; pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 μg.

[3] Prepared as 1 g of chromic oxide added to 4 g of ground corn.

[4] 1 g of Soybean bioactive peptide (SBP) product, (Fortide, Chengdu Mytech Biotech Co. Ltd., Chengdu, Sichuan, China) added to 4 g of soybean meal.

[5] In Exp. 1, SBP premix was added at the expense of soybean meal to supply 0, 1,2, 3, 4, or 5 g SBP/kg diet.

[6] In Exp. 2, SBP premix was added at the expense of soybean meal to supply 0, or 4 g SBP/kg diet.

Table 2.2 Primers used in real –time quantitative PCR

Genes	Primer sequence (5'- 3')	Gene Bank ID	Ref
Housekeeping gene			
GAPDH	F: TCCTAGGATACACAGAGGACCA R: CGGTTGCTATATCCAAACTCA	ENSGALG00000014442*	Grenier et al., 2015
Markers of inflammation			
IL-1β	F: GCATCAAGGGCTACAAGCTC R: CAGGCGGTAGAAGATGAAGC	NM_204524	Adedokun et al., 2012
IL-6	F: CTGCGAGAACAGCATGGAGA R: GAAAGGTGAAAAGCCCGCTG	XM_01,310,0522	Bai et al., 2018
IL-8	F: GCGGCCCCCACTGCAAGAAT R: TCACAGTGGTGCATCAGAATTGAGC	ENSGALG00000011670*	Grenier et al., 2015
IL-10	F: GCTGAGGGTGAAGTTTGAGG R: AGACTGGCAGCCAAAGGTC	ENSGALG00000000892*	Grenier et al., 2015
TGF-β	F: ACACGGACTGCAGACAAAGT R: GGTGCACAAACAAATGGCCT	XM_0,050,19359	Bai et al., 2018
TNF-α	F: AGATGGGAAGGGAATGAACC R: ACTGGGCGGTCATAGAACAG	AY765397	Adedokun et al., 2012
TLR-4	F: GTTCCTGCTGAAATCCCAAA R: TATGGATGTGGCACCTTGAA	NM_001030693	Adedokun et al., 2012
Markers of gut integrity			
Claudin-1	F: TGGAGGATGACCAGGTGAAGA R: CGAGCCACTCTGTTGCCATA	NM_001013611.2	Shao et al., 2013
Occludin	F: GATGGACAGCATCAACGACC R: CATGCGCTTGATGTGGAAGA	NM_205128.1	Wu et al., 2018
ZO-1	F: GCCTGAATCAAACCCAGCAA R: GCCTGAATCAAACCCAGCAA	XM_015278980.1	Wu et al., 2018
ZO-2	F: ATCCAAGAAGGCACCTCAGC R: CATCCTCCCGAACAATGCCT	NM_2040918	
Muc-2	F: GCTACAGGATCTGCCTTTGC R: AATGGGCCCTCTGAGTTTTT	XM_421035	Adedokun et al., 2012

F stands for forward primer; R stands for reverse primer; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IL = interleukin; TGF-β = transforming growth factor-β; TLR4 = toll-like receptor 4; TNF-α = tumor necrosis factor alpha; MUC2 = mucin 2; ZO-1 = zonula occludens 1; ZO-2= zonula occludens 2. *Sequence obtained from Ensembl chicken genome data resources.

Table 2.3 Growth performance of broiler chickens fed graded concentrations of soybean bioactive peptide (SBP) from d 1 to 22 post hatching[1].

	SBP, g/kg							*P*-value		
Item	0.0	1.0	2.0	3.0	4.0	5.0	SEM	Treatment	Linear	Quadratic
BW										
d 15	465	489	506	523	521	538	11.3	<0.01	<0.01	0.27
d 22	881	912	919	931	949	972	12.6	<0.01	<0.01	0.99
Gain, g/bird										
d 1 to 15	418	441	459	475	473	491	10.9	<0.01	<0.01	0.27
d 15 to 22	416	423	412	409	429	433	12.3	0.68	0.32	0.34
d 1 to 22	833	864	871	884	902	924	12.3	<0.01	<0.01	0.99
Feed intake, g/bird										
d 1 to 15	593	577	578	577	583	589	20.7	0.98	0.98	0.49
d 15 to 22	645	641	647	649	655	643	39.4	0.99	0.91	0.90
d 1 to 22	1237	1218	1225	1226	1237	1231	35.9	0.99	0.91	0.78
Gain:feed, g/kg										
d 1 to 15	705	767	797	828	825	836	22.1	<0.01	<0.01	0.06
d 15 to 22	648	668	646	663	689	692	49.4	0.96	0.42	0.79
d 1 to 22	674	712	715	731	733	755	24.5	0.26	0.01	0.70

[1] Data are least squares means of 8 replicate cages of 8 birds per cage.

Table 2.4 Ileal and total tract nutrient digestibility of broiler chickens fed graded concentrations soybean bioactive peptide (SBP) from d 1 to 22 post hatch[1,2].

	SBP, g/kg							*P*-value		
Item	0.0	1.0	2.0	3.0	4.0	5.0	SEM	Treatment	Linear	Quadratic
Jejunum										
VH, μm^2	1120	1151	1181	1210	1244	1229	43.03	0.24	0.01	0.72
CD, μm^2	153	142	135	127	123	128	6.51	0.01	<0.01	0.26
VH:CD	7.5	8.29	8.78	9.73	10.1	9.8	0.52	<0.01	<0.01	0.43
Ileum										
VH, µm	768	813	850	885	918	918	39.5	0.03	<0.01	0.48
CD, µm	154	149	144	139	135	135	11.17	0.70	0.10	0.79
VH:CD	5.3	5.9	6.1	6.5	6.9	6.9	0.50	0.10	<0.01	0.67
Immune organ weight, g/kg BW										
Liver	27.2	28.2	29.6	29.7	30.2	30.2	1.09	0.31	0.03	0.41
Pancreas	1.9	1.7	2.2	2.2	2.4	2.7	0.28	0.33	0.04	0.46
Spleen	1.03	1.05	1.07	1.08	1.09	1.13	0.02	<0.01	<0.01	0.81
Ileal digestibility										
DM, %	70.1	71.2	71.7	72.7	73.6	73.3	0.50	<0.01	<0.01	0.60
Energy, %	73.3	73.9	74.2	74.8	75.5	74.4	0.50	0.01	<0.01	0.77
IDE, kcal/kg	3,189	3,191	3,209	3,234	3,246	3,228	24	0.10	<0.01	0.89
Total tract metabolizability										
DM, %	73.5	73.3	74.1	74.0	74.2	74.1	0.30	0.21	0.03	0.43
Energy, %	76.5	76.5	76.7	76.6	77.1	76.8	0.20	0.62	0.16	0.92
AME, kcal/kg	3,331	3.299	3,278	3,281	3.316	3,292	11	0.02	0.14	0.02
AMEn, kcal/kg	3,113	3,083	3,055	3,061	3,098	3,077	10	<0.01	0.13	<0.01

[1] Data are least squares means of 8 replicate cages of 8 birds per cage.

[2] IDE = Ileal digestible energy; VH = Villus height; CD = Crypt depth

Table 2.5 Growth performance, ileal digestibility, and jejunal morphology of broiler chickens fed diets containing soybean bioactive peptide (SBP) concentration at 0 or 4 g/kg with or without coccidia challenge from d 13 to 21post hatch[1,2].

	0, g/kg SBP		4 g/kg SBP			*P*-value		
Item	Non-challenge	Challenge	Non-challenge	Challenge	SEM	SBP	Challenge	SBP × Challenge
d 13 to 21								
d 13 BW, g/bird	414	414	414	413	14.3	0.56	0.87	0.79
d 21 BW, g/bird	858	728	859	801	24.9	0.05	<0.01	0.06
Gain, g/bird	443	314	446	388	19.7	0.04	<0.01	0.06
Feed intake, g/bird	632	550	627	568	27.8	0.80	0.01	0.60
Gain: Feed, g/kg	701	577	707	685	20.2	<0.01	<0.01	0.01
Jejunal morphology[5]								
VH, µm	840	485	1067	815	25.1	<0.01	<0.01	0.05
CD, µm	151	319	115	155	5.61	<0.01	<0.01	<0.01
VH/CD	5.61	1.53	9.34	5.26	0.25	<0.01	<0.01	0.98
GC, cells/µm of villi	0.12	0.32	0.10	0.18	0.01	<0.01	<0.01	<0.01
Ileal digestibility								
DM, %	69.21	59.77	75.09	68.87	0.36	<0.01	<0.01	<0.01
Energy, %	71.19	58.11	76.57	67.05	0.53	<0.01	<0.01	<0.01
IDE, kcal/kg	3,148	2,564	3,341	2,925	25	<0.01	<0.01	<0.01

[1] On d 0 post hatch, birds were divided into two groups with 16 replicates each, and were fed control diet and SBP supplemented diets, each with an initial body weight of 42 g/bird. The final BW on d 13 post hatch was 382 and 373 g/bird for birds fed control diet and SBP supplemented diet respectively. The feed intake was 457 and 446 g/bird for birds fed control and SBP. The feed efficiency was 744 g/kg and 739 g/kg for birds fed control diet and SBP supplemented diets respectively.

[2] Data are least squares means of 8 replicate cages with 8 birds per cage.

[3] Each bird in challenged and non-challenged group were gavaged with 20× of coccidia vaccine, which is twice the manufacture's recommended dose of 25× /kg BW or distilled water respectively on d 14 and sampled on d 21 post hatch.

[4] *P*-value according to main effects of SBP, challenge, and a SBP × challenge interaction.

[5] VH= Villus height; CD = Crypt depth; GC = Goblet cell; IDE = Ileal digestible energy.

Table 2.6 Relative gene expression † of cytokines and tight junction proteins in jejunal mucosa and plasma cytokines and antibody of broiler chickens fed diets containing soybean bioactive peptide (SBP) concentration at 0 or 4g/kg with or without coccidia challenge at d 21 post hatch[1,2].

	0, g/kg SBP		4 g/kg SBP			*P*-value[3]		
Item[4]	Non-challenge	Challenge	Non-challenge	Challenge	SEM	SBP	Challenge	SBP × Challenge
Jejunal mucosa								
IL1β	1.00	3.32	1.21	1.88	0.31	0.06	<0.01	0.01
IL-6	1.00	5.05	0.64	1.90	1.05	0.11	0.01	0.20
IL-8	1.00	4.09	1.02	1.84	0.99	0.27	0.06	0.27
IL-10	1.00	7.91	0.69	2.25	1.56	0.06	0.01	0.09
Claudin-1	1.00	0.63	8.17	3.06	0.94	<0.01	<0.01	0.02
Occludin	1.00	0.23	5.20	0.80	0.49	<0.01	<0.01	<0.01
ZO-1	1.00	0.27	2.47	0.91	0.35	<0.01	<0.01	0.24
ZO-2	1.00	0.19	2.44	0.79	0.43	0.02	0.01	0.34
TNF-α	1.00	4.36	1.40	1.75	0.75	0.15	0.02	0.05
TLR-4	1.00	1.42	0.82	4.40	0.71	0.04	0.01	0.06
MUC2	1.00	1.91	0.75	1.13	0.25	0.06	0.02	0.30
TGF-β	1.00	9.32	1.46	2.58	1.59	0.06	0.01	0.03
Plasma								
IL-6, pg/mL	24.43	92.08	33.66	69.17	2.43	0.01	<0.01	<0.01
IL-10, pg/mL	12.15	178.99	60.68	114.37	3.57	0.03	<0.01	<0.01
TNF-α, pg/mL	24.42	935.8	152.31	511.24	19.7	<0.01	<0.01	<0.01
IgA, ng/mL	0.82	17.54	3.89	11.70	0.34	<0.01	<0.01	<0.01

[1] Data were least squares means of 8 replicate cages with 8 birds per cage.

[2] Each bird in challenged or non-challenged group was orally gavaged with 20× of coccidia vaccine, which is twice the manufacturer's recommended dose of 25× /kg BW or distilled water respectively on d 14, and sampled on d 21 post hatch.

[3] *P*-value according to main effects of SBP, challenge, and a SBP × challenge interaction.

†Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated with GAPDH as the endogenous control.

[4] IL = interleukin; TGF-β = transforming growth factor-β; TLR4 = toll-like receptor 4; TNF-α = tumor necrosis factor α; MUC2 = mucin 2; ZO-1 = zonula occludens 1; ZO-2= zonula occludens 2; IgA = Immunoglobulin A.

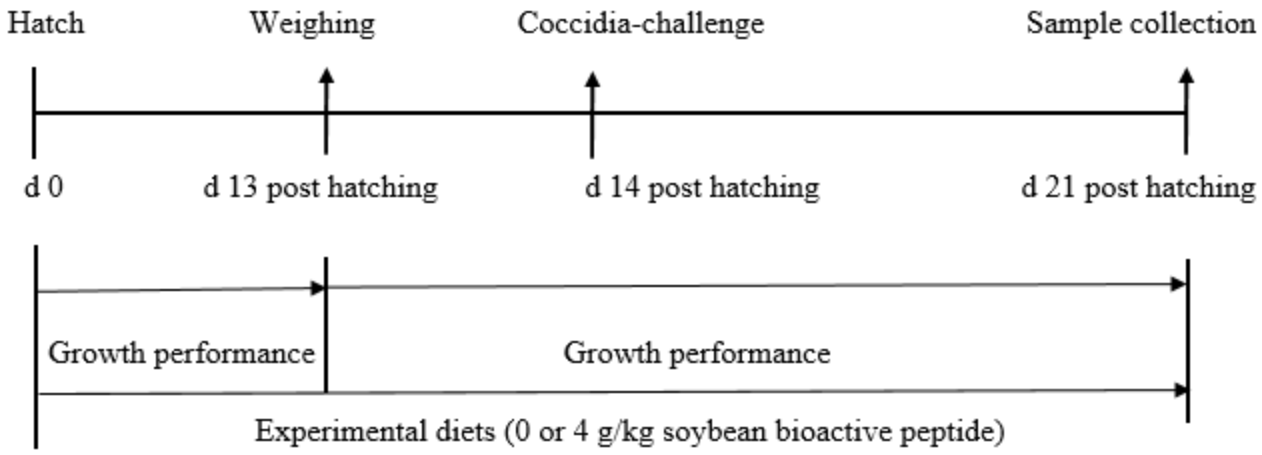

Figure 2.1 Diagram of experimental procedure. Broiler chickens were fed basal diet supplemented with 0 or 4 g/kg SBP from d 0 to 21 post hatch. They were weighed on d 13 and challenged with coccidia vaccine on d 14 post hatch. Growth performance was measured for d 0-13, and 13-24 post hatch. Ileal digesta and intestinal tissues were collected on d 21 post hatch.

CHAPTER 3. IMPACT OF DIETARY CHITOSAN OLIGOSACCHARIDE AND COCCIDIA CHALLENGE IN BROILER CHICKENS

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3.1 Abstract

Growth performance, nutrient digestibility, jejunal morphology, plasma cytokine and gene expression responses of broiler chickens (Cobb 500) to graded dietary concentrations of dietary chitosan oligosaccharide (COS) and coccidia challenge (CC) were investigated in 2 experiments. Experiment 1 was conducted to investigate the effect of graded dietary concentration of COS in the diets of broiler chickens using 8 replicate cages of one-day-old male broiler chickens in each of 6 diets. Corn-soybean meal-based diet was formulated to contain 0.0, 0.5, 1.0, 1.5, 2.0, and 2.5 g of COS/kg of diet. There were linear effects ($P < 0.05$) of graded COS concentration on body weight (BW), BW gain, feed intake, jejunal villus height and villus height to crypt depth ratio at d 22 post hatch. Between 0.50 and 1.18 g COS/kg diet was optimum for BW gain, jejunal villus height, and villus height to crypt depth ratio. There were linear increases ($P < 0.05$) in ileal dry matter and energy digestibility. Experiment 2 was conducted to investigate the immune-protective properties of COS on broiler chickens during CC. A total of 224 male broiler chicks were randomly assigned to 8 replicate cages in a 2 × 2 factorial arrangement of treatments with two COS concentrations (0 or 1 g of COS/kg of diet) and with or without CC. On d 18 post hatch, birds in the challenged group received 30×, which is twice the recommended coccidia vaccine dose of 25 doses/kg BW, whereas the nonchallenged group received physiological saline. Coccidia challenge reduced ($P < 0.05$) and dietary COS increased ($P < 0.05$) BW gain, and feed intake. Dietary COS

mitigated ($P < 0.05$) the CC-induced effects ($P < 0.05$) on gain:feed. Coccidia challenge reduced ($P < 0.05$) jejunal villus height whereas dietary COS supplementation increased ($P < 0.05$) jejunal villus height. Dietary COS supplementation attenuated the CC-induced effects ($P < 0.05$) on the expression of plasma interleukin-6 and 10, claudin-1 and occludin genes. In conclusion, dietary COS improved growth performance, nutrient digestibility, and jejunal morphology. In addition, dietary COS reduced the negative effect of coccidia challenge, and may provide immune protection for broiler chickens during coccidia infection.

Key words: Broiler chickens, chitosan oligosaccharide, coccidia challenge, concentration, gene expression

3.2 Introduction

Coccidiosis is a worldwide protozoan disease in broiler chickens caused by parasites of the genus *Eimeria*. These parasites can develop and multiply within the intestinal tract damaging the intestinal mucosa that can induce a severe inflammatory response (Metwaly et al. 2012; Dkhil et al. 2013). This tissue destruction is also associated with reduced performance and nutrient malabsorption (Amer et al. 2015). Coccidiosis therefore causes massive economic losses in the poultry industry (McDougald, 2003).

Concerns over the future use of anticoccidial drugs has led to an intense search for alternative feed additives capable of promoting growth and intestinal health. Recently, compounds that stimulate the non-specific immunity against coccidia have gained interest. Chitosan oligosaccharide (COS) is a compound obtained from chitin by chemical and enzymatic hydrolysis. Chitosan, the second most abundant polymer in nature (Singla and Chawla, 2001), is obtained by the deacetylation of chitin which is a component of exoskeleton of crustaceans, insects, and fungi

(Crini, 2005). The partial hydrolysis of chitosan yields COS which is a compound with low molecular weight, good solubility, and low viscosity (Chae et al., 2005). Chitosan oligosaccharide was proposed as an immune-stimulant due to some of its anti-inflammatory characteristics and antimicrobial activities (Koch et al., 2015). Its impact in stimulating cellular and humoral immune response allowed it to be used in the prevention of immune system disorders and inflammatory processes. Previous studies in poultry showed that COS improves growth performance (Huang, 2005), enhanced immune function and antimicrobial activities (Xiao et al., 2013; Swiatkiewicz et al., 2015). Chitosan oligosaccharide has been reported to show potent immunological effects such as activation of macrophages (Nishimura et al., 1984), stimulation of non-specific host resistance (Okawa et al., 2003), and protections against pathogenic infections (Rhoades et al., 2006).

According to currently available literature, this is the first study to use COS as an anticoccidia feed additive in broiler diet following a mild coccidia challenge. Therefore, we hypothesized that the increasing dietary concentrations of COS will not affect growth performance, digestive function, and immune functions in broiler chickens. The objectives of the current study are to: 1) determine an optimum dietary concentration of COS in broiler diets to support growth performance, digestive functions, intestinal morphology, and immune organs; 2) evaluate the immune-protective properties of COS on broiler chickens following coccidia challenge.

3.3 Materials and Methods

The Purdue University Animal Care and Use Committee (West Lafayette, IN) approved the experiment protocol.

3.3.1 Experiment 1

3.3.1.1. Chitosan Material

Chitosan oligosaccharide supplement used in both experiments was purchased from Qingdao Yunzhouo Biochemistry Co. Ltd. (Jimo, Qingdao, China). They were derived from shrimp shells and the degree of deacetylation was greater than 90%.

3.3.1.2. Birds, Diets, and Experimental Design

Three hundred and eighty-four one-d-old male broiler chicks (Cobb 500) were used in this study. All chicks were maintained in electrically heated battery cages (Alternative Design Manufacturing and Supply Inc.) in an environmentally controlled room until d 22 post hatch. The birds were given access to mash feed and water ad libitum during the 22-d experiment. The basal diet was formulated to contain standard broiler starter diet, based on corn-soybean meal in mash form. This was supplemented with 0.0, 0.5, 1.0, 1.5, 2.0, or 2.5 g/kg COS (Qingdao Yunzhouo Biochemistry Co. Ltd., Jimo, Qingdao, China; Table 1) in powdered form. Chromic oxide was added to the diets as an indigestible marker. All diets were formulated to meet the NRC (1994) nutrient requirements of broiler chickens. On d 1 post hatch, all birds were individually weighed and allotted to one of the six dietary treatments based on body weight (BW) in a randomized complete block design with eight cage replicates containing eight birds per cage.

3.3.1.3. Growth Performance and Nutrient Utilization

Individual BW of birds and feed intake by cage were determined at d 15, and 22 post hatch. Final BW, feed intake and feed efficiency for the weigh periods were measured for each period. On d 22 post hatch, ileal digesta was collected from the distal two-thirds of the ileum excised from each bird, contents were gently flushed with distilled, deionized water and pooled within cage

(Kluth et al., 2005). Excreta samples from d 19 to 21 post hatch were collected from trays under each cage and care was taken to minimize contamination with feed and feathers. Ileal digesta and excreta samples were stored at – 20˚C before further analyses. Pooled ileal and excreta samples were dried in a forced-air oven for 1 week at 56 ^{0}C. Diet, dried ileal digesta and excreta were finely ground using an electric coffee grinder and a mill grinder (Retsch ZM 100, GmbH & Co. K.C., Haan, Germany) respectively. Diet, ileal digesta, and excreta were analyzed for dry matter (DM), gross energy (GE), and nitrogen content. The concentration of DM in diets, ileal digesta, and excreta samples were measured by drying at 105°C for 24 h in a forced-air oven (The precision Scientific Co., IL; method 934.01; AOAC, 2006). Gross energy was determined using an isoperibol bomb calorimeter (Parr 6200, Parr Instruments, Moline, IL) standardized with benzoic acid. Nitrogen was determined using a Leco analyzer (TruMac N, Leco Corp., St. Joseph, MO) standardized with EDTA (method 990.03, AOAC International 2006). Chromic oxide was used as an indigestible marker, and its concentration in the diet, digesta, and excreta were determined according to the procedures of (Fenton and Fenton, 1979). Apparent ileal digestibility (AID) of DM, nitrogen, and energy were calculated using the index method as previously described (Olukosi et al., 2007).

3.3.1.4. Intestinal Morphological Analysis

The proximal ileum and mid jejunum from the median weight bird in each cage was collected on d 22 post hatching. The proximal ileum was defined as the section of the gut from the Meckel's diverticulum to the ileocecal junction, and 5-cm proximal to the Meckel's diverticulum was cut. The mid jejunum was defined as the section of the gut between the end of the duodenal loop and Meckel's diverticulum was collected, and a 5-cm section was cut at the middle). Excised intestinal segments were flushed in ice-cold 10% phosphate-buffered saline (VWR International,

Radnor, PA, USA), stapled to a cardboard strip, and fixed in 10% buffered formalin (VWR International, Radnor, PA, USA) for approximately 30 d. Samples were subsequently dehydrated with ethanol (VWR International, Radnor, PA), cleared with Sub-X® (Polysciences, Inc., Warrington, PA) and placed in paraffin (Polyfin paraffin, Sigma Polysciences, St. Louis, MO, USA). Segments of the ileum and jejunum were sliced (5 μm) and subjected to hematoxylin and eosin (H&E) stain, by the Purdue Histology and Phenotyping Laboratory (Purdue University, West Lafayette, IN USA). Villus height and crypt depth were measured from 4 complete, vertically oriented villi per slide and villus height to crypt depth ratio was calculated. Villus length is defined as the length from the villus tip to the valley between each villi while crypt depth is defined as the length between the crypt opening and base. All measurements were performed under a National binocular light microscope (National Optical and Scientific Instruments, Inc., Schertz, TX, USA).

3.3.1.5. Organ weight

Immediately following euthanasia, the median weight bird in each cage was selected, and the liver, pancreas and spleen were removed and weighed. The weights of liver, pancreas and spleen were expressed as g of organ/kg of BW.

3.3.1.6. Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) with treatment being assigned the fixed effect and block as random effect. The following statistical model was used in the analysis: $Yij = \mu + W_i + B_j + \varepsilon_{ij}$ where Y is the response criterion; μ is the overall mean; W_i is the effect of ith treatment (i = 1,2,3,…,6); B_j is the effect of jth block (k = 1, 2, 3,… 8); and ε_{ij} is the error term. If there was an effect of treatment, single-degree-of-freedom linear and quadratic contrasts were conducted. Broken-line regression analysis was conducted for

BW gain, gain to feed ratio, and villus height to crypt depth ratio measurements using the NLIN procedure of SAS as described by Robbins et al. (2006). Significant difference was defined as $P \leq 0.05$. Cage was denoted as the experimental unit.

3.3.2 Experiment 2

3.3.2.1. Bird Husbandry, and Dietary Treatments

In total, 288 one day-old male broiler chicks (Cobb 500) were used in this experiment. All chicks were housed in electrically heated battery cages, as in experiment 1, until d 24 post hatch. Battery cage temperature was maintained at 37 ± 1°C for the first week and gradually decreased to 27°C in the third week. The lighting schedule was 22L:2D throughout the experiment. Feed and water were provided *ad libitum*. Chicks were inspected daily for any health problems, and mortality was recorded as it occurred. Standard broiler starter diets in mash form were fed from d 0 to 24 post hatch. All birds were provided with a corn-soybean meal basal diet (free of antibiotics growth promoters and coccidiostats) formulated to meet or exceed all the NRC (1994) requirements for broilers. Dietary treatments were formulated by supplementing a basal diet with COS at 0 or 1 g/kg (Table 3-1).

3.3.2.2. Experimental Design and Procedure

On the first day of the experiment, all broiler chicks were individually tagged and weighed. Birds were assigned to 2 experimental diets with 2 levels of COS at 0 or 1 g/kg based on the optimum dietary COS established in experiment 1. There were 16 replicate cages of 0 g COS /kg and 16 replicate cages of 1 g COS /kg diet with 9 birds per cage. On d 17 post hatch, all birds were weighed, and feed was weighed back to calculate feed intake. At this time, average BW of birds was 608 g. All birds were re-randomized within each of 0 or 1 g COS/kg diet to equalize the

average body weight of the diets before coccidia challenge. In each of the 0 or 1 g COS/kg diet, 56 birds in 8 replicate cages of 7 birds per cage were orally gavaged with coccidia vaccine on d 18 post hatching for the challenged group (Figure 3-1). Also, in each of the 0 or 1 g COS /kg diet, 56 birds in 8 replicate cages of 7 birds per cage were orally gavaged with physiological saline for the non-challenged group. This resulted in a 2 × 2 factorial arrangement of treatments with 2 levels of COS at 0 or 1 g/kg and 2 coccidia challenge status (challenged or non-challenged) with 8 replicate cages of 8 birds per cage.

3.3.2.3. Coccidia Infection and Sampling

The manufacturer's recommended dose of the coccidia vaccine (Coccivac®-B-52, Merck Animal Health) for vaccinating a d-old broiler chick was estimated to be 25 doses/kg BW. Thus, we calculated the approximate recommended vaccination dose for birds at 608 g to be 15 doses (15×) per bird. However, in order to challenge the birds, we doubled the recommended vaccination dose which resulted to 30 doses (30×) per bird. Therefore, on d 18 post hatch, birds in the challenged group were orally gavaged with 1 mL of coccidia vaccine of 30 doses (30×) per bird while birds in the non-challenged group were gavaged with 1 mL of physiological saline. The vaccine was a live oocyst, isolated from chickens, and prepared from anticoccidia-sensitive strains of *E. acervulina, E. maxima, E. maxima MF, E. mivati, and E. tenella.* On d 24 post hatch, growth performance parameters were measured to evaluate BW, BW gain, feed intake (FI) and gain to feed ratio. Average FI and BW gain were corrected for mortality. In addition, all birds were euthanized (CO_2 asphyxiation) except 1 bird/cage (median weight bird) which was euthanized (CO_2 asphyxiation) separately, to prevent additional stressor, for blood collection, tissue sampling, and mucosal scrapping from the jejunum. Ileal digesta samples were collected from the remaining birds and pooled within cage for the determination of ileal digestibility of nutrients.

3.3.2.4. Chemical Analysis of Diet and Ileal digesta

Ileal digesta samples from birds within a cage were pooled for apparent ileal nutrient and energy digestibility measurements. Pooled ileal samples were dried in a forced-air oven for 1 wk at 56°C and the diet and ileal were subsequently ground to a homogenous mixture by passing through a 0.50 mm screen (Retsch ZM 100, GmbH & Co. K.C., Haan, Germany). Dry matter content of the diet and excreta were determined by overnight drying at 105°C. Diet and ileal gross energy was determined by isoperibol bomb calorimetry (Model 1261, Parr Instrument Co., Moline, IL) using benzoic acid as the calibration standard and nitrogen content was determined by the combustion method (Leco Model FP 2000, Leco Corp., St. Joseph, MI) using ethylenediaminete tetra acetic acid as the standard. Furthermore, diets and ileal samples were wet-ashed (nitric/perchloric wet ash, AOAC Method 935.13A) and chromium concentration was determined by using a spectrophotometer (Spectronic 21D, Milton Roy Co., Rochester, NY) according to methods described by Fenton and Fenton (1979). Apparent ileal digestibility (AID) was calculated using the formula:

$$AID, \% = 100 \times [1 - (Cr_i/Cr_o \times N_o/N_i)]$$

where C_i and C_o are concentration of chromium in the diet or ileal, respectively; N_i and N_o are concentration of nutrient or energy in the diet or ileal, respectively.

3.3.2.5. Oocyst Counting

Excreta samples were collected from each cage on d 5, and 6 post coccidia challenge. Briefly, excreta within each cage were pooled together, weighed (≈ 500 mg), passed and stirred through a sieve with 300 mL saturated NaCl solution. The oocysts in the mixture were counted microscopically using McMaster chamber as described as previously by (Levine et al., 1960). The number of oocysts was expressed as oocyst per gram of excreta.

3.3.2.6. Jejunal Morphology

Jejunal segments were collected at the midpoint between the bile duct entry and Meckel's diverticulum. Tissue was fixed in 10% buffered formalin and paraffin-embedded. Following tissue fixation, samples were dehydrated with ethanol and placed in paraffin. Tissue sections (5μm) were prepared and stained with Hematoxylin and Eosin, Alcian Blue and periodic acid-Schiff (AB/PAS) Stain by Purdue Histology and Phenotyping Laboratory. Villi height, crypt depth, and villi height to crypt depth ratio were measured as described in experiment 1. The average of the four villi were used, and density of goblet cells calculated as the number of goblet cells per micrometer of villi height. All measurements were performed under a National binocular light microscope (National Optical and Scientific Instruments, Inc., Schertz, TX).

3.3.2.7. Total RNA Extraction and Reverse Transcription

At d 24 post hatch, the mid jejunum from 1 bird/cage were placed in RNALater (Ambion, Waltham, MA, USA) overnight at 4°C and frozen at -80°C until RNA extraction. Total RNA was extracted from the tissues using TRIzol reagent (Invitrogen, Waltham, MA, USA) following the manufacturer's protocol. RNA concentrations were determined by NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA), and RNA integrity was verified by 1% agarose gel electrophoresis. To eliminate contaminating DNA, extracted RNA was purified with DNA-*free* DNase Treatment and Removal Kit (Ambion, Waltham, MA, USA). Then, 2 mg of total RNA from each sample was reverse transcribed into cDNA using the MMLV reverse transcription system (Promega, Madison, WI, USA), and cDNA was then diluted 1:10 with nuclease-free water (Ambion, Waltham, MA, USA) and stored at -20°C until use.

3.3.2.8. Quantitative Real-time PCR Analysis

Real-time PCR was performed with Bio-Rad iCycler with the Faststart SYBR green-based mix (Life Technologies Waltham, MA, USA). PCR programs for all genes were designed as: 10 min at 95°C; 40 cycles of 95°C for 30 s, primer-specific annealing temperature for 30s, and 72°C for 30s; followed by melting curve analysis. The primer sequences used in this study were listed in Table 3-2. Primer specificity and efficiency (90 to 100%) were verified. Samples were analyzed in duplicate, and a difference lesser than or equal to 5% was acceptable. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with normalization against the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as described in a previous study (Tan et al., 2014).

3.3.2.9. Plasma Collection and Analyses

Blood was collected from one bird per cage via cardiac puncture into tubes containing EDTA. Tubes were placed on ice, centrifuged for 20 min at 1,250 × *g* and 4°C to separate plasma, and stored at -80°C until analysis. Plasma concentrations of interleukin-6, interleukin-10, tumor necrosis factor-alpha, and immunoglobulin-A were measured using a sandwich enzyme linked immunosorbent assay (ELISA) kits (MyBiosource, Inc., San Diego, CA, USA). According to the instructions of the manufacturer (intra assay CV < 8%, inter assay CV < 10%), absorbance was measured at 450 nm. Each blood sample was measured in triplicate according to the instructions of manufacturers.

3.3.2.10. Statistical Analysis

Post-coccidia challenge data were analyzed using the MIXED procedure of SAS as a 2 × 2 factorial with 2 levels of coccidia challenge (unchallenged or challenged) and 2 levels of COS

(0 or 1 g/kg) and evaluated for main effects and interactions. Oocyst count data were analyzed separately for the effect of chitosan on challenged birds, because there was no oocyst detected in the non-challenged group. Statistical significance was declared at a probability of $P \leq 0.05$ and cage was the experimental unit.

3.4 Results and Discussion

3.4.1 Experiment 1

Growth performance of broiler chickens fed graded concentrations of COS from d 1 to 22 post hatch are presented in Table 3-3. Final BW, and BW gain showed a linear increase ($P < 0.05$) and a quadratic response ($P < 0.05$) with increasing concentrations of COS in the diet on d 1 to 22 post hatch. There was a linear decrease ($P < 0.05$) on feed intake and quadratic effect of treatment ($P < 0.05$) on gain: feed ratio on d 1 to 22 post hatch. The breakpoints for BW gain and gain:feed were 1.12 g of COS/kg and 0.52 g of COS/kg of diet at d 22 post hatch, respectively. From previous reports, the effects of COS on growth performance of broiler chickens, pigs or other livestock species have not consistent. Some studies in broiler chickens indicated that dietary COS increased weight gain and gain:feed compared with unsupplemented diets (Suk, 2004; Khambualai et al. 2008, 2009). Tang et al. (2005) also reported that COS could improve the growth performance and feed efficiency of piglets. However, Razdan et al. (1997) observed that dietary supplementation of 30 g/kg chitosan oligosaccharide reduced BW gain and FI in broiler chickens compared with those fed unsupplemented control diets. One possible explanation for these divergences was the varying doses of chitosan oligosaccharide used in feed. Due to the aforementioned details, the current experiment determined the adequate dose by administering different concentrations of COS. Growth performance peaked at around 1 g/kg, and showed signs of impairment at 2.5 g/kg inclusion. It can be assumed that such observation were because of the

amount of COS in diet. However, these results corresponds well with the results of reduced FI and BW in broiler chickens receiving a high dietary COS concentration (30 g/kg; Razdan et al. 1997). These reductions might have been caused by high viscosity and slow motility of COS in the gastrointestinal lumen, stimulating the satiety center of the brain. Therefore, low concentration of dietary COS between 0.5 and 1 g/kg in the present study could increase feed intake, resulting in improved BW gain and gain to feed ratio.

Intestinal morphological measurements of broiler chickens fed graded concentrations of COS are summarized in Table 3-4. Villus height to crypt depth ratio in the jejunum and ileum showed a quadratic response ($P < 0.05$) with increasing concentrations of dietary COS. The break point for jejunal and ileum villus height to crypt depth ratio was a 1.01 g, and 1.08 g of COS/kg of diet, respectively. Xu et al. (2013) reported that COS concentration quadratically increased villus height to crypt depth ratio in all parts of the small intestine. The villus height to crypt depth ratio is a useful tool in the evaluation of nutrient absorption potential and the epithelial cell turnover in the small intestine (de Verdal et al. 2010). The mechanism by which COS improves villus height is still unknown.

Relative weight of the liver and spleen linearly increased ($P < 0.05$) with increasing concentrations of COS in broiler diets (Table 3-4). There were no effects of COS on the pancreas. The liver and spleen are important immune organs of poultry, and the development status of immune organs directly influences the immune function (Fang et al. 2013). The spleen, a crucial non-specific peripheral lymphoid organ, has a dominant role in the generation of immune responses because of the absence of well-developed lymph nodes in most avian species, including the chicken. (Mast and Goddeeris, 1999). Wang et al. (2003) reported that dietary supplementation of 0.1% COS improved the relative weight of spleen and liver in broiler chickens. From the above

observations, it could be speculated that COS might have immune enhancing effects during pathogenic infection. In broilers, giving a low concentration of COS could increase the weight of immune organs (Zhu et al. 2003; Wang et al 2003). In the present study, the relative weight of immune organs increased in all treatments when the dietary level was not exceeding 1.0 g/kg.

Ileal digestibility of DM, energy, and ileal digestible energy showed a quadratic response ($P < 0.05$) with increasing concentrations of COS in the diet (Table 3-4). There was a quadratic effect of treatment ($P < 0.05$) on total tract metabolizability of DM and energy. Apparent metabolizable energy (AME) and nitrogen-corrected apparent metabolizable energy (AMEn) showed a quadratic response ($P < 0.05$) with increasing concentration COS in broiler diet. This result is in agreement with previous studies which indicated that dietary supplementation of COS was effective in increasing apparent total tract digestibility of nutrients in pigs and other farm animals (Lim et al. 2006; Liu et al. 2008; Chen et al. 2009). On the contrary, some authors did not observe any effects of COS on nutrient digestibility (Razdan and Pettersson, 1994, 1996; O'Shea et al. 2011). Inconsistent results on nutrients digestibility might be due to the different molecular weights of COS used in diets. Dietary inclusion rate of COS higher than 1 g/kg did not show any further improvements in the ileal digestibility of DM and energy. The results suggested that an increase in the digestion and absorption of nutrients might be responsible for the enhanced growth performance of broilers in response to dietary COS supplementation. However, further studies are required to identify the exact mechanism of action of COS that underlies these observations.

3.4.2 Experiment 2

Enhancing the ability of broiler chickens to resist diseases without the use of antibiotics is a crucial strategy to benefit the animal's health, and production efficiency. Chitosan oligosaccharide is a positively charged oligosaccharide that is easily obtained by hydrolysis of

chitosan. Compared with chitosan, COS has a good water solubility and low viscosity that enables it to be easily absorbed through the intestine and to quickly enter the bloodstream (Jeon et al., 2000; Chae et al., 2005). Chitosan oligosaccharide has been shown to possess multiple biological activities, such as antimicrobial (Zhang et al., 2003), and immune enhancing effects (Mori et al., 1998).

The results of the current experiment are in close agreement with previous observations showing that the performance of broiler chickens is impaired by CC (Lee et al., 2010; Allenz and Fetterer, 2002). The consistent challenge model applied created an acute or subclinical form of coccidiosis, without causing severe mortality. In other coccidia challenge trials, the concentration of the coccidia vaccine was determined based on the BW of birds relative to the manufacturer's recommendations. In order to induce acute or subclinical forms of coccidiosis, we doubled the manufacturer's recommendation for vaccination based on the BW. The procedure has been consistent with previous broiler chicken trials conducted by our laboratory where BW gain was reduced between 25 and 35% with CC.

The effects of coccidia challenge and COS supplementation on BW, BW gain, FI, and gain:feed are summarized in Table 3-5. Coccidia challenge decreased ($P < 0.05$) BW, BW gain, and FI while COS supplementation increased ($P < 0.05$) BW, BW gain, and FI. Chitosan oligosaccharide supplementation lessened the coccidia challenge-induced effect ($P < 0.05$) on gain to feed. Most common effects of coccidiosis in poultry are reduction in BW gain, diminished FI, and a concomitant adverse effect on gain:feed ratio (Cook, 1998; McDougald, 2003). In the current study, broiler chicks subjected to coccidia challenge benefited from the dietary provision with COS supplements in terms of performance enhancement. Birds in the control group fed 0 g/kg COS and challenged with coccidia vaccine had a 28.6% decrease in BW gain as compared with the non-

challenged group fed the same diet. Whereas birds fed 1 g/kg COS and challenged with coccidia vaccine had 13.1% decrease in BW gain. Comparing the reduction in gain:feed ratio as a result of CC, there was 7% decrease in birds fed diet supplemented with 1 g/kg COS. In a more severe challenge, Parker et al. (2007) reported reductions in average feed intake and weight gain of all treatments infected with mixed *Eimeria* spp by 21 and 45%, respectively, whereas the feed conversion ratio was increased by 43% compared with the average of the unchallenged control. In the current study, broilers fed diets with COS supplementation displayed a growth-promoting effect and improved feed efficiency. This agreed with findings from most studies showing that the use of COS has a positive effect on growth performance of broilers (Li et al., 2007). The improvements in BW, BW gain, FI, and gain:feed of broilers fed COS supplement were probably due to the increased feed consumption, or the improved nutrient digestibility (Huang et al., 2005). The reduced nutrient digestibility and increased immune costs associated with coccidia infection may be responsible for the reduced broiler performance. (Williams, 2005).

Birds in the CC group fed diet supplemented with COS had reduced ($P < 0.05$) oocyst count compared with those not supplemented with COS. Birds in the non-challenged group excreted no oocysts. Chitosan oligosaccharide supplementation reduced the oocyst count by 53.5% compared with the challenged group. Oocyst shedding have been shown to be a useful way to determine the level of coccidia infection (Du et al., 2005; Talebi and Mulcahy, 2005). Historically, the severity of experimental coccidia infection in chickens have been assessed by loss of BW gain, presence of intestinal lesion, and excreta oocyst (Lee et al., 2010). These parameters reflect host immunity status in avian coccidiosis (Lillehoj et al., 2007). Reduced loss of BW gain, and decreased excreta oocyst shedding by COS supplementation in coccidia challenge could be attributed to improved host immune response in the current study. A reduction in oocysts shed in

the excreta indicates improved resistance of broiler chickens to *Eimeria* species infection. This implied that COS supplementation impaired intracellular development and replication of *Eimeria* species in the intestinal epithelium of broiler chickens. This could be due to the ability of COS to penetrate and adhere to the intestinal mucosa and its ability to induce goblet cell hyperplasia (Thongborisute et al., 2006). The exact mechanism of the antimicrobial action of COS and its derivatives is still not completely elucidated, but different mechanisms have been proposed. It has been suggested that a positive charge on the NH_3^+ group of the glucosamine monomer allows interactions with negatively charged microbial cell membranes that lead to the leakage of intracellular constituents (Papineau et al., 1991; Sudarshan et al., 1992; Helander et al., 2001). Chitosan oligosaccharide supplementation decreased the excreta coccidia oocyst count at 6 d post-coccidia challenge and thus accelerated the clearance of coccidia.

Dietary COS supplementation increased ($P < 0.05$) villi height and villi height to crypt depth ratio, whereas CC decreased ($P < 0.05$) the villi height and villi height:crypt depth ratio in the mid-jejunum (Table 3-5). Dietary COS also mitigated the coccidia challenge effect ($P < 0.05$) on crypt depth and goblet cell density in the mid-jejunum. Intestinal morphology, including villus height and crypt depth, as well as villus height to crypt depth ratio, are important indices for intestinal health, recovery and function. Birds in the challenged group exhibited damaged gut morphological structure, suggesting that coccidia challenge exerted a harmful effect on gut health. However, broilers fed COS-supplemented diet showed improved gut morphology in the mid-jejunum, regardless of the coccidia challenge, indicating that supplementing COS exerted a positive role in controlling coccidia infection. Very little data have been published on the effects of COS on gut morphology during coccidia challenge, the exact mechanism for the improvement in villus height and villus:crypt depth ratio is unclear. Low molecular weight COS may enhance

intestinal morphology through cell proliferation as shown in mice (Torzsas et al., 1996). The small intestine is the main site for digestion and absorption of nutrients, and the intestinal mucosa plays an important role in these processes. *Eimeria* challenge can result in relatively quick changes in the intestinal mucosa morphological structure, which can lead to a reduction in villus height and an increase in crypt depth (Allenz and Fetterer, 2002). Abnormal intestinal morphological structure is usually associated with retarded growth during coccidia challenge. A shortening of the villus decreases the surface area for nutrient absorption, which leads to poor nutrient absorption and reduced performance (Xu et al., 2003). The crypt is the area where stem cells divide to permit the renewal of the villus, and a large crypt indicates fast tissue turnover and a high demand for new tissue (Hu et al., 2012). The ratio of villus height to crypt depth is a useful criterion for estimating the digestive and absorptive capacity in the small intestine (Montagne et al., 2003). In agreement with the improved performance in coccidia-challenged broiler chickens, dietary COS improved intestinal morphological structure, as indicated by the increased small intestinal villus height and the ratio of villus height to crypt depth, and the decreased small intestinal crypt depth of broiler chickens that were challenged in the current study. Similar results were reported by other investigators (Mrales-lopez et al; 2009; Cox et al., 2010). It is well known that pathogenic germs such as coliforms can destroy the normal morphology of small intestinal mucosa. Previous study indicated that dietary COS could inhibit the proliferation of *E. coli* in the intestine, and improve gut micro-ecology (Xu et al., 2012). Furthermore, COS provided a beneficial environment for the proliferation of enterocytes, preventing intestinal atrophy (Han et al., 2012). These studies showed that COS is an effective polysaccharide in ameliorating intestinal structure and function, which may be one of the reasons for the increased growth performance in the coccidia-challenged birds fed COS. This result suggests that the epithelial cells might be activated to a greater extent in the

COS group than in the control groups. The goblet cells synthesize and secrete mucus that cover's the gastrointestinal epithelium, forming a boundary between the luminal contents and mucosa (Deplancke et al., 2001). Furthermore, goblet cells have a pivotal role in regulating the localization of commensal bacteria in the intestinal lumen and in maintaining the homeostasis of the gut microenvironment by producing mucins (Goto et al., 2012). This study demonstrated that COS supplementation could increase intestinal epithelium and goblet cells. However, the mechanism by which COS interacts with intraepithelial cells and leads into immunological response is still unclear.

The effect of coccidia challenge and COS supplementation on ileal digestibility of DM, energy and ileal digestible energy are summarized in Table 3-5. Chitosan oligosaccharide supplementation ameliorated the *CC* effect ($P < 0.05$) on ileal digestibility of dry matter, energy and ileal digestible energy. During coccidiosis, sporozoites infect the cells of the intestinal lining causing tissue damage and trauma to the intestinal mucosa and submucosa (Perez-Carbajal et al., 2010). Furthermore, CC negatively influences the morphology of the intestine, especially the shortening of villi (Kettunen et al., 2001) and reduces digestive enzymes (Williams, 2005). The overall effect is reduction in the digestion and absorption of nutrients.

The jejunal mucosa gene expression of cytokines are presented in Table 3-6. Dietary COS supplementation increased ($P < 0.05$) mRNA expression of mRNA expression of zonula occludens-1 (ZO-1), zonula occludens-2 (ZO-2), claudin-1 and occluding, and reduced the mRNA expression of tumor necrosis factor-alpha (TNF-α), Interferon-gamma (IFN-γ), and toll-like receptor-4 (TLR-4) in coccidia challenged birds. The mRNA expression of IL-6, IL-10, and IL-1β was increased ($P < 0.05$), and IL-8 tended to be increased ($P < 0.10$) among CC birds. Overall,

dietary COS supplementation resulted in improved intestinal barrier function (tight junctions) and a mitigation of inflammation associated with *Eimeria* challenge.

Pro-inflammatory cytokine response to immunological challenge is an important criterion reflecting the extent of cellular immunity (Johnson, 1997). Pro-inflammatory cytokines or chemokine such as IL-1β, IL-8, and IL-6 were produced in response to experimental *Eimeria* infections (Hong et al., 2006; Lee et al., 2011). Specific cytokines such as IL-4 and IL-10 help in the down regulation of infection and inflammation whereas some cytokines, such as IFN-γ, IL-1β, and IL-6 are involved in the protection against coccidia infection by stimulating the hormonal and cellular immunity (Dalloul et al., 2007). The current study also showed that coccidia challenge increased key pro-inflammatory cytokines, such as IFN-γ and IL-6, in the jejunum, whereas COS supplementation significantly reduced IFN-γ.

Interferon-γ is a pro-inflammatory cytokine that is usually regulated after coccidia infection in broiler chickens (Dimier-Poisson et al.,1999). Interferon-γ is also important for the clearance of *Eimeria* is broiler chickens (Yun et al., 2000). The current findings suggest that down regulation of IFN-γ gene in the gut associated lymphoid tissue were involved in the COS reduction of *Eimeria spp* colonization in the gut. It may be also possible that COS prevented or decreased the cytokine producing immune system cell movement following coccidia challenge in the tissue, which resulted in the reduction of cytokine gene expression in broiler chickens fed dietary COS. Therefore, the results relating to IFN-γ in the current study suggest that COS potentially influence immune gene expression to regulate inflammatory responses in broiler chickens.

IL-6 is produced from monocytes and macrophages and serves as both pro-inflammatory and anti- inflammatory cytokine (Waititu et al., 2014). It is indicative of the initiation of an acute phase response occurring in avian cells in response to *Eimeria* invasion ((Lynagh et al., 2000). The

increased expression of IL-6 due to CC has been observed in several studies (Withanage et al., 2005; Fasina et al., 2008). Higher expression of IL-6 may be associated with strong pro-inflammatory immune responses. Dietary COS in the current study potentially amplified the acute phase response against *Eimeria spp* for the rapid inhibition of colonization.

Toll-like receptor (TLR) plays a crucial role in the defense against pathogens by activating the transcription factor NF-kB signaling pathway and induction of inflammatory cytokines such as TNF-α, IL-6, IL1β, and IFN-γ. Coccidia challenge increased proinflammatory cytokine IFN- γ mRNA levels compared with the non-challenged control, suggesting coccidia challenge induced and intestinal T-helper cell-1 inflammatory response. However, birds fed COS displayed a decrease in the mRNA expression of TLR4, in the intestines of broiler chickens compared with non-supplemented controls, showing that COS has the potential to suppress intestinal inflammation or not induce immune inflammatory responses or activate TLR-mediated signal pathway responses in the intestine. Furthermore, previous studies have showed that CC reduced intestinal inflammation in mice by downregulating expression of intestinal cytokines (Abdel-Latif et al., 2016). However, it has been also reported that due to the augmentation of TLR-4 signaling, there can be more regulation of local mucosal cell mediated immunity and promotion of gut barrier integrity (Gao et al., 2008; Ng et al., 2009).

Interleukin-10 is an anti-inflammatory and immune-regulatory cytokine that is involved in B-cell activation and antibody production (Saraiva and O'Garra, 2010). In the current trial, birds with CC showed upregulation of the IL-10 expression (Cyktor and Turner, 2011). Chitosan oligosaccharide supplementation showed potential anti-inflammatory effects and may have positive effects on B-cell activation and antibody production against coccidia infection.

Tight junctions are an essential component of intestinal mucosal barriers, providing for paracellular permeability (Ballard et al., 1995; Kucharzik et al., 2001). They comprise several unique proteins, such as claudin, occludin, and ZO-1. In the current study, relative abundances of jejunal transcripts of ZO-1, claudin-1, and occludin, increased in the birds fed diet supplemented with COS. A higher expression of claudin-1 within the tight junction was observed in the challenge group fed COS, compared with broiler chickens in the control group. The mechanism by which tight junctions are re-established by COS is known to involve the translocation of transmembrane protein JAM-1 (a transmembrane tight junction protein). This may act via its positive charge binding with negative charge in nucleic acid within the microbial cells. This inhibits the generation of harmful microorganisms such as protozoa parasites, *E. coli* and bacterial toxins, and may enhance claudin-1 and occludin protein expression and improve intestinal connectivity. Dietary COS recovered the alterations in intestinal structures or morphometric changes in CC birds by downregulating inflammatory responses by upregulating occludin protein expression intestinal tissue. *Eimeria* species has been reported to destabilize and dissociate claudin-1, occludin, and ZO-1 tight junction complexes (Suzuki, 2013), and increased tight junction protein expression in birds fed diets supplemented with COS concurs with the decreased oocyst populations.

Dietary COS supplementation attenuated the coccidia challenge-induced effect ($P < 0.05$) on the expression of plasma IL-6, IL-10, TNF-α, and IgA (Table 3-6). Antibodies are hallmark of host immune response to *Eimeria* parasites but are not involved in protection against coccidiosis (Lillehoj and Lillehoj, 2000). Amino groups of COS could be recognized by the immune system, then stimulate immune cells to proliferate and differentiate, and to release immunoglobulins (Tokura et al. 1999).

In conclusion, dietary chitosan improved growth performance, nutrient digestibility, and jejunal morphology. Between 0.50 and 1.18 g COS/kg diet was optimum for BW gain, jejunal villus height, and villus height to crypt depth ratio. Additionally, COS reduced the negative effects of coccidia challenge by improving gain to feed ratio, nutrient digestibility, inflammatory cytokine production, decreased crypt depth, and oocyst shedding post-coccidia challenge. Thus, COS may be a useful feed ingredient to combat coccidiosis in broiler chickens.

3.5 References

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Table 3.1 Ingredients and nutrient composition of experimental diets used in Exp. 1 and 2, g/kg as-fed basis.

Ingredients	
Corn	537.1
Soybean meal (47% CP)	326.2
Corn DDGS	30.0
Soybean oil	35.0
Monocalcium phosphate[1]	17.0
Limestone (38% Ca)	15.5
Salt	4.0
Vitamin-mineral premix[2]	3.0
DL-Methionine	3.5
L-Lysine.HCl	3.0
Threonine	0.7
Chromic oxide premix[3]	25
COS premix[4,5,6]	0
Total	1,000
Calculated Nutrients & Energy content	
CP, g/kg	220
ME, kcal/kg	3060
Ca, g/kg	9.6
P, g/kg	7.3
Non-phytate P, g/kg	4.7
Ca:Tp	1.3

[1] Contained 16% Ca, 21% P.

[2] Supplied the following per kilogram of diet: vitamin A, 5,484 IU; vitamin D_3, 2,643 IU; vitamin E, 11 IU; menadione sodium bisulfite, 4.38 mg; riboflavin, 5.49 mg; D-pantothenic acid, 11 mg; niacin, 44.1 mg; choline chloride, 771 mg; vitamin B_{12}, 13.2 μg; biotin, 55.2 μg; thiamine mononitrate, 2.2 mg; folic acid, 990 μg; pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 μg.

[3] Prepared as 1 g of chromic oxide added to 4 g of ground corn.

[4] 1 g of chitosan oligosaccharide (COS), (Qingdao Yunzhouo Biochemistry Co. Ltd., Jimo, Qingdao, China) added to 4 g of corn.

[5] In Exp. 1, chitosan oligosaccharide premix was added at the expense of corn to supply 0, 0.5, 1.0, 1.5, 2.0 or 2.5 g COS/kg diet.

[6] In Exp. 2, chitosan oligosaccharide premix was added at the expense of corn to supply 0, or 1.0 g COS/kg diet.

Table 3.2 Primers used in real –time quantitative PCR.

Genes	Primer sequence (5'- 3')	Gene Bank ID	Ref
Housekeeping gene			
GAPDH	F: TCCTAGGATACACAGAGGACCA R: CGGTTGCTATATCCAAACTCA	ENSGALG00000014442*	Grenier et al.,
Markers of			
IL-1β	F: GCATCAAGGGCTACAAGCTC R: CAGGCGGTAGAAGATGAAGC	NM_204524	Adedokun et al.,
IL-6	F: CTGCGAGAACAGCATGGAGA R: GAAAGGTGAAAAGCCCGCTG	XM_01,310,0522	Bai et al., 2018
IL-8	F: GCGGCCCCCACTGCAAGAAT R:	ENSGALG00000011670*	Grenier et al.,
IL-10	F: GCTGAGGGTGAAGTTTGAGG R: AGACTGGCAGCCAAAGGTC	ENSGALG00000000892*	Grenier et al.,
IFN-γ	F: GCATCTCCTCTGAGACTGGC R: GCTCTCGGTGTGACCTTTGT	NM205149	Adhhikari et al.,
TNF-α	F: AGATGGGAAGGGAATGAACC R: ACTGGGCGGTCATAGAACAG	AY765397	Adedokun et al.,
TLR-4	F: GTTCCTGCTGAAATCCCAAA R: TATGGATGTGGCACCTTGAA	NM_001030693	Adedokun et al.,
Markers of gut			
Claudin-1	F: TGGAGGATGACCAGGTGAAGA R: CGAGCCACTCTGTTGCCATA	NM_001013611.2	Shao et al., 2013
Occludin	F: GATGGACAGCATCAACGACC R: CATGCGCTTGATGTGGAAGA	NM_205128.1	Wu et al., 2018
ZO-1	F: GCCTGAATCAAACCCAGCAA R: GCCTGAATCAAACCCAGCAA	XM_015278980.1	Wu et al., 2018
ZO-2	F: ATCCAAGAAGGCACCTCAGC R: CATCCTCCCGAACAATGCCT	NM_2040918	
Muc-2	F: GCTACAGGATCTGCCTTTGC R: AATGGGCCCTCTGAGTTTTT	XM_421035	Adedokun et al.,

F stands for forward primer; R stands for reverse primer; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IL = interleukin; TGF-β = transforming growth factor-β; TLR4 = toll-like receptor 4; TNF-α = tumor necrosis factor alpha; MUC2 = mucin 2; ZO-1 = zonula occludens 1; ZO-2= zonula occludens 2; IFN-γ =Interferon gamma.
*Sequence obtained from Ensembl chicken genome data resources.

Table 3.3 Growth performance of broiler chickens fed graded concentrations of chitosan oligosaccharide (COS) from d 1 to 22 post- hatching[1].

Item	COS, g/kg						SEM	*P*-value		
	0.0	0.5	1.0	1.5	2.0	2.5		Treatment	Linear	Quadratic
BW										
d 15	479	484	488	471	462	451	11.9	0.07	0.01	0.15
d 22	899	919	950	916	901	865	15.3	<0.01	0.02	<0.01
BW Gain, g/bird										
d 1 to 15	434	440	443	425	417	407	11.2	0.07	0.01	0.15
d 15 to 22	419	435	462	446	439	414	9.0	0.01	0.67	<0.01
d 1 to 22	854	874	905	871	856	820	14.6	<0.01	0.02	<0.01
Feed intake, g/bird										
d 1 to 15	571	578	569	561	553	520	15.2	0.02	0.002	0.08
d 15 to 22	622	606	616	619	618	611	18.8	0.98	0.89	0.99
d 1 to 22	1193	1184	1185	1181	1171	1130	25.9	0.30	0.04	0.28
Gain/feed, g/kg										
d 1 to 15	760	762	781	759	757	787	17.5	0.74	0.51	0.72
d 15 to 22	676	723	761	720	711	684	25.6	0.20	0.86	0.02
d 1 to 22	716	740	767	738	731	728	13.2	0.11	0.99	0.02

[1] Data are least squares means of 8 replicate cages of 8 birds per cage.

Table 3.4 Ileal and total tract nutrient digestibility of broiler chickens fed graded concentrations of chitosan oligosaccharide (COS) from d 1 to 22 post hatching[1,2].

Item	COS, g/kg						SEM	*P*-value		
	0.0	0.5	1.0	1.5	2.0	2.5		Treatment	Linear	Quadratic
Jejunum										
VH, μm^2	1229	1316	1442	1299	1240	1134	83.11	0.20	0.23	0.03
CD, μm^2	167	152	131	158	204	239	27.5	0.01	<0.01	0.26
VH:CD	7.6	8.9	11.4	8.3	7.0	5.5	0.77	<0.01	<0.01	<0.01
Ileum										
VH, µm	858	903	957	874	836	811	42.5	0.21	0.15	0.08
CD, µm	165	145	128	145	163	171	8.7	0.70	0.10	0.79
VH:CD	5.4	6.4	7.5	6.1	5.4	4.9	0.45	<0.01	0.06	<0.01
Immune organ weight, g/kg BW										
Liver	26.8	28.1	28.6	30.2	32	30.2	1.09	0.31	0.03	0.41
Pancreas	1.9	1.7	2.2	2.2	2.1	2.2	0.28	0.33	0.24	0.46
Spleen	1.03	1.05	1.07	1.08	1.09	1.13	0.02	<0.01	<0.01	0.81
Ileal digestibility										
DM, %	68.9	71.2	73.2	71.1	69.6	67.8	0.71	<0.01	<0.03	<0.01
Energy, %	72.4	75.1	78.2	74.8	73.3	71.7	0.68	<0.01	0.02	<0.01
IDE, kcal/kg	3,270	3,554	3,602	3,454	3,337	3,276	30	<0.01	<0.01	<0.01
Total tract metabolizability										
DM, %	72.6	73.1	74.8	73.4	73.1	72.6	0.46	0.01	0.68	<0.01
Energy, %	76.3	77.3	79.9	77.5	76.7	76.4	0.32	<0.01	0.15	<0.01
AME, kcal/kg	3,444	3,661	3,679	3,581	3,491	3,490	15	<0.01	<0.01	<0.01
AMEn, kcal/kg	3,217	3,427	3,439	3,341	3,265	3,262	14	<0.01	<0.01	<0.01

[1] Data are least squares means of 8 replicate cages of 8 birds per cage.
[2] IDE = Ileal digestible energy; VH = Villus height; CD = Crypt depth.

Table 3.5 Growth performance, ileal digestibility, jejunal morphology, and excreta oocyst count of broiler chickens fed diets containing chitosan oligosaccharide (COS) concentration at 0 or 1g/kg with or without coccidia challenge from d 17 to 24 post hatching[1,2].

	0, g/kg COS		1 g/kg COS			*P*-value		
Item	Non-	Challeng	Non-	Challenge	SEM	COS	Challen	COS ×
d 17 to 24								
d 17 BW, g/bird	608	608	608	608	24.8	0.95	0.99	0.95
d 24 BW, g/bird	1013	897	1052	993	27.1	<0.01	<0.01	0.22
BW gain, g/bird	405	289	444	386	25.6	<0.01	<0.01	0.21
Feed intake,	624	553	645	606	22.1	0.04	<0.01	0.36
Gain: Feed, g/kg	647	524	686	637	34.1	<0.01	<0.01	0.01
Oocyst /g	ND	119	ND	55	2.6	<0.01	-	-
Jejunal								
VH, µm	1027	662	1281	913	18.4	<0.01	<0.01	0.92
CD, µm	168	389	126	161	4.8	<0.01	<0.01	<0.01
VH/CD	6.16	1.7	10.2	5.7	0.17	<0.01	<0.01	0.97
GC, cells/µm of	0.14	0.28	0.18	0.21	0.02	<0.01	0.01	0.01
Ileal digestibility								
DM, %	69.26	59.95	78.63	71.29	0.29	<0.01	<0.01	<0.01
Energy, %	72.38	59.25	76.62	66.99	0.36	<0.01	<0.01	<0.01
IDE, kcal/kg	3,299	2,478	3,338	2,807	17	<0.01	<0.01	<0.01

[1] On d 0 post hatching, birds were divided into two groups with 16 replicates each, and were fed control diet and COS supplemented diets, each with an initial body weight of 43 g/bird. The final BW on d 17 post hatch was 570 and 610 g/bird for birds fed control diet and COS supplemented diet respectively. The feed intake was 781 and 738 g/bird for birds fed control and COS. The feed efficiency was 674 g/kg and 770 g/kg for birds fed control diet and COS supplemented diets respectively.

[2] Data are least squares means of 8 replicate cages with 8 birds per cage; ND = not detected.

[3] Each bird in challenged or non-challenged group was orally gavaged with 30× of coccidia vaccine, which is twice the manufacturer's recommended dose of 25× /kg BW or distilled water respectively on d 18 and sampled on d 24 post hatching.

[4] *P*-value according to main effects of COS, challenge, and a COS × challenge interaction.

[5] VH= Villus height; CD = Crypt depth; GC = Goblet cell; IDE = Ileal digestible energy.

Table 3.6 Relative gene expression † of cytokines and tight junction proteins in jejunal mucosa and plasma cytokines and antibody of broiler chickens fed diets containing chitosan oligosaccharide (COS) concentration at 0 or 1 g/kg with or without coccidia challenge at d 24 post hatching[1,2].

	0, g/kg COS		1 g/kg COS			*P*-value[3]		
Item	Non-	Challenge	Non-	Challenge	SEM	COS	Challenge	COS ×
Jeiunal mucosa								
IL1β	1.00	3.87	1.35	2.08	0.35	0.05	<0.01	<0.01
IL-6	1.00	4.52	0.74	1.78	1.07	0.17	0.04	0.25
IL-8	1.00	3.76	1.18	1.94	0.98	0.41	0.09	0.31
IL-10	1.00	4.76	1.23	2.96	1.68	0.29	0.03	0.24
Claudin-1	1.00	0.72	6.45	4.29	0.82	<0.01	<0.01	0.01
Occludin	1.00	0.27	5.20	1.13	0.48	<0.01	<0.01	<0.01
ZO-1	1.00	0.35	3.02	1.35	0.31	<0.01	<0.01	0.12
ZO-2	1.00	0.21	3.30	1.09	0.52	<0.01	0.01	0.16
TNF-α	1.00	4.97	1.59	1.85	0.69	0.08	0.01	0.01
TLR-4	1.00	6.22	1.18	1.63	1.03	0.04	0.01	0.03
MUC2	1.00	2.15	0.88	1.21	0.30	0.09	0.02	0.19
IFN-γ	1.00	7.72	1.24	2.12	1.21	0.03	0.01	0.03
Plasma								
IL-6,	22.86	88.61	31.38	64.61	1.53	<0.01	<0.01	<0.01
IL-10,	15.06	158.35	55.54	108.74	1.62	0.01	<0.01	<0.01
TNF-α,	22.48	867.03	150.38	476.8	11.4	<0.01	<0.01	<0.01
IgA, ng/mL	0.91	16.44	5.51	10.64	0.28	0.02	<0.01	<0.01

[1] Data were least squares means of 8 replicate cages with 8 birds per cage.

[2] Each bird in challenged or non-challenged group was orally gavaged with 30× of coccidia vaccine, which is twice the manufacturer's recommended dose of 25× /kg BW or distilled water respectively on d 18 and sampled on d 24 post hatching.

[3] *P*-value according to main effects of COS, challenge, and a COS × challenge interaction.

†Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated with GAPDH as the endogenous control.

[4] IL = interleukin; IFN-γ = Interferon-gamma; TLR4 = toll-like receptor 4; TNF-α = tumor necrosis factor α; MUC2 = mucin 2; ZO-1 = zonula occludens-1; ZO-2= zonula occludens-2; IgA = Immunoglobulin A

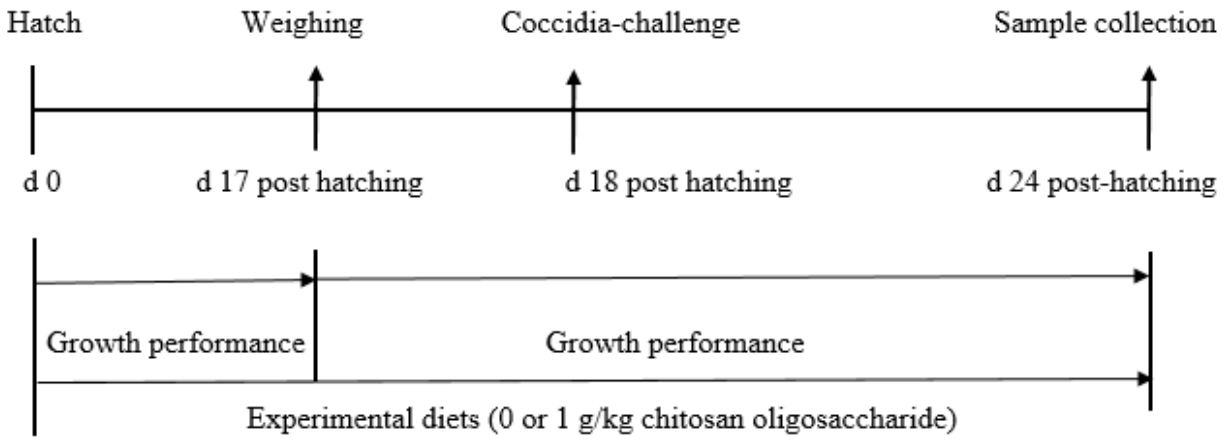

Figure 3.1 Diagram of experimental procedure. Broiler chickens were fed basal diet supplemented with 0 or 1 g/kg COS from d 0 to 24 post hatching. They were weighed on d 17 and challenged with coccidia vaccine on d 18 post hatching. Growth performance was measured for d 0-17, and 17-24 post hatching. Ileal digesta and intestinal tissues were collected on d 24 post hatching.

CHAPTER 4. CHITOSAN OLIGOSACCHARIDE SUPPLEMENTATION ALLEVIATES STRESS STIMULATED BY IN-FEED DEXAMETHASONE IN BROILER CHICKENS

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4.1 Abstract

This experiment was conducted to investigate the effect of dietary chitosan oligosaccharide (COS) on growth performance, nutrient digestibility, jejunal morphology, gene expression, and plasma antioxidant enzymes in male broiler chickens under experimentally induced stress via in-feed dexamethasone (DEX). On d 3 post hatching, male broiler chicks were assigned to 2 diets supplemented with COS at 0 or 1 g/kg in a randomized complete block design and fed to d 27 post hatching. Birds were pooled within each diet (0 or 1 g/kg COS) to equalize the average BW and fed 2 diets supplemented with 0 or 1 g/kg DEX, within each dietary COS, from d 20 to 27 post hatching. This resulted in a 2 × 2 factorial arrangement of treatments with 2 levels each of COS and DEX, 8 replicate cages and 7 birds per cage. On d 27 post hatching, birds were weighed and euthanized, and samples were collected. Dietary COS also decreased ($P < 0.05$) DEX-induced effects (interaction; $P < 0.05$) on BW, BW gain, and gain:feed. Dietary COS supplementation attenuated the DEX effects (interaction; $P < 0.05$) on villus height, crypt depth, villus height to crypt depth ratio, and ileal digestibility of DM and energy. The DEX-induced effect of relative mRNA expression of jejunal mucosa IL-6, IL-10, and claudin-1 was reduced by dietary COS supplementation (interaction; $P < 0.05$). Responses (interaction; $P < 0.05$) in the activity of plasma superoxide dismutase, catalase, and glutathione peroxidase to COS and DEX were similar to that observed with the relative mRNA expression. Chitosan oligosaccharide supplementation increased

($P < 0.05$) the mRNA expression of IL-8 and occludin. In conclusion, dietary COS decreased the DEX-induced effect by improving growth performance, nutrient digestibility, jejunal morphology, gene expression, and plasma antioxidant enzymes in broiler chickens. These implies that dietary COS may be useful for ameliorating the negative effect of stress on gut health in broiler chickens.

Key words: Broiler chickens, chitosan oligosaccharide, dexamethasone, gene expression, stress

4.2 Introduction

A multitude of stressors exists in the rearing of broiler chickens including immunological challenge, oxidative stress, and transportation. Stress has many detrimental effects on broiler chickens; for example, it reduces growth performance, increases susceptibility to disease, and impairs immune function (Lin et al., 2006). It has been reported that the hypothalamus- pituitary-adrenal axis is activated (Siegel, 1980) when an animal is under stress thus, there is secretion of glucocorticoid from the adrenal gland. A plethora of glucocorticoids is a hallmark of stress (Chrousos and Gold, 1992).

Dexamethasone (DEX) is a synthetic glucocorticoid used as an immunosuppressive agent. It has been used to induce oxidative stress and to investigate the stress responses in poultry species (Gao et al., 2010; Njagi et al., 2012). Dietary antioxidants could have positive effects and benefits in preventing oxidative stress and relieving the immunosuppression induced by DEX. In previous studies, glucocorticoids have proven to be involved in the alteration of redox balance in poultry (Lin et al., 2004; Mujahid et al., 2006), as well as modulating immune function and enteric mucosal integrity (Lin et al., 2000).

In the past, oxidative stress and immune suppression were controlled by using antibiotic growth promoters in broiler chickens (Williams et al., 2001). Currently, specific dietary

supplementation can offer a viable and practical alternative to antibiotics that alleviate these stressors in broiler chickens. Previous studies on poultry and swine have found that chitosan oligosaccharide (COS) improves growth performance (Huang, 2005), enhances immune function (Xiao et al., 2013), and increases antioxidant properties (Niu et al., 2013).

In the current experiment, DEX was administered to broiler chickens diet to induce stress. There are limited studies in broiler chickens that evaluate the effect of COS on antioxidant enzymes and immune response. This finding may provide a useful evidence for the application of COS in diets to mitigate immunological stress and improve antioxidant activity in broiler chickens. We therefore hypothesized that dietary COS supplementation will not mitigate the stress-induced effect by in-feed dexamethasone in broiler chickens. Our objective was to investigate the effect of dietary COS supplementation on anti-oxidative function and immune response induced by in-feed DEX supplementation.

4.3 Materials and Methods

The Purdue University Animal Care and Use Committee (West Lafayette, IN) approved the experiment protocol.

4.3.1 Chitosan and Dexamethasone Materials

Chitosan oligosaccharide supplement used in the current experiment was purchased from Qingdao Yunzhou Biochemistry Co. Ltd. (Jimo, Qingdao, China). They were derived from shrimp shells and the degree of deacetylation was greater than 90%. Dexamethasone was purchased from (Alfa Aesar, Maryland, United States). Both materials were in powdered form.

4.3.2 Birds and Diets

A total of two hundred and eighty-eight male broiler chicks (Cobb 500) were used in this study. The birds were maintained in cages in an environmentally controlled room. All birds received a basal diet consisting of antibiotic free corn-soybean based diet meeting the nutritional requirements of poultry recommended by National Research Council (1994). Four diets were formulated by supplementing a portion of the basal diet with two levels of chitosan oligosaccharide (COS) (0 or 1 g/kg) and two levels of dexamethasone (DEX) (0 or 1 mg/kg) Table 4-1.

4.3.3 Experimental Procedure and Design

On d 3 post hatching, 288 birds were individually tagged and weighed. All birds were randomly assigned to 2 experimental diets (basal diet supplemented with COS at 0 or 1 g/kg diet). The optimum dietary concentration of COS for broiler chickens was determined to be 1 g/kg (Osho and Adeola, 2019). There were 16 replicate cages of 0 g COS/kg and 16 replicate cages of 1 g COS/kg diet with 9 birds per cage. On d 20 post hatching, all birds were weighed, and birds were re-randomized within each of 0 or 1 g COS/ kg diet to equalize the average body weight of the birds before administration of in-feed DEX. In each of the 0 or 1 g COS/kg, birds in 8 replicate cages of 7 birds per cage were fed diet supplemented with either 0 or 1 mg DEX/kg diet. This resulted in a 2 × 2 factorial arrangement of treatments with 2 levels of COS at 0 or 1 g/kg and 2 levels of DEX with 8 replicate cages of 7 birds per cage.

4.3.4 Growth Performance and Nutrient Utilization

Individual BW of birds and feed intake were determined at d 20, and 27 post hatching. Final BW, feed intake and feed efficiency for the weigh periods were measured. On d 27 post hatching, all birds were euthanized and ileal digesta was collected from the distal two-thirds of

ileum excised from each bird, contents were gently flushed with distilled, deionized water and pooled within cage (Kluth et al., 2005). The samples were stored at – 20°C before further analyses. Apparent ileal digestibility (AID) of DM, nitrogen, and energy were calculated using the index method as described by (Olukosi et al., 2007)

Apparent ileal digestibility (AID) was calculated using the formula:

$$AID, \% = 100 \times [1 - (Cr_i/Cr_o \times N_o/N_i)]$$

where C_i and C_o were the concentration of chromium in the diet or ileal digesta, respectively while, N_i and N_o were the concentration of nutrient or energy in the diet or ileal digesta respectively.

4.3.5 Chemical Analysis

Pooled ileal digesta samples were dried in a forced-air oven for 1 week at 56 ^{0}C. Diets and dried ileal digesta were finely ground using an electric coffee grinder and thereafter analyzed for dry matter (DM) and gross energy (GE). The concentration of DM in diets and ileal digesta were measured by drying at 105°C for 24 h in a forced-air oven (The Precision Scientific Co., Chicago, IL; method 934.01; AOAC, 2006). Gross energy was determined using an isoperibol bomb calorimeter (Parr 6200, Parr Instruments, Moline, IL) standardized with benzoic acid. Chromic oxide was used as an indigestible marker. Diet and ileal digesta digesta chromium concentrations were determined according to the procedures of Fenton and Fenton, (1979).

4.3.6 Jejunal Morphology and Total RNA Extraction and Reverse Transcription

Jejunum tissue were excised (10-cm mid jejunum) from one bird per cage (median weight bird) on d 27 post hatching and processed as previously described (Osho et al., 2019). The tissues were gently flushed with cold sterile saline solution to remove intestinal contents and immediately placed in 10% neutral buffered formalin. Tissue sections (4 mm) were prepared and stained with

Hematoxylin and Eosin by the Purdue Histology and Phenotyping Laboratory and were examined using a light microscope (National Optical and Scientific Instruments, Inc., Schertz, TX). Villus height and crypt depth were measured from 4 well oriented villi per slide. Villus height was measured as the distance from the tip of the villus to the crypt mouth while crypt depth was measured from the base of the villi to the submucosa. Villus height to crypt depth ratio was calculated by dividing the villus height by crypt depth.

Total RNA was extracted from the jejunum using TRIzol reagent (Invitrogen) following the manufacturer's protocol and processed as previously described (Osho and Adeola. In Press). The concentration of the extracted RNA was determined using a NanoDrop spectrophotometer (ND-1000; NanoDrop Products) at an optical density of 260 nm whereas, RNA purity was verified by measuring absorbance at an optical density of 260/280. From each sample, 2 µg of total RNA were reverse transcribed into cDNA using the MMLV reverse transcription system (Promega), and cDNA was then diluted 1:10 with nuclease-free water (Ambion) and stored at -20°C until use.

4.3.7 Quantitative Real-time PCR Analysis

Expression levels of IL-6, IL-8 IL-10, TNF-α, Muc-2, and Claudin-1 genes were analyzed in the jejunum by quantitative real-time polymerase chain reaction (PCR). It was performed with Bio-Rad iCycler with the Faststart SYBR green-based mix (Life Technologies). The PCR programs for all genes were designed as: 10 min at 95°C; 40 cycles of 95°C for 30 s, primer-specific annealing temperature for 30s, and 72°C for 30s; followed by melting curve analysis. The primer sequences used in this study are listed in Table 4-2. Primer specificity and efficiency (90 to 100%) were verified. Samples were analyzed in duplicate, and a difference lesser than or equal to 5% was acceptable. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and

Schmittgen, 2001) with normalization against the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as described in a previous study (Tan et al., 2014).

4.3.8 Analysis of Plasma Samples

Blood samples (2 mL) were taken via cardiac puncture and kept on ice until plasma was separated by centrifugation for 10 min at 1,500 rpm. Plasma samples were stored at -80°C until assayed. The corresponding assay kits (MyBiosource, Inc., San Diego, CA) for plasma superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) concentration were determined according to the instructions of the manufacturer. Samples were added in duplicate to check intra assay variability. The inter- and intra-assay coefficients of variations were less than 10%.

4.3.9 Statistical Analysis

Post DEX-challenge data were analyzed using the MIXED procedure of SAS as a 2 × 2 factorial consisting of 2 levels of COS (0 or 1 g/kg) and 2 levels of DEX (0 or 1 mg/kg) and evaluated for main effects and interactions. Statistical significance was declared at a probability of $P \leq 0.05$ and cage was the experimental unit.

4.4 Results and Discussion

Broiler chickens are usually subjected to several potentially immunosuppressive stimuli during lifetime (Galha et al., 2008). Dexamethasone is an analog of the glucocorticoid secreted when animals are under stress. Glucocorticoids act by decreasing the population of both B and T lymphocytes, promoting a considerable immune-suppression. However, administration of DEX is satisfactory to mimic stress and provide a way to study the effects of stress conditions on enteric

mucosa integrity. In the current study, broiler chickens were initially visibly depressed, less active, and diarrheic during the immunological stress mimicked by in-feed DEX, in accordance with previous reports (Lin et al., 2004; Gao et al., 2008).

The effect of in-feed DEX and COS supplementation on growth performance are summarized in Table 4-3. In the group of birds fed diet supplemented with 0 g/kg COS, dietary DEX at 1 mg/kg decreased BW gain from 378 to 91 g. However, in birds fed diet supplemented with 1 g/kg COS, dietary DEX at 1 mg/kg decreased BW gain only from 477 to 243 g thus, resulting in a COS × DEX interaction ($P < 0.05$). In-feed DEX decreased ($P < 0.05$) feed intake (FI) whereas COS supplementation increased ($P < 0.05$) FI. Chitosan oligosaccharide supplementation decreased the DEX-induced effect (interaction; $P < 0.05$) on gain to feed ratio. It has been established that decreased feed intake is a primary cause of reduced growth rate in broiler chickens. The result of feed intake agrees with the report from Sapolsky et al., (2000), who observed that DEX reduced appetite. In broiler chickens, one of the most recognizable effect of glucocorticoid treatment on growth performance is a drastic reduction in BW gain (Puvadolpirod and Thaxton, 2000a), which was observed in the current experiment.

It is well known that glucocorticoid plays an important role in reduced anabolic and enhanced catabolic processes (Virden and Kidd, 2009). Perhaps the stressed broiler chickens used up more energy to adapt to the stress condition and less energy for growth. Meanwhile, supplementation with COS relieved the inhibitory effect of in-feed DEX on growth performance of broiler chickens, suggesting a potentially important role for COS to inhibit the adverse effects of immune stress in broiler chickens. This is similar to a previous report (Zhang et al., 2011), which showed that dietary COS supplementation remarkably prevented the decrease in BW gain caused by immune stress.

Dietary COS supplementation attenuated the DEX effects (interaction; $P = 0.02$) on villus height, crypt depth, and villus height to crypt depth ratio in the mid-jejunum (Table 4-3). The structure of intestinal mucosa can reveal some information on the absorptive ability of intestine to nutrients and is always associated with the performance of animals. The ratio of villus height to crypt depth reflects the comprehensive ability for intestinal nutrient absorption and function. The results indicate that glucocorticoids may decrease nutrient absorption, by regulating the nonspecific absorption of jejunum in broilers. Broiler chickens fed COS-supplemented diet showed improved gut morphology in the mid-jejunum, regardless of the DEX treatment, indicating that supplementing COS exerted a positive role in controlling immunological stress. The improvement in the structure of the intestinal mucosa in COS treatment groups might lead to prolific nutrient absorption, increased disease resistance, and decreased diarrhea-producing factors. Few data are available on the effects of COS on gut morphology during DEX challenge. However, low molecular weight COS may enhance intestinal morphology through cell proliferation as shown in mice (Torzsas et al., 1996).

Dietary COS supplementation ameliorated DEX effects (interaction; $P < 0.05$) on ileal digestibility of dry matter and energy (Table 4-3). Previous research has described the physiology of stress in chickens (Siegel, 1985). However, descriptions of digestion and metabolism inherent to stress in chickens are limited. By considering the many effects of immunological stress on metabolism and broiler performance, we speculate that the potential exists for a reduction in the detrimental effects of stress. Puvadolpirod and Thaxton, (2000b) observed that broiler chickens given adrenocorticotropic hormone (ACTH) had significantly lower nutrient digestibility than broilers in the non-stressed control group. These researchers concluded that the reduction in digestion of nutrient was most likely due to an increase in feed passage rate in the presence of the

stressor because birds treated with ACTH displayed polydipsia and polyuria during and after stress. Similarly, in the current study, we observed poor absorption of nutrients, which could be as a result of decreased villus height in birds fed diet supplemented with DEX.

The jejunal mucosa gene expression of cytokines are presented in Table 4-4. Dexamethasone-induced effect on relative mRNA expression of jejunal mucosa on IL-6, IL-10, and claudin-1 were ameliorated by dietary COS supplementation (interaction; $P < 0.05$). Chitosan oligosaccharide decreased the mRNA expression of IL-8, TNF-α, and occludin, whereas DEX decreased the expression of MUC-2. Glucocorticoids inhibit many of the initial events in an inflammatory response that impact both the innate and adaptive immune responses (Busillo et al., 2011). Thus, the primary anti-inflammatory action of glucocorticoids is to repress a plethora of pro-inflammatory genes encoding cytokines and chemokines, to resolve the inflammatory process and restore homeostasis. Interestingly, we found increased mRNA expression of IL-10 in birds that were fed diets supplemented with DEX. Interleukin-10 is known to be an inhibitor of pro-inflammatory cytokines, it is possible that the beneficial effect of DEX is partly results from upregulation of IL-10 producing cells.

Glucocorticoids can inhibit inflammation by abrogating the activity of transcription factors (such as nuclear factor-κB and activator protein-1) that controls the production of pro-inflammatory cytokines by interacting with glucocorticoid-responding elements when bound to glucocorticoid receptors (Dejager et al., 2014). Glucocorticoids affect Toll-like receptor signaling pathways and promote T helper type 2 responses by increasing the production of interleukin 4 (IL-4) and IL-10 (Brunetti et al., 1995; Kwabe et al., 2012). The epithelial tight junction complex includes the proteins claudin, occludin and ZO-1. Some tight junction proteins such as claudin-1 and occludin are important in the assembly and maintenance of tight junctions (Amasheh et al.,

2009). In chronically stressed and repeatedly corticosterone-injected rats, a significant reduction in claudin-1, and occludin protein expression were observed (Zheng et al., 2013). The mechanism underlying down-regulation of these tight junction proteins remains largely unknown. A higher expression of claudin-1 within the tight junction was observed in the challenge group fed COS compared with broiler chickens fed diet without COS. The mechanism of the activity of reversible tight junction opening, induced by COS, is known to be involved in the translocation of transmembrane protein JAM-1 (a transmembrane tight junction protein).

Dietary COS supplementation mitigated the DEX-induced effect ($P < 0.05$) on the activity of plasma superoxide dismutase, catalase, and glutathione peroxidase (Table 4-4). In previous studies by Gao et al., (2010), glucocorticoids have been proven to be involved in the altered redox balance in poultry. The maintenance of redox balance depends on the production and extinguishing of reactive oxygen species. The antioxidative systems, responsible for the quenching of reactive oxygen species, consist of an enzymatic system and non-enzymatic antioxidants. The enzyme defense system consists of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Superoxide dismutase provides the efficient dismutation of superoxide radicals into less toxic hydrogen, while CAT and GPx reduce hydrogen peroxide into oxygen and water (Ahmad *et al*., 2012).

Indeed, COS has shown beneficial effects in relieving oxidative stress induced by certain drugs or under various physiological and pathophysiological conditions (Anandan et al., 2013). Previous studies showed that supplementation of COS in diets resulted in the improvement of serum GPx, SOD and CAT activities in piglets (Li et al., 2013) and serum SOD activity in beef cattle (Li et al., 2015). In agreement with those findings, the current results showed that dietary supplementation with COS increased plasma SOD, GPx, and CAT activities, which are

representative enzymatic antioxidants in broiler chickens. A well-known function of COS is its ability to mitigate stress and improve antioxidant activity (Feng et al. 2008). One possible mechanism that explains this potential could be that COS and its derivatives react with free radicals due to the active hydroxyl and amino groups present on their chains. The hydroxyl and amino group in COS can serve as hydrogen donors to the proxy radicals, and react with unstable free radicals, hence protecting cells from damage (Feng et al. 2008). Indeed, in our study, COS supplementation in diets had pre-protective effects against oxidative stress induced by DEX in broiler chickens, suggesting that COS might contribute to the enhancement of anti-oxidative functions.

In conclusion, the results of our study indicate that stress mimicked by in-feed DEX treatment could significantly increase BW loss, induce oxidative stress, and suppress immune function. However, dietary COS improved growth performance and immune function for broiler chickens, especially in the presence of stress, which can be partially ascribed to the ability of COS to decrease catabolism and oxidative injury of tissues. Inclusion of COS in broiler diet was effective in improving growth performance by improving villus structure, sustaining a balanced intestinal barrier function, and decreasing stress response. Therefore, COS supplementation may be a potential agent to relieve oxidative stress in immuno-suppressed broiler chickens.

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Table 4.1 Ingredients and nutrient composition of experimental diets, g/kg as-fed basis.

Ingredients, g/kg	Control	COS (1 g/kg)
Corn	538.1	528.1
Soybean meal (48.5% CP)	345.2	345.2
Soybean oil	45.0	45.0
Monocalcium phosphate[1]	17.0	17.0
Limestone (38% Ca)	15.5	15.5
Salt	4.0	4.0
Vitamin-mineral premix[2]	3.0	3.0
DL-Methionine	3.5	3.5
L-Lysine.HCl	3.0	3.0
Threonine	0.7	0.7
Chromic oxide premix[3]	25	25
COS premix[4]	0	10
DEX premix[5,6]	0	0
Total	1,000	1,000
Calculated Nutrients & Energy content		
CP, g/kg	220	220
ME, kcal/kg	3115	3112
Ca, g/kg	9.7	9.7
P, g/kg	7.3	7.3
Non-phytate P, g/kg	4.8	4.8
Ca:Tp	1.3	1.3

[1] Contained 16% Ca, 21% P.

[2] Supplied the following per kilogram of diet: vitamin A, 5,484 IU; vitamin D_3, 2,643 IU; vitamin E, 11 IU; menadione sodium bisulfite, 4.38 mg; riboflavin, 5.49 mg; D-pantothenic acid, 11 mg; niacin, 44.1 mg; choline chloride, 771 mg; vitamin B_{12}, 13.2 μg; biotin, 55.2 μg; thiamine mononitrate, 2.2 mg; folic acid, 990 μg; pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1mg; Zn, 44.1 mg; Se, 300 μg.

[3] Prepared as 1 g of chromic oxide added to 4 g of ground corn.

[4] 1 g of chitosan oligosaccharide (COS), (Qingdao Yunzhouo Biochemistry Co.Ltd., Jimo, Qingdao, China) added to 9 g of ground corn.

[5] Prepared as 0.01 g of dexamethasone (DEX), (Alfa Aesar, Maryland, United States) added to 9.99 g of ground corn.

[6] Dexamethasone premix was added at the expense of corn to supply 0, 1 mg DEX/kg diet.

Table 4.2 Primers used in real –time quantitative PCR

Genes	Primer sequence (5'- 3')	Gene Bank ID	Ref
Housekeeping gene			
GAPDH	F: TCCTAGGATACACAGAGGACCA R: CGGTTGCTATATCCAAACTCA	ENSGALG00000014442*	Grenier et al., 2015
Markers of inflammation			
IL-6	F: CTGCGAGAACAGCATGGAGA R: GAAAGGTGAAAAGCCCGCTG	XM_01,310,0522	Bai et al., 2018
IL-8	F: GCGGCCCCCACTGCAAGAAT R: TCACAGTGGTGCATCAGAATTGAGC	ENSGALG00000011670*	Grenier et al., 2015
IL-10	F: GCTGAGGGTGAAGTTTGAGG R: AGACTGGCAGCCAAAGGTC	ENSGALG00000000892*	Grenier et al., 2015
TNF-α	F: AGATGGGAAGGGAATGAACC R: ACTGGGCGGTCATAGAACAG	AY765397	Adedokun et al., 2012
Markers of gut integrity			
Occludin	F: GATGGACAGCATCAACGACC R: CATGCGCTTGATGTGGAAGA R: CATCCTCCCGAACAATGCCT	NM_205128.1	Wu et al., 2018
Muc-2	F: GCTACAGGATCTGCCTTTGC R: AATGGGCCCTCTGAGTTTTT	XM_421035	Adedokun et al., 2012
Claudin-1	F: TGGAGGATGACCAGGTGAAGA R: CGAGCCACTCTGTTGCCATA	NM_001013611.2	Shao et al., 2013

F stands for forward primer; R stands for reverse primer; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IL = interleukin; TNF-α = tumor necrosis factor alpha; MUC2 = mucin 2. *Sequence obtained from Ensembl chicken genome data resources.

Table 4.3 Growth performance, ileal digestibility, and jejunal morphology of broiler chickens fed diets containing chitosan oligosaccharide (COS) concentration at 0 or 1 g/kg with or without dexamethasone (DEX)[1,2].

	0, g/kg COS		1 g/kg COS			*P*-value[3]		
Item	No-DEX	DEX	No-DEX	DEX	SEM	COS	DEX	COS × DEX
d 20 to 27								
d 20 BW, g/bird	750	750	750	750	21.2	0.85	0.95	0.85
d 27 BW, g/bird	1127	841	1226	993	11.4	<0.01	<0.01	0.03
Gain, g/bird	378	91	477	243	18.8	<0.01	<0.01	0.02
Feed intake, g/bird	619	399	708	526	30.6	<0.01	<0.01	0.55
Gain: Feed, g/kg	627	236	674	487	20.2	<0.01	<0.01	<0.01
Jejunal morphology[4]								
VH, µm	926	742	902	862	34.1	0.07	<0.01	0.02
CD, µm	76	144	74	101	7.9	0.01	<0.01	0.02
VH/CD	12.3	5.55	12.2	8.65	0.621	0.02	<0.01	0.02
Ileal digestibility								
DM, %	65.1	58.2	67.2	63.7	0.68	<0.01	<0.01	0.02
Energy, %	69.6	62.8	71.7	68.0	0.70	<0.01	<0.01	0.04
IDE, kcal/kg	3,561	3,142	3,655	3,375	37	<0.01	<0.01	0.07

[1] On d 0 post hatching, birds were divided into two groups with 16 replicates each, and were fed control diet and COS supplemented diets, each with an initial body weight of 61 g/bird. The final BW on d 13 post hatching was 707 and 720 g/bird for birds fed control diet and COS supplemented diet respectively. The feed intake was 857 and 879 g/bird for birds fed control and COS. The feed efficiency was 755 g/kg and 750 g/kg for birds fed control diet and COS supplemented diets respectively.

[2] Data are least squares means of 8 replicate cages with 7 birds per cage.

[3] *P*-value according to main effects of COS, DEX, and a COS × DEX interaction.

[4] VH= Villus height; CD = Crypt depth; IDE = Ileal digestible energy.

Table 4.4 Relative gene expression † of cytokines, tight junction proteins in jejunal mucosa, plasma antioxidant enzymes of broiler chickens fed diets containing chitosan oligosaccharide (COS) concentration at 0 or 1g/kg with or without dexamethasone (DEX)[1,2].

	0, g/kg COS		1 g/kg COS			*P*-value[3]		
Item[4]	No-DEX	DEX	No-DEX	DEX	SEM	COS	DEX	COS × DEX
Jejunal mucosa								
IL-6	1.00	4.74	0.88	2.20	0.621	<0.01	0.02	0.03
IL-8	1.00	0.72	3.8	1.31	0.753	0.04	0.08	0.15
IL-10	1.00	7.27	2.91	3.04	1.504	0.44	0.04	0.05
Occludin	1.00	0.90	3.37	2.11	0.551	<0.01	0.23	0.30
Claudin-1	1.00	0.47	3.10	2.55	0.585	<0.01	<0.01	0.02
TNF-α	1.00	3.51	0.68	2.45	0.653	<0.01	0.30	0.57
MUC2	1.00	1.53	0.59	1.38	0.332	0.40	0.05	0.70
Antioxidant enzymes								
SOD, U/mL	174.08	25.23	231.99	110.53	1.567	<0.01	<0.01	<0.01
CAT, U/mL	45.05	15.49	70.77	34.06	0.753	<0.01	<0.01	<0.01
GPx, U/mL	22.49	7.51	42.40	15.18	0.665	<0.01	<0.01	<0.01

[1] Data were least squares means of 8 replicate cages with 7 birds per cage.

[2] *P*-value according to main effects of COS, DEX, and a COS × DEX interaction.

[3] IL = interleukin; TNF-α = tumor necrosis factor α; MUC2 = mucin 2; SOD = Superoxide dismutase; CAT = Catalase; GPx = Glutathione peroxidase.

†Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated with GAPDH as the endogenous control

CHAPTER 5. GRWTH PERFORMANCE, GUT MORPHOLOGY, AND CECAL MICROFLORA RESPONSES OF BROILER CHICKENS TO DIETARY SUPPLEMENTATION WITH GARLIC-DERIVED DIALLYL DISULFIDE AND DIALLYL TRISULFIDE

5.1 Abstract

Bioactive components of garlic contain high amounts of sulfur compounds, which can impact gastrointestinal dynamics, immune function, and antimicrobial activity. The objectives of this study were to determine the effect of dietary supplementation of garlic-derived diallyl disulfide (DADS) and diallyl trisulfide (DATS) on growth performance, gut morphology, and cecal microflora in broiler chickens. On d 1 post hatch, male broiler chickens were allotted into treatment groups in a randomized complete block design to one of six treatments that consisted of 0, 15, 30, 45, 60, or 75 mg of DADS + DATS per kg of feed with 8 replicate cages per treatment and 10 birds per cage. The experimental diets were administered from d 1 to 22 post hatch. Growth performance was recorded, on d 8, 15, and 22 post hatch, the median weight bird in each cage was euthanized and the mid jejunum was excised for histological measurements and cecal content was collected on d 8 and 22 post hatch for microbial analyses. Dietary concentrations of garlic-derived DADS + DATS between 0 and 75 mg/kg had no effect on growth performance and gut morphological responses examined. There was a quadratic effect ($P < 0.05$) of DADS + DATS on $\log_{10}$ number of copies of cecal *Escherichia coli* at d 8 post hatching. However, *Lactobacillus spp* $\log_{10}$ number of counts were not affected by dietary supplementation of garlic derived DADS + DATS. Results from the current study indicates that dietary supplementation of garlic derived DADS + DATS may decrease *Escherichia coli* population in cecal content of broiler chickens. Further research should be carried out to investigate the efficacy of garlic derived DADS + DATS through a challenge model in broiler chickens.

Key words: broiler chicken, garlic, growth performance, gut morphology, intestinal microflora

5.2 Introduction

Phytochemical feed additives are plant-derived products that have bioactive functions (Windisch et al., 2008). Increasing interest on the use of phytochemical feed additives has led to a more developed understanding of the relationship between gastro-intestinal (GI) health and growth efficiency. There is also an increased interest in finding alternatives to the use of growth promoting antibiotics in livestock diets. Garlic bioactive components have antioxidant, immune modulatory, antimicrobial and anti-parasitic properties (Amagase, 2006).

Garlic bioactive components improve growth performance in poultry by decreasing the incidence of sub-clinical enteropathogenic infections (Brennan et al., 2003). Plant-derived compounds impact microbial ecology by disrupting bacteria membranes and modulation of microflora resulting in higher concentrations of beneficial microbes in the gut lumen (Diaz-sanchez et al., 2010). Dietary inclusion of some phytogenic plants was shown to reduce *Escherichia coli* population in broiler cecal content (Jamroz et al., 2003). Murugesan et al., (2015) reported that phytochemical feed additive significantly reduced the cecal population of coliforms and fortified the gut microbiota with beneficial bacterial such as *Lactobacillus spp.*

Outcomes of garlic supplementation in poultry diets have shown varying results. Cross et al. (2016) showed garlic product supplementation improved broiler chicken growth performance at d 7 d post hatching, whereas Olukosi and Dono (2014) showed no improvement in broiler chicken performance with dietary supplementation of a crude garlic preparation. Garlic associated improvements in growth performance may be due to enhanced gastrointestinal morphology (Tatara et al., 2008). From an economic efficiency standpoint, Raeesi et al., (2010) showed that, dietary supplementation of 14 g/kg of garlic powder resulted in optimal feed efficiency in broiler chickens.

The intact garlic bulb contains compartmentalization of bioactive AA precursors, such as alliin, and enzymes, such as allinase. Immediately after crushing or grinding of the garlic bulb, alliin comes in contact with allinase and the unstable intermediate allicin is formed. Amagase (2006) reported that allicin spontaneously degrades to stable bioactive phytochemicals such as the oil-soluble derivatives diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) and water-soluble derivatives such as s-allyl-cysteine (SAC).

In a recent study, we observed that daily oral gavage of DADS + DATS for a period of 6 d improved broiler chicken growth performance, ileal morphology, and total-tract digestibility and determined that daily oral gavage of 2.51 mg DADS + DATS per kg BW maximized BW gain and villus height (Horn et al., 2016). However, no information exists on in-feed application of DADS + DATS in broiler chickens diet. Therefore, we hypothesized that dietary supplementation of DADS + DATS will improve growth performance, gut morphology, and cecal microflora. The objectives of this study were to determine growth performance, jejunal morphology, and cecal microbial count responses of broiler chickens to various dietary doses of DADS + DATS.

5.3 Materials and Methods

5.3.1 Garlic Analysis

The garlic-derived product was donated by Biomatrix (Princeton, MN), analyzed at Purdue University Metabolite Profiling Facility (West Lafayette, IN) by HPLC and determined to contain 39.4% DADS, 25.2% DATS, and 4.6% Allyl methyl trisulfide.

5.3.2 Experimental Design

All procedures used in the current study were approved by the Purdue University Animal Care and Use Committee. Male broiler chicks (Ross 308) were purchased from a local hatchery

on d 1 of post hatch, weighed, and tagged for identification. Birds were housed in electrically heated battery cages (Alternative Design and Manufacturing and Supply, Inc., Siloam Springs, AR) and allowed ad-libitum access to feed and water throughout the experiment. Brooder temperature was kept at 35.5 ˚C, 31 ˚C, and 27 ˚C from d 1 to 8, d 9 to 15, and d 16 to 22 post hatching, respectively. At d 1 post hatching, male broiler chickens were allotted to treatments in a randomized complete block design, on the basis of body weight, to one of six treatments that consisted of 0, 15, 30, 45, 60, or 75 mg DADS + DATS per kg of feed with 8 replicates per treatment and 10 broiler chickens per cage. The ingredient composition of the starter diet is presented in Table 5-1.

5.3.3 Sample Collection

Body weight and feed intake were measured at d 8, 15 and 22 post hatch. At the end of the experiment, the bird with median weight in each cage was selected and euthanized by CO_2 asphyxiation, mid-jejunum was also excised for histological analyses. Ceca contents were collected aseptically from two birds per cage pooled at d 8 and one bird per cage at d 22 post hatch.

5.3.4 Histological Measurements

The small intestine of the median weight bird from each cage was removed and the jejunum was collected. A 10-cm section of the mid-jejunum was excised, gently flushed with cold sterile saline solution to remove intestinal contents and immediately placed in a 10% neutral buffered formalin. Samples of jejunum were sectioned transversely approximately 1 cm at the midpoint and then fixed in a cassette and placed in a 10% neutral buffered formalin. Following tissue (jejunum) fixation, samples were dehydrated with ethanol (VWR International, Radnor, PA), cleared with Sub-X® (Polysciences, Inc., Warrington, PA) and placed in paraffin (Polyfin paraffin,

Sigma Polysciences, St. Louis, MO). Segments of the jejunum were sliced using a microtome and stained with hematoxylin and eosin as previously described (Osho et al., 2017). Villus height and crypt depth were measured and villus height to crypt depth ratio was calculated. Average from at least 5 villi per segment were analyzed for differences. Villus height is defined as the length from the villus tip to the valley between villi and crypt depth is defined as the length between the crypt opening and base.

5.3.5 Microbiological Analyses

At d 8 and 22, broiler chicks were euthanized by CO_2 asphyxiation. Ceca content were collected aseptically from two birds per cage were pooled at d 8, and one bird per cage at d 22. The samples were weighed and diluted 10 times in peptone water. Microbial populations were determined by serial dilution (10^{-1} to 10^{-7}) of samples in PBS before inoculation into petri dishes of sterile agar. The selective media for *Lactobacillus* was prepared using Man Rogosa, and Sharpe agar (Difco Laboratories, Detroit, MI). Agar used for total coliforms was McConkey agar (Difco Laboratories, Detroit, MI). Pink colored colonies formed on MacConkey Agar were presumptively identified as *Escherichia coli*. Such isolates were then transferred to eosin-methylene blue medium (EMB; Becton Dickinson and Company, Sparks, MD) and violet red bile agar (VRB; Becton Dickinson and Company, Sparks, MD) for further confirmation. The plates were incubated at 37ºC anaerobically (73% N, 20% CO_2, 7% H_2) for *Lactobacillus* and aerobically for total coliforms. Plates were counted between 24 and 48 h after inoculation.

5.3.6 Statistical Analysis

Data was analyzed using the General Linear Model procedure of SAS (SAS Inst., Inc., Cary, NC) with the fixed effect of treatment and the random effect of block. The following

statistical model was used in the analysis: $Y_{ij} = \mu + A_i + B_j + \varepsilon_{ij}$ where Y is the response criterion [Growth performance, Villi height, Crypt depth, villus height crypt depth ratio, *Lactobacillus spp* (Log_{10}CFU/g), *Escherichia coli* (Log_{10}CFU/g)]; μ is the overall mean [Growth performance, Villi height, Crypt depth, villus height crypt depth ratio, *Lactobacillus spp* (Log10 CFU/g) and, *Escherichia coli* (Log_{10}CFU/g)]; A_i is the effect of i^{th} treatment (i = 1, …,6); B_j is the effect of j^{th} block (k = 1, 2, 3,… 8); and ε_{ij} is the error term. Statistical significance was set at an α of 0.05.

5.4 Results and Discussion

The objectives of the current study were to determine the impact of dietary supplementation of DADS + DATS on growth performance, gut morphology and cecal microflora of broiler chickens. The doses used (0, 15, 30, 45, 60 and 75mg DATS + DATS/kg diet) were chosen based on a previous study in which we observed that daily oral gavage of 2.51 mg DADS + DATS per kg BW gain and villus height (Horn et al., 2016). The daily oral dose of 2.51 mg DADS + DATS per kg BW translates to 60 mg DADS + DATS per kg of diet.

There was no effect of dietary supplementation of DADS + DATS on growth performance of broiler chickens at d 8, 15 and 22 post hatching (Table 5-2). Numerically, broiler chickens fed diet without DADS + DATS supplementation had the highest BW, weight gain, and feed intake at d 8, 15 and 22 post hatching. Broilers fed diet supplemented with 45 mg DADS + DATS per kg of diet also showed the highest numerical gain to feed ratio at d 22 post hatching. The trend observed in this study agreed with previous studies which reported that herbs, plant extracts, essential oil showed no effect on growth performance (Demir et al., 2003). Previous studies have shown conflicting results following dietary inclusion of garlic botanicals in broiler chickens. While Lewis et al. (2003) reported improved feed efficiency and body weight gain in broilers fed garlic, Konjufca et al. (1997) showed that dietary garlic had no affect the body weight gain and feed

efficiency. Olukosi and Dono (2014) also reported no effect on growth performance of broiler chickens fed with 10 g garlic meal per kg diet.

These lack of effects on growth performance of broilers fed with dietary treatment in this study may be due to differences in the garlic botanical product used; the nature of the additive employed and the concentration of active components, which differ considerably between experiments; and the complicated chemistry of garlic; or a combination of these factors. Another reason for the lack of response noticed could be due to the pungent odor of the garlic botanical which could interfere with feed intake, compounding the treatment effect. It could take a while before the birds got adapted to the feed, which suggests an allowance of some adaptation period in subsequent studies. The difference noticed between results of the previous research reported by Horn et al. (2016) and the current study could result from the possible dilution effect contributed by the components of the diets. The dilution effect could reduce the potency of the garlic derivatives. In the previous experiment, the birds were orally gavaged for 6 d, which may have had direct effect on the bird's GI-tract. Therefore, orally-gavaged birds might have limited variations in terms DADS + DATS intake, as compared to in-feed application. this may have had an impact on performance of the birds.

Gastrointestinal health is key factor to enhanced productivity in all farm animals and poultry. These digestive functions could be considered the most limiting factor in growth performance. Gut surface area affects nutrient utilization, specifically longer villi support greater nutrient absorption because of increased surface area, whereas deeper crypt can indicate greater cell turnover in response to normal cellular sloughing or inflammatory response (Murakami et al., 2007). In the current study, there was no effect of dietary supplementation of DADS + DATS on the villus height, crypt depth, and villus height to crypt depth ratio at d 22 post hatching (Table 5-

3) in broiler chickens. This is in line with Olukosi and Dono (2014), which showed no impact of garlic meal on jejunal morphology. Numerically, broiler chickens fed diet supplemented with 30 mg DATS + DATS per kg had the highest villi height (Table 5-3). This numerical increase in villi height also impacted a numerical highest final body weight (797 g).

One of the objectives to use garlic botanicals in broiler diets is to increase GI beneficial organisms and to reduce pathogenic organisms which may cause infectious diseases (Higgins et al., 2010). Indeed, garlic extracts have exhibited a wide spectrum of antibacterial activity including *Escherichia coli* and *Lactobaccillus spp* (Uchida et al., 1975). The result obtained in the present study showed that there was a quadratic effect ($P < 0.05$) of DADS + DATS on $\log_{10}$ number of copies of cecal *Escherichia coli* at d 8 post hatching (Table 5-3). Quadratically, highest *Escherichia coli* population obtained with d 8 d broilers fed with 75 mg/kg DADS + DATS might be due to changes in gut profile to a population of coliform bacteria potentially beneficial to growth (Ravindran et al., 2006). However, *Lactobacillus spp* $\log_{10}$ number of counts were not affected by dietary supplementation of garlic derived DADS + DATS. The present findings suggested that dietary supplementation of garlic derived DADS + DATS at 30 and 45 mg/kg DADS + DATS may decrease cecal *Escherichia coli* population in cecal content of d 8 broiler chickens. Similarly, Kumar et al. (2010) indicated that the inclusion of garlic at 250 ppm in the broiler diet reduced the count of *Escherichia coli* when compared with the control. In broiler chicken production, the cage environment has been shown to be best in reducing bacterial growth (Permin et al., 1999), whereas floor litter provides a substrate for pathogenic bacterial growth (Pope and Cherry, 2000). Lee et al. (2003) concluded that responses to phytochemicals may be greater in a more challenging environment such as commercial production.

In conclusion, the current study showed that dietary supplementation of garlic-derived DADS + DATS in d 8, 15, and 22 broiler chickens diet showed no effect on growth performance and gut morphology. In d 8 broilers, quadratic reduction occurred in the cecal *Escherichia coli* count. These lack of effects on growth and morphology may be due to reduced feed intake, and hormetic action (when there is a stimulatory or beneficial effect at low doses and an inhibitory or toxic effect at high doses of phytochemicals) which may induce biologically opposite effects at different doses. Additional studies are needed on stability of garlic-derived DADS + DATS in broiler chickens diet. One of the possible solutions is to coat the garlic product, which could improve product efficacy. Further research should also be carried out to investigate the efficacy of garlic derived DADS + DATS through a disease challenge model in broiler chickens.

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Table 5.1 Ingredients and chemical composition of experimental diets (Starter diet) on an as-fed basis.

	DADS + DATS, mg/kg					
Ingredient, g/kg	0	15	30	45	60	75
Corn	520.2	510.2	500.2	490.2	480.2	470.2
Soybean meal (48.5% CP)	356.0	356.0	356.0	356.0	356.0	356.0
Soybean oil	50.0	50.0	50.0	50.0	50.0	50.0
Monocalcium phosphate[1]	17.0	17.0	17.0	17.0	17.0	17.0
Limestone (38% Ca)	17.0	17.0	17.0	17.0	17.0	17.0
Salt	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin-mineral premix[2]	3.0	3.0	3.0	3.0	3.0	3.0
DL-Methionine	3.8	3.8	3.8	3.8	3.8	3.8
L-Lysine, HCL	2.9	2.9	2.9	2.9	2.9	2.9
L-Threonine	1.1	1.1	1.1	1.1	1.1	1.1
Chromic oxide- ground corn premix[3]	25	25	25	25	25	25
DADS + DATS-premix[4]	0.0	10	20	30	40	50
Total	1,000	1,000	1,000	1,000	1,000	1,000
Calculated nutrients and energy content						
CP, g/kg	224.7	224.7	224.7	224.7	224.7	224.7
ME, kcal/kg	3191	3191	3191	3191	3191	3191
Ca, g/kg	10.3	10.3	10.3	10.3	10.3	10.3
P, g/kg	7.3	7.3	7.3	7.3	7.3	7.3
Non-phytate P, g/kg	4.8	4.8	4.8	4.8	4.8	4.8
Ca:P	1.4	1.4	1.4	1.4	1.4	1.4

[1] Contained 16% Ca, 21% P.

[2] Supplied the following per kilogram of diet: vitamin A, 5484 IU; vitamin D_3, 2643 IU; vitamin E, 11 IU; menadione sodium bisulfite, 4.38 mg; riboflavin, 5.49 mg; D-pantothenic acid, 11 mg; niacin, 44.1 mg; choline chloride, 771 mg; vitamin B12, 13.2 μg; biotin, 55.2 μg; thiamine mononitrate, 2.2 mg; folic acid, 990 μg; pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 μg.

[3] Prepared as 1 g of chromic oxide added to 4 g of ground corn.

[4] Prepared as 3 g of DADS + DATS added to 2000 g of ground corn.

Table 5.2 Growth performance of broiler chickens fed dietary supplementation of garlic derived DADS + DATS from d 1 to 22 post hatch 1.

Item	DADS + DATS, g/kg						SEM[2]	*P*-value
	0	15	30	45	60	75		
Body weight, g/bird								
d 1 to 8	140	136	139	138	137	136	2.4	0.82
d 1 to 15	396	371	369	385	375	377	9.4	0.36
d 1 to 22	797	760	796	785	777	780	18.6	0.73
Weight gain, g/bird								
d 1 to 8	96	92	95	95	94	92	2.4	0.81
d 1 to 15	256	235	230	246	237	240	7.1	0.14
d 1 to 22	693	655	669	675	664	674	19.3	0.82
Feed intake, g/bird								
d 1 to 8	134	124	125	124	131	122	5.6	0.65
d 1 to 15	344	326	317	334	322	328	7.1	0.14
d 1 to 22	975	934	961	944	939	951	22.5	0.80
Gain/feed, g/kg								
d 1 to 8	721	754	764	762	744	756	25.8	0.86
d 1 to 15	743	719	729	739	736	731	14.9	0.89
d 1 to 22	710	701	696	715	706	708	10.9	0.87

[1] Data are means of 8 replicate cages with 10 birds per cage.
[2] Standard error of mean.

Table 5.3 Responses of broiler chickens fed dietary supplementation of garlic-derived DADS + DATS on jejunal morphology and cecal microbial count (Log_{10} CFU/g of cecal content)[1].

Item	DADS + DATS, g/kg						SEM[2]	*P*-value
	0	15	30	45	60	75		
d 22 Jejunal morphology								
Villus height (VH), µm	1071	1191	1212	1121	1090	1026	69.2	0.47
Crypt depth (CD), µm	123	120	119	126	117	113	7.9	0.90
VH:CD	8.7	10.2	10.3	8.9	9.6	9.1	0.4	0.16
d 8 Cecal microbial count Log_{10} CFU/g								
Escherichia coli	2.9	3.4	2.7	2.4	3.4	3.8	0.31	0.04
Lactobacillus spp	5.4	5.6	5.4	5.5	5.2	5.2	0.23	0.88
d 22 Cecal microbial count Log_{10} CFU/g								
Escherichia coli	6.8	6.1	6.7	6.8	6.7	6.6	0.36	0.75
Lactobacillus spp	8.9	8.2	8.7	8.9	8.4	8.4	0.21	0.04

[1] Data are means of 8 replicate cages with 10 birds per cage.
[2] Standard error of mean.

CHAPTER 6. SUMMARY

Improvements in nutrition have had a dramatic impact on the efficiency and productivity of broiler chickens in the poultry industry. As a result of the continuous changes to dietary and health regulations, consumer preferences, feed ingredients and additives, and much more, the goal in poultry production is to improve the health and productivity of the bird to benefit the producer, consumer. , Ultimately,advancing the science for improved bird health and productivity is top priority. One area of research that has received much attention recently is the health of the gastro-intestinal tract. Considering 20% of a bird's energy goes towards maintenance of the GIT, factors such as disease or diet that could disrupt the natural healthy balance of the intestine become extremely important avenues to explore to insure optimal health and efficient production of birds.

As mentioned earlier, it should be the goal of poultry nutritionist to not only improve the nutrition of birds, but to understand the other factors of poultry management that ultimately have an effect on nutrition. Because a healthy intestine is necessary for adequate nutrient digestion, enteric diseases such as coccidiosis that compromise the integrity of the intestinal tract are of great importance when considering how to improve intestinal health through nutrition. Equally important is the way nutritional strategies affect disease control methods. In the past, in-feed coccidiostat medication was the primary method used to control coccidiosis. However, as consumer preferences change, vaccinating broilers against coccidiosis is becoming increasingly more common. Unfortunately, the use of live oocyst vaccines is associated with an initial set-back in performance. Therefore, the objective of the studies reported in the thesis was to understand the efficacy of some alternative feed additives to enhance performance and intestinal health in broiler chickens.

In chapter 2, two experiments were conducted to determine the optimum dose for dietary SBP supplementation in broiler chickens diet from d 1 to 22 post hatch based on growth performance and intestinal morphology, and the optimum dose was used to test the efficacy of SBP during a coccidia challenge in broiler chickens. In Experiment 1, 384 birds were assigned in a randomized complete block design. Corn-soybean meal-based diet was formulated to contain 0, 1, 2, 3, 4, 5, and 6 g of SBP/kg of diet. Results indicated that there were linear effects ($P < 0.05$) of graded SBP concentration on body weight (BW), BW gain, feed intake, jejunal villus height and villus height to crypt depth ratio at d 22 post hatch. In experiment 2, dietary SBP attenuated the CC-induced effects ($P < 0.05$) on the mRNA expression of expression interleukin-1β, transforming growth factor-β, claudin-1 and occludin genes.

Similarly, in chapter 3, two experiments were conducted to determine the optimum dose for dietary COS supplementation in broiler chickens diet from d 1 to 24 post hatch based on growth performance and intestinal morphology, and the optimum dose was used to test the efficacy of SBP during a coccidia challenge in broiler chickens. In Experiment 1, 384 birds were assigned in a randomized complete block design. Corn-soybean meal-based diet was formulated to contain 0.0, 0.5, 1.0, 1.5, 2.0, and 2.5 g of COS/kg of diet. Results indicated that there were linear effects ($P < 0.05$) of graded COS concentration on body weight (BW), BW gain, feed intake, jejunal villus height and villus height to crypt depth ratio at d 22 post hatch. Between 0.50 and 1.18 g COS/kg diet was optimum for BW gain, jejunal villus height, and villus height to crypt depth ratio. In experiment 2, COS mitigated the effects of coccidia challenge on intestinal morphology and mucosa gene expression.

The study reported in chapter 4 was conducted to answer the question whether dietary supplementation of COS will ameliorate stress stimulated by in-feed dexamethasone in broiler

chickens. Results indicated that dietary COS also decreased ($P < 0.05$) DEX-induced effects (interaction; $P < 0.05$) on BW, BW gain, and gain:feed. The DEX-induced effect of relative mRNA expression of jejunal mucosa IL-6, IL-10, and claudin-1 was reduced by dietary COS supplementation (interaction; $P < 0.05$). Responses (interaction; $P < 0.05$) in the activity of plasma superoxide dismutase, catalase, and glutathione peroxidase to COS and DEX were similar to that observed with the relative mRNA expression. Chitosan oligosaccharide supplementation increased ($P < 0.05$) the mRNA expression of IL-8 and occludin. Chitosan oligosaccharide alleviated the immunological stress stimulated by in-feed dexamethasone.

Lastly, in chapter 5, all birds were randomly assigned to six dietary treatments (0, 15, 30, 45, 60, or 75 mg DADS + DATS per kg of feed). Growth performance, histological measurements and microbiological analyses was assessed weekly. There was no effect of dietary supplementation of DADS + DATS on growth performance and jejunal morphology. There was a quadratic effect ($P < 0.05$) of DADS + DATS on $\log_{10}$ number of copies of cecal *Escherichia coli* at d 8 post hatch. The result suggested that dietary supplementation of garlic derived DADS + DATS at 30 and 45 mg/kg DADS + DATS may decrease cecal *Escherichia coli* population in cecal content of d 8 broiler chickens.

Maintaining a healthy gut in our birds would continue to be a challenge for the near future. This will not be as a result of our inability to come up with products that will, to a reasonable extent, fill in the gap left by the withdrawal of antimicrobial growth promoters, but rather coming up with a product that the consumers will readily accept. Based on current trends, this product must be a "natural" product. If this trend continues, our ability to be able to accurately identify and extract products that are able to protect and enhance the development of the GIT will be crucial.

The additives used in the experiments presented in this dissertation were derived from natural products. The results indicates that dietary supplementation of SBP or COS has many potential benefits including improvement in growth performance, digestion and absorption of nutrients, modification of birds' metabolism, immunomodulation, and in the functioning and health of the gut. Soybean bioactive peptides are relatively nonspecific in their action and this may account for why the exact mechanism of action during coccidia challenge are largely unknown. Further research is needed to investigate the mode of action of SBP. Specifically, an understanding of how SBP influences NFkB pathway activation and intestinal microflora would give insight into the mode of action of SBP. In addition, a better understanding on the mode of action of SBP on gastrointestinal permeability and intestinal microflora is needed. The mechanism by which COS interacts with intraepithelial cells leading into immunological response is still unclear. However, further study is needed to elucidate the mechanism of the interaction between COS and macrophages by which COS improves immune function in broiler chickens. Thus, considering the potentials of COS in improving BW and feed efficiency, it is noteworthy to investigate the influence of COS on mRNA gene expression of nutrient transporters as well as insulin-like growth factor-1 receptors in the GIT of broiler chickens, the aforementioned parameters will provide more clarity on the on the growth promoting effect of COS in broiler chickens. A well-developed antioxidation system could prevent oxidative injury in animals caused by stress. Therefore, further research should be conducted to investigate the mechanism of regulating antioxidation system by COS. In future studies, it will be pertinent to validate the potency of the oocyst in the coccidiosis vaccine that will be used, considering the coccidia challenge model that was developed for the experiments conducted in this dissertation. Lastly, for the garlic-derived DADS + DATS used in the experiment conducted in this dissertation, more *in-vitro* studies are needed to determine its

stability in broiler chickens diets with a follow-up gut simulation trial. Further research should also be carried out to investigate the efficacy of garlic derived DADS + DATS through a stressor or disease challenge model in broiler chickens.

Generally, additional studies, which would explore various combinations of these alternatives with specific target to enhance improvement in poultry production, are needed. Maintaining the integrity of the GIT may require more than one product but a cocktail of products that could exhibit immunomodulatory potentials on the GIT. For example, exploring the synergistic effects of COS or SBP with other natural agents and compounds such as direct-fed microbials. Furthermore, future poultry breeding and selection programs should include genes responsible for the bird's ability to resist an infection, as well as the ability of the birds to be resilient in the face of a high pathogen load. The selection criteria should include those genes that make some of these birds resistant or resilient to gastrointestinal challenges. This means a holistic approach through novel strategies is necessary to minimize the impact of these stressors on poultry gut health.

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