INTERVENTIONS FOR MITIGATION OF COCCIDIOSIS AND NECROTIC ENTERITIS IN BROILER CHICKENS

A Dissertation

by

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ABSTRACT

The objective of this research was to investigate antibiotic alternatives to controlling Eimeria and necrotic enteritis in chickens. Eimeria are apicomplexan intestinal parasites ubiquitous to commercial poultry production. Because of their ubiquitous nature, control of coccidia is required in essentially every commercial poultry facility. Coccidia replicate intracellularly in the host intestinal epithelium, which not only causes nutrient malabsorption, but predisposes the birds for necrotic enteritis. Necrotic enteritis and coccidiosis cost the poultry industry a combine US $9 Billion annually. With consumer preference shifting towards antibiotic-free animal production, developing and implementing viable alternatives will continue to be crucial to sustainable agriculture. Antibiotic-free poultry production is continuing to grow in market share and in order to maintain the low-cost of the world's most popular protein, viable antibtioic alternatives need to be elucidated.

My first aim was to evaluate the potential of functional feed ingredients as interventions to mitigate necrotic enteritis in broiler chickens experimentally co-infected with *Eimeria* spp. and *C. perfringens.* Because of their antimicrobial activities, functional feed ingredients, including dietary prebiotics and botanical extracts, are widely seen as potential alternatives to antibiotics in the prevention and management of gastrointestinal disease in poultry. We administered a dietary prebiotic or a botanical extract blend to broilers under experimental co-infection with *Eimeria spp.* and *C. perfringens* and evaluated growth performance, gross intestinal lesions, and gastrointestinal counts of *C. perfringens.*

My second aim was to profile the longitudinal response of commercial broiler operations to bioshuttle administration. Referred to as a bioshuttle, ionophores can be administered post-vaccination to mitigate *Eimeria* vaccine-related performance losses. Although vaccine administration has been demonstrated to restore drug-sensitive *Eimeria* populations, it is unknown whether similar population shifts occur as a result of commercial bioshuttle use. In order to address this unmet need we took field data to profile the response of bird performance and coccidia presence before, during, and after bioshuttle application using aggregate production data (e.g., live weights, adjusted FCR, etc.) over 12-months from a large commercial broiler integrator as indicators of anticoccidial drug sensitivity.

My last aim was to investigate the potential of Acetyl CoA-Carboxylase (ACC) as a novel target for the development of anticoccidial drugs *in vivo*. Acetyl-CoA Carboxylase (**ACCase**), responsible for conversion of Acetyl-CoA to Malonyl-CoA, is an essential metabolic reaction. Because of low conservation in structure between apicomplexans and vertebrates, inhibitors of apicomplexan ACCase are predicted to have low toxicity in chickens. We administered ACCase inhibitors to broilers chicken experimentally infected with *Eimeria* spp. and evaluated growth performance. From the studies, we determined administration of phenoxy carboxylic acid inhibitors of apicomplexan ACCase inhibit coccidia *in vivo* and mitigated coccidiosis in experimentally infected broiler chickens.

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NOMENCLATURE

AUC	Area under the curve
BW	Body weight
BWG	Body weight gain
FC	Feed consumption
FCR	Feed consumption ratio
LS	Lesion score
NE	Necrotic enteritis
OC	Oocyst count
OPG	Oocyst per gram feces
UIC	infected un-medicated control
UUC	Uninfected un-medicated control
WG	Weight gain

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Enteric pathogens, such as *Eimeria* and *C. perfringens*-associated necrotic enteritis pose significant threats to animals produced for meat. Damage from coccidia replication causes reduction in feeding, digestive processes, nutrient absorbption, dehydration and suscpetibility to other diseases such as necrotic enteritis (154). Necrotic enteritis (NE), induced by prolieration of pathogenic *Clostridium perfringens*, is also associated with varying levels of intestinal tissue damage and reduced acquistion of nutrients (178, 211). Necrotic enteritis and coccidiosis cost the poultry industry a combined US $9 Billion annually from preventative costs and performance losses (150, 239). Losses from coccidiosis have been reduced with in feed anticoccidials or live coccidia vaccines while *C. perfringens*/NE is primarily controlled by antibiotics. Some products such as ionophores, have both anticoccidial and anticlostridial properties, and their inclusion reduced coccidiosis and necrotic enteritis (112, 144).

In-feed subtherapeutic antibiotics have been used in poultry production for the past 50 years. The antibiotics helped to improve feed efficiency as well as reduce mortality from diseases such as NE (77). When European countries removed antibiotics from poultry production, NE incidence increased (117). There has been research conducted on many alternatives with variable levels of success (103, 110, 202, 220) for controlling NE.

Eimeria are apicomplexan intestinal parasites ubiquitous to commercial poultry production. Because of their ubiquitous nature, control of coccidia is required in essentially every commercial poultry facility. Coccidia replicate intracellularly in the host intestinal

epithelium, which not only causes nutrient malabsorption, but predisposes the birds to subsequent diseases such as necrotic enteritis (40).

Most commercial poultry production facilities have some amount of Eimeria and with large flocks of birds raised in confined housing, these parasites have created a niche that make them virtually impossible to eliminate (154).

Historically, ionophores were included in broiler diets, serving as an anticoccidial and antibiotic (38). As more poultry producers remove antibiotics from production, the use of these products is limited. It seems unlikely to find a single product that will replace ionophores, so it is important to discover efficient anticoccidials and products which control *C. perfringens*. This review focuses on current and future control of *Eimeria* and necrotic enteritis.

Nine species that have been identified to infect the domestic chicken: *E. acervulina, E. brunetti, E. hagani, E. maxima, E. mitis, E. mivati, E. necatrix, E. praecox,* and *E. tenella.* Seven of these species are most commonly associated with clinical coccidiosis in commercial chicken production. *E. hagani* has been considered a doubtful species because of the incomplete original description (154). *E. mivati* has also been debated as a species and was most recently demonstrated to be a variation of *E. mitis* (238). *Eimeria* species disseminate throughout the intestinal tract and can cause simultaneous infection, representing a disease complex (249). Eimeria infect different portions of the intestines, but interspecies competition is observed in heavy infections (247, 248).

Poultry *Eimeria* do not have intermediate hosts (152) and unlike Apicomplexa parasites such as Toxoplasma, they are self-limiting. The life cycle of *Eimeria* are a series of stages, with a specific number of schizont generations depending on species (93). **Figure 1.1** shows the life cycle of *E. tenella*. When sporulated oocysts are ingested, the gizzard mechanically ruptures the

oocyst wall, releasing sporocysts. Trypsin and bile released in the duodenum promote ex-cystation of sporozoites. Depending on the species, sporozoites penetrate site-specific host mucosal cells in different segments of the intestinal tract. Some sporozoites of *Eimeria* (*E. brunetti and E. praecox*) develop within the same cells they initially penetrated (218). Other species' sporozoites (*E. acervulina, E. maxima, E. necatrix and E. tenella*) are transported via the crypt epithelium and develop in different sites (129, 134). The sporozoites become trophozoites and undergo rapid fission schizogony. This multiplication is asexual, so daughter cells are genetically identical. The daughter cells (merozoites), rupture and individually penetrate more mucosal cells. The merozoites grow into second and third generation schizonts. Because merozoites individually infect cells, each generation causes significant damage to the infected intestinal segment. Clinical signs, such as bloody feces during *E. tenella* or *E. necatrix* infections, are associated with this schizont maturation.

As merozoites mature, they sexually differentiate into either macrogamonts or microgamonts. Microgamonts are the male form and produce microgametes. Microgametes are motile and fertilize macrogamonts (female form) by penetrating host cells (218). Upon fertilization, a zygote is produced which will mature into an oocyst. Each fertilization results in a new oocyst, so infection of a naive host with one oocyst has the potential to generate thousands of oocysts to be passed in feces. *Eimeria* species vary in their reproductive indices, for example *E. acervulina* has a high reproductive potential where *E. maxima* has a much lower potential *(252)*. After maturation, the oocysts are released into the intestinal lumen. The unsporulated oocysts are shed in the feces and un-infective until sporulated. Sporulation of the oocyst requires adequate access oxygen, a temperature range of 10°C- 30°C, with 30°C being optimal (179). The sporulated oocysts are resistant to many disinfectants and can remain infective for up to 602 days

in a chicken house (182). Being raised in high-density and coprophagy being a normal behavior in chickens, non-motile oocysts are frequently ingested.

To control coccidiosis, the poultry industry has used three main approaches: 1) anticoccidials 2) vaccination 3) improving management practices. Each approach has shown to help production, but none seem to be without flaws. Anticoccidial drugs have been used extensively as affordable coccidia control (172). Vaccination with live coccidia hasn't historically been used as much as anticoccidials, but with drug resistance concerns and removal of ionophores from many production facilities, this alternative has begun to grow in popularity (35, 249, 255). While reducing oocysts on a farm would reduce coccidia issues, it has proven to be near impossible. The indoor rearing conditions for a modern broiler promoted *Eimeria* proliferation and these parasites are now considered ubiquitous in commercial facilities (34, 137).

Sulfanilamide was the first drug used as an anticoccidial to treat chicken coccidiosis in 1939 (133). A few years later, it was determined that low levels of sulfaquinoxaline could be included in chicken diets for prophylaxis of coccidiosis (90). The use of low-dose anticoccidials was beneficial for the expansion of intensive poultry and was a catalyst to the rapid growth capabilities of the industry. Anticoccidial compounds are affordable (less than $0.01/bird) and consistent, which also contributed to their wide-spread use (151). Because of the growth of the industry, and the wide-spread use of anticoccidials, it is not surprising, the United States broiler industry spends an estimated $127 million annually on anticoccidial drugs (33).

Anticoccidials can be described as coccidiostats or coccidiocidals. Coccidiostats arrest the growth of intracellular life stages and so parasite development will continue after drug withdrawal. Coccidiocidals on the other hand, destroy the parasite, preventing continuation of

development after drug removal. Some anticoccidials, such as ionophores, have both coccidiostatic and coccidiocidal properties.

There are three main categories of anticoccidial drugs (172):

1. Synthetic compounds, also known as 'chemicals': These drugs have a specific action against parasite metabolism (ex: nicarbazin, robenidine).
2. Ionophores or polyether antibiotics: These antibiotics are produced by the fermentation of Streptomyces or Actinomadura spp. They act by rupturing the parasite via osmotic balance interference. Ionophores work by creating lipophilic complexes that transport ions into the parasite's cell, offsetting the cross-membrane gradient. They affect influx of ions with different charges and so are broken into three categories and more details in **Table 1.1**:
    a. Monovalent Ionophores (monensin, narasin and salinomycin)
    b. Monovalent glycosidic ionophores (maduramicin and semduramicin)
    c. Divalent Ionophores (lasalocid)
3. Mixed products: combination of chemical and ionophore or two chemicals (nicarbazin/narasin, meticlorpindol/methylbenzoquate respectively.

A factor that has allowed the ionophores to continuously be effective is "leakage", where the drugs work on the majority of parasites infecting birds, but a few bypass the drug, and allow the bird to develop natural immunity (91). Another benefit of some ionophores is gram-positive antibacterial activity against *Clostridium perfringens*, the causative agent of necrotic enteritis (187). However, this characteristic has resulted in them being classified as antibiotics, and their future use is in question as more poultry producers grow more chickens without any antibiotics.

Varying amounts of reduced sensitivity and resistance have been observed for all commeriaclly available anticoccidial drugs (19, 30, 153, 174). Most anticoccidials don't prevent development of immunity, which could explain the continued success of these drugs (120). Producers also rotate drugs throughout a grow-out (shuttle) and rotate between flocks (rotation), which help to reduce the resistance pressure.

Antiparasitical and Anticoccidial drug discovery budgets have drastically been reduced in major pharmaceutical companies (79). Some research universities have filled this gap and placed emphasis on drug discovery with new molecular techniques. However, there have only been 2 new anticoccidial drugs introduced in the past 20 years.

Several compounds have been described and tested in vitro or in vivo, with varying levels of success (135, 196, 203, 207, 245). The World Association for the Advancement of Veterinary Parasitology outlined guidelines for evaluating anticoccidial efficacy (105). These guidelines were aimed to set international standards for evaluating anticoccidials. Research testing novel anticoccidials should use this guideline for experimental design and for evaluating already published data.

With consumer preference shifting, inclusion of anticoccidial compounds may be more restricted in the future. Because ionophores are antibiotics, their use has been reduced as more poultry producers move to antibiotic-free production. Chemical anticoccidials are being used more frequently in antibiotic-free production, so resistance may become even more of an issue.

Plentiful drug alternatives have been investigated for anticoccidial properties. Varying results have led to confusion and no clear "silver bullet". However, with consumers demanding poultry raised without antibiotics, producers have been forced to find viable alternatives. Some

of these ingredients such as probiotics, essential oils and organic acids, have been tested for anticoccidial properties and are described below.

Probiotics, which are live strains of bacteria, have become more popular as chicken producers move away from antibiotic-use (101). Most of the research done with probiotics has focused on the efficacy of the bacteria to exclude the bad or pathogenic bacteria, but some of the research done with *Eimeria* and probiotics has shown promising results. It has known for many years that *Eimeria* and host bacteria have an interaction, whether through the increase severity of coccidial infections in conventional chickens when compared with gnotobiotic chickens, or as a predisposing factor of necrotic enteritis (40, 72, 188, 189). Some research has shown pro- and prebiotics alleviate the negative impact *Eimeria* infections has on body weights (23, 65). Results of selected published research with *Eimeria* and probiotics are summarized in **Table 1.3**. The exact mechanism of interaction has not been determined and may be different for each probiotic and species of *Eimeria*. There are several proposed mechanisms of the interaction of probiotics and *Eimeria*:

1. Probiotics produce early immune stimulation, resulting in greater resistance to *Eimeria* infections (59, 60, 215).
2. Probiotics inhibit *Eimeria* sporozoite penetration in vitro (100, 219).
3. Probiotics may reduce severity of lesions, but allow oocyst cycling, promoting immunity development (23).

Several researchers have investigated a probiotic mix with *Enterococcus faecium, Bifidobacterium animalis* and *Lactobacillus salivarius* both in water and in feed with varying ability to reduce Eimeria lesions, oocyst output and improve performance (7, 84, 185). The

varying anticoccidial results with probiotics may limit their use in the field and could potentially be more effective with an optimized dosage and delivery system.

Essential oils are another alternative that have been marketed to have anticoccidial properties. Essential oil products extracted from a variety of plants and have been shown to have mixed anticoccidial properties (111). **Table 1.2** summarizes some of the research done with essential oils and *Eimeria* challenges. Artemisia, tea tree, thyme and have been demonstrated to have oocysticidal activity (184). The oocyst spends very little time in the chicken though, so this application may have more efficacy if used as a disinfectant. The hydrophobic properties of the essential oils are thought to disrupt the oocyst walls (111).

Aloe vera supplementation resulted in reduction of *E. maxima* lesion scores and oocyst shedding, which the researchers attributed to potential increase in cell-mediated responses (257). *Artemisia annua* has been demonstrated to have anti-parasitical properties against a variety of parasites. *A. annua* was shown to have significant anticoccidial properties when birds were challenged with *E. tenella,* reducing lesion scores and oocyst output (15). It is hypothesized that the mechanism of this essential oil is through iron-free radical production that results in oxidative stress of the parasite (14). Essential oil from *Beta vulguris* (sugar beet), has the active ingredient betaine, which has been shown to maintain osmotic pressure in cells (4). It has been shown to reduce sporozoites penetration and helps promote intestinal structure during an *Eimeria* infection, reducing the damages from parasite replication (166). Tumeric has been used as an anticoccidial-additive in developing countries because of its antioxidative, anti-inflammatory and immunomodulatory properties (3). Reduced lesion score and oocyst counts were observed in *E. maxima* infections but not in *E. tenella* infections (14). The effects of the oil may vary in efficacy depending on the location of the parasite infection, so should be screened with other species.

Oregano oil, carvacol and thymol, had anticoccidial effects when supplemented in feed to E. *tenella* infected birds (83). *Saccharum officinarum* is a sugar cane extract that has been shown to have protective effects against E. tenella infections through immune-stimulation.

Essential oils have gained popularity as more poultry producers move to antibiotic-free production. Unlike drugs which had to go through extensive research before they were put onto market, essential oils have little to no regulation. Many of the claims have not been consistently shown to have true anticoccidial properties, but rather secondary effects when a coccidia vaccine was used. Therefore, interpreting this data should be taken with caution.

Organic acids have been shown to have anticoccidial properties. The mechanism is thought to be disruption of the intracellular parasite's pH after the acid penetrates the intestinal cells (2). Administration of Acetic acid (3%) to *E. tenella* infected birds, resulted to lesion reduction comparable to Amprolium (6). Butyric acid has become much more popular and is marketed to reduce necrotic enteritis as well as having anticoccidial-properties. Because free butyrate disappears in the upper GI tract, encapsulation has allowed for the acid to be absorbed throughout (132). When used alone or with Clopidol, encapsulated in-feed butyric acid was shown to significantly decrease *E. maxima* lesion scores and improve performance parameters compared to the infected control (11). However, when challenged with *E. acervulina* and *E. tenella*, encapsulated butyrate was lower than the controls, but not similar to Salinomycin (217). Because butyrate increases the intestinal integrity and increase epithelial cell turnover, it may provide more secondary effects than act as a true anticoccidial.

Currently vaccines are the only practical alternative to anticoccidial drugs for control of coccidiosis in commercial poultry (37). Low doses of live coccidia are administered to chicks on day of hatch; through which oral ingestion and subsequent immunity is developed through

infection. Full immunity is not developed from the initial infection, and so vaccines rely on low-dose re-infection to provide future protection and minimize intestinal damage (182). Coprophagy is common in chickens, so utilizing this behavior promotes ingestion of feces with low levels of oocysts when vaccinated which can induce immunity without significant intestinal damage. In addition, low-dose re-infections have been shown to generate a stronger, longer lasting immunity than single infections (115).

Eimeria do not show cross protection, and so infection and subsequent immunity to *E. tenella* does not result in protection from any of the other species. The species of *Eimeria* also vary in their immunogenicity. *E. maxima* is highly immunogenic, where *E. acervulina* requires several infections to develop immunity (115). Although highly immunogenic, *E. maxima* has been demonstrated to show significant antigenic variability and so lack of cross protection within the species has been observed (209). Martin et al (145) showed that five E. *maxima* isolates from across the United States, formed 3 distinct immunological groups that could not confer immune protection. Because of this antigenic variation, most commercial coccidia vaccines have multiple strains of *E. maxima* to provide encompassing immunity.

When vaccination is optimized, birds develop immunity to coccidia and resistance to a subsequent challenge. This can be observed by a reduction in macroscopic lesions, a decrease in oocyst production and performance of birds (172). The low numbers of oocysts administered to vaccinated birds help maintain performance during a grow out, but can make optimum cycling for development of immunity difficult (13). “Trickle down” or multiple low-dose exposure of vaccines help to maintain immunity. When a vaccine regularly cycles through chickens, the immunity developed is stronger and longer lasting than immunity developed from fewer larger infections (114, 115).

Cycling vaccines with drugs has been shown to reinstate anticoccidial sensitive coccidia populations (107, 148). It has been demonstrated in laboratory settings that mixed population of sensitive and resistant *Eimeria tenella*, the sensitive parasites tended to dominate in the absence of medication (136). Comparing sensitivity of isolates from facilities with either live vaccine or anticoccidial drugs, the former showed significant increases in sensitivity to diclazuril and monensin (173). It would be logical to hypothesize that in the field, drug-sensitive coccidia outcompete the drug-resistant ones from a biological standpoint of resource allocation, resulting in more sensitive populations (172). Unlike vaccination, changing the drugs has not been shown to reintroduce coccidia sensitivity. Therefore, the intermittent use of live vaccines can provide a unique benefit by reintroducing drug sensitive coccidia to poultry houses.

Necrotic enteritis (NE) is one of the costliest diseases to the commercial broiler industry, estimated to cost producers worldwide US $6 billion annually (239). The etiological agent associated with necrotic enteritis is *Clostridium perfringens*. This bacterium is also associated with other diseases in poultry, such as gizzard erosions and gangrenous dermatitis (235). Infection with *C. perfringens* alone will not induce necrotic enteritis, but rather is described as a multifactorial disease. In addition to the commonly associated coccidial infection and diet components, management also plays a role in the disease onset. Successful induction of the disease requires sloughing of intestinal epithelium resulting from intestinal damage, and subsequent leakage of protein into the intestinal lumen, setting up for *C. perfringens* proliferation and toxin production (62). Other bacteria, although not more prevalent than the dominant *C. perfringens*, have been isolated from field cases of necrotic enteritis, including *Escherichia coli* and *Proteus mirabilis* (162). The aforementioned opportunistic bacteria are able to proliferate because of the gut epithelium damage caused during a necrotic enteritis episode (167). Counts of

C. perfringens have not been found to correlate with disease severity and high numbers have been detected in normal birds (162, 170). Although 75%-95% of commercial chickens are colonized by *C. perfringens*, only a small portion of these birds may manifest NE symptoms (149). Therefore, NE can be described as dysbiosis because of proliferation of *C. perfringens* out-competing other bacteria. Since *C. perfringens* is found in normal flora of healthy chickens, pre-disposing factors must be present to induce NE disease (256). To support this, researchers demonstrated administration of *C. perfringens* alone did not alter microbial diversity, while addition of fishmeal and *Eimeria* did promote colonization of *C. perfringens* (214).

C. perfringens, a Gram-positive anaerobic spore-forming bacterium, associated with necrotic enteritis, most commonly manifested as small intestinal necrosis (158). Over-growth of toxin producing *C. perfringens* is thought to induce NE, but predisposing factors are required to promote the *C. perfringens* proliferation. *C. perfringens* is one of the fastest growing bacterial-pathogen, and as a result NE onset is often rapid (205).

C. perfringens has been isolated from all stages of poultry production including breeders, hatchery, farms, processing and even after chilling (51, 52, 54, 235). Not all types of *C. perfringens* induce NE, and the disease is associated most commonly with *C. perfringens* Type A and Type C (176). Although most *C. perfringens* strains isolated from healthy and sick birds are type A, it hasn't been possible to relate a specific subtype with ability to induce necrotic enteritis (235). Attempts have been made to induce NE with *C. perfringens* isolated from healthy birds, but no disease was observed, where *C. perfringens* isolated from birds showing symptoms of NE, was able to induce in the disease in naïve laboratory birds (221). Many *C. perfringens* isolates from field outbreaks have been able to induce NE in naïve laboratory chickens, but some isolates were avirulent to chickens (28, 47, 210, 224). Extensive research has been conducted to

determine what characteristics of a *C. perfringens* strain allow for NE induction and results from this research are highlighted in **Table 1.3** with references in favor or against the mentioned hypothetical characteristics. Although significant progress has been made in determining the NE inducing characteristic, a definite trait unique only to NE producing *C. perfringens* has yet to be elucidated.

The Bacterial Enteritis Global Impact Assessment (BEGIA) reported in 2010 that NE is a global threat to the poultry industry with an 69% prevalence in the United States and Canada (233, 234). Molecular characterization of *C. perfringens* from field cases of NE has shown proliferation of only one or two strains (69), where up to five types of *C. perfringens* have been isolated from healthy birds (159).

NE disease can be acute clinical or subclinical. Although clinical NE can result in mortality of 10-40% of an infected flock, subclinical NE impacts the welfare and performance of a far greater number of birds, many of which go untreated (149). Subclinical NE costs producers significant losses in feed cost and reduced bird performance (208). Since subclinical NE is difficult to diagnose and requires sacrificing random birds from a suspected flock. Confirmation of the subclinical form of the disease comes from identifying intestinal or liver lesions (167). Lesions from subclinical NE are self-limiting and while the lesions are recovered at five weeks post hatch, reduced performance and increased feed conversion can be observed through the entire grow-out of the broiler (167). NE lesions can be some of the most severe of any disease in the chicken intestine (256).

Wet litter is associated with subclinical NE, and in some instances it is the symptom farmers use to initiate therapeutic medication (251). Wet litter should be noted with caution due to the many issues such as feed quality, mycotoxins, ventilation, and infection with viruses,

bacteria or coccidia have been associated with the symptom (39). Due to the differences in manifestation of subclinical NE, histological examination is necessary for proper diagnosis (104, 162).

NE does not follow Koch's postulate that a disease-causing organism would not be present in a healthy individual, so one must manage predisposing factors of this multifactorial disease to reduce incidence (231). Some of these pre-disposing factors include *Eimeria* infection, stress, high dietary protein and wheat or barley-based diets in the presence of pathogenic *C. perfringens* (157, 167, 186). A pathogenic *C. perfringens* is required to manifest the disease (222).

Coccidia has been known for quite some time to be a common predisposing factor to NE (8, 40, 251). Intestinal damage caused by coccidia infection has been shown to predispose birds to necrotic enteritis. Infection with coccidia is associated with increased gut stasis, giving rise to a nutrient rich environment for possible *C. perfringens* proliferation (199). Coccidial infections have been shown to decrease intestinal pH, which has been shown to decrease proliferation of *C. perfringens* (190, 232).

C. perfringens is found in the ceca of healthy chickens; however, necrotic enteritis lesions are characterized by intestinal *C. perfringens*. Research with birds co-infected with *E. necatrix* and *C. perfringens*, showed a significant increase in intestinal *C. perfringens* (18). It therefore seems plausible that *Eimeria* damage promotes intestinal colonization of the opportunistic *C. perfringens*. It should be noted that *C. perfringens* was not increased when *E. tenella* was administered to birds (125). This may explain why intestinal species of *Eimeria* such as *E. necatrix* and *E. maxima*, are more likely to predispose birds to necrotic enteritis than *E. tenella*.

Coccidial infections induce T-cell mediated responses in birds, resulting in increased mucogenesis(222). This increased mucogenesis has been shown to promote the proliferation of *C. perfringens* and subsequently the pathogenesis of NE (222). The increase in mucous in the gut provides a nutritional substrate for *C. perfringens*, subsequently favoring proliferation (40).

Some associate the increase use of live coccidia vaccines to increase of NE. This may be explained by the subclinical form of coccidiosis occurring from vaccination is enough intestinal damage to promote NE. It may also be explained that in addition to vaccine use increasing, in-feed antibiotics and anticoccidials are continuously being removed from the poultry production cycle. These drugs have been shown to provide relief from NE, and when they aren't included in diets, *C. perfringens* is able to more readily replicate (144).

Williams et al. (253) hypothesized that clinical coccidiosis exacerbates NE, where subclinical coccidiosis protects from NE. Clinical infections with *E. necatrix* and *C. perfringens* showed increased edema which was absent in single infections of either pathogen (18). Coccidial vaccines have been shown to mitigate the pathogenesis of NE (20, 229, 253). An explanation for this observation could be that controlled infection with *Eimeria* allows for early immunity development. This immunity in-turn provides minimal gut damage when the birds are exposed to high numbers of *C. perfringens* at 14-21 days of age, which may in turn minimize the manifestation of NE (251). It should be noted that these studies took place in controlled settings where application of the vaccine was orally given to respective birds. In field studies, where coverage may not be 100%, coccidial vaccines have been observed to predispose to NE (70).

It should be noted that although coccidia is commonly associated with NE, clinical coccidiosis cases have been reported without necrotic enteritis and vis versa (251). Coccidiosis should therefore be viewed as a component to the multifactorial NE.

Historically in-feed antibiotics, also known as antibiotic growth promoters (AGP) have provided cheap and reliable NE control. When antibiotic growth promoters were used, NE was not a common issue for poultry producers (118). However, as more countries remove these products from the market, NE incidence has consequentially rose (235). These bans will inevitably change the bacterial microflora in commercial poultry intestinal tracts and require antibiotic alternatives to control disease (128, 202, 235). Heavy research has been placed on antibiotic alternatives and many products have demonstrated variable efficacy (16, 80, 202, 235).

Gaucher et al (78), conducted a survey comparing performance and bird health between drug-free and conventional poultry production facilities, and showed clinical and subclinical necrotic enteritis was seen in 27.45% and 49% of the drug-free flocks and none of the conventional flocks.

Inclusion of subtherapeutic levels of some antibiotics has been demonstrated to prevent NE (68, 168, 206). Some coccidiostatic drugs, such as ionophores, have been shown to prevent *C. perfringens*-associated necrotic enteritis (253). Their future use remains uncertain as more and more producers shift to antibiotic free production.

Management has been another avenue used to reduce NE incidence (228). Tsiouris et al. (230), showed that feed restriction reduced necrotic enteritis lesions in an experimental model Since feed restriction improves blood circulation to intestinal mucosa, the researchers concluded this circulation may help intestines from becoming necrotic (230).

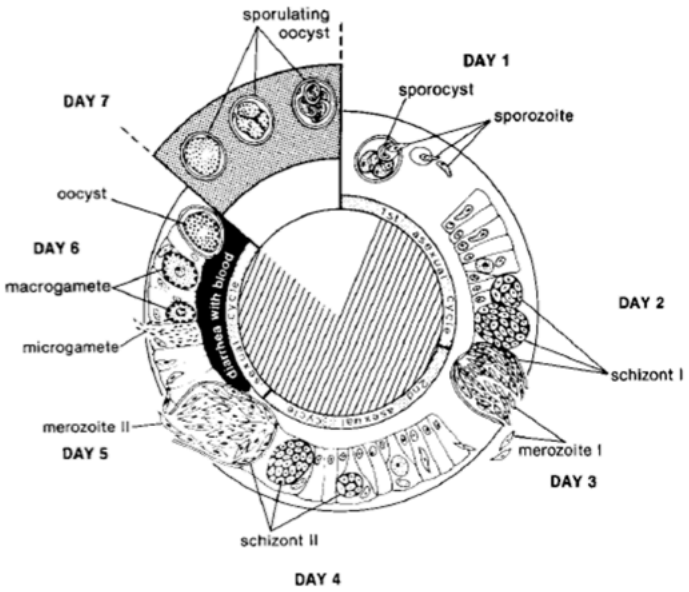

Figure 1.1. Life Cycle of *Eimeria*, taken from (151) with Permission from Wiley Publishing.

Table 1.1 Anticoccidial Chemicals (modified from (32, 172))

Drug	Trade Name	Target(s)	Mode of Action
Amprolium	Amprol	1st generation schizont	Inhibits thiamin (vitamin B1) absorption
Clopidol	Coyden	sporozoite	Allows parasite penetration into host cell but prevents further development- coccidiostat effect (242)
Decoquinate	Deccox	sporozoite	Affect parasite metabolism- blocks electron transport in mitochondria (193)
Diclazuril	Clinacox	multiple stages	Unknown
Halofuginone	Stenorol	asexual stages	Inhibit parasite penetration and further development in host cells (126)
Nicarbazin	Nicarb	sporozoite	Not defined but thought to affect parasite's ability to generate energy, directed against developing second-generation schizonts
Nitrobenzamides	Zoalene	asexual stages	Thought to stop asexual replication of parasite
Robenidine	Robenz	multiple stages	Prevent asexual replication in *E. tenella* (192)

Table 1.2 Anticoccidial Properties of Botanicals Modified from **(4)**

Botanicals	Active ingredients	Mode of action	Species studied	Affected parameters
Ageratum conyzoides	flavonoids	oxidative stress	*E. tenella*	↓ OC ↑ WG
Carcia papaya	Papaine	Sporozoite degradation	*E. tenella*	↓ OC
Cyamopsis Tetragonoloba	Glacatomannans and saponins	Binding with sterol molecules present on cell membrane	*E. tenella*	↓ OC
Linum usitatissimum	N-3 fatty acids	Oxidative stress	*E. tenella*	↓ LS
Leninus edodes and *Tremella fuciformis*	Polysaccharide extracts	Immune stimulation	*E. tenella*	↑ WG
Olea euopea	Maslinic acid	Anti-inflammatory & antioxidant properties	*E. tenella*	↓ OC, LS ↑ WG
Pinus radiate	35% condensed tannins	Damage of sporozoite cytoplasm	*E. acervulina*, *E. maxima*, *E. tenella*	↓ Sporulation
Passum sativum	Antibody fragments	Inhibition of sporozoites	*E. tenella*	↓ Sporozoite infectivity
Tulbaghia	Cysteine sulphoxide, bis-disulphide	Oxidative stress	Mixed infection	↓ OC
Vitis vinifera	Tannins	Oxidative stress	*E. tenella*	↓ M, LS ↑ WG
Vernonia amygdalina	Vernoside	Oxidative stress	*E. tenella*	↓ OC

Table 1. 3: Probiotic in vivo Effects on Eimeria Infections

Probiotic strain	Infeed/water	Eimeria	Result	Reference
Bifidobacterium animalis DSM 16284, *Lactobacillus salvarius* DSM 16351, *Enterococcus faecium* DSM 21913	Water	*Eimeria acervulina*, *E. maxima* ,*E. tenella*	↓OC, ↓Jejunum LS	(185)
Lactobacillus acidophilus, Lactobacillus casei, Enterococcus faecium and *Bifidobacterium bifidium*	Feed	*E. acervulina, E. maxima, E. tenella, E. mitis, E. brunetti,* and *E. praecox*	↓LS, ↑WG	(23)
Bacillus subtilis (15AP4, Bs27, Bs278)	Feed	*E. maxima*	↓LS, ↑WG	(130)
Pedococcus acidilactici, Saccharomyces boulardii	Feed	*E. acervulina, E. tenella*	↓OC	(131)
Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium thermophilum, Enterococcus faecium	Feed	*E. acervulina*	↓OC	(59)
Bifidobacterium animalis DSM 16284, *Lactobacillus salvarius* DSM 16351, *Enterococcus faecium* DSM 21913	Feed	*E. acervulina, E. maxima and E. tenella*	↓FCR	(7)
Bifidobacterium animalis DSM 16284, *Lactobacillus salvarius* DSM 16351, *Enterococcus faecium* DSM 21913	Feed /water	*E. acervulina, E. maxima, and E. tenella*	↓OC	(84)
Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium thermophilum, Enterococcus faecium	Feed	*E. acervulina, E. tenella*	↓OC, ↓LS	(217)
Lactobacillus plantalum CMU-FP002	Feed	*E. tenella*	↓OC	(29)

Table 1.4. Virulence Factors of NE-associated *C. perfringens*

Factor		References	
		Supporting	[Against]
Toxins	Genes		
α-toxin	*plc*	(9, 47, 73, 104, 139)	(122)
NetB	*netB*	(82, 121, 123, 204, 210, 213)	(46)
Perforin	Perforin	(225)	
Beta 2 toxin	*cpb*2	(26, 76, 99, 127, 143)	(46, 56, 69, 146, 226)
Entero toxin		(55)	(69)
Tle gene present	*tle*	(27, 48)	
Other			
Intraspecific growth-inhibition abilities	Bactericins	(21, 221)	
Collagenolytic enzymes	Damage extracellular matrix of host	(163)	
Adhesion to extracellular matrix molecules (ECMM)	*cnaA*	(147, 240)	
Quorum sensing-toxin production	Agr-like QS sensing	(259)	

CHAPTER II

EVALUATION OF INTERVENTIONS IN EXPERIMENTAL NECROTIC ENTERITIS MODEL

Introduction

Necrotic enteritis (NE) is a multifactorial disease typically characterized by an over-growth of *Clostridium perfringens* (142). This Gram-positive, spore forming bacterium is a common inhabitant in the ceca of healthy chickens (235). However, pre-disposing stressors including specific dietary changes and *Eimeria* infection may cause a shift in the intestinal environment and allow *C. perfringe*ns to establish itself in proximal segments of the intestine. The toxins produced during opportunistic toxicoinfection by *C. perfringens* have been demonstrated to cause significant nutrient malabsorption and intestinal necrosis (47, 157, 199, 222). Clinical NE outbreaks often result in significant mortality, while the subclinical form of this disease has been demonstrated to be associated with reduced performance (46, 167).

Dietary prebiotics are non-digestible feed ingredients that selectively promote the growth or activities of beneficial microorganisms in the host's gastrointestinal tract (85). One example of a commercially available prebiotic for poultry is a yeast cell wall product. Dietary inclusion of yeast cell wall prebiotics has been shown to improve broiler performance (75, 169, 195, 261). These products are attractive feed additives because they have been demonstrated to have immunomodulatory characteristics and provide competitive binding sites for pathogenic bacteria (12, 75). Administration of prebiotics in-feed has been shown to improve immune responses to

Eimeria tenella infections (74). It was shown the yeast cell product improved performance as well improve immune function during coccidial infections (86).

Botanical additives are blends of plant essential oils extracted from aromatic plants using non-aqueous solvents (24). These oils were shown to have anti-protozoal characteristics against *Plasmodium*, the causative agent of malaria (164). Botanical *Artemisia annua, Quisqualis indica*, *Sophora flavescens*, grape seed extract, and oregano oils have been shown to have activity against *Eimeria* in vivo through immunomodulation and amelioration of intestinal damage from coccidial infections (15, 83, 244, 258).

NE was a relatively rare disease when subtherapeutic antibiotics were included in poultry feed (103), as producers were able to prevent disease and manage losses using antibiotics. As consumer preferences shift and changes in regulation of antibiotic-use, the antibiotic-free poultry market has rapidly grown. Because of their antimicrobial activities and effects in improving intestinal health, functional feed ingredients, including dietary prebiotics and botanical extracts, are widely seen as potential alternatives to antibiotics in the prevention and management of gastrointestinal disease in poultry (16, 58, 202, 235). In this study, we evaluated the effect of the administration of a Mannanoligosaccharide dietary prebiotic derived from inactivated *Saccharomyces cerevisiae* and a botanical essential oil extract on growth performance, gross intestinal lesions, and gastrointestinal counts of *C. perfringens* in broiler chicks experimentally co-infected with *Eimeria* spp. and *C. perfringens.*

Materials and Methods

288 day of hatch male broiler chicks (Cobb) were weighed, randomly assigned to treatment pens of similar starting weight, and placed into battery brooder pens with 6 birds per pen. There were six treatments with eight replicate pens of each treatment. The control treatments were: uninfected with *Eimeria* or *C. perfringens*, infected with only *C. perfringens*, infected with both *Eimeria* and *C. perfringens*. The other three treatments were all infected with *Eimeria* and *C. perfringens* but one was given BMD in feed (40 g/ton), one prebiotic (453 g/ton) and the last one was given botanical Essential Oil Extract (680 g/ton). Chicks were fed a commercial-type broiler mash diet formulated to meet or exceed NRC requirements (1). Birds were placed on experimental diets starting Day 0. Experimental diets were prepared as single basal ration with experimental treatments blended as directed by the manufacturers' recommendations. Feed and water were administered *ad libitum*. Body weight, body weight gain, feed consumption, feed conversion, and mortality were monitored during the 28-day course of the study. All mortality was necropsied and inspected for necrotic enteritis. Research animals were given access to feed and water ad libitum and cared for in accordance with the Texas A&M University Institutional Animal Care and Use Committee (IACUC). Birds were raised in battery cages in BSL-2 isolation rooms at Texas A&M Vet Med Park.

Necrotic enteritis was induced using co-infection with *Eimeria* and *C. perfringens.* Broiler chicks were administered 10^3 oocysts/mL using a commercial vaccine containing non-attenuated live oocysts of *Eimeria acervulina*, *E. maxima* and *E. tenella* at 14 d post-hatch and 3 mL of *C. perfringens* culture (10^7 CFU mL^{-1}) at 19, 20, and 21 d post-hatch by oral gavage (103). Sterile water or culture broth were used as mock infections as appropriate. *C. perfringens*

was cultured using fluid thioglycolate medium (FTM) anaerobically (Coy Laboratory Products, Inc).

At 21 d post-hatch, three broilers from each pen were selected randomly, euthanized, weighed, and necropsied for scoring of gross intestinal lesions. If there are fewer than 3 birds remaining, all remaining birds in the pen were selected. Gross intestinal lesions were scored in the jejunum at Meckel's diverticulum for gross intestinal lesions. The scoring were based on a 0 to 3 score: 0 = normal; 1 = thin-walled or friable; 2 = focal necrosis or ulceration; and 3 = large necrotic patches/ "Turkish towel" appearance (262).

Sections of the jejunum and ileum were dissected from each sampled broiler; pooled by pen; homogenized and diluted using FTM; and plated for enumeration using Tryptose Sulfite Cycloserine – Egg Yolk (TSC-EY) agar incubated anaerobically at 37^{o}C. *C. perfringens* was selectively enriched using FTM and Iron Milk Medium (IMM).

Data were analyzed using ANOVA with the pen as the experimental unit. Significant differences between treatment groups were determined using Duncan's multiple-range test using $\alpha = 0.05$. *Clostridium perfringens* counts were $\log_{10}$ transformed prior to statistical analysis. Samples for which no colonies appeared on the enumeration plates but were positive by selective enrichment were assigned the limit of detection for the assay (2 $\log_{10}$ CFU g^{-1}).

Results

There were no significant differences in body weights observed between any treatment on Day 0 (P=0.189), 14 (P=0.666), or 19 (P=0.111) (**Table 2.1**). However, a significant effect of the treatments on body weight was observed on Day 21(P=0.002) and Day 28 (P=0.0397). On Day 21, body weights were significantly lower for the Control Infection Group (co-infected) and for broilers receiving the prebiotic and botanical extract when compared to the uninfected broilers, while the body weight of broilers infected with only *C. perfringens* and treated with BMD were not significantly different than the body weights of uninfected birds. On Day 28, body weights of only the Control Infection (co-infected) and BMD bird body weights were significantly different from each other, while the remaining treatment groups were similar to both treatments.

The treatments were observed to have a significant effect on feed conversion ratio (FCR) of broilers for Day 0-21 (P = 0.047) and Day 0 – 28 (P=0.045) (**Table 2.2**). FCR was observed to be significantly higher for Control Infection (co-infected) birds compared to the uninfected, *C. perfringens* only-infected, and BMD treated birds on D 0-21 (P=0.0002) (**Table 2.2**). The FCR for D 0-21 in prebiotic and botanical birds were not observed to be different for any treatment. The FCR for D 0-28 was significantly higher in the prebiotic birds compared to the uninfected, *C. perfringens* only birds and BMD birds (**Table 2.2**). No significant differences in FCR D 0-28 was observed between co-infected birds and any other treatment.

There were no NE-related mortalities in the un-infected birds (MOCK). No significant differences in NE mortality were observed between the *C. perfringens* only (CPI), BMD and un-infected birds (MOCK). The mean NE-related mortality was significantly higher in the co-infected (CON) (30% mortality) and the botanical (BOT) (27% mortality) birds compared to the

C. perfringens only (CPI) and BMD birds (P<0.05) (**Figure 2.1**). No differences were observed in mean NE mortality of the prebiotic birds compared to the botanical, or co-infected birds.

Over all, lesion scores were higher in the jejunum than the duodenum for all treatments. One exception was the *C. perfringens* only birds, which had higher scores in the duodenum compared to the jejunum.

The treatments were observed to have a significant effect on gross duodenal lesions was observed (P<0.001) (**Figure 2.3A**). No significant differences in mean duodenum NE lesions were observed in *C. perfringens* only-infected (CPI), BMD and un-infected birds (MOCK) (**Figure 2.3A**). However, the aforementioned treatments had significantly lower mean lesions than the co-infected (CON), prebiotic (PRE) and botanical (BOT) treatment birds (P<0.001) (**Figure 2.3A**).

The treatments were observed to have a significant effect on gross jejunal lesions (P<0.001) (**Figure 2.3B).** No significant differences in mean jejunum NE lesions were observed in *C. perfringens* only-infected (CPI), BMD and un-infected birds (MOCK) (P<0.001) (**Figure 2.3B**). No differences were reported in the co-infected (CON), prebiotic (PRE) and botanical (BOT) treatments mean jejunum NE lesion scores.

No lesions were observed in the un-infected birds (MOCK) in both the duodenum and jejunum.

In general, *C. perfringens* counts were higher in the jejunum than in the duodenum, among all treatments.

Treatments were observed to have a significant effect on duodenal *C. perfringens* counts ($P<0.001$) (**Figure 2.2A**). Duodenal *C. perfringens* counts in co-infected (CON), prebiotic (PRE) and botanical (BOT) treatments (**Figure 2.2A**). *C. perfringens* only birds (CPI) had significantly different duodenal *C. perfringens* than all other treatments ($P<0.001$) (**Figure 2.2A**). BMD and un-infected birds (MOCK) did not significantly differ in duodenal *C. perfringens* but were lower than all other treatments ($P<0.001$) (**Figure 2.2A**).

Treatments were observed to have a significant effect on jejunal *C. perfringens* counts ($P<0.001$) (**Figure 2.2B**). Jejunal *C. perfringens* counts were significantly lower in BMD and un-infected birds compared to other treatments (MOCK) ($P<0.001$) (**Figure 2.2B**). The *C. perfringens* only birds had jejunal *C. perfringens* counts that were significantly lower than the botanical and co-infected birds, but similar to prebiotic ($P<0.001$) (**Figure 2.2B**).

All birds that were not infected with *C. perfringens* had no colonies were observed on the plates. All birds that had been infected with *C. perfringens* had a positive for enrichment, even if no colonies were observed on the plates.

Discussion

Necrotic enteritis (NE) is a multifactorial disease often characterized by an over-growth of *Clostridium perfringens* (142). This bacterium is a common inhabitant of the ceca of healthy chickens. However, stressors including dietary changes and *Eimeria* infections may cause a shift in the intestinal environment which allows *C. perfringe*ns to establish itself in proximal segments of the intestine and cause significant nutrient malabsorption from toxin production and intestinal necrosis (47, 157, 199, 222). Clinical NE may result in significant morality, while the subclinical form of this disease is associated with reduced performance (46, 167).

Since NE is a multifactorial disease, understanding the relationships between each predisposing factor is crucial to developing relevant experimental models (157, 177, 236). Not all *C. perfringens* isolates are able to induce NE, so using a pathogenic strain is important to inducing the disease (21, 210, 224). Therefore, we used *C. perfringens* isolated from a natural necrotic enteritis outbreak. We also used coccidia, a common predisposing factor, which induces intestinal damage and allows for colonization of *C. perfringens* (251).

The *Clostridium perfringens* used in this model has alpha-toxin and Net-B genes. Both of these toxins have been associated with NE-inducing *C. perfringens* (27, 121). The weights and FCR of *C. perfringens* only birds were not significant from uninfected birds at any point. However, there was mortality associated with NE in this treatment, although it was significantly lower than the co-infected birds. The CFU counts in the duodenum and jejunum in *C. perfringens* were significantly different than the uninfected birds and the co-infected birds. Due to the lack of performance differences, but NE mortality in *C. perfringens* only birds, it would seem this presentation of NE is more acute. Both chronic and acute forms if the disease have been reported (47, 149, 235).

Although birds were infected with 10X of a non-attenuated *Eimeria* vaccine, no weight differences between the ones infected with coccidia and those without (Day 19-one week after *Eimeria* administration). The *Eimeria* infection was a week before birds were weighed at D 19, which would be enough time for weight reduction from *Eimeria* (154). This infection was mild, so no significance in performance loss from *Eimeria* was observed, but enough intestinal damage to predispose to NE. This is important for the NE model because if there is too heavy infection with coccidia, *C. perfringens* will not have an intestinal epithelium to bind to and subsequently cause NE (251).

Treatments were observed to have a significant effect on body weights on D 21 and D 28. The uninfected controls were significantly heavier than the co-infected birds on D 21. Previous researchers have demonstrated similar results when birds are infected with both *Eimeria* and *C. perfringens* (40, 177, 253). The *C. perfringens* only and BMD birds were similar weights to the uninfected controls on D 21. On Day 21, when the levels of *C. perfringens* would be the highest due to three *prior C. perfringens* inoculations, the birds were similar weights with the uninfected and the *C. perfringens* only birds. BMD has been shown to significantly reduce *C. perfringens* levels even without a challenge (66, 67, 216).

Birds only infected with *C. perfringens* were similar in body weights on Day 21 and 28 to the uninfected controls. Previous research with 5 different strains of *C. perfringens* showed significant differences in weights and FCR in only one of the strains compared to the un-infected controls (28). Other researchers have observed more severe weight suppression and necrotic lesions in birds given only *C. perfringens* (10, 25, 49). Perhaps the virulence of the bacteria dictates some of the weight reduction in treatments only given *C. perfringens*. The toxins in the

broth with administered *C. perfringens* could also be a factor that allows induction of disease in the absence of predisposing factors (9).

The co-infected birds had significantly higher FCR compared to the *C. perfringens* only, uninfected and BMD birds during D 0-21. The prebiotic and botanical were not significantly different from either of those groups. BMD birds had similar FCR to the uninfected and *C. perfringens* only birds. The FCR in co-infected birds were similar to all treatments D 0-28. The only treatments that were significantly different FCR D 0-28 were BMD, uninfected, *C. perfringens* only compared with the prebiotic. This could be explained due to the recovery period D 21-28 and surviving bids were able to over-come the challenge, resulting in similar FCR. In our study, *C. perfringens* alone did not have an impact on FCR compared to uninfected birds D 0-21. This has been observed by others as well, and has been suggested to indicate a subclinical form of necrotic enteritis (81).

C. perfringens counts in both the duodenum and jejunum were significantly lower in the *C. perfringens* only birds compared to those co-infected with *Eimeria*. This is makes sense since intestinal damage is required for successful colonization of the *C. perfringens* in those regions of the intestine (253). *C. perfringens* is commonly found in the ceca and ileum of birds, but in much lower numbers in the duodenum, because of the low pH which doesn't favor colonization (176).

Bacitracin Methylene Dialicylate (BMD) was effective at reducing *C. perfringens* counts. Based on these results and the reduction in NE lesions, it seems that the *C. perfringens* strain used in our study is sensitive to BMD, but in vitro studies could be done to confirm this.

The essential oil botanical did not appear to reduce *C. perfringens* counts in duodenum or jejunum. Birds given some essential oil blends have been shown to have lower *C. perfringens* counts (156). However, these birds were not experimentally infected, but rather just naturally

exposed to *C. perfringens* through the litter. It seems plausible these products work more effectively when the challenge is low, and perhaps even on a subclinical level of NE. Other researchers demonstrated significant reduction in NE mortality in birds given an essential oil blend, and NE was induced through dietary fishmeal, coccidia vaccine and *C. perfringens* administration (223). A difficulty with using essential oils, is the variation in proprietary blends used. Understanding individual effects in reducing NE of each compound in a blend may help to better optimize the use of these products.

Birds that were not infected with *C. perfringens* did not have any NE lesions or NE associated mortality. These had significantly lower *C. perfringens* counts, which indicates there wasn't significant cross contamination between the battery cages. *C. perfringens* is ubiquitous in the soil, environment and commonly isolated in chicken intestines (52, 53, 160, 199). However, induction of NE requires predisposing factors and presence of *C. perfringens* alone doesn't reliably produce the disease (58).

The prebiotic additive did not appear to have a positive effect on gross NE lesion scores. Our results were similar to other researchers that showed NE mortality, FCR, BW and lesion score was not different in positive control and prebiotic-fed birds in a NE model system (103). The model used in the previously mentioned research was similar to our model with *Eimeria* infection a week before *C. perfringens* inoculation but they included dietary fishmeal as well (103).

With 30% mean NE mortality in the co-infected controls, we can say that the NE model was strong and successful. The *C. perfringens* only birds did have some NE mortality, but was statistically similar to the uninfected controls. This would be in line with what other researchers identify as subclinical NE, focal necrosis in intestines with little to no mortality and performance

may not be significantly influenced (9, 104, 235). While it does not seem likely that one single intervention will be able to replace BMD or other in-feed antibiotics for prevention of NE, it did appear that the two products tested may help to improve performance during and after a NE challenge. BMD was the only tested product that significantly reduced NE mortality, *C. perfringens* counts or gross lesions. The bird weights were similar on Day 28 in botanical, prebiotic and BMD treatment. FCR was similar between the botanical, prebiotic and BMD treatment for D 21 and 28. So while the products tested may not reduce *C. perfringens* counts, NE lesions or NE mortality, they do promote weights in surviving birds similar to that of BMD. All products added, were included in the feed from day of hatch, so none were used on a treatment basis. However, the challenge in this model is very strong, and so testing potential additive effects of the products may improve the NE parameters examined. As antibiotic free poultry production becomes more common, the search for viable alternatives is crucial.

Table 2.1 Average Body Weight of Broiler Chickens

Treatment		Body Weight (kg)				
Product	Infection	D 0	D 14	D 19	D 21	D 28
Untreated	Uninfected	0.043	0.415	0.786	0.873[a]	1.351[ab]
Untreated	Clostridium[4]	0.044	0.416	0.718	0.861[ab]	1.264[ab]
Untreated	Co-infection[5]	0.044	0.414	0.694	0.761[c]	1.107[b]
BMD[1]	Co-Infection	0.044	0.435	0.724	0.861[ab]	1.352[a]
Prebiotic[2]	Co-Infection	0.044	0.422	0.705	0.766[c]	1.093[ab]
Botannical[3]	Co-Infection	0.044	0.420	0.699	0.795[bc]	1.154[ab]
	P–value	0.189	0.666	0.111	0.002	0.0397
	Pooled SEM	0.000	0.01	0.024	0.022	0.0343

[a-c] Different superscripts indicate means are significantly different ($P \leq 0.05$)

[1]Bacitracin Methylene Dialicylate; [2] PREBIOTIC; [3]BONTANICAL EXTRACT

[4] Infection with *C. perfringens*; [5]Coinfection with *Eimeria* spp. and *C. perfringens*

Table 2.2. Feed Conversion of Broiler Chickens

Treatments		FCR (Feed:Gain)	
Product	Infection	D 0 -21	D 0 -28
Untreated	Uninfected	1.263[b]	2.776[b]
Untreated	Clostridium[4]	1.263[b]	2.659[b]
Untreated	Co-infection[5]	1.505[a]	2.993[ab]
BMD[1]	Co-Infection	1.226[b]	2.555[b]
Prebiotic[2]	Co-Infection	1.370[ab]	4.038[a]
Botannical[3]	Co-Infection	1.376[ab]	3.053[ab]
	P-value	0.0002	0.0048
	Pooled SEM	0.047	0.045

[a-c] Different superscripts within a column indicate means are significantly different ($P \leq 0.05$)
[1]Bacitracin Methylene Dialicylate; [2] PREBIOTIC;
[3]BONTANICAL EXTRACT
[4] Infection with *C. perfringens*; [5]Coinfection with *Eimeria* spp. and *C. perfringens*[5]

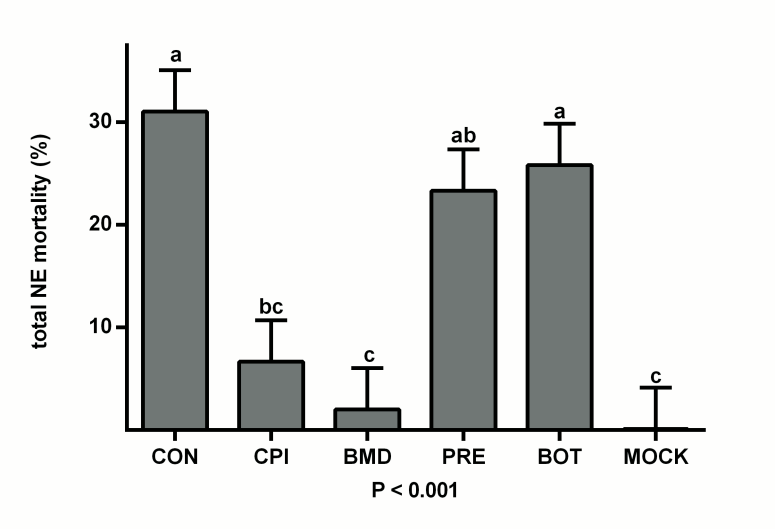

Figure 2.1. **Necrotic Enteritis Mortality.** Mortality (%) due to necrotic enteritis post experimental infection. Mortality reported as mean ± SEM from 8 pens per treatment. CON, control infection; CPI, *C. perfringens*-only infected; BMD, BMD treated; PRE, prebiotic treated; BOT, botanical extract treated; MOCK, mock infection. Different letters above bars indicates means are significantly different ($P < 0.05$).

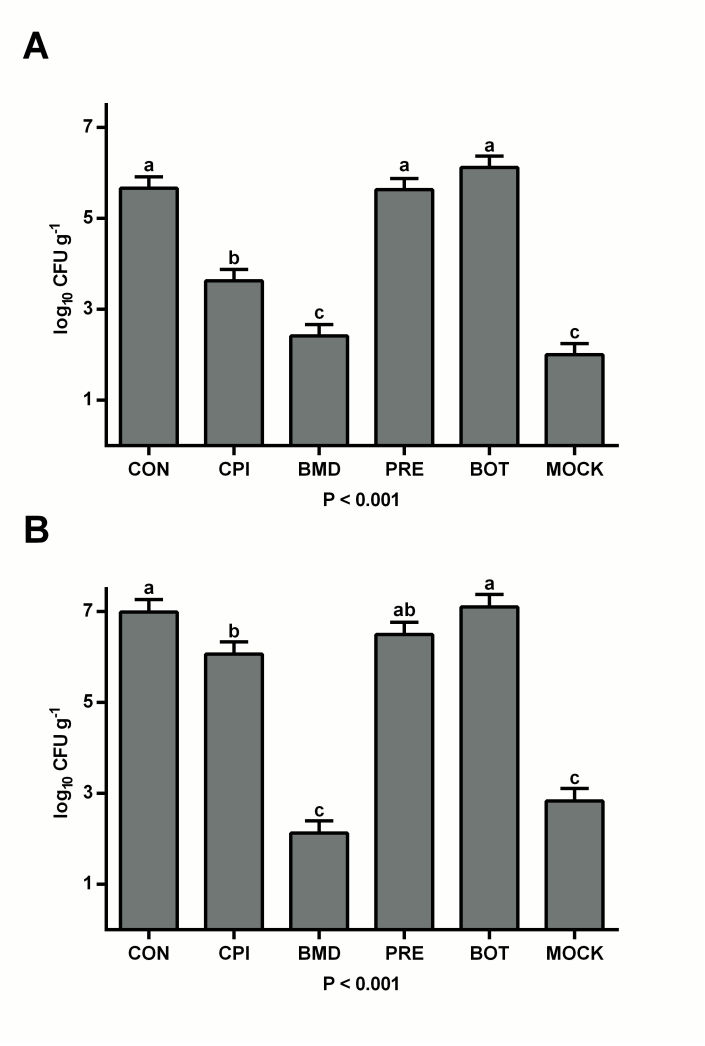

Figure 2.2. **Enumeration of *Clostridium perfringens* from Broiler Chickens.** *C. perfringens* were enumerated from (A) duodenum and (B) jejunum of broiler chickens at 8 hours post infection on day 21 post-hatch. Counts are reported as the mean ± SEM $\log_{10}$ CFU g^{-1} digestive from pooled contents from 3 broilers pen from 8 pens per treatment. CON, control infection; CPI, *C. perfringens*-only infected; BMD, BMD treated; PRE, prebiotic treated; BOT, botanical extract treated; MOCK, mock infection. Different letters above bars indicate means are significantly different ($P < 0.05$).

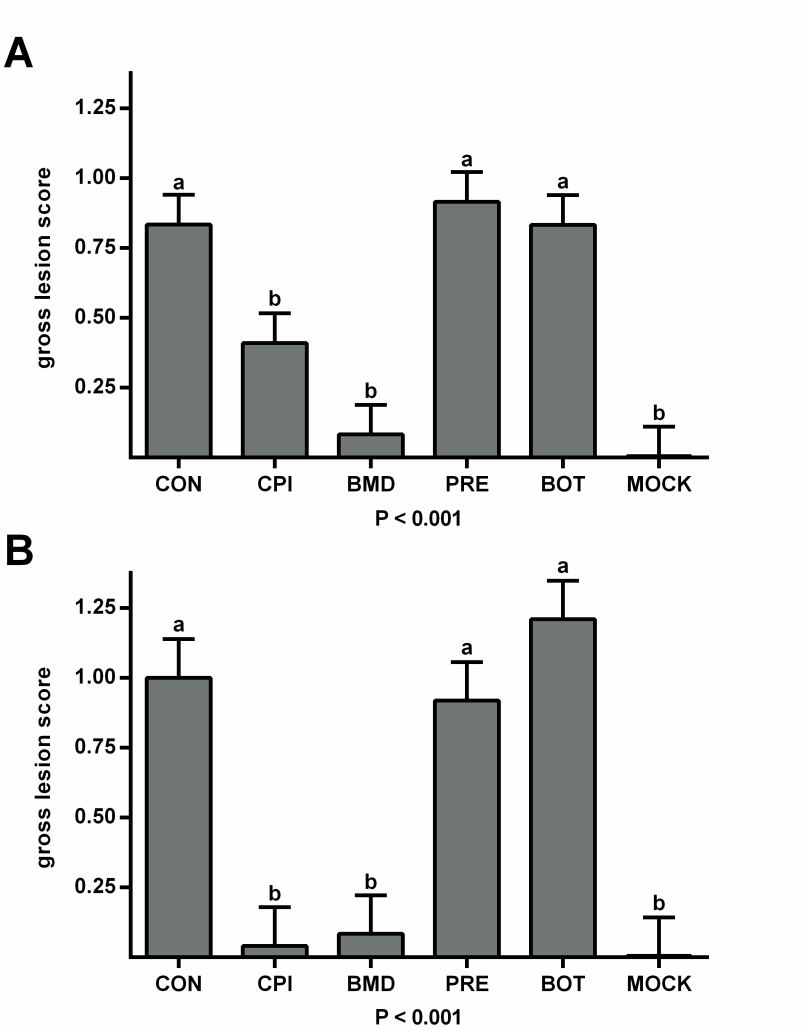

Figure 2.3. **Necrotic Enteritis Lesion Scores.** Gross intestinal lesions in the (A) duodenum and (B) jejunum were scored 8 hours post infection on day 21 post-hatch. Lesion scores are reported as the mean ± SEM from 3 broilers pen from 8 pens per treatment. CON, control infection; CPI, *Clostridium*-only infected; BMD, BMD treated; PRE, prebiotic treated; BOT, botanical extract treated; MOCK, mock infection. Different letters above bars indicate means are significantly different ($P < 0.05$).

CHAPTER III

LONGITUDINAL RESPONSE OF COMMERICAL BROILER OPERATIONS TO BIOSHUTTLE ADMINISTRATION

Introduction

Coccidiosis, caused by intracellular intestinal parasitic *Eimeria* spp., poses a substantial cost to the poultry industry which has been estimated to have a total worldwide annual cost of $3 billion (USD). The sources of costs or economic losses associated with coccidiosis include the costs of coccidiosis control measures; predisposition to secondary disease such as necrotic enteritis; and morbidity and mortality (179, 254).

Coccidia are primarily controlled through the use of in-feed anticoccidial drugs or vaccination with live *Eimeria* oocysts (172). In-feed anticoccidials were used widely, but due to the few products commercially available, concerns over increases in drug resistance have continued to rise (5). Because they are composed of drug sensitive *Eimeria*, interest in the use of coccidia vaccines to potentially combat drug resistance has increased (150, 249). The use of live-*Eimeria* vaccines alone has been demonstrated to restore anticoccidial drug sensitive *Eimeria* populations on poultry farms (36, 148, 171). This succession of *Eimeria* populations is thought to be the result of competition between drug sensitive vaccine strains and drug resistant *Eimeria* previously present in the litter.

Although they can be used alone, vaccines can also be administered as part of a bioshuttle program in which an ionophore, or other low-dose anticoccidial drug, is administered in-feed during the grower and finisher diet phases or later. The ionophores are not administered until the grower ration, so as to not interfere with initial cycling of the vaccine *Eimeria*. Bioshuttle programs can be administered to mitigate *Eimeria* vaccine-related performance losses and potentially reduce the risk of secondary diseases such as necrotic enteritis. However, very little research has been performed on the effects of bioshuttle use. Additionally, although the use of vaccines alone has been demonstrated to restore populations of drug sensitive *Eimeria,* it is unknown whether drug-sensitive *Eimeria* populations are similarly restored during the use of a bioshuttle in commercial settings. In this study, we collected data from a commercial integrator to develop a longitudinal profile of the response of broiler chicken production before, during, and after bioshuttle application as part of the drug/vaccine program of a commercial integrator over a 12-month period.

Materials and Methods

The overall drug program used by the integrator through 2016 (**Table 4.1**) was divided into three periods: Pre/Before the bioshuttle; During the bioshuttle, and Post/after the bioshuttle. Before the bioshuttle, the birds were placed on a chemical program: nicarbazin in the starter diet, decoquinate in the grower and finisher one diets, and no anticoccidial drug in the finisher two diet. During the bioshuttle program, the birds were vaccinated (Coccivac B52, Merck Animal Health, Millsboro, DE) for coccidia in the hatchery, and administered an ionophore program: Salinomycin (Huvepharma, Peachtree City) in the grower and finisher one diets, and no anticoccidial drug in finisher two diet. After the bioshuttle, the anticoccidial program was narasin with nicarbazin in the starter, grower and finisher one diet and nothing in the finisher two.

Aggregate production data including live weights, adjusted FCR, and livability from ten broiler complexes were provided by a commercial integrator. Performance data was collected from each complex before, during and after the bioshuttle. The time period sampled was chosen to ensure that all birds processed were only exposed to one coccidia control method. Body weights collected were live body weight measured at the processing plant. Mortality corrected FCR was calculated from farm weights and feed consumption. Livability was calculated from birds placed on Day of hatch and those that arrive live at the plant. Standard cost deviations were calculated from the subtracting the final cost of the sold bird from the cost it took to raise it. A negative value indicates more money was made from selling the bird than it cost to raise the bird.

Coccidiosis lesion scores (i.e., gross lesions and microscopic *E. maxima* scores) were collected from posting sessions at all complexes during the 12-month data collection period. Six birds from each farm were sampled and examined for coccidia. These birds were randomly selected from a house of approximately 10,000 broilers. Ten farms with birds of different ages

were examined for each complex, for a total of 60 birds scored per complex before, during and after the bioshuttle. Gross lesion scores were determined using Johnson and Reid Method (1970) (113). Because of the low prevalence of gross *E. maxima* scores, microscopic scores were reported instead (22). Scrapings from the intestine near Meckel's diverticulum were collected from each bird during posting session and were wet mounted onto a slide. Only *E. maxima* developmental stages were accounted for in the micro-scores. Briefly, 0 indicated no stages; 1, 1–20 stages per 10X objective field; 1: 1–20 stages; 2: 21–50 stages; 3: 51–100 stages; and 4: too numerous to count. Approximately 10 fields of vision were observed per bird.

Performance data from ten broiler complexes was collected for one week of processing before, during and after the integrator's bioshuttle program. Coccidiosis lesions were reported for posting sessions during respective time points. Data was compiled from each complex and analyzed using ANOVA, and significantly different means were separated using Duncan's Multiple Range Test using $\alpha = 0.05$. Additionally, data from super bird (processed average 9 pounds) and tray-pack bird (processed average 7 pounds) complexes were analyzed separately. Body weights were averaged for each complex and reported as mean ± SEM lbs of body weight at processing. . ADG is shown as mean ± SEM lbs per bird per day of weight gain.

Results

A significant effect of the drug program period was observed on body weights (BW) of super birds (P<0.001) (**Figure 3.1A**) and tray-pack birds (P<0.001) (**Figure 3.1C**). For both super and tray-pack birds, BWs were lowest for birds processed during the bioshuttle application and greatest for birds processed in the period after bioshuttle application.

A significant effect of the drug program period was also observed for average daily gain (ADG) of super birds (P<0.001) (**Figure 3.1B**) and tray-pack birds (P<0.001) (**Figure 3.1D**). ADG was lowest for both super and tray-pack birds during the bioshuttle application period. ADG was similar in the periods before and after bioshuttle application for super birds. However, for tray-pack birds, ADG was greater during the period after the bioshuttle was administered than the period before the bioshuttle was administered.

A significant effect of drug program period was observed on mortality corrected feed conversion ratio (FCR) in super birds (P<0.001) (**Figure 3.2A**) and tray-pack birds (P<0.001) (**Figure 3.2B**). The FCR were significantly higher during bioshuttle, than before and after in both super and tray-pack birds.

A significant effect of drug program period was observed on livability of super birds (P=0.0324) but was not observed for tray-pack birds (P=0.179) (**Figure 3.3**). The mean livability of super birds was significantly higher before compared to during the bioshuttle, but livability after bioshuttle application was similar to the other periods.

There was no observed effect of drug program period in standard cost deviation of super birds (P=0.988) (**Figure 3.4A**). However, there was an observed effect in tray-pack birds (P<0.001) (**Figure 3.4B**). Mean standard cost deviation was significantly higher during bioshuttle compared to before and after.

A significant effect of the drug application period was observed on the total prevalence of *Eimeria acervulina* lesions (P=0.05) (**Figure 3.5A**). and in the distribution of lesion scores (P=0.02) (**Figure 3.5B**) for super birds. The prevalence of *E. acervulina* lesions was significantly higher in super birds for the periods during and after bioshuttle application than for the period before the bioshuttle was administered. The highest lesion score reported across all periods for super birds was +2, and no significant difference was observed in the percentage of super birds with +2 lesion during any time point. However, a significant difference was observed in the percentage of birds with a score of +1 was higher during and after the bioshuttle compared to the period before. A significant effect of the drug treatment period on *E. acervulina* lesion prevalence was not observed before, during or after the bioshuttle in tray-pack birds (P=0.803) (**Figure 3.5C**). The highest lesion score reported in these birds was a +3. However, there were no differences in the percentage of tray-pack birds with any lesion score value (**Figure 3.5D**).

A significant effect on drug program period was observed on prevalence of microscopic *E. maxima* in super birds (P=0.045) (**Figure 436A**). Prevalence was significantly higher in super birds during the bioshuttle compared to before and after. All scores (0-4) were reported in super birds. However, the distribution was only significant in lesion score of +1. The percentage of birds with lesion score +1 was significantly higher in super birds during the bioshuttle (P-0.006) (**Figure 3.6B**). There was a significant effect on drug program period observed on prevalence of microscopic *E. maxima* in tray-pack birds (P=0.036) (**Figure 3.6C**). The prevalence was significantly higher during the bioshuttle compared to before and similar to after the bioshuttle in tray-pack birds. All scores (0-4) were reported in tray-pack birds. The only significant differences in lesion distribution were observed for lesion score of +2. The percentage of birds with +2 lesion score was significantly higher during the bioshuttle compared to before and similar to after bioshuttle in tray-pack birds (P=0.023) (**Figure 3.6D**). No other significant differences were observed in the other lesions.

Discussion

Due to their ubiquity in commercial poultry production, control of these parasites is required for [sustainable poultry production. Traditionally this parasite has been controlled using in-feed anticoccidials or vaccination (154). Vaccination with live coccidiosis vaccines has become much more popular (35). Administration of vaccines exposes birds to low number of oocysts in order to stimulate development of protective immunity. The *Eimeria* oocysts used in live vaccines are drug sensitive, so vaccine use allows introduction of drug sensitive *Eimeria* that could potentially out-compete the drug-resistant *Eimeria* already present in the chicken house (35, 36, 148). Indeed, the use of live-*Eimeria* vaccines alone has been demonstrated to restore anticoccidial drug sensitive *Eimeria* populations on poultry farms (36, 148, 171).

Recently some poultry producers have begun to use a bioshuttle program, in which birds are vaccinated with a coccidia vaccine at day of hatch and then given an in-feed anticoccidial during the grower ration (usually the second week of life). Some researchers demonstrated Salinomycin administration almost completely suppressed oocyst production when oocyst vaccination alone had been implemented 4 flocks prior, indicating restoration of drug sensitivity (36). However, what wasn't determined was if isolates that were concurrently exposed to Salinomycin and coccidia vaccines, known as a bioshuttle, would be sensitive to anticoccidial drugs in subsequent flocks. We wanted to determine the broiler performance and coccidiosis lesions before, during and after a bioshuttle and potential drug sensitivity in a field setting. To evaluate sensitivity of the *Eimeria* to the drugs, monitoring of bird performance as well posting birds to examine coccidia levels is commonly done by poultry producers. Although these variables are not stand-alone indicators of drug sensitivity, we used both to develop a profile for this particular broiler integrator.

Evaluating the lesions and performance before, during and after a bioshuttle program could therefore indicate potential increased drug sensitivity in a field setting.

Different measurements of anticoccidial sensitivity have been debated as superior in the past (105, 187, 241). Generally gross lesion scores, weight gain and fecal oocyst counts are used to evaluate anticoccidial efficacy (102, 105, 113). Some debate the value of using lesion score alone because of differences in correlation with each *Eimeria* or mixed species (22, 42). However, in a field setting, measuring weight gain alone is difficult to make conclusions on anticoccidial sensitivity because of the confounding variables. In this study, we chose to incorporate performance parameters as well as coccidia lesions scores to evaluate drug sensitivity before, during and after a bioshuttle program.

Broiler performance (BW and ADG) in super and tray-pack birds before and after bioshuttle was significantly higher than during the bioshuttle. Super and tray-pack birds were significantly heavier after the bioshuttle compared to during and before. ADG was significantly increased in tray-pack birds compared to before and during the bioshuttle, but this was not seen in super-birds. The mortality adjusted FCR in super and tray-pack birds were significantly higher during the bioshuttle compared to before and after. Livability was significantly higher in super birds before the bioshuttle compared to during. No differences were observed after the bioshuttle. For tray-pack birds, no significant differences were observed in livability during any drug program period. Overall, measures of growth performance were worse during bioshuttle application than they were before and after.

Currently no data has been published during a bioshuttle. Additionally, no data has been published comparing broiler response before, during and after a bioshuttle. This limits our ability

to compare to other research, but since many poultry producers are beginning to use bioshuttle programs, data on the effects of this program should become more available.

In addition to being on a coccidia vaccine, birds during the bioshuttle are being grown during southern summer heat. Heat stress has been long known to be associated with poor bird performance (180). Therefore, it makes it difficult to determine the exact influence weather and coccidia vaccine have on performance during this time. During the spring and fall seasons, the birds do not have the weather pressure, which could also explain the improvement in weights and FCR before and after the bioshuttle. However, the winter time is most commonly associated with coccidiosis issues because of reduced ventilation, promoting better environmental factors for coccidia oocysts (96).

Drug program period did not have an influence on standard cost deviation in super birds. However, in tray-pack birds, the standard cost deviation was highest in birds during the bioshuttle compared to the other periods. In other words, the lower the cost deviation, the more economical it was to raise the birds. This observation does not appear to be coccidia related because prevalence of *E. acervulina* and microscopic *E. maxima* lesions were higher after the bioshuttle when compared to during. Therefore, it seems that this observation was related to weather and warm summers in the Southeastern United States would increase standard cost deviation.

Although they were generally similar, the differences in the super and tray-pack birds could be the differences in growing a chicken to 9 pounds verses 7 pounds and the physiological implications that could have. For instance, the older the bird gets, the more the FCR increases, decreasing economic value (97). This could explain why the standard deviation costs were higher in the super compared to the tray-pack birds during all time points.

Before the bioshuttle, the birds were on a chemical program (nicarbazin and decoquinate). Chemicals have a strong selection pressure than ionophores, and nicarbazin is has been shown to be a strong anticoccidial (19, 31, 212). This explains why the *E. acervulina* and microscopic *E. maxima* lesion prevalence was significantly lower in both super and tray-pack birds before the bioshuttle.

Although not significant during and after, the prevalence of *E. acervulina* lesions was highest after the bioshuttle, when the birds were on a combination ionophore and chemical program. This was not predicted because during the bioshuttle, when the birds are vaccinated with live oocysts, would we expect to see the highest prevalence of *E. acervulina* lesions. This may be due to general anticoccidial activity of narasin and nicarbazin compared with the salinomycin (61, 191, 246).

Prevalence of *E. acervulina* lesions did not correlate with performance parameters in super birds during the sample periods. Prevalence of *E. acervulina* was the highest after the bioshuttle while body weights and ADG were significantly higher and mortality adjusted FCR the significantly lower during this period. The prevalence of *E. acervulina* lesions were not significantly different in the tray-pack birds, but the trends were similar to super birds. Although significant infection of *E. acervulina* can impair growth in birds, researchers have shown a lack of correlation with *E. acervulina* infection and bird performance reduction (42).

Prevalence of *E. maxima* microscopic lesions did correlate with performance parameters in super birds during the sample periods. When prevalence of *E. maxima* was highest, during the bioshuttle, the body weights and ADG were significantly lower. This relation in *E. maxima* prevalence and performance, although not significant, was also seen in the tray-pack birds. *E.*

maxima is more pathogenic than E. acervulina and infections with this species often correlate with reduction in performance (42, 198).

With the improvement in weight seen after the bioshuttle, it is tempting to predict sensitive coccidia out-competed the field strains, subsequently causing performance improvement when the winter coccidia control program was started.

However, there was an increase in *E. acervulina* lesions after the bioshuttle. Although not significant, the prevalence was numerically higher after the bioshuttle. This higher prevalence of *E. acervulin*a lesions would not indicate that drug sensitivity was restored after administration of bioshuttle.

Prevalence of microscopic *E maxima* was significant lower before and after the bioshuttle, with performance being the best during these times (BW, ADG, mort. adj. FCR). Because these species are more commonly associated with reduction in performance, we speculate these results indicate a drug sensitive *Eimeria* population after the bioshuttle.

The drug program period had a significant effect on performance, with the best bird performance after the bioshuttle in both super and tray-pack birds. The prevalence of *E. acervulina* lesions was not correlated with broiler performance in either the super or tray-pack birds. However, the prevalence of microscopic *E. maxima* appeared to be related to the broiler performance in both super and tray-pack birds. This should be investigated with more sampling points to determine if the interaction is significant. Utilizing production data allowed a larger scale analysis of a bioshuttle program in the field. In addition to this work, examining the drug sensitivity of *Eimeria* isolates in a laboratory setting would help to determine drug sensitive populations, and further indicate an effect the bioshuttle may have on *Eime*ria populations. This would also take out the confounding variable of the weather during the different growing times.

Table 3.1. Drug[1]/Vaccine Program (2016)

Program phase (months)[2]	Pre Bioshuttle (Jan – Apr)	Bioshuttle (May – Aug)	Post Bioshuttle (Sep – Dec)
Diet Phase			
Starter (d 0 – 14)	Nicarbazin BMD[3]	Coccivac-B52[4] Virginiamycin	Nicarbazin Narasin
Grower (d 14 – 27)	Deccox BMD	Salinomycin Virginiamycin	Nicarbazin Narasin
Finisher 1 (d 28 - 48)	Deccox BMD	Salinomycin	Nicarbazin Narasin
Finisher 2 (d 48 - d59)	Virginiamycin	Virginiamycin	Virginiamycin

[1] all drugs administered in-feed; [2]4 month phases (inclusive);

[3] bacitracin methylene disalicylate; [4] vaccination in-hatchery

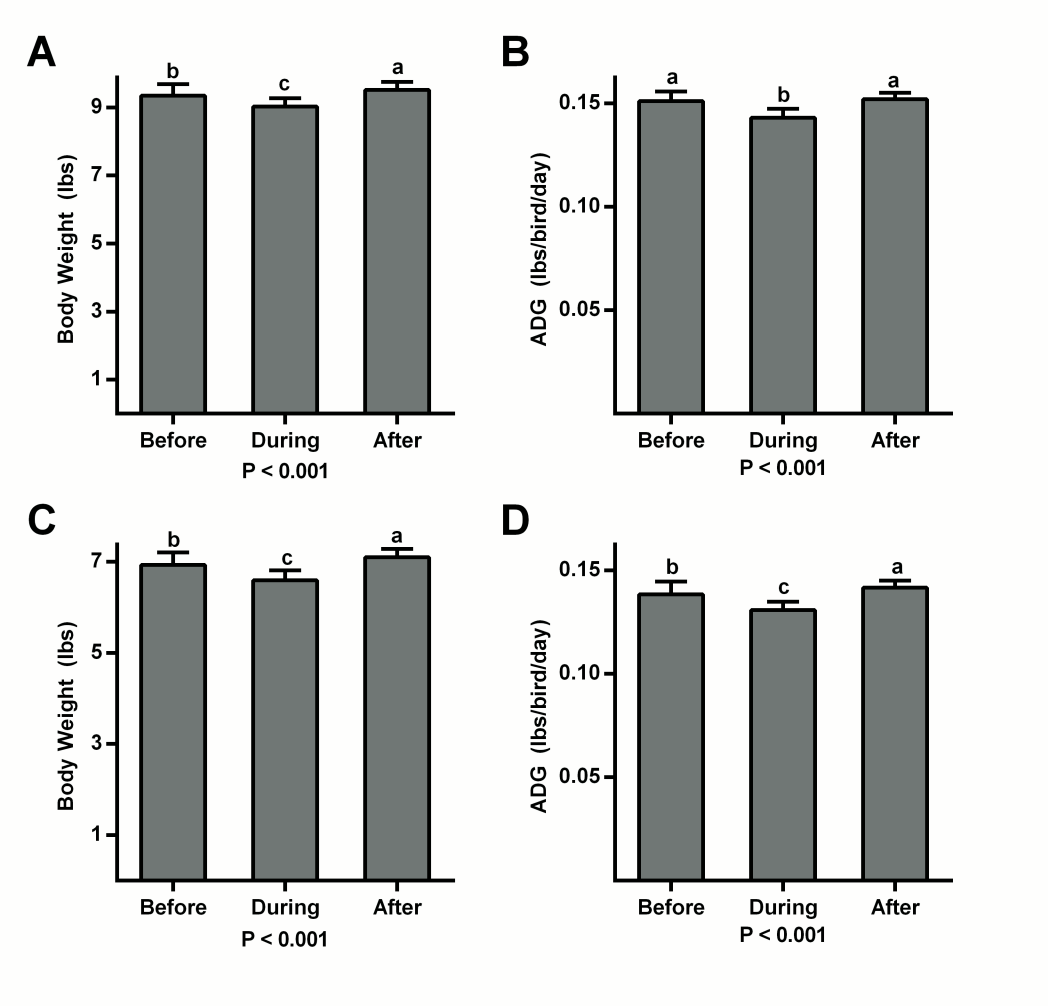

Figure 3.1: Body Weights and Gain. (A) Live Body Weights and **(B)** Average Daily Gain (ADG) of super birds**(C)** Body weight and (D) ADG of tray-pack birds. Body weight is shown as mean ± SEM lbs of body weight at processing. ADG is shown as mean ± SEM lbs per bird per day of weight gain. Different letters above bars indicate means are signficiantly different ($P < 0.05$).

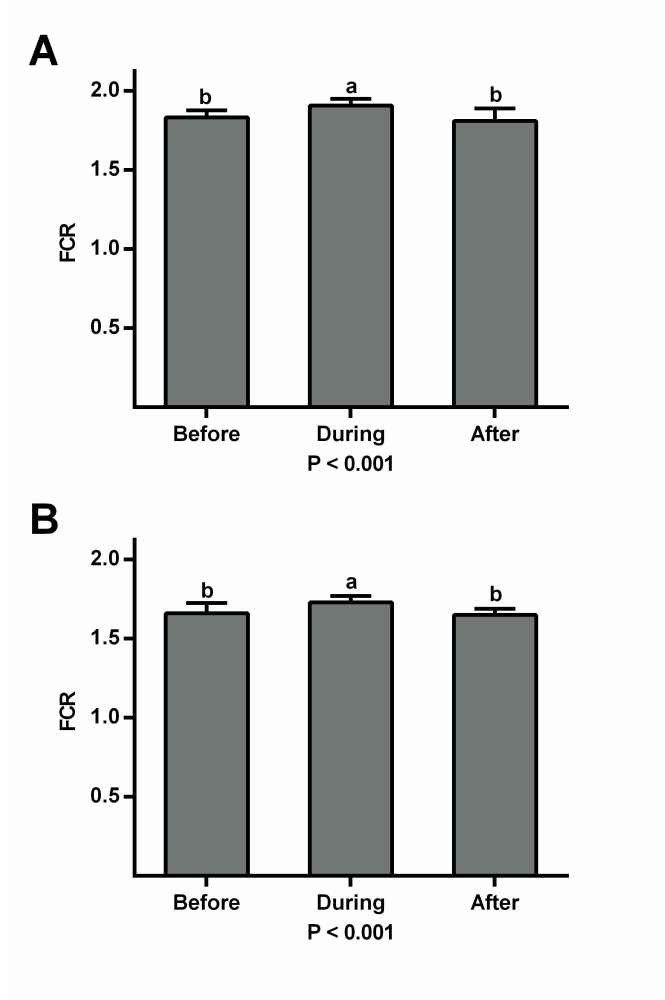

Figure 3.2: Feed Conversion Ratio. Mortality corrected feed conversion ratio (feed:gain) of **(A)** super birds and **(B)** tray-pack birds. FCR is shown as mean ± SEM feed:gain. Different letters above bars indicate means are signficiantly different ($P < 0.05$).

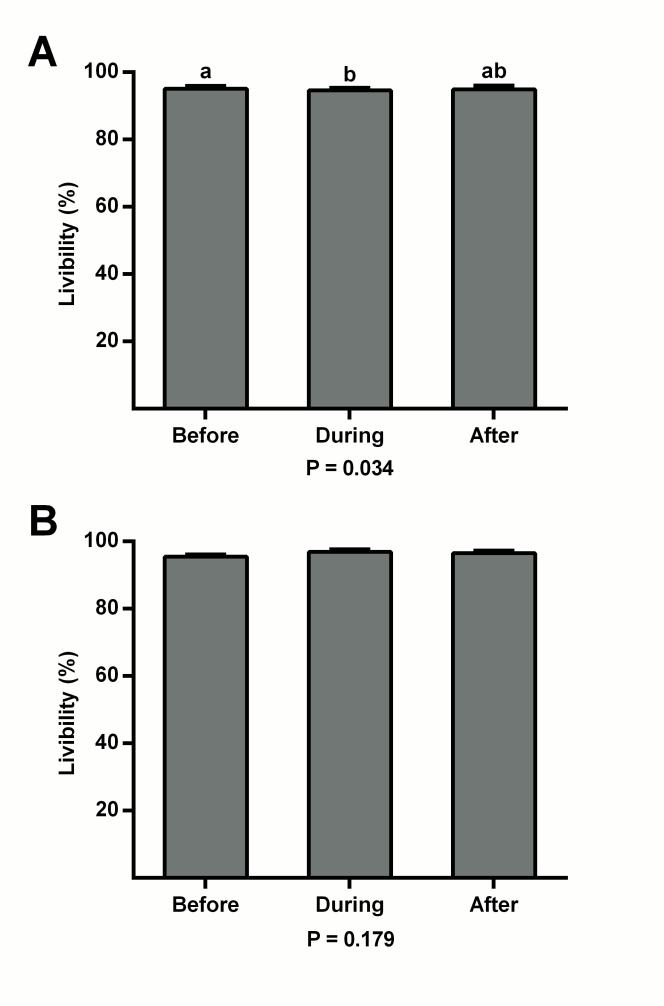

Figure 3.3: Livability. Livability (%) of **(A)** super birds and **(B)** tray-pack birds. Mean ± SEM % livability shown. Different letters above bars indicate means are signficiantly different ($P < 0.05$).

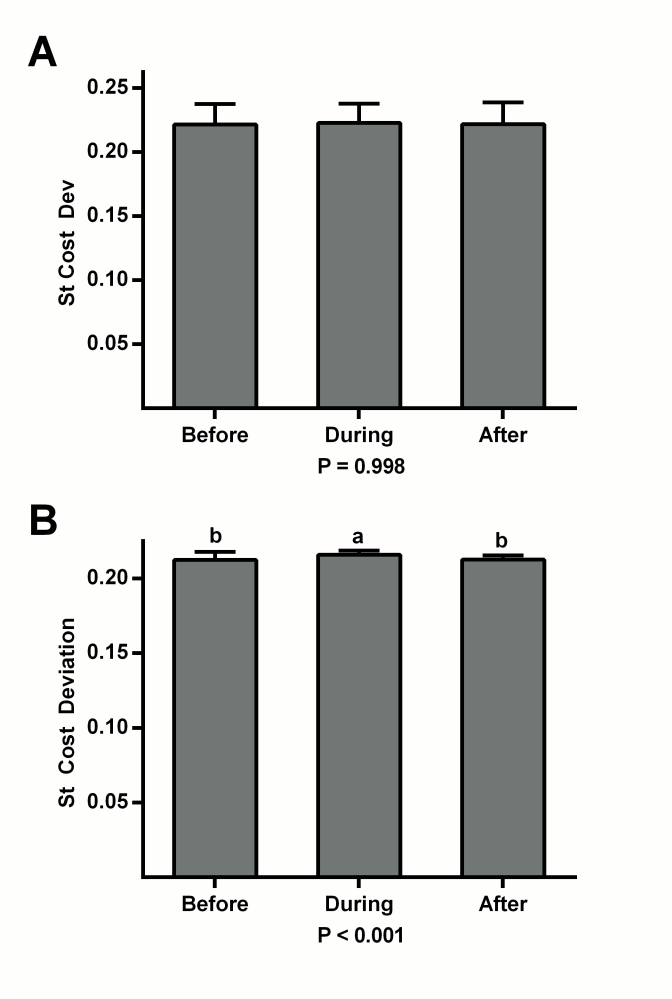

Figure 3.4: **Standard Cost Deviation.** Standard cost deviation of **(A)** super birds and **(B)** tray-pack birds. Mean ± SEM % deviation is shown. Different letters above bars indicate means are signficiantly different ($P < 0.05$).

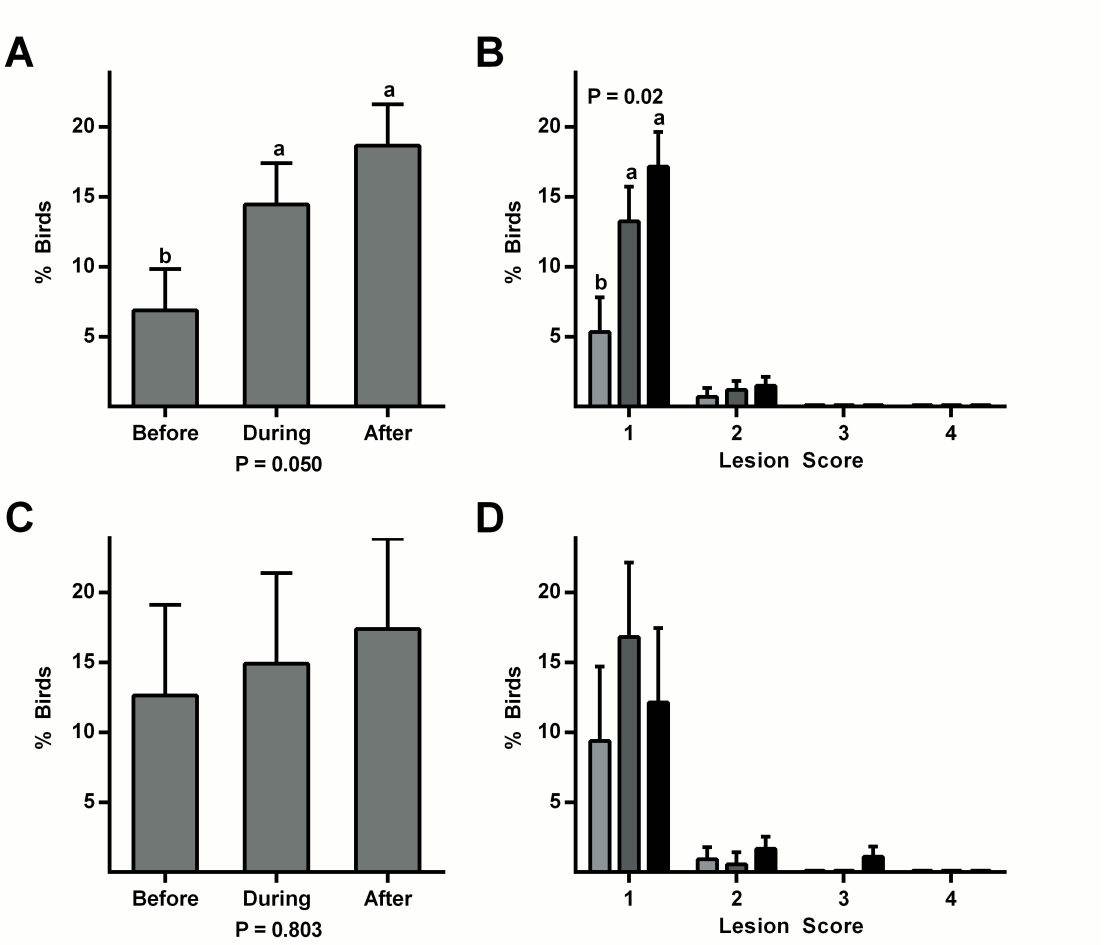

Figure 3.5: Gross *Eimeria acervulina* Lesions. (A) % of total birds with lesions and **(B)** distribution of lesion scores for super birds. **(C)** % of total birds with lesions and **(D)** distribution of lesion scores for tray-pack birds. Light gray bars, before; dark gray bars, during; and black bars, after. Different letters above bars indicate means are signficiantly different ($P < 0.05$).

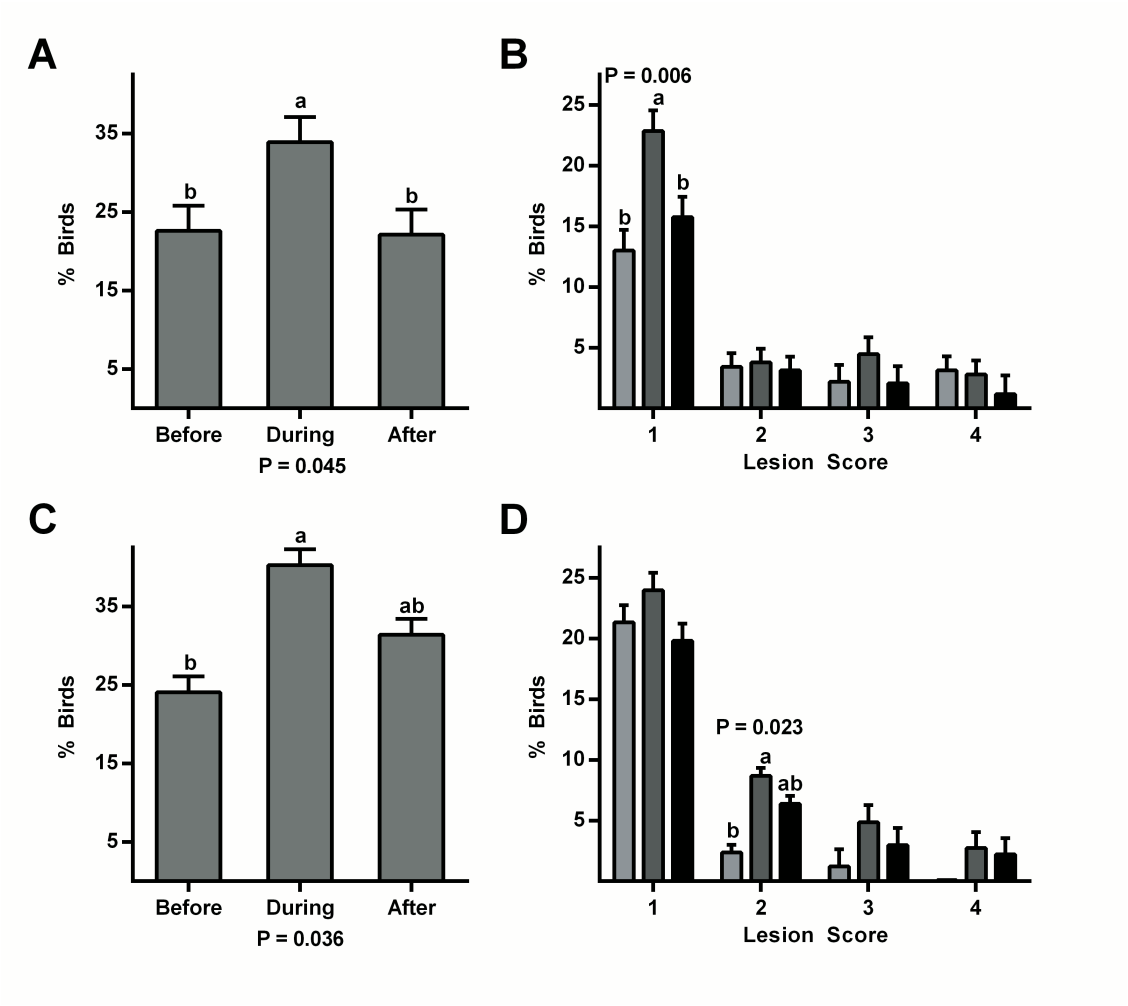

Figure 3.6: Microscopic *Eimeria maxima*. (A) % of total birds with lesions and **(B)** distribution of lesion scores for super birds. **(C)** % of total birds with lesions and **(D)** distribution of lesion scores for tray-pack birds. Light gray bars, before; dark gray bars, during; and black bars, after. Different letters above bars indicate means are signficiantly different ($P < 0.05$).

CHAPTER IV

EVALUATION OF THE POTENTIAL OF ACETYL COA-CARBOXYLASE AS A NOVEL TARGET FOR ANTICOCCIDIAL DRUGS IN BROILER CHICKENS

Introduction

Eimeria spp., the causative agent of coccidiosis in poultry, are apicomplexan intestinal parasites ubiquitous in commercial production environments. Intracellular replication of coccidia in the host intestinal epithelium results in intestinal damage, nutrient malabsorption, and bacterial dysbiosis which predispose the birds to necrotic enteritis (40). There are seven species of *Eimeria* known to infect chickens with varying levels of pathogenicity (154). The three species most commonly isolated from commerical broiler facilites are *Eimeria acervulina*, *Eimeria maxima* and *Eimeria tenella*. Each species has a tropisim towards specific segments of the intestinal track, and several species may infect chickens simultaneously potentially resulting in wide-spread intestinal damage (109, 153, 198).

Currently, coccidiosis is controlled through inclusion of anticoccidial drugs in feed and/or administration of vaccines containing live *Eimeria* at day of hatch. Anticoccidial drugs have been heavily used in commerical poultry production, and although drug rotation has been used to reduce and/or delay the development of resistance, reduced drug sensitivity and drug resistant *Eimiera* have been observed in the field (19, 30, 153, 174). There are very few anticoccidal drugs commerically available which can be rotated to manage the development of drug resistance.

Discovery of new anticoccidial compounds would reduce pressure on the current drugs and potentially delay development of resistance., Pharmaceutical companies have historically

conducted large screens for potential drugs. However, with reductions in budgets and advances in technology, anticoccidial drug discovery has been more consolidated and focused on drug targets. Because of the differences in gene conservation between chickens and *Eimeria,* recent research of new anticoccidial drugs has investigated potential targets in biochemical pathways (45). Several commercially available anticoccidial drugs currently target biochemical pathways in *Eimeria* spp. including such as the mannitol cycle, hypoxanthine and guanine acquisition, and the urea cycle (44, 197, 243). Since *Eimeria* are obligate intracellular parasites, determining the biochemical requirements of these parasite is difficult and research is scare. However, biochemical pathways or specific enzymes not conserved between *Eimeria* and chickens are the most promising for investigation as potential drug targets (88, 140).

Acetyl-CoA carboxylase (ACCase) catalyzes the conversion of acetyl-CoA to malonyl-CoA and is necessary for fatty acid synthesis in the apicoplast associated with host cell penetration (63). In related apicomplexan parasites including *Toxoplasma*, *Plasmodium*, and *Cryptosporidium,* broad-spectrum arylphenoxy carboxylic acids inhibitors of ACCase have been demonstrated to be effective in reducing parasite infections in mice and calves (88, 108). Analysis using X-ray crystallography has demonstrated derivatives of arylphenoxypropionate, aryloxyphenoxyacetate, and aryloxyphenylacetate to target the carboxyltransferase (CT) subunit of *Mycobacterium tuberculosis* ACCase (181). Computational analysis of homology-based structural models of the CT domain of *Eimeria* ACCase suggests these compounds may also target orthologous sites in *Eimeria* (194). Thus, ACCase may be a potentially important target for the development of anticoccidial drugs. In this study, we investigate the potential of ACCase as a target for the development of anticoccidial drugs. We will evaluate the effect of broad-spectrum arylphenoxy carboxylic acid inhibitors of apicomplexan ACCase in reducing gross intestinal lesions in broiler chickens experimentally infected with *Eimeria*.

Materials and Methods

Small molecule compounds predicted to have broad-spectrum inhibitory activity against acetyl CoA carboxylase (ACCase) from the arylphenoxy carboxylic acids including arylphenoxypropionate, aryloxyphenoxyacetate, and aryloxyphenylacetate, and their derivatives were chemically synthesized, purified, and analyzed for purity in the laboratory of Dr. James C. Sacchettini (Texas A&M University). Additionally, double amine substituents were also synthesized in order to evaluate non-orally absorbed derivatives of the phenoxy ACCase inhibitors. Individual candidate compounds were prepared for administration by oral gavage as a 10% (%w/v) DMSO solution and diluted in order to deliver 20 mg dose in 1 mL of vegetable oil suspension. For in-feed administration, candidate compounds were prepared as a pre-mix in limestone (1:10) and added to feed at 280 mg kg^{-1} feed to achieve a cumulative dose of 180 mg per experimental animal during the treatment period based on predicted feed consumption from the breeder. Compounds were mixed at a 1:10 ratio of commercial limestone. These premixes were then added to the respective diets for an inclusion of 20/mg/bird/day. In the study evaluating highly and lowly absorbed, the concentrations were decreased to 10/mg/bird/day. In order to protect the proprietary structures of these compounds, each candidate compound will be designated using a unique single letter code which will remain the same throughout all the studies.

Male broiler chicks (Cobb) were used in all experiments, housed in coccidia-free battery brooders, and provided feed and water *ad libtum* for the duration of each study. Animal care was provided in accordance with protocols approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC). Experimental rations were formulated to meet or exceed NRC requirements for broiler chickens (161). When appropriate salinomycin was added

to feed according to the manufacturer's instructions (60 ppm), while ACCase inhibitor/limestone compound premixes were added to achieve the desired dose.

Broiler chickens were experimentally infected by oral gavage with 10^6 oocysts using a commercial vaccine (Huvepharma, Inc.) containing non-attenuated live oocysts of *Eimeria acervulina*, *E. maxima* and *E. tenella.*

Two sets of birds were used: those orally administered the novel compound at inclusion rate of 20 mg/kg bird and those administered 40 mg/kg bird. Another group of birds were given the compound in the feed, and then infected with *Eimeria* (100,000 oocysts) to determine if infection altered absorption. 300 uL of blood was taken from 3 birds/time point/drug inclusion level in the first study and from 5 birds/time point/*Eimeria* infection in the second study.

Collected whole blood samples were collected, inverted. and stored on ice prior to separation of plasma using centrifugation. 20 uL of plasma was removed and added with 2.5 uL Internal Standard solution of methanol and Warfarin (1 ug/mL), 500 uL of methanol and 0.1% Formic acid. The solution was centrifuged at 14,000 rpm for 5 minutes (194). This was repeated and the supernatant was removed and pellet dried in speed vacuum at 40 C (194). Samples were reconstituted in 100 µL of acidified methanol, transferred into clean vial inserts, and 10 µl were subjected to LC-MS analysis (194).

A 10 µL sample solution was injected into a Kinetex 2.6 um EVO C18 100A LC Column 100 x 4.6 mm and separated by a mobile phase gradient elution of acetonitrile (A) and water (B) both containing 0.1% formic acid at a flow rate of 0.5 mL/min. An Agilent 1200 Series LC system was used to start acetonitrile at 10% and increase linearly to 100% from 0 to 8 minutes, hold B at 100% from 8 – 12 minutes, decrease linearly from 100% to 10% from 12 – 14 minutes, and hold at 10% for an additional 3 minutes (total 17 minutes). A Bruker Daltonics micrOTOF-Q

II LC/MS with an electrospray ionization system was operated in positive mode, at a source temperature of 220^0 C, dry nitrogen gas flow of 11 L/min, and under 3.5 bar of pressure. A 0.4 mg/mL sodium formate external calibration solution was directly injected at 13.0 minutes for future data processing and calibration. Quantitation was based on integrating peak area corresponding to the elution of internal standard and target compound in the extracted product ion chromatograms.

Standard calibration curves for each compound were prepared in acidified methanol in concentrations ranging from 1.95 to 1000 ng/mL using Warfarin (25 ng/mL as an internal standard. A series of 10 calibration standards and *in vivo* samples were assayed together along with 4 quality control samples. Data was collected using Bruker Hystar Software 4.1 and extracted ion chromatograms were created for each compound and Warfarin. Standard curves were constructed by plotting drug/internal chromatographic peak area ratio against the known drug concentration in each calibration standard. Calibration curves were fitted in a cubic curve with the y-intercept forced to zero.

Broiler chicks were placed on untreated feed and water and allow to acclimate for 10 days upon arrival to research facilities. On Day 10, birds were weighed, wing-banded, randomly assigned to treatment pens with similar starting weights, and provided their respective treatment diets. On Day 12, birds were experimentally infected with *Eimeria* (10^6 oocysts $bird^{-1}$) by oral gavage, while uninfected control birds were administered a mock infection containing water. On Day 18, the studies were terminated and all experimental animals were euthanized. Individual body weights were measured on Day 10 and Day 18, and feed consumption was determined on a pen basis. Sections of the duodenum, jejunum and cecum were dissected and gross intestinal lesion scored were scored using the method of Johnson and Reid (1970)(113).

One study, using 20 birds per treatment, was conducted to evaluate oral absorption of 2 candidate ACCase inhibitors administered by oral-gavage or in-feed. A second study was conducted to determine the effect of two orally-administered ACCase inhibitors in reducing *Eimeria*-induced lesions using 20 birds per treatment and two replicate pens. A third study evaluating the effect of administering 9 candidate ACCase inhibitors administered in–feed in reducing *Eimeria-induced lesions* was performed using 24 total birds per treatment across 4 replicate pens. A fourth study comparing the effects of absorbed and non- absorbed ACCase inhibitors administered in-feed was conducted using 36 total birds per treatment across 6 replicate pens.

Data was analyzed using one-way ANOVA General Linear Model (GLM). Significantly different means were separated using Duncan's multiple range test with $\alpha = 0.05$.

Results

Test compound A was used to evaluate the potential intestinal absorption of the phenoxy acid derived ACCase inhibitors administered by oral gavage and in-feed during *Eimeria* infection. Plasma concentration of Compound A from three birds per time point was evaluated over 24 hours post administration by oral gavage with 20 mg or 40 mg of compound administered per bird (**Figure 4.1**). The total plasma absorption of Compound A, represented using the area under curve (AUC)for was 4.685 and 5.270 ug hr mL^{-1} for birds receiving a 20 mg and 40 mg dose respectively. While the maximum plasma concentration (C_{max}) was 0.794 µg/mL and 1.013 µg/mL for birds receiving the 20 mg and 40 mg doses, respectively. Thus, increasing the dose from 20 mg to 40 mg resulted in a 20% increase in total absorption and a 25% increase in maximum plasma concentration for Compound A. Additionally, no mortality or clinical symptoms of toxicity were observed in any of the birds.

The potential effects of infection by mixed *Eimeria spp*. on drug absorption was evaluated (**Figure 4.2**). Compound A was administered in feed at a dose of 280 mg/kg feed to infected and uninfected broilers and plasma concentration was evaluated at 2.5 and 5.0 hours post administration. No difference in average plasma concentration between infected and uninfected broilers was observed at 2.5 hr post administration, but plasma concentrations of infected broilers was significantly lower than that of uninfected broilers at 5 hr post administration (**Figure 4.2A**). Additionally, because feed consumption varies and individual feed consumption was not recorded, drug absorption of individual birds was also evaluated (**Figure 4.2B**). Plasma concentrations of the drug were variable across individuals for both uninfected and infected birds. The plasma concentration of one uninfected bird was almost double the concentration of the other four uninfected birds the at 5 hours post drug

administration. Overall, plasma concentration was generally lower in the infected birds as compared to the uninfected birds. While plasma concentrations were higher at 5 hours post drug administration in the uninfected birds, plasma concentrations were higher or equal in only 3 of the 5 infected birds.

A preliminary evaluation of the effect of AACase inhibitor administration using oral-gavage on *Eimeria* infection in broiler chickens was performed (**Figure 4.3**). The efficacy of both ACCase inhibitors in reducing coccidiosis lesions was variable across each segment of the intestine of experimentally infected broiler chickens (**Figure 4.3)**. Compound B significantly reduced *Eimeria* lesion scores in the duodenum ($P<0.001$) as compared to unmedicated broilers (infected control) but did not reduce lesion scores in the jejunum or cecum (**Figure 4.3**). Although Compound A was not observed to a significantly reduce *Eimeria* lesions in any segment of the intestine, lesion scores were numerically lower in the ceca and duodenum as compared to the infected control (**Figure 4.3**). Salinomycin, was observed to significantly reduce *Eimeria* lesions in the duodenum ($P<0.001$) and ceca ($P<0.001$) (**Figure 4.3**). However, *Eimeria* lesion scores in the jejunum were higher for broilers administered salinomycin that for the infected controls ($P=0.009$) (**Figure 4.3**).

BWG was significantly greater when broilers were administered salinomycin greater ($P<0.001$) when compared to the other treatments (**Figure 4.4**), and no differences in BWG were observed between the other treatments (**Figure 4.4**).

The effect of in feed administration of 9 novel ACCase inhibitors in reducing coccidiosis lesion scores was evaluated in experimentally infected broilers (**Figure 4.5**). Administration of the various phenoxy ACCase inhibitors had a significant effect on the development of *Eimeria* lesion scores in the duodenum ($P = 0.007$), jejunum ($P = 0.003$), and the cecum ($P < 0.001$), with

the individual compounds having varying efficacy in reducing *Eimeria* lesions in each segment of the intestinal tract of broiler chickens.

In the duodenum (**Figure 4.5A**), lesion scores of broilers administered the phenoxy ACCase inhibitors were lower than the unmedicated infected control (UIC) or at least similar to the Salinomycin treated broilers. Although administration of Compounds D and F was not observed to significantly reduce lesion scores when compared the control infection, lesion scores were similar to the Salinomycin treated broilers. Administration of the remaining compounds was observed to significantly reduce lesions scores when compared to the control infection. In the jejunum (**Figure 4.6B**), administration of salinomycin did not reduce lesions when compared to the control infection. However, lesions scores in the jejunum were significantly reduced compared to the control infection when broilers were administered compounds D,E, F, G and I (**Figure 4.5B**). In the cecum (**Figure 4.5C**), *E. tenella* lesion scores were lower than the control infection only for broilers administered Compound B and Salinomycin.

Body weight gain of experimentally infected broilers was significantly lower than for the uninfected control broilers regardless of the treatment administered. However, Salinomycin was the only treatment to significantly increase the body weight gain when compared to the Untreated-Infected Control broilers (**Figure 4.6**). Salinomycin was also the only treatment to improve FCR compared to the UIC (**Figure 4.6**).

The effect of the administration of double amine substituents was evaluated in order to determine whether intestinal absorption of phenoxy ACCase inhibitors was required to reduce the effects of *Eimeria* infection in broiler chickens. No significant differences were observed in *E. acervulina* lesions, including the unmedicated control (P=0.082) (**Figure 4.7A**). However, there was a trend observed that salinomycin was lower than Compound K, a low-soluble

ACCase inhibitor. No differences were observed with *E. maxima* lesions in the jejunum, including the unmedicated control (**Figure 4.7B**) (P=0.938). Salinomycin, highly absorbed Compound B and low-absorbed Compound K significantly reduced *E. tenella* lesion scores in the ceca compared to the infected control (**Figure 4.7C**) (P<0.001). No significant differences were observed between the unmedicated control and low-absorbed Compound J.

A significant effect of coccidia infection was observed on BWG from D10-D18 (**Figure 4.8A**) (P<0.001). Salinomycin bird weights were significantly different from the uninfected and the unmedicated control (**Figure 4.8A**) (P<0.001). Compound J and K were not different from the unmedicated control (**Figure 4.8A**). Compound B was significantly lighter than the unmedicated controls (**Figure 4.8A**) (P<0.001). A significant effect of coccidia infection was observed in FCR D10-19 (**Figure 4.8B**) (P<0.001). The uninfected controls had significantly lower FCR than all treatments D10-18 (**Figure 4.8B**). No significant differences in FCR D10-18 were observed between salinomycin and the unmedicated control (**Figure 4.8B**). Compound B FCR D10-18 was significantly higher than the unmedicated control (**Figure 4.8B**) (P<0.001).

Discussion

Apicomplexan parasites are distinguished by the presence of an apicoplast, an organelle thought to have arisen from endosymbiosis of an algal cell which had incorporated a cyanobacterium (141). Enzymes in pathways expressed in the apicoplast may serve as promising drug targets because they are not conserved in the cells of these parasites' hosts (87). Additionally, because of the apicoplast's likely plant origin, herbicides and their derivatives have been shown to be efficacious drugs against some Apicomplexan parasites.

Arylphenoxy acid derivatives have been used widely as herbicides for the control of some broadleaf plants in crop production (89). The "phenoxy" herbicides inhibit Acetyl CoA-Carboxylase (ACCase) localized in the chloroplasts of Graminaceae plants (95). The insensitivity of ACCases of other plants, mice, and humans to the phenoxy herbicides allows their wide-spread commercial application (200). ACCase catalyzes the conversion of acetyl-CoA to malonyl-CoA which is the first committed step of fatty acid synthesis. Because it is necessary for fatty acid synthesis in the apicoplast and is associated with host cell penetration, ACCase is a potentially important target for the development of therapeutic drugs against apicoplast parasites (227).These drugs have been shown to target the carboxyltransferase (CT) domain of apicoplastic ACCase, but have no effect on the cytosolic form of the enzyme (88). In vitro studies with Apicomplexan *Toxoplasma gondii*, administration of a selected ACCase resulted in 70% inhibition of parasite growth (263). Importantly, phenoxy acids are produced inexpensively. In 2005, the estimated price of one gram of clodinafop, an effective inhibitor of *T. gondii*, was less than 15 cents (87). Commercial production of these drugs to the scale useful for the poultry industry, could prove affordable for poultry producers.

Compound A was administered to broiler chickens by oral gavage (**Figure 4.2**) and in-feed (**Figure 4.2**) in order to model intestinal absorption of phenoxy acid derivative ACCase inhibitors. We have demonstrated that Compound A was absorbed intestinally when administered in-feed or as a bolus by oral gavage, suggesting that other phenoxy acid derivatives would also likely be absorbed intestinally. Pharmacokinetic data has demonstrated high intestinal absorption of most all commercially available anticoccidials such as Amprolium and Salinomycin (17, 92, 98, 124, 165, 175). However, decoquinate is the only commercially available anticoccidial in the United States that has low-absorption rates (50). Absorption of Compound A was comparable to commercially available anticoccidials.

Additionally, oral absorption of Compound A was compared between *Eimeria* infected and uninfected birds. Absorption of Compound A was higher in uninfected birds compared to *Eimeria* infected birds. Individual drug serum levels were variable in both *Eimeria* infected and uninfected birds. The absorption of Compound A in *Eimeria* infected birds was lower than uninfected cohorts at 5 hours post feed administration. This may be related to decreased absorption of nutrients common to *Eimeria* infections (116, 201). On the other hand, decreased feed intake is often associated with *Eimeria* infection, which subsequently would reduce in-feed drug ingestion (183). However, some research has shown that reduced feed intake results in increased drug absorption because of gastric emptying (155). Total absorption (AUC values) of the chemical anticoccidial Amprolium has been demonstrated to be greater when birds were fasted compared with those under non-fasting conditions (92).

Lesion scores for all regions of the intestine were numerically lower when broilers were administered ACCase inhibitor compounds when compared to unmedicated controls in the study where birds were given ACCase inhibitors through oral gavage. However, except for *E.*

acervulina lesions when compound B was administered, none of the reductions were significant. Greater numbers of *E. acervulina* oocysts are included in the coccidia vaccine used compared to *E. maxima* and *E. tenella*, so this could explain why there were more differences in lesions observed between treatments (Kimminau, unpublished data). No differences in growth performance were observed between either ACCase inhibitor and the infected controls. We hypothesize that the daily oral gavaging of birds contributed to the performance differences, as Salinomycin was administered in the feed. This experiment was designed as a preliminary evaluation of the anticoccidial properties of these compounds, and the subsequent experiments included more replicates, more birds. and administration of the drugs in- feed.

We determined the mixability into feed/water and stability at room temperature of phenoxy herbicide derivatives was sufficient for their application in feed (not shown). The importance of these characteristics for anticoccidial drugs was outlined by Edgar in 1970 (64). Because anticoccidials are more commonly administered in-feed, this is the drug delivery method we chose for all subsequent experiments (154). Although drug premix formulas (Type A medicated article) are proprietary, anticoccidials are typically formulated as a mixture containing limestone, mineral oil, calcium silicate, silicon dioxide and micro-tracers (119, 237). From the results of the pharmacokinetics studies, we can conclude that our compounds were able to be absorbed when administered to the bird diets.

We conducted an initial evaluation of 9 candidate phenoxy acid derivative ACCase inhibitors administered in feed to experimentally infected broilers. The uninfected controls were significantly heavier than infected controls, demonstrating a heavy coccidial challenge.

Only one of the compounds other than Salinomycin reduced *E. tenella* lesions, while several of the compounds reduced *E. acervulina* lesions. Anticoccidials have variable

anticoccidial efficacy against different *Eimeria* species. For example, Amprolium is highly efficacious against *E. tenella* and *E. necatrix*, but is less efficacious against species such as *E. maxima* and *E. acervulina* (57). Although *E. acervulina* is not considered highly pathogenic, it is commonly found in most commercial broiler production facilities and is known for high reproductive potentials (106, 138, 248). Therefore, the compounds with efficacy against *E. acervulina* would be beneficial for commercial broiler production.

Because the *E. maxima* gross lesion scores for were low (0.5 average) in the infected control group, it is difficult to interpret anticoccidial efficacy against this species. Single species infections would give more insight to efficacy against *E. maxima* without the *E. acervulina*-crowding that is likely the case in this study (248).

In this study, the inclusion of the phenoxy compounds (140 mg/kg feed) was more than double for Salinomycin (60 mg/kg feed). The extreme drug dose, could explain no significant body weight gain in the phenoxy compound treatments. High levels of Salinomycin has been shown to reduce body weight of birds (94). Many attribute the continued success of current anticoccidials to their ability to allow "leakage" of some coccidia, which reduces the genetic pressure for resistance as well as allows immunity development (32). Determining the dose of the ACCase inhibitors that sufficiently reduces coccidia while allowing some leakage to stimulate for immune development is important to the potential development of these compounds as drugs.

Another reason for the lack in performance differences is the activity of the compounds against specific *Eimeria*. Some *Eimeria*, such as *E. maxima*, require fewer oocysts to reduce performance (42). Others such as *E. tenella*, are pathogenic, but require more oocysts to reduce performance (42). Some of the novel compounds that demonstrated reduction of *E. tenella*

lesions, may not necessarily improve performance comparable to the UUC treatment. Future research with single species infections would allow better comparison of anticoccidial activity for each *Eimeria* species.

These experiments were designed to evaluate the ability of ACCase inhibitors to reduce coccidiosis lesions. The *Eimeria* challenge was strong, and in one of our studies, caused mortality. However, the study did not account for compensatory gain that is seen after *Eimeria* infection (250). Lowering the dose of drug as well as extending the time period of the study would allow for better understanding of the ACCase inhibitors ability to reduce coccidiosis lesions as well as improve performance when compared to the control infection treatment.

Salinomycin did not reduce *E. maxima* lesions but was higher than the unmedicated controls in all of the in-vivo studies. Previous research with mixed *Eimeria* infections showed *E. maxima* lesions were not significantly different with inclusions of Salinomycin (66 ppm) or unmedicated controls (41). Research with single *E. maxima* infections and Salinomycin (66 ppm) demonstrated a reduction in lesion scores, but was not as efficacious as semduramicin (325 ppm) (43). Because the *Eimeria* isolates used were from a coccidia vaccine, which are sensitive to anticoccidials, so resistance is not likely. However, research has shown co-infection with *E. acervulina* can interfere with reproductive potentials of *E. maxima, E. brunetti, E. necatrix* and *E. tenella* infections (248). Salinomycin is very effective at reducing *E. acervulina* (61, 260). It may be possible that the *E. acervulina* from the vaccine was reduced enough by Salinomycin, to give vaccine *E. maxima* more opportunity to infect.

Anticoccidials have a varying level of intestinal absorption. Some such as Amprolium, Salinomycin and Toltazuril are highly absorbed while others such as decoquinate are not (50, 71). A benefit of lowly absorbed drugs is that they have lower toxicity. Researchers administered

nequinate, a quinolone related to decoquinate, at 100 times the therapeutic level with no adverse effects of the birds (193). For example, Decoquinate is not absorbed, with 97.5-99% recovery of after oral administration (50, 71). Others such as Salinomycin are more bioavailable and readily absorbed (17).

In our study, it did not appear that the lower-absorbed drugs were more efficacious in reducing lesion scores. Although not significant, the body weights of the birds with the higher absorbed drug (Compound B), were lower than the lower-absorbed drugs.

In an earlier study, Compound B significantly reduced lesions for *E. acervulina* and *E. tenella* but not *E. maxima*. The inclusion levels of the drug in that study were 280 mg/kg feed, and 140 mg/kg feed in this study. Since there was no significant reduction in the lesions when a lower level of drug was included, a dose response study would give more insight to an optimum amount.

Research in plants with these ACCase inhibitor herbicides has shown that variation in residues where the herbicide binds, may account for variable sensitivity (87). Therefore, it seems plausible that modifying these dimer binding sites to target Apicomplexa specific residues, could drastically improve the efficacy of these inhibitors against specific parasites such as *Eimeria*. It seems ACCase is a promising drug target and these inhibitors warrant further research as anticoccidials.

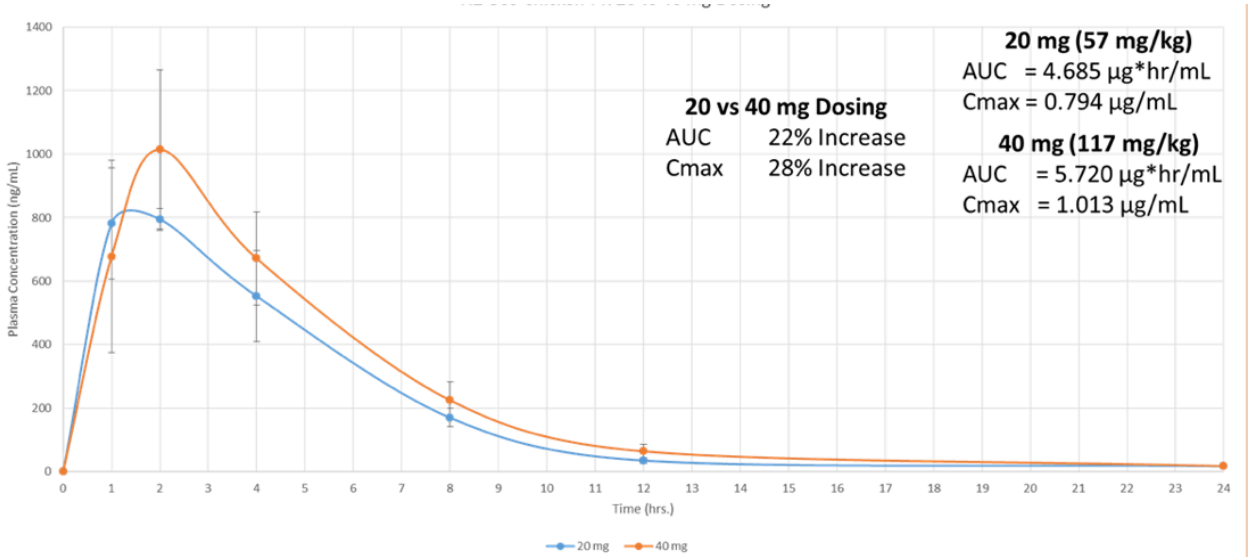

Figure 4.1: Pharmacokinetics of Orally Gavaged Compound A. Drug given at 20 mg/kg bird and 40 mg/kg bird.

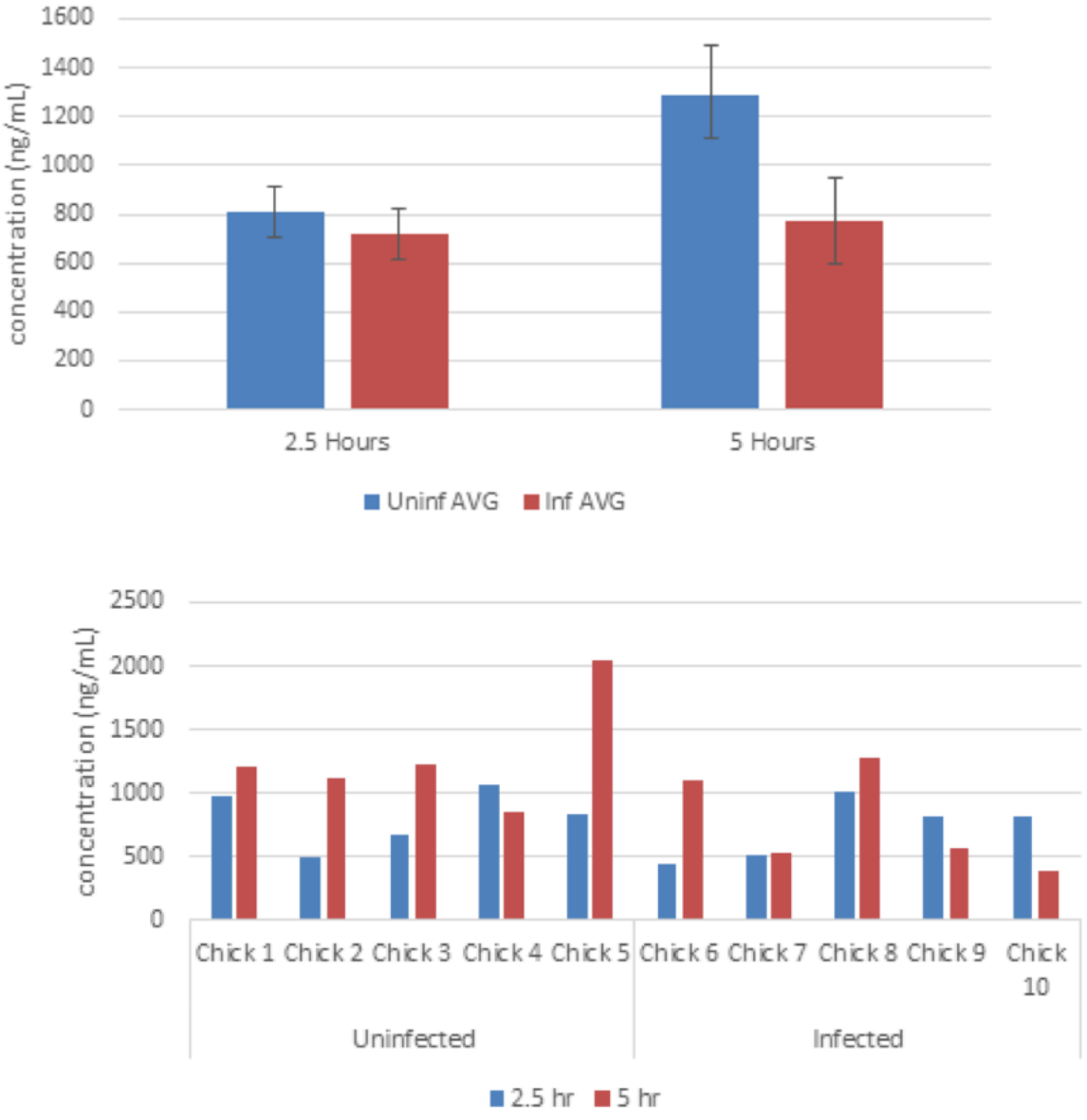

Figure 4.2: Drug Plasma Concentration. A-Top:. Average drug plasma concentrations in *Eimeria* infected (Inf) or uninfected (Uninf) birds 2.5 or 5 hours after given feed with 280 mg compound A /kg feed. **B-Bottom**: Individual plasma drug concentrations of Compound A in *Eimeria* infected or uninfected birds 2.5 or 5 hours after given feed with 280 mg compound /kg feed.

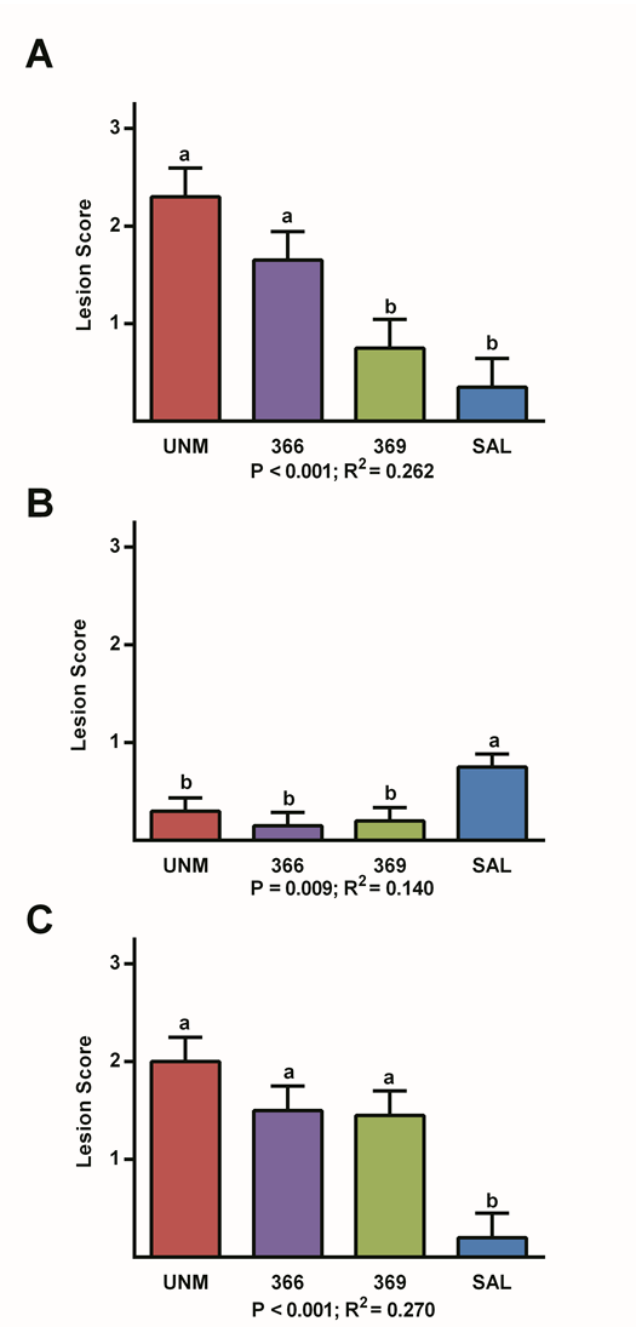

Figure 4.3: Lesion Scores. (A) Gross intestinal lesions in the **(A)** duodenum caused by *E. acervulina*, **(B)** jejunum caused by *E. maxima* and, **(C)** cecum caused by *E. tenella*. Lesion scores expressed as the mean ± SEM from 20 broilers per treatment. Different letters indicate means are significantly different ($P < 0.05$). UNM is unmedicated, infected control. SAL is Salinomycin. 366 and 369 are two compounds used.

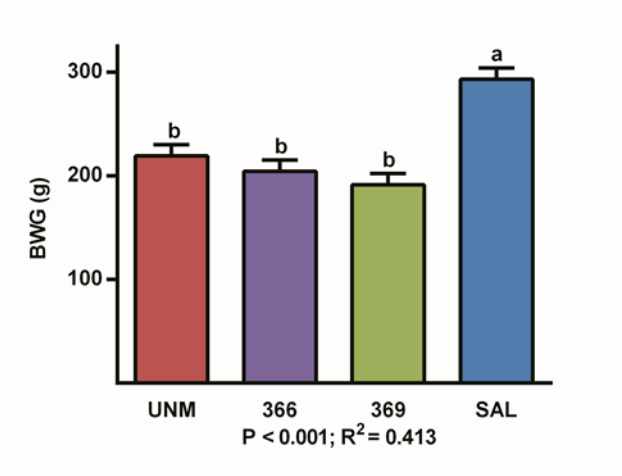

Figure 4.4: Body Weight Gain (10 – 18D). BWG expressed as mean ± SEM from 20 broilers per treatment. Different letters indicate significantly different means ($P < 0.05$). UNM is unmedicated, infected control. SAL is Salinomycin. 366 and 369 are two compounds used.

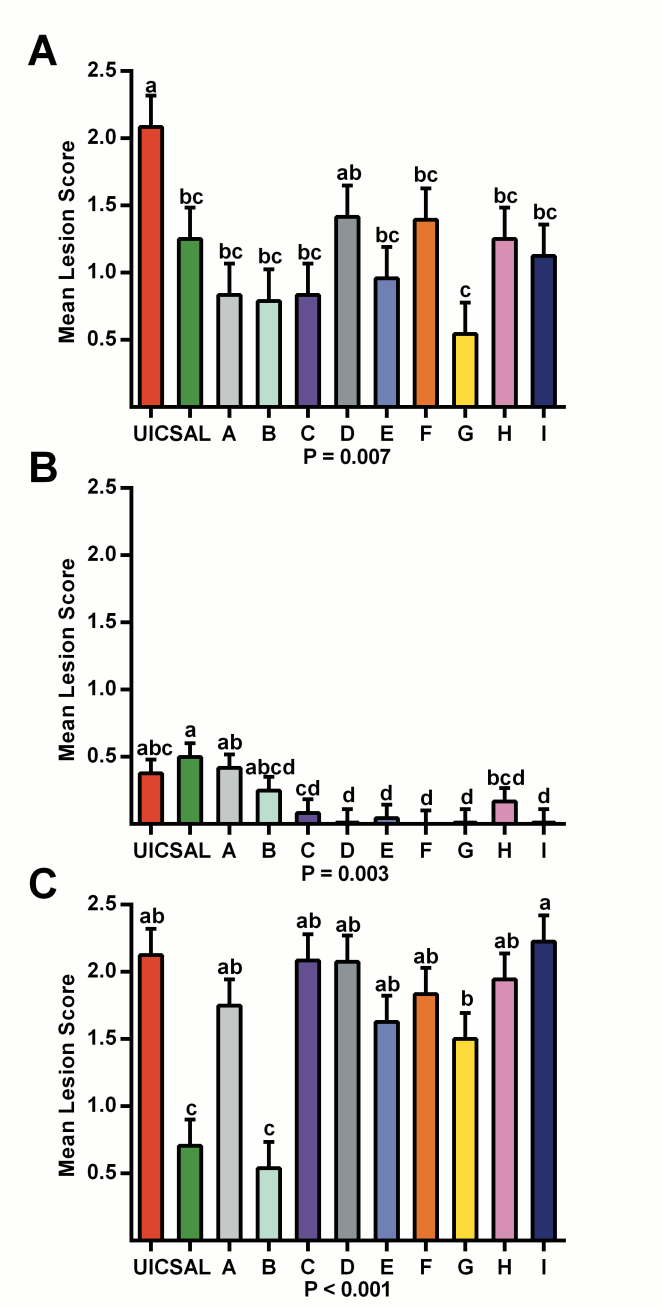

Figure 4.5: Lesion Scores. Gross intestinal lesions in the **(A)** duodenum caused by *E. acervulina* **(B)** jejunum caused by *E. maxima* and, **(C)** cecum caused by *E. tenella*.; UIC, untreated infected control; SAL, salinomycin. Lesion scores expressed as the mean ± SEM from 4 pens per treatment. Different letters indicate significantly different means ($P < 0.05$). UUC is unmedicated, uninfected control. UIC is unmedicated, infected control. S is Salinomycin. A, B, C, D, E, F, G, H and Iare respective compounds used.

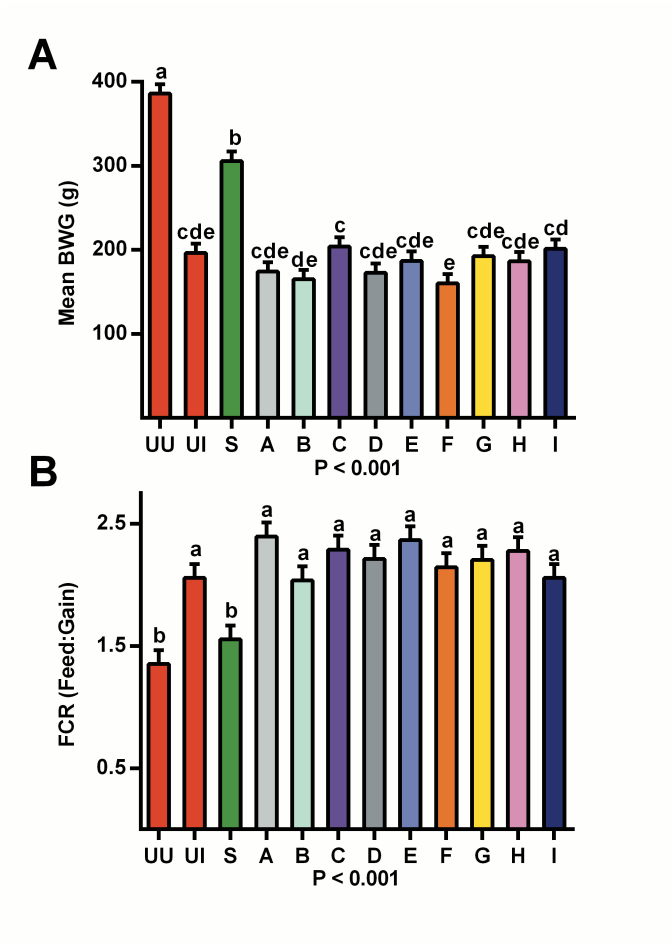

Figure 4.6: Broiler Performance Results. (A) Body weight gain D10 –D18. **(B)** Average Pen FCR D10-D18. UU, untreated uninfected control; UI, untreated infected control; S, salinomycin. Both expressed as mean ± SEM from 4 pens per treatment. Different letters indicate significantly different means ($P < 0.05$). UUC is unmedicated, uninfected control. UIC is unmedicated, infected control. S is Salinomycin. A, B, C, D, E, F, G, H and I are respective compounds used.

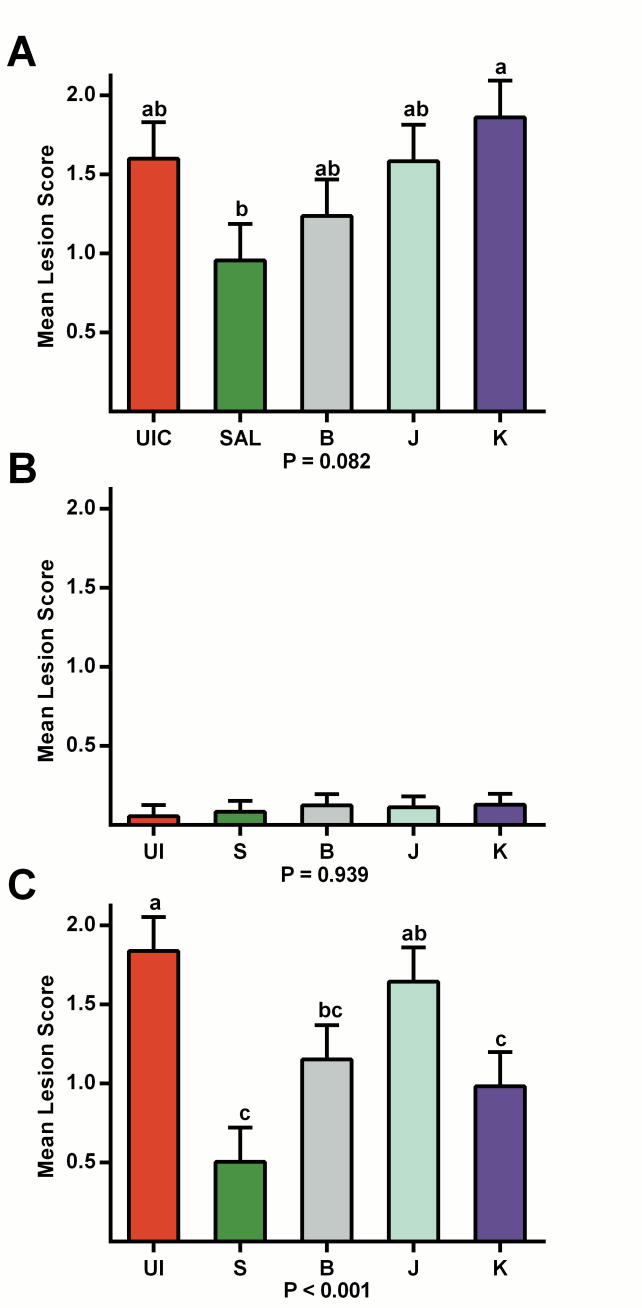

Figure 4.7: Lesion Scores. Gross intestinal lesions in the **(A)** duodenum caused by *E. acervulina*, **(B)** jejunum caused by *E. maxima* and, **(C)** cecum caused by *E. tenella*. Lesion scores expressed as the mean ± SEM from 4 pens of 6 broilers per treatment. Different letters indicate significantly different means ($P < 0.05$). UUC is unmedicated, uninfected control. UIC is unmedicated, infected control. SAL is Salinomycin. B, J and K are respective compounds used.

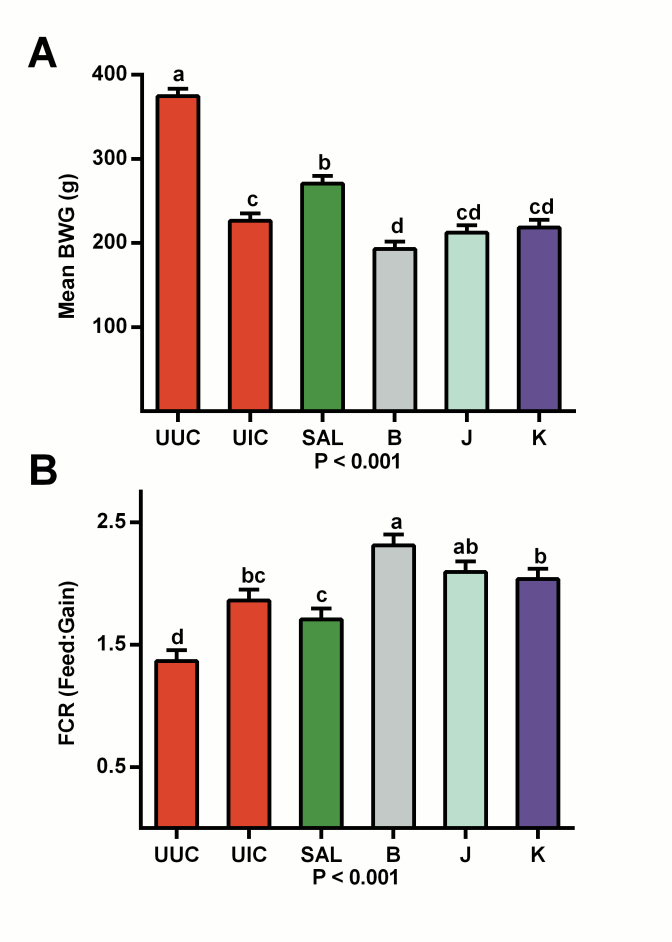

Figure 4.8: Broiler Performance Results. Average individual body weight gain D10-D18. BWG expressed as mean ± SEM from 36 broilers per treatment. Different letters indicate significantly different means as determined using Duncan's multiple range test ($P < 0.05$).UUC is unmedicated, uninfected control. UIC is unmedicated, infected control. SAL is Salinomycin. B,J and K are respective compounds used.

CHAPTER V

CONCLUSION

Control of coccidiosis and necrotic enteritis is a significant cost to poultry producers world-wide. Because of the ubiquity of *C. perfringens* and *Eimeria* in poultry production environments, it is unlikely these pathogens will be eradicated. Thus, emphasis must be placed on improving current control methods as well as researching new drugs and additives as a part of overall management programs to help mitigate intestinal damage.

The research presented here investigated antibiotic free, conventional and exploratory interventions. The future control of coccidiosis and necrotic enteritis will rely not only on optimizing current interventions but development of new ones as well. As more producers move from antibiotic-use during poultry production, alternatives will continue to be required. For poultry producers that continue to use non-human antibiotics, such as ionophores, understanding the dynamics of concurrent use of these products and vaccines is also important.

Each intervention evaluated showed variable efficacy in reducing coccidiosis and necrotic enteritis. The DFM tested with a coccidia vaccine demonstrated some efficacy in reducing vaccine-related performance losses. Future research into other DFM will potentially find products with greater efficacy in reducing performance losses from coccidia vaccine use. In addition, novel ACCase inhibitor compounds showed promising results as anticoccidials.

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