DIETARY SAPONINS AND IMMUNE ACTIVATION BY *EIMERIA* SPECIES IN
BROILERS

BY

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THESIS

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ABSTRACT

Coccidiosis is an intestinal parasitic disease caused by protozoans of the genus *Eimeria* and substantially impacts the poultry industry economically worldwide by diminishing animal growth. The prophylactic use of anticoccidial drugs in order to ameliorate the detrimental effects of an *Eimeria* infection has been the most successful control measure in broilers. However, due to an increase in resistance to anticoccidial drugs and a growing public concern for the development of resistant microorganisms, dietary intervention as a nutritional alternative to antibiotics and anticoccidial drugs have become of specific interest to combat coccidiosis in broilers. Because of this, an experiment was conducted to determine if dietary *Yucca*-derived saponin supplementation could mitigate the immune and growth responses of broilers during a mixed coccidian challenge. Dietary treatments were corn-soybean meal-based and included: 1) control diet + sham-inoculated (Ucon), 2) control diet + Eimeria oocyst challenge (Icon), 3) control diet with 250 mg/kg Yucca-derived saponin product + Eimeria oocyst challenge (ISap250), and 4) control diet with 500 mg/kg of Yucca-derived saponin product + Eimeria oocyst challenge (ISap500). Growth performance, oocyst shedding, histopathological morphometrics and lesion scoring, differential blood cell counts, immunophenotyping of cecal-derived T-cell profiles, and intestinal inflammatory cytokine gene expression were evaluated in broilers inoculated with a mixture of *E. acervulina*, *E. maxima,* and *E. tenella* oocysts. The birds challenged with *Eimeria* showed clear signs of a successful infection, as evidenced by oocyst counts of excreta samples collected from each pen. The birds challenged orally with the *Eimeria* mixture displayed a reduction in growth compared with birds not challenged with the *Eimeria* mixture, but there was no detectable difference due to dietary treatment when comparing

amongst infected birds alone. Histopathological analysis identified a treatment effect for mucosal thickness in the jejunum, where infected birds fed an inclusion rate of 250 mg/kg of diet of saponin supplementation and the un-infected control diet birds were not significantly different from each other. Histopathological lesion scoring exhibited no detectable differences due to dietary treatment within the *Eimeria*-infected groups. Seven days following *Eimeria* inoculation, lymphocytes as a percentage of white blood cells were elevated in all infected treatment groups, but birds fed saponin supplementation at 250 mg/kg of diet did not differ from the un-infected control diet birds. Saponin supplementation in conjunction with an *Eimeria* challenge had no measureable effect on cecal-derived T-cell profiles. Expression of cecal and duodenal *IFN-γ* increased with infection when compared to the sham-inoculated birds. Inoculation with *Eimeria* also increased expression of *IL-1β*, but saponin supplementation at 250 mg/kg and 500 mg/kg of diet ameliorated *IL-1β* expression in the cecal tonsils and duodenum to levels not different from sham-inoculated birds. Overall, this research demonstrates that dietary intervention in the form of saponin supplementation may serve as an effective strategy in mitigating the resulting inflammatory response following exposure to *Eimeria* in broilers as evidenced by lymphocyte responses, changes in intestinal structure, and alterations in cecal and duodenal inflammatory cytokine mRNA expression.

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CHAPTER 1: INTRODUCTION

Coccidiosis is a major parasitic disease affecting the poultry industry and causes severe economic loss worldwide of more than $3 billion USD (Dalloul & Lillehoj, 2006; Williams, 1999). The disease is caused by a protozoan in the genus *Eimeria*, an intracellular parasite whose predilection site is along the gastrointestinal tract (GIT) of broiler chickens (McDougald & Fitz-Coy, 2008). Coccidiosis is manifested by a destruction of the intestinal epithelia and elicits an immune response within the host, thereby leading to increased maintenance costs, decreased absorption of nutrients, and ultimately causes diminished animal growth (Turk & Stephens, 1967). Because of the pathogenicity of *Eimeria* species and current management practices within the poultry industry, a variety of control measures have been developed to mitigate the detrimental effects of coccidiosis. The most successful of these measures being prophylactic chemotherapeutic agents or anticoccidial drugs (Dalloul & Lillehoj, 2006), in addition to the use of vaccination programs (Chapman, 2014). Importantly, a shift in *Eimeria* susceptibility to anticoccidial drugs was noted soon after discovery of the drugs, and evidence of global coccidial resistance to chemotherapeutic agents has been demonstrated for almost all anticoccidial drugs (Abbas et al., 2011). Public concern regarding the resistance of microorganisms to control strategies (Cosby et al., 2015; Endtz et al., 1990) and implications in human health have been increasing over the past decade. The affirmation that science and technology will continuously create novel, more efficient drugs to counteract microorganismal resistance has dwindled (Gold & Moellering, 1996). As the industry heads towards an antibiotic-free era brought on by pressure from both consumers and regulatory agencies, dietary intervention and the discovery of alternatives for antibiotics and anticoccidial drugs without detriments in growth performance has

become of upmost importance to the poultry industry. A plethora of alternatives have been introduced to the poultry industry, all of which seek to maintain optimal growth performance while promoting overall bird health (Gadde et al., 2017). Phytogenic compounds are non-antibiotic feed additives of plant origin and are available as solids, extracts, and essential oils (Yitbarek, 2015). Saponins are one type of phytogenic compounds that have been suggested as an alternative to anticoccidial drugs due to their antiprotozoal activity (Cheeke, 2000). Saponins are natural detergents that consist of a lipophilic nucleus, either steroid or triterpenoid in structure depending on plant origin, with one or more water-soluble carbohydrate side chains (Cheeke, 2000; Francis et al., 2002). Saponins have the ability to bind to membrane cholesterol of protozoan cells, thus creating saponin-cholesterol complexes that can modify membrane function and structure, leading to eventual cell lysis and cell death (Francis et al., 2002). Saponins also have the ability to improve nutrient digestibility (Johnson et al., 1986), growth performance (Sahoo et al., 2015; Sun et al., 2017), and odor control (Çabuk et al., 2004). Phytogenic compounds, specifically saponins, may prove to be an effective alternative to antibiotics and anticoccidial drugs due to their antiprotozoal activity and ability to enhance nutrient absorption and growth of broilers. These products offer a natural alternative to prophylactic chemotherapeutic agents in addition to staying in the realm of governmental regulations while assuaging to the growing public concern for microorganismal resistance.

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CHAPTER 2: LITERATURE REVIEW

Coccidiosis in Poultry

Introduction and Impact

Parasitic infections cause severe economic damage to the poultry industry and can be attributed to two general groups, coccidia and mastiogophora (McDougald, 1998). Infection from the more common coccidia results in coccidiosis, which continuously and substantially affects the cost of poultry production. Globally, coccidiosis causes a severe economic impact by costing producers up to $3 billion USD in annual losses (Dalloul & Lillehoj, 2006; Williams, 1999).

The prevalence of coccidiosis in today's flocks is not a new problem afflicting the poultry industry. Walter T. Johnson first published on avian coccidiosis (Johnson, 1923) over 95 years ago. Today, it is rare to find a poultry flock that has not been infected or exposed to coccidia, which can be found wherever chickens are raised (Mcdougald et al., 1986; Williams, 1998). Due to subclinical exposure and a depletion in traditional rearing space (near 15 birds/m^2), birds are ingesting and shedding coccidian oocysts in a confined area making it easy for birds raised on litter to be continually re-exposed to active coccidia oocysts. Exposure to large quantities of oocysts have the potential to overwhelm the avian immune system, and activate an immune response within the birds (H.S. Lillehoj & Lillehoj, 2000; Williams, 2001). These exposure patterns, coupled with the rise in anticoccidial drug resistance due to extensive use (Sundar et al., 2017), have lead producers to seek other means of action to control and combat coccidiosis.

Coccidiosis is caused by a protozoan parasite that belongs to the genus *Eimeria.* Within the genus *Eimeria*, seven different species are widely encountered within the poultry industry, all of which are intracellular parasites that rely on both external (environment) and internal (host animal) developmental stages (H.S. Lillehoj & Lillehoj, 2000). These include *E. tenella, E.*

acervulina, E. maxima, E. brunetti, E. mitis, E. praecox, and *E. necatrix* (McDougald, 1998). Of the seven species, all are pathogenic, but only *E. acervulina, E. brunetti, E. maxima, E. necatrix,* and *E. tenella* yield quantifiable gross lesions in varying spots along the gastrointestinal tract and their severity ranges from moderate-to-severe, respectively (Allen & Fetterer, 2002). Mortality due to a coccidian infection is rare, but has been reported during infection with *E. tenella* at a dose of 1×10^4 or more sporulated oocysts, making it the most pathogenic species found in chickens (McDougald & Fitz-Coy, 2008).

The preferred site of habitation varies by *Eimeria* species along the gastrointestinal tract (GIT) of broiler chickens. *Eimeria acervulina* targets the duodenum, *E. maxima* the ileum, and *E. tenella* inhabits the ceca (McDougald & Fitz-Coy, 2008). Subjectively viewing gross lesions along the intestines of birds enables producers to identify which species of coccidia may be infecting their flock, which can aid in controlling the disease. Coccidiosis is manifested by a destruction of the intestinal epithelia, thereby leading to increased maintenance costs for tissue repair and decreased absorption of nutrients (Turk & Stephens, 1967). Weight loss and poor feed conversion have been attributed to both anorexia and other general factors, such as malabsorption (Russell & Ruff, 1978). Anorexia induced by an *Eimeria* challenge accounted for 30-70% of the suppression in body weight gain, while the remainder was accredited to a disruption in nutrient absorption as determined in pair-wise feeding studies (Preston-Mafham & Sykes, 1970).

Pathogenesis

Coccidia are unique in that they are self-limiting and must undergo a continual cycling both inside and outside of a host (McDougald & Fitz-Coy, 2008). Infection begins with ingestion of sporulated oocysts, which can be derived from contaminated litter, feed, or water. An oocyst

remains inert until it becomes sporulated in the environment, and sporulation occurs when adequate levels of moisture, oxygen, and heat are attained, typically over a 24-48–hour period (Austic & Nesheim, 1990). These three characteristics, oxygen, moisture, and heat, are also crucial variables regulated in commercialized poultry facilities, making it the ideal environment for oocysts to sporulate and thrive. Once sporulated, oocysts remain viable for an extended period, with *E. acervulina* oocysts found to be infective for up to 86 weeks after being extracted from excreta and placed on outdoor soil plots (Farr & Wehr, 1949; Fayer, 1980). After ingestion of sporulated oocysts, they are degraded in the gizzard to expose sporocysts (McDougald, 1998). These sporocysts come in to contact with bile and trypsin found in the small intestine causing a release of sporozoites that invade enterocytes along the GIT. Once embedded in the epithelia lining of the GIT, sporozoites undergo schizogony, a process of growth and division accumulating hundreds of daughter cells called merozoites. After two or three generations of schizogony, merozoites have two fates, spreading to invade additional host cells or forming either *micro*gametes or *macro*gametes. Macrogametes are fertilized by the released motile microgametes in a sexual fission to form a zygote, which undergo maturation to form a new oocyst that is excreted into the environment (Austic & Nesheim, 1990; McDougald, 1998).

Each *Eimeria* species undergoes a life cycle similar to that described above, but the timing and reproductive potential varies depending on oocyst pathogenicity. For more pathogenic species, like *E. tenella,* the potential to reproduce is lowered, whereas, less pathogenic species, like *E. acervulina*, exhibit higher potentials to reproduce (McDougald, 1998). The process from initial oocyst infection to final zygote excretion generally takes 4 to 5 days, with maximum oocyst shedding occurring 6-to-9 days after initial infection (Allen & Fetterer, 2002). Considering the cyclic reproductive cycle of *Eimeria* and scale of the broiler

industry, one infected bird can shed thousands of new oocysts and initiate an exponential spread of oocysts in a very short time, making avoidance of reinfection nearly impossible.

Invasion of *Eimeria* in the host causes an array of metabolic, physiologic, and immunologic issues. At the tissue level, *Eimeria* destroys enterocytes and degrades the protective mucosal lining of the GIT, which reduces nutrient absorptive capacity, induces ulceration, predisposes birds to pathogenic bacteria, elicits an immune response, and in severe cases, results in internal bleeding (Austic & Nesheim, 1990). Ulceration and destruction of mucous membranes reduces the primary physical barrier to subsequent infection by bacteria such as *Clostridium perfringens,* a major predisposing factor of necrotic enteritis (Van Immerseel et al., 2004). In severe cases, a coccidian infection has the ability to invade and destroy enterocytes at the tips of villi, thereby leaving a barren GIT with severely reduced absorptive capacity (McDougald & Fitz-Coy, 2008). Intestinal lesions can physically be seen along the GIT of a bird that has been infected with coccidiosis. Severity of the lesions depends largely on the *Eimeria* species, oocyst load, age of the bird, and the original immune state of the bird. Additionally, these lesions can be indicative of other parameters induced by infection, such as reduced weight gain. Lesion scoring immediately after euthanasia has been reported using a scale of 0-4 where 0=normal and 4=most severe in addition to histopathological analysis in order to quantify the coccidian state to which a bird has been exposed (Johnson & Reid, 1970; McDougald & Fitz-Coy, 2008).

As a result of tissue damage, diarrhea and decreased skin and shank pigmentation are among some of the clinical signs of coccidiosis infection in a flock. Michael Ruff and Henry Fuller (1975) demonstrated that birds orally inoculated with sporulated *E. acervulina* and *E. tenella* oocysts, 1,000,000 and 100,000, respectively, had notably reduced plasma carotenoids,

thus changing the skin and shank pigmentation of infected birds. The most detrimental effect economically of coccidiosis in chickens is reduction in weight gain and feed efficiency due to depressed feed intake, malabsorption, and diarrhea as a result of subclinical exposure (Williams, 1999). A meta-analysis conducted by Kipper et al. (2013) compiled data from 69 publications, around 44,000 birds, to quantify the variation in feed intake and weight gain across multiple species of *Eimeria*. Their results showed that a variation in feed intake quadratically influenced the impairment of weight gain, and the severity of this effect was dependent on many factors including, sex, age, *Eimeria* species, and environmental conditions (Kipper et al., 2013). Mortality due to coccidia is rare, making it difficult for a producer to physically see if their flock is infected until it is too late and an outbreak has occurred. Thus, it is imperative that farmers take preventive measures to reduce the effects of a coccidian infection.

Control Measures

Based on the reproductive cycle of *Eimeria* species and management practices within the poultry industry, a variety of control measures have been developed to mitigate the detrimental effects of coccidiosis. The most successful of these measures, by far, is the use of prophylactic chemotherapeutic agents, or anticoccidial drugs, which have been deemed as "absolute requirements to control the disease" (Dalloul & Lillehoj, 2006). Anticoccidial drugs can be classified as chemicals (e.g., amprolium, clopidol decoquinate, halofuginone), which directly affect parasite metabolism, or polyether ionophores (e.g., monensin lasalocid, salinomycin, narasin), which alter osmotic balance and ion transport of potassium and sodium in the GIT of the birds to make the intestinal environment less habitable (Chapman, 2001, 2014; McDougald, 1998). When an anticoccidial drug is used to directly destroy coccidian populations, they are termed coccidiocidal, whereas the prophylactic use of an anticoccidial drug is termed as

coccidiostatic (Kant et al., 2013). Determining the criteria to evaluate the efficacy of an anticoccidial drug should be based on the objective of the experiment, but most often weight gain (global indicator of infection severity), lesion scores (pathology of infection), serum parameters (alterations in absorption of infection), and feed intake (anorexic behavior of infection) are clinical outcomes taken into consideration (Chapman, 1998).

Prophylactic use of anticoccidial drugs have proven to be extremely beneficial for producers, however, due to the extensive use of these drugs worldwide, coccidia have been able to develop a tolerance, thus limiting the effectiveness of anticoccidial drugs (Abbas et al., 2011). Grumbles et al. (1948) first reported on the beneficial effects of prophylactic use of an anticoccidial drug (sulfaquinonxaline) to control coccidiosis and less than ten years later, evidence of resistance was reported by Cuckler et al. (1955). Because of the growing concern for the diminishing effectiveness of anticoccidial drugs due to evolved resistance, implementation of shuttle or rotation programs have been in heavy use by the broiler industry to prolong the benefits of anticoccidial drugs. A shuttle program consists of using two or more drugs, usually with different modes of action, whereas a rotation program utilizes different drugs in successive flocks (Chapman, 2014). A study of the use of anticoccidial drugs in the U.S. broiler industry analyzed data compiled from 1995 to 1999 and concluded that the use of chemical compounds may be superior to prophylactic ionophores in reducing resistance to control agents (Chapman, 2001).

Anticoccidial drug resistance has forced the industry to seek alternative methods for treating coccidiosis, and recent efforts have focused on vaccine development. The use of live vaccines takes advantage of the protective immunity a bird develops early on in life, allowing vaccinated birds to become immune to species-specific coccidiosis prevalent in the vaccine

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(Dalloul & Lillehoj, 2006). Coccidian vaccines can either be virulent (non-attenuated), derived from laboratory or field strains with their original integrity, or attenuated, with artificially-reduced virulence, and the difference of these two is reflected in the clinical outcomes of the birds. R. B. Williams (2002) compiled literature on broiler growth performance data available for commercially-available anticoccidial vaccines (Paracox®, Coccivac®, Immucox®, Livacox®, and Nobilis COX ATM®), both attenuated and nonattenuated, in which vaccinated birds did not outperform non-vaccinated birds in 32 of the 43 studies . Despite this evidence, there is still a push for the use of vaccines over anticoccidial drugs in managing coccidiosis due to other benefits, such as animal welfare, absence of drug residuals, and avoidance of toxicity (Williams, 2002).

Growth performance loss due to mild *Eimeria* challenges via a vaccine, may result in a decrease in performance. Nevertheless, this may be recovered through compensatory gain, a process in which the bird compensates for the loss of growth with a general increase in feed consumption and weight gain and is especially apparent in birds taken to a large target weight (McDougald & McQuistion, 1980). However, producers cannot always be reliant on compensatory gain to ameliorate adverse effects of a coccidiosis vaccination, but it is because of this, nutritional intervention during the recovery phase of a transient *Eimeria* infection has become a crucial role in combatting an *Eimeria* infection in chickens.

The Avian Immune System and Response to *Eimeria* Infection

Innate and Adaptive Immune Systems

Immunology is a science that defines the way an organism protects and defends itself from external and internal danger signals. Protection against pathogens relies on several different

levels of defense without exerting unwarranted damage to host cells, while generating immunological memory to recognize the same pathogen with higher efficiency in the future. This requires recognition, development, regulation, and differentiation processes to act in collaboration and ensure a successful immune response to counteract pathogens. These responses are coordinated by two major defense mechanisms, the innate and adaptive arms of immunity, which work in cooperation to overcome infection (Murphy & Weaver, 2016).

The innate immune system is comprised of both anatomic barriers, such as the skin, oral mucosa, respiratory epithelium, and intestinal lining, and chemical barriers, such as complement and secretion of antimicrobial peptides and polymorphonuclear leukocytes. The innate immune system is activated immediately following breach of these barriers with a foreign pathogen, making its defense mechanisms non-specific, and is mediated by monocyte-derived macrophages, granulocytes, natural killer (NK) cells, and serum proteins (Dalloul & Lillehoj, 2006). Granulocytes include heterophils (i.e., the equivalent of mammalian neutrophils) that possess phagocytic capability to engulf and destroy pathogens, in addition to basophils, eosinophils, and mast cells, but in smaller proportions compared to heterophils (Schat et al., 2013). Innate immune cells are outfitted with pattern recognition receptors (PRRs), such as toll-like receptors, in order to recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Murphy & Weaver, 2016). Once activated, these PRRs then initiate transcription of chemokines and cytokines, small proteins that are crucial to an immune response and are triggered for release by bacteria. Chemokines act as chemoattractants to stimulate the migration of cells to the site of infection and activate phagocytic cells and lymphocytes while cytokines can affect the behavior of other cells (Murphy & Weaver, 2016). Detection of a pathogen via PRRs initiates a rapid recruitment of additional

innate immune cells that secrete cytokines and chemokines to alter the behavior of surrounding cells through signaling, attract additional immune cells to the site of infection, and induce inflammation (Murphy & Weaver, 2016).

Following activation of the innate immune response, development of the adaptive antigen-specific memory immune response is reciprocated. This process can be mediated either humorally by B-lymphocytes (B-cells) or cellularly by T-lymphocytes (T-cells) (H.S. Lillehoj & Lillehoj, 2000). T-cell generation occurs in the thymus where subsets can gain functional identity by differentiating into either $CD4^+CD8^-$ or $CD4^-CD8^+$ T-cells, whereas B-cell generation takes place in the bursa of Fabricius (Dalloul & Lillehoj, 2006). After differentiation in their respective primary lymphoid organ, B and T cells migrate to secondary lymphoid tissues like the spleen, lymph nodes, gut-associated lymphoid tissue (GALT), or mucosal-associated lymphoid tissue (MALT) (Dalloul & Lillehoj, 2006). Secondary lymphoid tissue acts as a hub for lymphocytes to congregate and await activation via antigen presentation cells, which stimulate the lymphocytes to differentiate even further into their effector cells.

Antigen-presenting cells, like macrophages and dendritic cells, form the bridge between innate and adaptive immunity because they are able to detect, process, and present an antigen to lymphocytes (Murphy & Weaver, 2016). They reside in healthy peripheral tissues and migrate from the site of infection after detection of PAMPs or DAMPs to secondary lymphoid tissue via lymphatics to activate naïve lymphocytes that are then deployed to the site of infection. Mature $CD4^+CD8^-$ (CD4) are known as *helper* T-cells and recognize antigens presented by major histocompatibility complex (MHC) class II molecules on the cell membranes of macrophages and dendritic cells and then signal to the cell to become activated (Murphy & Weaver, 2016). Likewise, mature $CD4^-CD8^+$ (CD8) are known as *cytotoxic* T-cells and recognize antigens

presented by MHC class I molecules and directly kill the infected cell. For B cell activation, B-cell receptors on the cell membrane bind to antigens or peptides derived from endocytosed antigens presented on the cell membrane in affiliation with MHC class II molecules. Helper T-cells also recognize these MHC class II molecules, bind to the B-cell, and then aid in binding CD40 (on B-cell) to CD40L (on helper T-cell). B-cell receptor bonding to antigens coupled with CD40 binding to CD40L activates the naïve B-cell to promote immunological memory, cytokine production by macrophages and dendritic cells, and immunoglobulin secretion (Schat et al., 2013).

Macrophages possess phagocytic and cytotoxic activity to clear pathogens as well as secrete cytokines, such as interleukin-1β (*IL-1β*) and interleukin-12 (*IL-12*), in order to initiate an immune response (Murphy & Weaver, 2016). The pro-inflammatory cytokine, *IL-1β*, is introduced into peripheral circulation by activated macrophages and elicits systemic metabolic, behavioral, and cellular changes (Klasing & Leshchinsky, 1999). Injection of recombinant rat *IL-1β* was comparable in strength to administration of lipopolysaccharide when comparing reductions in body weight due to sickness-like behaviors (Dantzer et al., 1993). Secreted *IL-12*, or NK-cell stimulatory factor, activates NK cells, which are important in protecting the host from pathogens because they have the ability to induce cell lysis and secrete the cytokine interferon-gamma (IFN-γ) (Murphy & Weaver, 2016). In turn, *IFN-γ* increases the expression of MHC class II antigens and activates lymphocytes and macrophages, thus serving as a mediator of acquired immunity (H.S. Lillehoj & Lillehoj, 2000). Interferon- γ is crucial in the control of infections induced by intracellular pathogens (Schat et al., 2013), like *Eimeria*, in addition to other parameters that will be detailed below.

Immune Response to Eimeria Infection

The immunogenicity of *Eimeria* is dependent on species, making protective immunity, once attained for a specific strain, extremely specific and long lasting, but offers little help in protection against other species of *Eimeria* (Yun & Lillehoj, 2000). Although immunity is species-specific, the chicken's immune system exerts three inhibitory effects during three stages of the life cycle found in all *Eimeria* species. First, the avian immune system is able to detect an infection of parasites and works to inhibit invasion of intestinal epithelial cells (Jeurissen et al., 1996). Second, it inhibits the interaction of parasites with intraepithelial lymphocytes embedded in the GIT. And lastly, it disassociates penetration of the lamina propria (Jeurissen et al., 1996). This suggests that chickens are not able to inhibit an invasion, but rather inhibit development of *Eimeria*. Susceptibility to *Eimeria* is heightened in young chicks (i.e., less than 14 days post-hatch) lacking immunocompetence and this is especially pertinent for *E. maxima*, which is able to cause infection with a relatively small number of oocysts compared with other *Eimeria* species (Dalloul & Lillehoj, 2006; Yun & Lillehoj, 2000).

Due to its enteric nature, the majority of an *Eimeria*-induced immune response is compartmentalized to the GALT, including the intestinal mucosal layer, bursa of Fabricius, aggregates in the urodeum and proctodeum, Peyer's patches, and cecal tonsils (Befus et al., 1980). The GALT has three main functions during an enteric infection: process and present antigens, produce intestinal antibodies, such as IgA, and activate cell-mediated immunity (Dalloul & Lillehoj, 2005). Evidence suggests cell-mediation is the major factor driving an adaptive immune response during an *Eimeria* infection in broilers (Allen & Fetterer, 2002; Yun & Lillehoj, 2000). Further evidence solidifies this theory when bursectomized chicks were still able to develop protective immunity against coccidiosis, revealing that a cellular-mediated

response was likely the driving force for acquired immunity to *Eimeria* while the humoral

immune response plays only a minor role (Long & Pierce, 1963). A cell-mediated response is

characterized by antigen specific and non-specific activation to stimulate diverse cell populations

including NK cells, macrophages, and T lymphocytes, with the latter two accounting for the

majority of intestinal leukocytes during an *Eimeria* infection (Lillehoj & Trout, 1996; Vervelde

et al., 1996).

The cell-mediated response to an *Eimeria* infection is demonstrated by secretion of

various cytokines, and T helper cells differentiate based on the profile of cytokines they secrete.

The dichotomy of helper T cells has not fully been established within chickens, but type I helper

cells (T_H1) govern phagocyte-dependent protective responses, and activate macrophages, in

addition to secretion of pro-inflammatory cytokines like *IFN-γ*, interleukin-2 (*IL-2*), and tumor

necrosis factor-α (*TNF-α*) (Romagnani, 1999; Erf, 2004). In contrast, type II helper cells (T_H2)

are responsible for phagocyte-independent protective responses and inhibition of macrophages,

in addition to secretion of anti-inflammatory cytokines like interleukin-10 (*IL-10*), and

interleukin-13 (*IL-13*), thus acting as a regulator of an immune response (Romagnani, 1999; Erf,

2004). Evidence suggests that T_H1 immune responses are dominant over T_H2 immune responses

during an *Eimeria* infection. The primary cytokine secreted during a T_H1 response is *IFN-γ*, and

is one of the immunological parameters that is indispensable for host protection from *Eimeria* in

addition to lymphocytes (Chapman et al., 2013). Birds subjected to an *E. acervulina* challenge

exhibited local expression of *IFN-γ* mRNA in the intestine (Choi et al., 1999). Splenic

lymphocytes isolated from *Eimeria*-challenged birds also exhibited an elevated concentration of

IFN-γ protein consequent to *ex vivo* mitogen stimulation (Martin et al., 1994). When birds were

infected with *E. acervulina* and treated with injections of chicken-IFN-γ, birds that received

chicken-*IFN-γ* treatment lost less weight and recovered more quickly (Lowenthal et al., 1997).
These studies demonstrate the reliance of IFN-γ-producing T_H1 cells during *Eimeria* challenges
and further attest to cell-mediated immune responses being the driving force for combatting an
Eimeria infection.

Cytokines and chemokines also play a crucial role during the immune response to an
Eimeria infection. Secretion of *TNF-α*, a pro-inflammatory cytokine, from splenic macrophages
was significantly increased 3-6 days after *Eimeria* inoculation (Byrnes et al., 1993). Expression
of *IL-2*, a potent growth factor cytokine for T-cell proliferation, was increased in spleen
lymphoblasts following primary and secondary infections with *E. acervulina.* This increase in
expression of *IL-2* coincided with an increase in splenic γδ T-cells, suggesting favorable growth
of T-cells via increased production of *IL-2* following secondary infection with *Eimeria* (Choi &
Lillehoj, 2000). Seven days following an *E. tenella* infection, RNA was isolated from cecal
tissue and the chemokines K203 and macrophage inflammatory factor 1β were upregulated by
200-fold and 80-fold, respectively (Laurent et al., 2001).

An acute phase response (APR) is a non-specific innate immune response and is integral
in early-defense against pathogens (Cray et al., 2009). An APR causes systemic disturbances in
homeostasis such as anorexia, fever, and cytokine production, but most importantly, are the
changes in plasma proteins called acute-phase proteins (APP) (Schat et al., 2013). Any protein
whose plasma concentration is altered by 25% or more succeeding an inflammatory stimuli is
classified as an APP and host defense APPs include C-reactive protein, mannan-binding lectin,
and fibrinogen (Schat et al., 2013). Following an infection with *Eimeria*, acute-phase proteins in
the serum were found to be altered using a proteomics approach (Gilbert et al., 2011). Georgieva
et al. (2010) also saw a significant increase in APPs (fibrinogen and ceruloplasmin) following

inoculation with *E. tenella.* The relevance of APPs during an *Eimeria* infection may not be well understood, but stimulation of increased levels of APPs is evident and further research to pinpoint their role during an *Eimeria*-induced immune response is of importance.

Health-related Feed Additives

Importance

Antibiotics are utilized in a variety of ways in animal agriculture and the possible implication this has on human health has become a major concern in the past decade for both producers and consumers. The Food and Drug Administration's Center for Veterinary Medicine approves the use of antibiotics in food animals for disease treatment, control, prevention, and nutritional efficiency (Institute, 2018). It is the latter of the four uses that has caused consternation amongst the food animal industry and has led to the implementation of new regulations on the use of antibiotics in food animal production.

The use of antibiotics to promote nutritional efficiency was first reported in 1946 when Moore and colleagues published evidence on the growth promoting effects of subtherapuetic levels (i.e., less than amount required) of sulfasuxidine, streptothricin, and streptomycin in the diets of chicks. Since then, antibiotics have been incorporated into the diets of livestock species as antibiotic growth promotors (AGPs) and are attributable to the economic effectiveness and sustainability of livestock production (Wierup, 2000). In an attempt to quantify the net effect of AGPs, Thomke & Elwinger (1998) proposed as much as a 3-4% increase in broiler growth performance may result from increased feed efficiency and weight gain when compared with broilers fed unsupplemented control diets. However, some of the antibiotics used at sub-therapeutic levels in food animals as growth promotors are identical to the ones utilized in the

human health field, begging the question of whether antibiotic resistance in humans begins with animal agriculture (Cosby et al., 2015; Endtz et al., 1990; Gadde et al., 2017). Evidence of the transmission of resistance from animal to human was first observed when identical clones of *E. coli* isolates were present in fecal samples from both broilers and broiler farmers (van den Bogaard et al., 2001). Although evidence exists, the correlation between antimicrobial resistance in food animals and humans has never been clearly defined, leading to an ongoing discussion amongst scientists, government officials, and the public.

Because of this, World Health Organization met in 1997 to determine whether the use of AGPs in animal agriculture had any impact on the escalation of antimicrobial resistance in humans (World Health Organization, 1997). Shortly thereafter, small groups of antibiotics being used as growth promotors in the Europe Union (EU) were banned, inevitably leading to their complete demise in January 2006, when AGP sale and marketing was prohibited entirely in the EU (Regulation No. 1831/2003) (European Commission, 2003).

Following suit of the EU, the U.S. Food and Drug Administration took their first official regulatory step in December, 2013, by publishing Guidance for Industry #213, a non-binding recommendation that strongly encouraged animal drug sponsors to stop marketing antibiotics as growth promotors and that the remaining use of AGPs be brought under veterinarian supervision by December, 2016 (US Food and Drug Administration, 2013). Drugs that were once over-the-counter and incorporated into the diets of food animals soon transitioned to requiring a veterinary feed directive as of January 1, 2017. Some of these drugs include aureomycin, penicillin, erythromycin, and sulfaquinoxaline (Administration, 2016). These changes essentially make it more difficult for a producer to incorporate AGPs into the diets of their animals, although they have not been officially banned in the U.S..

The poultry industry has relied heavily on the use of anticoccidial drugs when trying to alleviate the detrimental effects of coccidiosis. The use of an anticoccidial drug was first documented by Levine (1939) when he investigated the drug sulfanilamide and its ability to inhibit coccidiosis. Anticoccidial drugs are a kind of antibiotic that specifically counteract *Eimeria* and are quantified based on their efficacy of coccidiostatic activity (Reid et al., 1969). Inevitably, after their discovery, a shift in the susceptibility of an anticoccidial drug to mitigate coccidiosis was noted and an acquisition of resistance to all anticoccidial drugs has been demonstrated globally (Abbas et al., 2011).

In the past, bacterial resistance to antimicrobial drugs was met with the development of novel classes of drugs or chemical alterations to existing drugs to circumvent antimicrobial drug resistance. Presently, there is no assurance that the development of novel drugs and chemical alterations will be able to keep up with the ability of bacterial pathogens to develop drug resistance (Gold & Moellering, 1996). Because of this, and the growing pressure from regulatory bodies and public concern, there exists a strong impetus to develop alternatives for antibiotics and anticoccidial drugs that do not allow for significant detriments in broiler growth performance.

Alternatives to antibiotics and anticoccidial drugs: Phytogenic compounds

The Animal Health Institute in 1998 estimated that the U.S. would require an additional 452 million chickens, 23 million more cattle, and 12 million more pigs in order to maintain the present level of animal production without the use of growth promoting antibiotics (Hughes & Heritage, 2004). In addition, the National Chicken Council reported that chicken consumption per capita has increased nearly every year since the mid 1960's, and Americans, in particular, purchase more chicken than any other meat source (Council, 2017). These two powerful

statistics demonstrate the pressure the poultry industry is under to keep up with the ever-growing demands of consumers for poultry, while shifting to an unavoidable antibiotic-free era. Due to an increase in resistance to anticoccidial drugs and also because of increased public concern about the increase in antibiotic resistance to microorganisms, evidence of nutritional alternatives, specifically phytogenic compounds, to combat coccidiosis is becoming more relevant.

Numerous alternatives have been introduced to the poultry industry that strive to maintain optimal growth performance while promoting overall bird health. Some of the class of alternatives include probiotics, prebiotics, synbiotics, organic acids, enzymes, hyper immune egg yolk antibodies, clays, bacteriophages, and lastly, phytogenic compounds (Gadde et al., 2017). Feed International conducted a world-wide nutrition and feed survey to investigate how poultry farmers are formulating their poultry diets for antibiotic-free production. Thirty-five percent of respondents reported using phytogenic compounds/essential oils as alternatives for AGPs in their feed formulations (Roembke, 2016).

Phytogenic compounds are a group of non-antibiotic feed additives of plant origin that are incorporated into animal feed and are available in the form of solids, extracts, or essential oils (Yitbarek, 2015). When compared with inorganic chemicals and synthetic antibiotics, it has been suggested that phytogenic compounds are natural and less toxic additives that possess antimicrobial and antifungal properties (Yitbarek, 2015). The efficacy of a phytogenic compound varies greatly depending on plant parts used (e.g., seed, root, flowers, buds, and bark), geographical origin of the source plant, and harvest season (Muthusamy & Sankar, 2015). Other potential factors can affect the overall bioactivity of a phytogenic compound such as extraction method (Handa et al. , 2008), housing conditions (Pirgozliev et al., 2014), and synergistic effects when combining compounds (Yang et al., 2015). Classes of phytogenic compounds include

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alkaloids, glycosides, flavonoids, phenolics, saponins, tannins, terpenes, anthraquinones, essential oils, and steroids (Doughari, 2012).

Plants that possess an array of phytogenic compounds have been utilized as potential alternatives to anticoccidial drugs in order to ameliorate the effects of coccidiosis in chickens. Coccidiosis was alleviated in chickens when extracts from *Sophora flavescnes,* Oregeno, and *Astragulus membranaceus* were used (Applegate, 2009). Dried *Artemesia annua*, a plant that contains phytogenic compounds like flavonoids and saponins (Kumar Ashok & Upadhyaya, 2013), when incorporated into the diet at a 5% inclusion rate over a three week period, reduced *E. tenella*-induced cecal lesions (Allen et al., 1997). An extract derived from the herb *U. macrocarpa* increased survival rate and decreased lesion scores in *Eimeria* challenged birds (Youn & Noh, 2001). Saponins are one class of phytogenic compounds that are of plant origin and may prove to be beneficial in terms of mitigating coccidiosis in chickens.

Saponins

Dietary intervention as an alternative to antibiotics and anticoccidial drugs is gaining interest, specifically plant extracts that contain powerful secondary metabolites, or phytochemicals. One such phytochemical, saponin, has been suggested as an alternative strategy for controlling effects of coccidiosis (Galli et al., 2018). Saponins are naturally occurring steroids or surface-active triterpenoid glycosides whose name is derived from the ability of a saponin to form stable, soap-like foams in aqueous solutions, thus making them strong surfactants (Francis et al., 2002). A majority of saponins are produced by plants, but they can also originate from lower marine organisms (Riguera, 1996). Desert plants are abundant in saponins; the two most common commercial sources are the Mexican desert derived *Yucca schidigera* and the Chile derived *Quillaja saponaria* (Cheeke, 2000). Saponins are also found in wild plants and have

been detected in soybeans, beans, peas, horse chestnut, oats, and ginseng (Francis et al., 2002).

Structurally, a saponin consists of a lipophilic nucleus, either steroid or triterpenoid in structure

depending on plant origin, with one or more water-soluble carbohydrate side chains (Cheeke,

2000). The classification of a saponin is dependent on nucleus structure, thus classifying the

Yucca schidigera derived saponins as steroidal and the *Quillaja saponaria* derived saponins as

triterpenoidal (Francis et al., 2002). Isolation of saponins has proven rather difficult due to their

particular polar, amphiphilic nature that allows them to occur as complex mixtures with other

similar surrounding secondary metabolites and the absence of a chromophore imposes further

hindrance (Marston et al., 2000). Bark and wood from *Quillaja saponaria* must be subjected to

boiling, condensation, and evaporation techniques, whereas the trunk of *Yucca schidigera* must

be subjected to maceration and evaporation techniques for isolation of saponins (Cheeke, 2000).

Although isolation of saponins is difficult, recent technologic advancements and modernization

of techniques have allowed for nearly complete isolation of saponins via chromatographic

techniques, like high-performance liquid chromatography, within reasonable quantities for an

overall quality control test of a product (Marston et al., 2000).

The unique chemical structure of a saponin enables it to have different biological effects

on animal health. Saponins extracted from *Yucca schidigera* have proven to be especially

beneficial for controlling ammonia and odor emissions in animal rearing facilities. This is

especially pertinent to poultry rearing facilities because chickens excrete uric acid, a compound

that can be converted to ammonia, as the principal nitrogenous constituent of their excreta due to

their lack of complete urea cycling (Stevens, 1996). Birds fed a diet containing 120 mg of *Yucca

schidigera*/kg of diet significantly decreased the ammonia concentration measured in broiler

houses (Çabuk et al., 2004). The exact mode of action by which saponins reduce ammonia

emissions is poorly understood, but Makkar et al. (1999) demonstrated that *Yucca schidigera* was more effective than *Quillaja saponaria* in binding to and therefore reducing concentrations of ammonia. Duffy and colleagues (2001) suggested that both the non-butanol-extractable fraction (i.e., mainly non-saponin carbohydrates) and the butanol-extractable fraction (i.e., saponins) of *Yucca* extract are attributable for nitrogen-metabolism, indicating that the active constituents are present in both fractions and working collectively, rather than one or the other.

In addition to their odor controlling capability, saponins also possess antiprotozoal and antibacterial activities due to their ability to disrupt cellular membranes (Cheeke et al., 2006). Saponins exhibit lytic action on protozoal cell membranes, but the exact molecular mechanisms for doing so are poorly understood. Probable modes of action for saponins and their ability to modify membrane function include the formation of insoluble saponin-cholesterol complexes with protozoal cells, formation of phospholipid breakdown products, and alterations in the organization of sarcolemma membrane phospholipids, all of which can modify protozoal membrane structure and function leading to eventual cell lysis (Francis et al., 2002). Protozoal diseases that undergo a large majority of their life cycle in the gastrointestinal tract act in response to the antiprotozoal activity of saponins (Cheeke et al., 2006). The target membrane and orientation of a saponin can also affect the efficacy of its ability to disrupt the membrane interaction (Francis et al., 2002). Steroidal saponins, like the ones found in *Yucca schidigera*, have been shown to exhibit antibacterial and antiprotozoal effects in ruminal microorganisms (Wallace et al., 1994). A powdered preparation of *Yucca schidigera* was effective in killing tropozoites from *Giardiaintestinalis*, a common protozoal intestinal pathogen similar in nature to that of *Eimeria* (McAllister et al., 2001). Addition of *Yucca* extract at 350 ppm enhanced a protective effect against coccidiosis for its ability to decrease the presence of oocysts in excreta

(Galli et al., 2018). Guar meal, a high protein by-product of galactomannan gum, is saponin-rich (5-13% by weight of dry matter) and when included at a dietary concentration of 5%, reduced the number of oocysts per gram in excreta when broilers were challenged with *E. tenella* (Hassan et al., 2008). These results demonstrate the antiprotozoal activity of saponins and their ability to inhibit the growth of coccidiosis as seen by a decrease in oocyst shedding following an *Eimeria* challenge.

Studies indicate that saponins may also improve nutrient digestibility. Johnson et al. (1986) established *in vitro* that saponins, both triterpenoid and steroidal, readily increased permeability of the mucosal small intestine cells. This was established by showing inhibition of active transport through a carrier-mediated galactose transporter, which in turn impeded uptake of nutrients that the small intestine was otherwise impermeable by simultaneously increasing the uptake of passively transported L-isomer glucose. An increase in nutrient absorption has the potential to improve growth performance through increases in feed efficiency. When incorporated in broiler diets at 100 mg/kg, *Yucca schidgera* extract increased average daily gain and feed efficiency in broilers during the finisher phase (d 29 to d 42) (Sun et al., 2017). Sahoo et al. (2015) also reported an improvement in the feed conversion ratio of broilers when supplemented with 125 mg/kg of diet of a *Yucca* supplement, in addition to a decrease in agonistic behavior like pecking and avoidance of other birds. In contrast, saponins have the ability to interact with protein in the diet and decrease digestibility, dependent on protein source. When *Quillaja*-derived saponins were fed to gerbils in conjunction with two different protein sources, casein and isolated soy protein, serum cholesterol concentrations were decreased by 32% in casein-fed gerbils. These results suggest that *Quillaja*-derived saponins can modify blood lipids in the presence of casein by altering digestion of proteins, resulting in a slower release of

protein when compared to ingestion of these protein sources alone (Potter et al., 1993).

Therefore, protein source should be taken into consideration when formulating a diet that

incorporates saponins. If used to their potential, saponins may prove to be a natural alternative to

antibiotics and anticoccidial drugs due to their antiprotozoal activity and ability to enhance

nutrient absorption and growth in broilers.

Due to limitations of current strategies to control coccidiosis, and because there are

significant economic losses due to decreased body weight gain and feed efficiency in affected

animals, identifying immunomodulating compounds to limit coccidiosis and improve

performance in poultry is of great value. Collectively, the use of dietary saponins may serve as

an effective strategy in mitigating the resulting inflammatory response following exposure to

Eimeria in broilers.

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CHAPTER 3: EFFECTS OF *YUCCA*-DERIVED SAPONIN SUPPLEMENTATION DURING A MIXED *EIMERIA* CHALLENGE IN BROILERS

Abstract

An experiment was conducted to determine if dietary *Yucca*-derived saponin supplementation could ameliorate the immune and growth responses of broilers during a mixed coccidian challenge. A total of 576 two-day-old male Ross 308 broiler chicks were housed in galvanized starter batteries and randomly assigned to 1 of 4 dietary treatment groups (12 replicate cages of 7 birds). Dietary treatments were corn-soybean meal-based and included: 1) control diet + sham-inoculated (Ucon), 2) control diet + *Eimeria* oocyst challenge (Icon), 3) control diet with 250 mg/kg *Yucca*-derived saponin product + *Eimeria* oocyst challenge (ISap250), and 4) control diet with 500 mg/kg of *Yucca*-derived saponin product + *Eimeria* oocyst challenge (ISap500). On study d 14, birds were orally inoculated with 1.5 ml of tap water containing *E. acervulina*, *E. maxima*, and *E. tenella* (100,000, 40,000, and 30,000 oocysts/dose, respectively), or sham-inoculated with 1.5 ml of tap water. *Eimeria*-challenged birds exhibited a reduction in growth compared with uninfected birds ($P < 0.001$); however, there were no detectable differences due to dietary treatment among *Eimeria*-challenged groups. Mucosal thickness in the jejunum was increased in all infected groups ($P < 0.05$), but there were no differences among infected groups, however, saponin supplementation included at 250 mg/kg was not significantly different from the uninfected birds. Lymphocytes as a percentage of total white blood cells were increased ($P < 0.05$) in all *Eimeria*-challenged groups at 7 days post inoculation compared with uninfected birds, but birds supplemented at 250 mg/kg were not different from uninfected birds. Saponin supplementation in conjunction with an *Eimeria* challenge had no effect on cecal-derived T-cell profiles ($P > 0.05$). Cecal and duodenal *IFN-γ* expression increased with infection when compared with sham-inoculated birds. Cecal and

duodenal *IL-1β* expression increased due to infection and ISap250 and ISap500 treatments ameliorated *IL-1β* expression to levels not different from sham-inoculated birds ($P < 0.05$). Interleukin-12 β expression increased in the duodenum of cocci-infected birds compared to uninfected birds ($P < 0.05$), but dietary treatments had no measureable effects. These results suggest that saponin supplementation may provide some immunomodulatory effects during a mixed coccidian challenge as evidenced by lymphocyte responses, changes in intestinal structure, and alterations in cecal and duodenal inflammatory cytokine mRNA expression.

Introduction

Avian coccidiosis is a major parasitic disease affecting the poultry industry resulting from intestinal parasitic infection by the protozoan species of genus *Eimeria* and is said to be found wherever chickens are raised (Williams, 1998). There are seven different species widely encountered within the poultry industry, but only *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, and *E. tenella* yield quantifiable gross lesions in varying spots along the gastrointestinal tract (GIT), the preferred site of habitation in broilers (Allen & Fetterer, 2002). Coccidiosis is manifested by a destruction of the intestinal epithelia and has the ability to initiate an immune response from the host, thereby leading to increased maintenance costs, decreased absorption of nutrients, and ultimately diminished animal growth (Preston-Mafham & Sykes, 1970). Coccidiosis is one of the most economically significant diseases for poultry producers worldwide due to its cost of control measures, prevalence in poultry rearing facilities, and subsequent consequences on growth performance parameters (Williams, 1999).

Because of the pathogenicity of *Eimeria* species, reproductive life cycle, and current management practices within the poultry industry, a variety of control measures have been

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developed to mitigate the detrimental effects of coccidiosis. The poultry industry has successfully relied on the use of prophylactic chemotherapeutic agents, or anti-coccidial drugs, which can directly affect parasite metabolism, osmotic balance, and ion transport in the GIT of birds to make the intestinal environment less habitable (Chapman, 2001, 2014; McDougald, 1998). However, due to an increase in resistance to anti-coccidial drugs (Abbas et al., 2011) and also because of increased public concern about the increase in antibiotic resistance microorganisms (Cosby et al., 2015), alternatives to combat coccidiosis in order to ameliorate the economic losses due to decreased growth performance in affected chickens has become of great importance.

Numerous phytogenic compounds have been introduced to the poultry industry as alternatives that strive to maintain optimal growth performance while promoting overall bird health (Gadde et al., 2017). One such phytogenic compound, saponins, have been reported to improve nutrient digestibility (Johnson et al., 1986), growth performance (Sahoo et al., 2015; Sun et al., 2017), and odor control in broilers (Çabuk et al., 2004). Saponins are natural detergents that have the ability to bind to membrane cholesterol of protozoan cells leading to eventual cell lysis and cell death, giving rise to anti-protozoal properties as well (Francis et al., 2002). Based on previous literature indicating that saponins have beneficial effects, the objective of the current study was to determine if dietary supplementation of *Yucca*-derived saponins could ameliorate the immune and growth responses of broilers during a mixed coccidian challenge.

Materials and Methods

All animal care and experimental procedures were approved by the University of Illinois Institutional Animal Care and Use Committee before initiation of the experiment.

Bird Husbandry

Five hundred and seventy-six male Ross × Ross 308 broiler chicks were obtained 2 d post-hatch from a commercial hatchery and placed in thermostatically-controlled batteries with raised wire floors in an environmentally-controlled room with continuous lighting. Upon arrival, birds were weighed, wing-banded, and assigned randomly to 1 of 4 dietary treatment groups, and allotted to pens (99 cm x 34 cm) such that average initial group weights and weight distributions were similar across treatments. Each treatment was replicated with 12 battery cages (n=12) each containing a total of 12 chicks at study initiation. Birds were provided free access to water and experimental diets (Table 1) that met or exceeded requirements for this age of broilers (NRC, 1994). All diets were corn-soybean meal based and saponin supplementation (Micro-Aid® Feed Grade Concentrate; DPI Global, Porterville, CA) was included on top of the formulation (i.e., no space reserved in the formulation) as having negligible nutritive contribution to the overall diet. The following diet names were assigned to the experimental treatment groups:1) control diet + sham-inoculated (Ucon), 2) control diet + *Eimeria* oocyst challenge (Icon), 3) control diet with 250 mg/kg of *Yucca*-derived saponin product + *Eimeria* oocyst challenge (ISap250), and 4) control diet with 500 mg/kg of *Yucca*-derived saponin product + *Eimeria* oocyst challenge (ISap500). Bird and feeder weights were recorded on the day of arrival, and then bi-weekly until study completion for calculation of body weight gain, feed intake, and feed efficiency to assess growth performance. At 16 d of age (d 14 of study), birds were weighed and the number of birds in each pen was adjusted to leave 7 birds per cage for the inoculation period. The 5 birds that were chosen to be removed were chosen such that when the 12 birds were placed in order of descending weight, the lightest 3 birds and heaviest 2 birds were culled. One bird was selected from each replicate cage on days 14, 21, and 28 of study (0, 7, and 14 days post-inoculation

(DPI)) and humanely euthanized for sample collection. The bird chosen for sample collection was based on weight such that when the birds were placed in descending order by weight, the bird closest to the average weight was chosen. Mortality during the study was less than 8% and was unrelated to treatment. Feed efficiency and feed intake were corrected to include weight gain of birds that died during the trial.

Proximate Analysis of Experimental Diets

A sample of each experimental diet was used to analyze the nutrient composition of each individual diet (Table 2). Diets were analyzed for dry matter (DM) (method 934.01, AOAC International, 2002) and organic matter, which was done by first determining percent ash (method 942.05, AOAC International, 2002) and subtracting that from 100. Crude fat was determined by using the traditional Soxhlet extraction method with diethyl ether (method 920.39, AOAC International, 2006) and crude protein was determined by measuring nitrogen using a Leco analyzer (TruMac N, Leco Corp., St. Joseph, MI) standardized with EDTA (method 990.03, AOAC International, 2006). Fiber analysis was determined by quantifying neutral detergent fiber and acid detergent fiber (method 2002.04, 973.18, AOAC International, 2002). Gross energy was determined using an adiabatic bomb calorimeter (Parr 6200, Parr Instruments, Moline, IL) standardized with benzoic acid.

Eimeria Challenge

Three strains of *Eimeria,* acervulina, maxima, tenella, were originally obtained from the University of Arkansas, Fayetteville, AR (courtesy of Dr. David Chapman) and were maintained in our laboratory by periodic passage (every 3 to 5 months) through broiler chickens. Oocysts were isolated from excreta and allowed to sporulate in 2.5% $K_2Cr_2O_7$ at 28°C under forced aeration before storage at 4°C. Immediately before inoculation, sporulated oocysts were washed

free of $K_2Cr_2O_7$ and diluted with distilled water to desired concentrations. At 16 d of age (0

DPI), birds were challenged orally with 1.5 ml of tap water containing a mixture of *E. acervulina*

(100,000 oocysts per dose), *E. maxima* (40,000 oocysts per dose), and *E. tenella* (30,000 oocysts

per dose) or sham-inoculated (unchallenged) with 1.5 ml of tap water orally. These inclusion

levels of *Eimeria* oocysts were derived from a recent study conducted in the lab and served to

induce a mild-to-moderate reduction in growth performance of the birds. Birds infected with

Eimeria were housed in batteries separated from the unchallenged birds. After the coccidial

challenge, all personnel were required to conduct procedures on unchallenged birds before

working on the *Eimeria*-infected birds to maintain biosecurity. Infection was confirmed by

oocyst counts of excreta samples collected from pans beneath each pen on days 7 and 14 DPI for

oocysts per gram outcomes. On the day of collection, samples were homogenized, and *Eimeria*

oocysts were enriched then diluted based on their infection status, and counted using a McMaster

counting chamber (Chalex Corporation, Ketchum, ID).

Histopathological Morphometries & Lesion Scoring

At 14 DPI, independent sub-samples from the jejunum and the duodenum were collected

from one bird in each pen. The bird chosen for sample collection was based on weight such that

when the birds were placed in descending order by weight, the bird closest to the average weight

was chosen. Immediately following sample collection of fresh tissue from the bird, the

duodenum and jejunum were immersed in 10% neutral buffered formalin and sent to an external

histopathology laboratory for morphometrical analysis by a board-certified histopathologist

(Veterinary Diagnostic Pathology, LLC, Fort Valley, VA). Sections of tissue (2 mm) were

trimmed from the submitted tissue, placed into cassettes, and prepared for paraffin-embedded 5

µm sections stained with hematoxylin and eosin (H&E). In order to ensure uniformity of

assessment, intestinal sections were kept intact in circular form. The duodenal sub-sample was also utilized for histopathological lesion scoring. A lesion scoring system developed for commercial poultry production gut assessment and inclusive to the findings of the project were used and lesions were semi-quantitatively scored for severity as 0, normal; 1, minimal severity; 2, mild severity; 3, moderate; 4, marked; and 5, severe (APPENDIX).

Differential Blood Cell Counts

On 0, 7 and 14 DPI, one bird per pen was euthanized for collection of blood. The bird chosen for sample collection was based on weight such that when the birds were placed in descending order by weight, the bird closest to the average weight was chosen. Chicks were bled by cardiac puncture into evacuated tubes. A sample (~2 mL) of blood was submitted to the Veterinary Clinical Pathology Laboratory at the University of Illinois at Urbana-Champaign for measuring the total and differential blood cell counts at each time-point.

Immunophenotyping

Immunophenotyping of T-cell populations was conducted at 14 DPI using cecal tonsils (same bird used for differential blood cell counts). Cecal tonsils were collected and placed on ice until cell isolation was conducted. Briefly, mononuclear cells were isolated by crushing cecal tonsils through a cell strainer to allow cells to flow through and onto a density gradient (Histopaque; Sigma Aldrich, St. Louis, MO). Following centrifugation (1800 rpm $\times$ 20 min.; 25°C; without a brake), mononuclear cells were separated, washed twice, and their concentrations were adjusted to 1×10^6 total cells. Cells were then surface-stained using the following antibody clones and conjugated fluorochromes: anti-CD3-FITC (clone: CT-3), anti-CD4-PE (clone: CT-4), and anti-CD8α-APC (clone: CT-8) (Southern Biotech, Birmingham, AL). Once stained with theses surface markers, cells were washed three times and then

permanently fixed with 2% paraformaldehyde for 10 minutes at room temperature, and then washed an additional three times. Cells were left at 4° C overnight until analysis the following day. The relative percentage of different phenotypes of T cells (i.e., single stain for CD4 or CD8 double positive T-cells) were determined using multi-color flow cytometry (BD LSR II Flow cytometry analyzer, Roy J. Carver Biotechnology Center, University of Illinois, Urbana-Champaign, IL).

Inflammatory Cytokine mRNA Expression

Samples of cecal tonsils and duodenum were collected at 7 DPI for quantitative real-time polymerase chain reaction (PCR) in order to quantify relative gene expression of the pro-inflammatory cytokines interferon-γ (IFN-γ), interleukin -1β (*IL-1β*), and interleukin-12β (*IL-12β*). Samples were collected and immediately submerged in RNAlater stabilization solution (AM7020; Thermo Fisher Scientific Inc., Waltham, MA) and placed at room temperature overnight to allow its penetration into the tissues. Subsequently, 24-h following collection, samples + RNAlater were then moved to -80°C freezer pending further analysis. Upon analysis, cecal tonsil and duodenum tissue samples, (50 to 100 mg), were placed into a 2 mL microcentrifuge tube along with 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA) and a 5 mm steel bead. Samples were homogenized through tissue disruption for 2 minutes at 30 Hz (TissueLyser II, Qiagen, Valencia, CA) and RNA was extracted following manufacturer instructions. Extracted RNA was quantified using a spectrophotometer, with samples having a 260:280 absorbance ratio of 1.8 or higher (NanoDrop ND-1000, Nano-Drop Technologies, Wilmington, DE). The extracted RNA was transcribed to complementary DNA (cDNA) using a high capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific Inc., Waltham, MA). The samples were placed in a thermocycler (Bio-Rad, Hercules, CA) set to 25°C for 10 minutes,

37°C for 120 minutes, 85°C for 5 minutes, and then cooled at 4°C and held there until the cDNA samples were removed and stored at -20°C until plating. The TaqMan Gene Expression Assay (Thermo Fisher Scientific Inc., Waltham, MA) was used to perform quantitative real-time PCR to quantify relative gene expression of pro-inflammatory cytokines *IFN-γ* (NM_205149.1), *IL-1β* (NM_204524.1), and *IL-12β* (NM_213571.1). Amplification was achieved by PCR for both target (*IFN-γ*, *IL-1β*, *IL-12β*) and reference (GAP-DH, NM_204305.1; Hong et al. 2006) chicken genes. Sample cDNA was amplified using TaqMan (Thermo Fisher Scientific Inc., Waltham, MA) oligonucleotide probes containing 5′ fluorescent reporter dye (6-FAM) and 3′ non-fluorescent quencher dye, and fluorescence was determined using a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). Gene expression was normalized through parallel amplification of endogenous GAPDH for each sample. Relative gene expression was calculated using the comparative threshold cycle (Ct) method (Livak & Schmittgen, 2001) and results are expressed as fold-change relative to sham-inoculated broilers fed the control diet.

Statistical Analyses

The experimental design was a completely randomized block design. Data were subjected to an analyses of variance using a MIXED procedure of SAS (version 9.4). A 1-way ANOVA was used to determine whether the model was significant, and when appropriate, means separation was conducted. Least-square means and the standard error of the mean estimates were derived from this 1-way ANOVA. Replicate pen served as the experimental unit for all outcomes, and significance was accepted with a *P*-value of less than 0.05.

Results

Growth Performance

All growth performance data are shown in Table 3. Oocysts per gram for treatment Ucon yielded positive results, but because of values near that of zero, a high dilution factor, and possible cross contamination of just excreta pans, not the birds themselves, from previously-conducted studies, these positive values were viewed as negligible and an anomaly when compared with the other treatment groups and were excluded from the final dataset. Birds challenged orally with the *Eimeria* mixture exhibited a reduction in growth compared with birds not challenged with the *Eimeria* mixture. During the first 14 days of the study, birds in treatment ISap500 had increased feed intake compared with birds in all other treatments. Birds assigned to ISap500 also had a decrease in gain:feed during the first 14 days of study.

Histopathological Morphometrics

For the two parameters crypt depth and the villus height to crypt depth ratio (villus:crypt ratio), a difference between *Eimeria*-infected and non-infected groups was observed (Table 4). However, when comparing within infected groups, there were no statistical differences noted for crypt depth, though there was a reduction in the villus:crypt ratio for birds assigned to the ISap250 treatment when compared with other dietary treatments. A treatment effect was noted for mucosal thickness in the jejunum, where ISap250 and UCon yielded similar results, with a numeric reduction in mucosal thickness in birds fed ISap250 compared with the other *Eimeria*-infected groups.

Histopathological Lesion Scoring

From lesion scoring data alone (Table 5), the birds challenged orally with the *Eimeria* mixture exhibited an increase in the following outcomes when compared with sham-inoculated

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birds: incidence of lamina propria lymphocytes and plasma cells, intraepithelial leukocytes, enteritis index, and total enteritis index. However, there were no detectable differences due to dietary treatment within the *Eimeria*-infected groups.

Differential Blood Cell Counts

At 0 DPI, total protein for ICon and ISap250 were elevated compared to Ucon and ISap500, which did not differ from each other (Table 6). At 7 DPI, hematocrit and heterophils showed differences only between uninfected and *Eimeria*-infected birds, and not between treatments within infected groups. Band heterophils and monocytes/azurophilic granules were elevated for ISap250 and ISap500 when compared with other treatment groups and basophils measurements of ISap250 were significantly higher when in comparison to all other treatment groups. Lymphocytes were elevated in all *Eimeria*-infected treatment groups and did not differ from one another when compared with UCon, but ISap250 was not significantly different than the Ucon birds. At 14 DPI, there was no detectable difference in differential blood cell counts amongst the dietary treatment groups. Taken together, uninfected birds outperformed birds that were *Eimeria*-infected; however, birds fed an inclusion rate of 250 mg/kg of diet of saponin supplementation did express similar lymphocyte percentages to that of the uninfected birds at 7 DPI.

Immunophenotyping

All immunophenotyping data are shown in Table 7. There was no significant difference between immunophenotyping of different subsets of T-cells derived from mononuclear cells from chicken cecal tonsils fed different levels of saponin supplementation during a coccidial oocyst challenge.

Gene expression

Gene expression data are shown in Table 8 and are reported as a fold-change relative to the sham-inoculated birds fed the control diet. In the cecal tonsils, *IFN-γ* mRNA expression increased in Icon, ISap250, and ISap500 when compared to Ucon ($P < 0.001$). Expression of cecal mRNA *IL-1β* was increased only for the treatment Icon, but not for the ISap250 and ISap500 treatments ($P < 0.01$). Treatments ISap250 and ISap500 significantly decreased expression of *IL-1β* in comparison to treatment Icon. Duodenal *IL-1β* mRNA expression increased with infection, but remained unchanged with saponin supplementation at both the 250 and 500 mg/kg inclusion rates. Duodenal *IL-12β* mRNA expression was increased with infection, however, birds fed ISap250 were not different from the Ucon birds ($P < 0.05$). Duodenal *IFN-γ* mRNA expression was also increased with infection ($P < 0.001$).

Discussion

Based on previous literature indicating that saponins have beneficial effects during parasitic infections, the objective of the current study was to determine if dietary supplementation of saponins via an extract from *Yucca schidigera* could ameliorate the immune and growth responses of broilers during a mixed coccidian challenge. We observed that saponin supplementation during a mixed coccidian challenge had no beneficial effects on growth performance, however, it may influence the immune response to *Eimeria* infection as evidenced by lymphocyte responses, changes in intestinal structure, and alterations in cecal and duodenal inflammatory cytokine mRNA expressions.

Growth performance responses to saponin supplementation appear to be highly variable. Studies by Johnston et al. (1980) and Su et al. (2016) both report saponin supplementation via an

extract from *Yucca schidigera* serves as an effective growth promoter in broilers, while

Sariozkan et al. (2015) reported *Yucca* extract had no significant influence on growth

performance of broilers. Interestingly, Balog and collegues (1994) demonstrated a significant

decrease in body weights with *Yucca* feeding. These studies measured performance in

unchallenged broilers. When saponin supplementation was used in conjunction with an *Eimeria*

challenge, Alfaro et al. (2007) recorded broilers had higher BWG, however, this study also used

a commercial coccidiosis vaccination at the initiation of the study, suggesting that improvements

in BWG may have been due to a synergistic effect between the supplementation and coccidiosis

vaccine. Herein, we report that birds challenged orally with the *Eimeria* mixture exhibited a

reduction in overall growth compared with birds not challenged with the *Eimeria* mixture. From

these results, we concluded that sham-inoculated birds outperformed *Eimeria*-infected birds, but

there were no detectable differences due to dietary treatment among *Eimeria*-infected groups. A

possible explanation for no detectable differences amongst dietary treatments in growth

performance may be due to the reduction in bio-efficacy of saponins when used during an

immune challenge. Recently, antibiotic treatment was shown to alter microbiome-independent

changes in host metabolites during infection, which in turn inhibited the antibiotic's efficacy

(Yang et al., 2017). As a possible alternative to antibiotics, saponins bio-efficacy could have

been dampened due to infection with *Eimeria*, thus warranting no outwardly improvements in

growth performance amongst dietary treatments.

Evidence of a successful *Eimeria* challenge was made possible through quantification of

oocysts in excreta collected from birds. Birds challenged with *Eimeria* were shedding oocysts up

to 622,695 oocysts/g of excreta at d 21 of study, but there was no treatment effect of saponin

supplementation noted among *Eimeria*-inoculated treatment groups. This finding is contrary to

previous studies in which guar meal, a high protein by-product of galactomannan gum rich in

saponins, reduced the number of oocysts per gram in excreta when broilers were challenged with

E. tenella (Hassan et al., 2008). Histopathological lesion scoring of the duodenum further

solidified evidence of a successful coccidian challenge due to birds being challenged showing

increased total enteritis indexes (representation of inflammation and repair) in comparison to the

control birds. Nonetheless, much like our growth performance parameters demonstrated, there

were no detectable differences due to dietary treatment within the *Eimeria*-infected groups and

sham-inoculated birds showed a trend toward a reduction in lesion scoring. It should also be

noted that lesions identified in the duodenum broadly represent those common to commercial

poultry production and were of generally minimal to moderate overall severity as reported by the

histopathologist.

Despite the inability for saponin supplementation to improve growth performance,

diminish oocyst shedding, and alleviate coccidian induced lesions, our results show they modify

morphological parameters of the jejunum 14 days following *Eimeria* infection. The

multifunctional intestinal mucosal layer serves as a barrier between harmful pathogens and the

underlying epithelial cells and when damaged, can result in inflammation, uncontrolled immune

responses, and unbalanced organic homeostasis (Kitessa et al., 2014). It is crucial to maintain the

integrity of the mucosal barrier in order to promote the overall adequacy of the GIT mucus layer

which aids in protection from external environment, lubrication for gut epithelium, and nutrient

transport (Montagne et al., 2004). Our study showed treatment effects for mucosal thickness in

the jejunum with a numeric reduction in mucosal thickness in birds supplemented with saponins

at 250 mg/kg of diet and no differences compared with the sham-inoculated groups, but there

was no difference when comparing among infected groups. Saponin supplementation may

promote regeneration and reconstitution of the intestinal mucosal layer back to normal levels as evidenced by ISap250 birds not being significantly different than the unchallenged birds. This is important because an entire turnover of the mucosa could take up to 96 hours, which is equal to about 10% of the lifetime of a market-ready broiler (Gottardo et al., 2016). If a dietary intervention like saponin supplementation can diminish the time it takes for an immune-challenged bird to return to homeostatic levels, it may prove beneficial to overall bird health during a mixed *Eimeria* challenge as evidenced by alterations in intestinal structure.

Acquired immunity to an *Eimeria* infection in broilers has already been demonstrated to be almost entirely driven by cell-mediation, which is characterized by antigen specific and non-specific activation to stimulate diverse cell populations including lymphocytes (Allen & Fetterer, 2002; Lillehoj & Trout, 1996). Although reactive lymphocytes can be detected in small numbers in healthy birds, an increase in lymphocytes is often viewed following exposure to infectious diseases and is an indicator that antigenic stimulation is occurring (Mitchell & Johns, 2008). Our results show an increase in total lymphocytes in all cocci-infected birds 7 DPI when compared to the un-infected birds, suggesting evidence of antigenic stimulation and development of immunological memory. Lymphocyte-mediated immunological memory is imperative to a bird's livelihood following continual exposure to a reoccurring pathogen, resulting in reduced nutritional costs overtime as the bird is able to recognize the same pathogen with higher efficiency in the future (Klasing & Leshchinsky, 1999; Schat et al., 2013). Although imperative, initial development of immunological memory is complex, has nutritional expense, and even when not in use, has high maintenance costs (Klasing & Leshchinsky, 1999). It is crucial to find the balance between inducing an immune response in order to generate acquired immunity without exerting unwarranted damage to the host. Cocci-infected birds did have elevated

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lymphocytes levels 7 DPI and did not differ from one another, yet birds supplemented at 250 mg/kg of diet expressed similar lymphocyte percentages to that of the sham-inoculated birds. These findings suggest that saponin supplementation may possess some measureable immunomodulatory effects when analyzing lymphocyte responses and may curtail some of the undue costs an *Eimeria*-induced immune response has on a bird.

Although evidence of cell-mediation being the driving force behind generating protective immunity to *Eimeria* is well documented (Rose & Hesketh, 1979), we were unable to show differences in subsets of T-cell populations derived from mononuclear cells of chicken cecal tonsils via immunophenotyping. A possible explanation for this finding could be the timeframe in which T-cell populations were isolated from the cecal tonsils. Vervelde and colleagues (1996) showed an increase in $CD4^+$ cells just two days after inoculation with *E. tenella,* whereas we quantified T-cell populations fourteen days post inoculation with *Eimeria*. Peaks in $CD4^+$ T-cell populations were seen at three and eleven and $CD8^+$ at four days post inoculation with *E. maxima* (Rothwell et al., 1995). Our quantification of T-cell populations could have been outside the window of an acquired immune response mounted by cell-mediation following an *Eimeria* challenge. Total lymphocyte percentages quantified from the blood fourteen days post inoculation in our study also showed no difference between the cocci-infected and uninfected birds, possibly indicating that antigenic stimulation and lymphocyte-mediated immunological memory were longer occurring. An alternative could also be that saponins may not exhibit their effects on cell-mediated pathways in non-mammalian species. Saponins have been used in non-living adjuvants to produce a more robust immune response to generate protective immunity to an antigen, but in non-mammalian species this response is not as pronounced (Francis et al., 2002).

Expression of inflammatory genes, including *IL-1β*, in cecal tonsils and duodenal tissues of broilers at 7 DPI differed due to dietary saponin supplementation. While *IL-1β* elicits systemic metabolic, behavioral, and cellular changes that are crucial in initiating an immune response, this cytokine also has the ability to induce sickness-like behavior in animals resulting in retardation of growth comparable in strength to administration of lipopolysaccharide (Dantzer et al., 1993; Klasing & Leshchinsky, 1999). Expression of *IL-1β* following immune stimulation is unavoidable, but suppressing the amount expressed in a timely fashion in order to mitigate reductions in growth often associated with an immune response may prove to be beneficial, thus modifying the way in which a bird copes with coccidiosis. Our results suggest that saponins may possess some measureable immunomodulatory-anti-inflammatory-like properties. However, anti-inflammatory polyphenolics like resveratrol and yuccaol C isolated from *Yucca schidigera* extract have been shown to inhibit NFκB activation, suggesting that the nature of our saponin supplementation derived from *Yucca schidigera* may account for the suppression in *IL-1β*, rather than saponin supplementation alone (Cheeke et al., 2006; Marzocco et al., 2004). As for *IFN-γ* production, our findings corroborate with previous reports of increased gene expression of intestinal *IFN-γ* due to *Eimeria* infection in chickens, although the timing and magnitude of response have varied considerably in the literature (Hong et al., 2006; Laurent et al., 2001; Rochell et al., 2016; Yun et al., 2000). Interferon-γ is another key pro-inflammatory cytokine that has been shown to exert direct inhibitory effects against *Eimeria* (Lillehoj & Choi, 1998), so it was of no surprise when our results showed an increase in *IFN-γ* 7 DPI in the cecal tonsils and duodenum. Because *IL-12β* can activate NK cells, which are important in protecting the host from pathogens and have ability to induce cell lysis and secrete *IFN-γ*, the significant increase in

expression of *IL-12β* in the duodenum is probably directly related to the increase in *IFN-γ* production (Murphy & Weaver, 2016).

In conclusion, these results suggest that saponin supplementation may influence the immune response of birds challenged with mixed coccidian oocysts. *Eimeria*-challenged birds exhibited a reduction in growth compared with uninfected birds, but there was no detectable differences due to saponin supplementation within *Eimeria*-challenged groups. Lymphocyte counts were increased in all *Eimeria*-challenged groups at 7 DPI compared with uninfected birds, but birds given saponin supplementation at 250 mg/kg were not significantly different from uninfected birds. Mucosal thickness in the jejunum was increased in all infected groups and there was no difference between infected groups; however, saponin supplementation included at 250 mg/kg was not significantly different when compared to uninfected birds. Expression of *IL-1β* in the cecal tonsils and duodenum was increased following infection, but saponin supplementation at 250 and 500 mg/kg of diet reduced the expression of *IL-1β* to that of an unchallenged bird. These results suggest that saponin supplementation may possess some measurable immunomodulatory effects during infection as evidenced by lymphocyte responses, changes in intestinal structure, and alterations in cecal and duodenal inflammatory cytokine mRNA expressions. Collectively, the use of dietary saponins may serve as an effective strategy in mitigating the resulting inflammatory response following exposure to *Eimeria* in broilers.

Tables

Table 1. Ingredient composition of the experimental basal diet[1,2]

Ingredient, g/kg	Concentration, g/kg
Corn	444.5
Soybean meal	420.0
Soy Oil	90.0
Salt	4.0
Limestone	14.0
Dicalcium Phosphate	20.0
Vitamin Premix[3]	2.0
Mineral Premix[4]	1.5
DL-Methionine	2.0
Choline chloride	2.0
Calculated composition	
Protein, g/kg	230.1
ME, kcal/kg	3301
Ca, g/kg	11.0
Total P, g/kg	7.5
nPP, g/kg	5.0
Ca:tP	1.5
Ca:nPP	2.2
Total AA, g/kg	
Arg	16.3
His	6.4
Ile	10.1
Leu	20.1
Lys	13.8
Met	5.6
Met + Cys	9.4
Phe	11.6
Phe + Tyr	20.9
Thr	9.1
Trp	3.4
Val	11.1

[1]Abbreviations: nPP = non-phytate phosphorus; tP = total phosphorus; ME = metabolizable energy; DM = dry matter.

[2]The following diet names have been assigned to a respective experimental treatment group: Ucon: control diet + sham-inoculated; Icon: control diet + *Eimeria* oocyst challenge; ISap250: 250 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge; ISap500: 500 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge. Saponin supplementation (Micro-Aid® Feed Grade Concentrate; DPI Global, Porterville, CA) was included in ISap250 at 250 mg/kg and ISap500 at 500 mg/kg on top of the diet as having negligible nutritive contribution to the overall diet.

[3]Provided per kilogram of complete diet: retinyl acetate, 4,400 IU; cholecalciferol, 25 μg; dl-α-tocopheryl acetate, 11 IU; vitamin B_{12}, 0.01 mg; riboflavin, 4.41 mg; d-Ca-pantothenate, 10 mg; niacin, 22 mg; and menadione sodium bisulfite complex, 2.33 mg.

[4]Provided per kilogram of complete diet: Mn, 75 mg from MnO; Fe, 75 mg from $FeSO_4 \cdot 7H_2O$; Zn, 75 mg from ZnO; Cu, 5 mg from $CuSO_4 \cdot 5H_2O$; I, 0.75 mg from ethylene diamine dihydroiodid; and Se, 0.1 mg from Na_2SeO_3.

Table 2. Proximate analysis of experimental treatment diets[1]

Item	Dietary Treatment			
	Ucon	**Icon**	**ISap250**	**ISap500**
Dry matter, %	88.08	88.00	87.91	87.90
Organic matter, % of DM	92.09	92.14	92.35	91.85
Crude fat, % of DM	11.07	10.39	11.25	11.27
Crude protein, % of DM	26.32	27.18	26.25	24.12
Neutral detergent fiber, % of DM	15.60	16.24	16.33	12.48
Acid detergent fiber, % of DM	3.26	3.70	3.21	3.96
Gross energy, Kcal/kg of DM	4,870	4,852	4,908	4,909

[1]Abbreviations: DM, dry matter. The following diet names have been assigned to a respective experimental treatment group: *Ucon:* control diet + sham-inoculated; *Icon:* control diet + *Eimeria* oocyst challenge; *ISap250:* 250 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge; *ISap500:* 500 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge.

Table 3. Growth performance and oocyst shedding for broilers fed different levels of saponin supplementation and exposed to an *Eimeria* oocyst challenge[1]

| | Uninfected | Cocci-infected | | | Pooled | Model |
	Ucon	Icon	ISap250	ISap500	SEM	*P*-value
Body weight, g/bird						
d 14	391	386	393	391	9.7	0.80
d 21	895[a]	723[b]	737[b]	737[b]	19.1	< 0.001
d 28	1,495[a]	1,305[b]	1,314[b]	1,324[b]	30.1	< 0.001
Body weight gain, g/bird						
d 1-14	357	352	359	357	9.1	0.79
d 14-28	1,091[a]	903[b]	906[b]	929[b]	22.8	< 0.001
d 1-28	1,460[a]	1,271[b]	1,280[b]	1,290[b]	29.6	< 0.001
Feed intake, g/bird						
d 1-14	407[b]	411[b]	413[b]	433[a]	9.4	0.046
d 14-28	1,384[a]	1,256[b]	1,274[b]	1,282[b]	26.7	< 0.001
d 1-28	1,491[a]	1,391[b]	1,403[b]	1,447[ab]	26.0	0.008
Gain:feed, g/kg						
d 1-14	875[a]	857[a]	870[a]	825[b]	10.8	0.011
d 14-28	789[a]	718[b]	711[b]	716[b]	8.4	< 0.001
d 1-28	980[a]	902[b]	912[b]	892[b]	11.2	< 0.001
Oocysts per gram[2]						
d 21	-	506,253	521,871	622,695	65,853.0	0.41
d 28	-	13,648	18,294	17,233	3,879.4	0.62

[ab]Means without a common superscript letter differ within a row (*P* < 0.05).
[1]Values are least-square means initially derived from 12 replicate pens of 12 birds at 2-d post-hatch (mean initial weight 34 ± 5 g). The following diet names have been assigned to a respective experimental treatment group: *Ucon:* control diet + sham-inoculated; *Icon:* control diet + *Eimeria* oocyst challenge; *ISap250:* 250 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge; *ISap500:* 500 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge.
[2]Oocysts per gram for Ucon treatment was near zero for d 21 and d 28.

Table 4. Histopathology of the duodenum and jejunum of broilers fed different levels of saponin supplementation fourteen days post *Eimeria* oocyst challenge[1,2]

Item	Uninfected Ucon[3]	Cocci-infected Icon[3]	ISap250[3]	ISap500[3]	Pooled SEM	Model *P*-value
Duodenum, μM						
Villi height	2468	2316	2169	2386	90.4	0.12
Crypt depth	259.5[b]	315.8[ab]	363.3[a]	328.5[a]	21.2	0.012
Villus:crypt ratio	10.11[a]	7.63[b]	6.37[c]	7.58[b]	0.450	< 0.001
Mucosal	2727	2631	2533	2714	101	0.48
Jejunum, μM						
Villi height	1011	1327	1182	1250	81.1	0.054
Crypt depth	182.5	229.2	201.8	219.4	16.7	0.18
Villus:crypt ratio	6.01	6.27	6.11	6.16	0.383	0.97
Mucosal	1194[b]	1556[a]	1384[ab]	1469[a]	91.0	0.042

[abc]Means without a common superscript letter differ within a row ($P < 0.05$).

[1]The following values were derived at a magnification level of 40x.

[2]Values are least-square means initially derived from 1 bird per pen, 12 replicate pens per treatment.

[3]The following diet names have been assigned to a respective experimental treatment group: *Ucon:* control diet + sham-inoculated; *Icon:* control diet + *Eimeria* oocyst challenge; *ISap250:* 250 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge; *ISap500:* 500 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge.

Table 5. Histopathological lesion scoring of the duodenum of broilers fed different levels of saponin supplementation fourteen days post coccidial oocyst infection[1,2]

Item	Uninfected Ucon	Cocci-infected Icon	ISap250	ISap500	Pooled SEM	Model P-value
Coccidia	0	0	0	0	_[3]	_[3]
Villus shortening	0	0	0	0	_[3]	_[3]
Crypt hyperplasia	0	0	0	0	_[3]	_[3]
Lamina propria lymphocytes and plasma	0.292[a]	1.083[b]	1.500[b]	1.417[b]	0.264	0.005
Bacteria[4]	1.000	0.500	0.250	0.250	0.266	0.16
Cystic crypts	0	0.167	0	0.250	0.092	0.11
Intraepithelial leukocytes	0.956[a]	1.583[b]	2.250[c]	1.750[bc]	0.267	0.002
Other[5]	0	0.250	0.091	0.250	0.108	0.27
Coccidia index[6]	0	0	0	0	_[3]	_[3]
Enteritis index[7]	2.236[a]	3.583[b]	4.360[b]	4.167[b]	0.495	0.003
Total enteritis index[8]	2.232[a]	3.667[b]	4.539[b]	4.167[b]	0.494	0.002

[a,b,c]Means without common superscript letter do differ within a row ($P < 0.05$).

[1]The duodenum was examined microscopically for lesions and parasites. A lesion panel developed for commercial poultry production gut assessment and inclusive to the findings of the project was used and lesions were semi-quantitatively scored for severity as 0, normal; 1, minimal severity; 2, mild severity; 3, moderate; 4, marked; and 5, severe.

[2]Values are least-square means initially derived from 12 replicates per treatment. The following diet names have been assigned to a respective experimental treatment group: *Ucon:* control diet + sham-inoculated; *Icon:* control diet + *Eimeria* oocyst challenge; *ISap250:* 250 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge; *ISap500:* 500 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge.

[3]Absense of statistical analysis due to infinite likelihood.

[4]Bacteria on tips or sides of villi and dysbacteriosis.

[5]Includes increased goblet cells and/or mucus, attenuated enterocytes, misshapen villus tips, heterophils in the lamina propria, or contracted villi.

[6]Calculated by summing the coccidian scores for duodenum only, meaning coccidian index is equal to coccidian lesion score.

[7]Calculated by summing all lesion scores.

[8]Calculated by subtracting the coccidia index from the enteritis index, representing inflammation and repair.

Table 6. Effects of saponin supplementation fed at different levels during a *Eimeria* oocyst challenge on total and differential blood cell counts in broilers[1]

Item	Uninfected Ucon[2]	Cocci-infected Icon[2]	Cocci-infected ISap250[2]	Cocci-infected ISap500[2]	Pooled SEM	Model *P*-value
Hematocrit, %						
0 DPI	28.8	30.7	29.0	28.9	1.22	0.67
7 DPI	30.7ᵃ	23.0ᵇ	24.3ᵇ	24.9ᵇ	1.96	0.027
14 DPI	31.1	31.1	30.4	31.6	0.877	0.83
Total protein, g/dL						
0 DPI	2.93ᵇ	3.27ᵃ	3.28ᵃ	2.92ᵇ	0.106	0.037
7 DPI	3.10	3.40	3.32	3.23	0.167	0.52
14 DPI	3.05	3.33	3.30	3.32	0.148	0.50
WBC count, $\times 10^3/\mu$L						
0 DPI	16.1	17.3	15.5	12.6	2.89	0.70
7 DPI	12.7	11.6	14.8	15.6	2.16	0.55
14 DPI	23.3	23.1	24.5	22.1	3.39	0.97
Heterophils, % of WBC						
0 DPI	18.0	19.3	21.8	19.0	4.76	0.95
7 DPI	44.8ᵃ	27.2ᵇ	22.0ᵇ	18.0ᵇ	3.61	< 0.001
14 DPI	45.7	39.8	40.0	38.2	4.11	0.58
Band heterophils, % of WBC						
0 DPI	0.167	0.167	0	0.333	0.158	0.54
7 DPI	0ᵇ	0ᵇ	0.833ᵃ	0.400ᵃᵇ	0.206	0.021
14 DPI	0.800	0	0.167	0.667	0.229	0.06

ᵃᵇMeans without a common superscript letter differ within a row (*P* < 0.05).

[1]Values are least-square means initially derived from 1 bird per pen, 6 replicate pens per treatment. Abbreviations: DPI = days post-inoculation; WBC = white blood cell (i.e., leukocytes).

[2]The following diet names have been assigned to a respective experimental treatment group: *Ucon:* control diet + sham-inoculated; *Icon:* control diet + *Eimeria* oocyst challenge; *ISap250:* 250 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge; *ISap500:* 500 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge.

Table 6. (cont.)

Item	Uninfected Ucon[2]	Cocci-infected Icon[2]	ISap250[2]	ISap500[2]	Pooled SEM	Model *P*-value
Lymphocytes, % of WBC						
0 DPI	73.3	73.3	69.8	74.3	5.28	0.94
7 DPI	41.8[b]	60.8[a]	51.0[ab]	57.3[a]	4.49	0.014
14 DPI	39.7	49.2	49.0	50.2	4.27	0.22
Eosinophils, % of WBC						
0 DPI	2.33	1.67	0.83	1.17	0.744	0.53
7 DPI	5.33	5.00	5.83	7.17	1.91	0.86
14 DPI	1.17	1.50	0.667	1.67	0.508	0.54
Basophils, % of WBC						
0 DPI	4.00	3.17	3.20	2.20	0.687	0.33
7 DPI	7.83[b]	5.83[b]	12.7[a]	8.50[b]	1.46	0.010
14 DPI	7.00	4.67	5.83	4.00	1.10	0.22
Monocytes/azurophilic granules, % of WBC						
0 DPI	2.17	2.33	2.00	1.17	0.447	0.23
7 DPI	0.17[b]	1.17[b]	7.67[a]	7.50[a]	1.40	0.001
14 DPI	5.17	4.83	4.33	2.83	1.08	0.46

[ab]Means without a common superscript letter differ within a row (*P* < 0.05).

[1]Values are least-square means initially derived from 1 bird per pen, 6 replicate pens per treatment. Abbreviations: DPI = days post-inoculation; WBC = white blood cell (i.e., leukocytes).

[2]The following diet names have been assigned to a respective experimental treatment group: *Ucon:* control diet + sham-inoculated; *Icon:* control diet + *Eimeria* oocyst challenge; *ISap250:* 250 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge; *ISap500:* 500 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge.

Table 7. Effects of saponin supplementation fed at different levels during a coccidial oocyst challenge on immunophenotyping of different subsets of T-cells derived from mononuclear cells from chicken cecal tonsils[1]

| Item | Uninfected | Cocci-infected | | | Pooled | Model |
	Ucon	Icon	ISap250	ISap500	SEM	*P*-value
Total T cells, % of isolated cells (CD3+)[2]	36.59	31.72	34.16	38.71	2.35	0.22
Helper T cells, % of total T cells (CD3+CD4+)[3]	38.09	41.49	48.72	43.79	3.13	0.14
Cytotoxic T cells, % of total T cells (CD3+CD8+)[3]	41.06	36.94	32.51	38.21	3.10	0.21
Memory T cells, % of total T cells (CD3+CD4+CD8+)[3]	2.08	4.21	3.51	5.40	1.41	0.36

[1]Values represent least square means of 1 bird per pen, 6 replicate pens per treatment group with collection of cecal tonsils occurring at 14 days post-inoculation. The following diet names have been assigned to a respective experimental treatment group: *Ucon:* control diet + sham-inoculated; *Icon:* control diet + *Eimeria* oocyst challenge; *ISap250:* 250 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge; *ISap500:* 500 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge.

[2]Percent of total lymphocytes that are positive for cell-surface marker CD3.

[3]Percent of CD3-positive lymphocytes that are also positive for cell-surface markers CD4, CD8, or CD4/CD8.

Table 8. Gene expression of the cecal tonsils and duodenum of broilers fed different levels of saponin supplementation seven days post coccidial oocyst infection[1,2]

Item	Uninfected Ucon	Cocci-infected Icon	ISap250	ISap500	Pooled SEM	Model P-value
Cecal tonsils						
IL-1β	1.00[a]	2.07[b]	1.48[a]	1.26[a]	0.196	0.008
IL-12β	1.00	1.67	1.46	1.65	0.265	0.22
IFN-γ	1.00[a]	9.87[c]	5.12[b]	5.50[b]	1.009	< 0.001
Duodenum						
IL-1β	1.00[a]	1.52[b]	1.00[a]	1.17[ab]	0.157	0.039
IL-12β	1.00[a]	2.13[b]	1.66[ab]	2.32[b]	0.221	0.003
IFN-γ	1.00[a]	6.10[b]	5.31[b]	4.64[b]	0.958	< 0.001

[abc]Means without a common superscript letter differ within a row ($P < 0.05$).

[1]Values are least-square means initially derived from 1 bird per pen, 6 replicates pens per treatment. Abbreviations:; *IL-1β*=interleukin 1 beta; *IL-12β*=interleukin 12 beta; *IFN-γ*=interferon gamma. The following diet names have been assigned to a respective experimental treatment group: *Ucon:* control diet + sham-inoculated; *Icon:* control diet + *Eimeria* oocyst challenge; *ISap250:* 250 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge; *ISap500:* 500 mg/kg of fed saponin supplementation in diet + *Eimeria* oocyst challenge.

[2]Values are reported as fold-change.

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CHAPTER 4: GENERAL CONCLUSIONS

The overall focus of this study was to evaluate if dietary supplementation of *Yucca*-derived saponins could ameliorate the immune and growth responses of broilers during a mixed coccidian challenge. We concluded that while sham-inoculated birds outperformed *Eimeria*-infected birds, there were no detectable differences due to dietary treatment of saponin supplementation within the *Eimeria*-infected groups. Saponin supplementation modified the way in which lymphocytes responded to infection. Total lymphocytes as a percentage of white blood cells increased in all *Eimeria*-challenged groups at 7 DPI when compared with uninfected birds, but birds fed 250 mg/kg of diet of saponin supplementation were not significantly different than uninfected birds. An inclusion rate of 250 mg/kg of diet of saponin supplementation also altered intestinal structure of the jejunum. Mucosal thickness in the jejunum was increased in all *Eimeria*-infected groups and there was no difference between infected groups; however, saponin supplementation included at 250 mg/kg was not significantly different from the uninfected birds. Inflammatory cytokine gene expression within the duodenum and cecal tonsils was impacted by saponin supplementation. In the cecal tonsils and duodenum, *IFN-γ* expression was increased with infection compared to unchallenged birds. Expression of cecal *IL-1β* was increased only in the infected birds while saponin supplementation reduced the expression to match that of the uninfected birds. Contrarily, duodenal *IL-1β* expression increased with infection, but remained unchanged with saponin supplementation.

These results revealed that saponin supplementation may influence the immune response during infection with *Eimeria* due to its effects on lymphocyte responses, changes in intestinal structure, and alterations in inflammatory cytokine gene expression. Due to limitations of current

strategies to control coccidiosis, and because there are significant economic losses due to decreased BW gain and feed efficiency in affected animals, identifying compounds to limit coccidiosis and improve performance in poultry is of great value. Saponin supplementation offers a natural alternative to prophylactic chemotherapeutic agents in addition to staying in the realm of governmental regulations while assuaging to the growing public for microorganismal resistance. Collectively, the use of dietary *Yucca*-derived saponins may serve as an effective alternative strategy in ameliorating the resulting inflammatory response following exposure to *Eimeria* in broilers. Future research should be directed towards discovering the exact mechanistic pathway for which saponins are able to exhibit their effects during a mixed coccidian challenge.

APPENDIX: MICROSCOPIC LESION SCORING

Standard Operating Procedure Form for Histopathological Lesion Scoring
Prepared by: Dr. Fred Hoerr, Veterinary Diagnostic Pathology, LLC

0-5 Scoring 12/17/16

Coccidia

Eimeria acervulina
0, absent;
1, 1 cluster per section entire gut section;
2, 2 clusters;
3, 3 clusters;
4, 4 clusters;
5 , 5+ clusters.

Eimeria maxima and *E. mivati*
0, no coccidia observed;
1, 0-20 coccidia/entire gut section;
2, up to 50;
3, up to 75;
4, up to 100;
5, >100 coccidia/section

Eimeria tenella
0, absent;
1, small cluster of infection;
2, scattered small clusters of coccidia;
3 scattered large clusters of coccidia;
4, large clusters of coccidia, some confluence;
5, multiple confluence to diffuse infection

Small Intestine Lesion Scoring
Scoring was on a 0-5 scale with 0 being no lesions, 1 = rare or slight 2 = mild, 3 = moderate, 4 = marked, 5 = severe. Villus atrophy was scored and checked by see and sort method.

Villus shortening
0 - tallest in the set,
1 = 20% loss in height,
2 = 40% loss in height,
3 = 60% loss in height,
4 = 80% loss in height,
5=80% or more

Crypt hyperplasia
0 = none,
1 = minimal
2 = mild
3 = moderate, 2X normal;
4 = marked (3X normal)
5= severe more than 3x normal

GALT hyperplasia
0 = no increased cell population in the lamina propria;
1 = sparse population of LP with lymphocytes, plasma cells and small mononuclear cells with up to 2 small lymphoplasmacytic cluster;
2 = multifocal to diffuse mild infiltrates with 3-5 or more small lymphocytic clusters;
3 = confluent mild diffuse infiltrates of the LP involving crypts and villi;
4 = moderate diffuse infiltrates in LP of crypts and villi;
5 = marked, diffuse infiltrates in LP of crypts and villi.

Cystic Crypts
0 = none;
1 = 1;
2 = 2-3;
3 = 4-6;
4 = 7-10;
5 = >10.

Small intestine, other
1(mild) or 2 points (moderate) for each for any of the following, to a maximum of 5:
- Heterophils in the lamina propria
- Increased numbers/prominence of goblet cells
- Mucus streaming from the sides and tips of villi
- Misshapen, flattened, irregular, or eroded or micro-ulcerated tips of villi
- Contracted villi
- Other

Pancreas
Extramedullary hematopoiesis/myelopoiesis or LC Pancreatic foci
0 = none,
1 = 1- 2 lymphoid foci or few to mild granulocytic infiltrates,
2 = 3-5 small to medium or mild to moderate;
3 = more than 6 to coalescing
4 Confluent
5 Confluent to diffuse
Cecum

Eimeria tenella
0, absent;
1, small cluster of infection;
2, scattered small clusters of coccidia;
3, scattered large clusters of coccidia;
4, large clusters of coccidia, some confluence;
5, multiple confluence to diffuse infection

Lamina propria lymphoid hyperplasia
0 = no increased cell population in the lamina propria;
1 = sparse population of LP with lymphocytes, plasma cells and small mononuclear cells with up
to 2 small lymphoplasmacytic cluster;
2 = multifocal to diffuse mild infiltrates with 3-5 or more small lymphocytic clusters;
3 = confluent mild diffuse infiltrates of the LP involving crypts;
4 = moderate diffuse infiltrates in LP of crypts;
5 = marked, diffuse infiltrates in LP of crypts.

Bacterial adherence to mucosa
0 = non-adherence
1 = to 20% of mucosa
2 = to 40%
3 = to 60%
4 = to 80%
5 > 80%

Cecum, other

1 (mild) or 2 points (moderate) for each for any of the following, to a maximum of 5:
- Lamina propria apoptosis
- Heterophil inflammation
- Necrosis, increased apoptosis, or microulceration of dome epithelium
- Cystic glands (crypts),
- Increased intraepithelial leukocytes
- Other