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Removal of Dietary Antimicrobials and Effects of their Replacement with Bacillus Subtilis on the Growth and Intestinal Health of Male Broilers

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By

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Removal of dietary antimicrobials and effects of their replacement with Bacillus subtilis on the growth and intestinal health of male Broilers

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The effects of dietary antimicrobial removal and *Bacillus subtilis* supplementation on the growth and intestinal health of male broilers were investigated. Birds were fed either a control, antimicrobial, or a *B. subtilis* probiotic diet at different feeding phases. Birds were challenged with a 10 × dose of a coccidiosis vaccine. Supplementation of *B. subtilis* in for antimicrobials in the late grower and early finisher phases improved growth similar to birds fed antimicrobials until withdrawal, while antimicrobial removal without *B. subtilis* supplementation in those periods hindered growth. The improved growth suggests that the probiotic was able to alleviate the stress of the challenge compared to antimicrobial removal. Processing yields were improved with antimicrobial removal and *B. subtilis* supplementation in late grower and early finisher phase. Intestinal health was improved with lower intestinal lesions when antimicrobial were removed and *B. subtilis* supplemented suggesting the reduction of *Eimeria* species from colonizing the intestine.

DEDICATION

I would like to dedicate this thesis to my family for their constant love, support, and encouragement.

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CHAPTER I

INTRODUCTION

Antibiotics have been used for over fifty years in the poultry industry (Jones and Ricke, 2003). With their continued use, the poultry industry has been able to improve bird growth and feed utilization, and provide them with a defense against diseases (Gustafson and Bowen, 1997). Over the years there has been an increase in the amount of antibiotics used in the poultry industry. In an estimate of antibiotic consumption by farm animals in 2010, the poultry, beef, and cow industry have consumed over 63 tons of antibiotics, and that number will increase by 67% by 2030 (Van Boeckel et al., 2015). Consumption of antibiotics to that magnitude can cause problems for the industry because there are growing concerns by consumers over antibiotic resistance in bacteria and antibiotic residue in poultry meat (Donoghue, 2003; Singer and Hofacre, 2006). These concerns have forced the industry to shift their view on antibiotics and begin looking at antibiotic alternatives.

There are many antibiotic alternatives to choose from that benefit poultry production and health. One of these alternatives is probiotics. Probiotics are live microorganisms which benefit the health and gastrointestinal tract of the host. Probiotics protect the birds in many ways, including: the enhancement of epithelial barriers, which would improve intestinal health; competitive exclusion, which would reduce the chances

of pathogenic bacteria from colonizing the intestine; and stimulation of the immune system (Kabir, 2009).

By positively affecting the gastrointestinal tract, the bird's overall growth and immune system may also have been affected. Hrncar et al. (2016) reported that when birds are given a mixture of probiotics in the diet that the birds had higher individual body weights and improved livability than birds given a control diet (Hrncar et al., 2016). Bird given probiotics can also have an improved body weight, not only when given in feed, but also through the water, when compared to birds given antibiotics and challenged with *E. acervulina*, *E. maxima*, and *E. tenella* (Ritzi et al., 2014). Jayaraman et al. (2017) observed that when necrotic enteritis was induced in birds when orally inoculated with *Eimeria* and *Clostridium perfringens*, supplementation of the probiotic *B. subtilis* were able to reduce the amount of *C. perfringens* in their intestines compared to infected controls. They also experienced less intestinal damage by *Eimeria* species and *C. perfringens* when compared to infected controls (Jayaraman et al., 2017).

Coccidiosis is a global poultry disease caused by a parasite from the genus *Eimeria* (Peek and Landman, 2011). Coccidiosis species are easy to carry from farm to farm and once *Eimeria* species get into a poultry house it is impossible to remove the *Eimeria* oocysts, leaving vaccines and anticoccidials medications as the only option to control them. Controlling coccidiosis costs the industry over three billion dollars annually worldwide (Chapman et al., 2002; Dalloul et al., 2006; Chapman, 2007). *Eimeria* species cause growth reduction, increased malabsorption and mortality, and increased intestinal lesions in birds (Dalloul and Lillehoj, 2006, Conway and McKenzie, 2007).

With growing concerns over antibiotics and anticoccidials and the benefits of probiotic species being reported, to our knowledge there is no research indicating when to remove antibiotics from feed and supplement probiotic *B. subtilis* without compromising the welfare, growth, and health of poultry when stressed by coccidiosis.

CHAPTER II

LITERATURE REVIEW

Antibiotic Use in Poultry Diets

The use of antibiotics in the poultry diet began during World War II. The poultry industry was growing during this period due to improvements in genetics and nutrition. By 1951, antibiotics could be used without the approval of a veterinarian (Jones and Ricke, 2003). The poultry industry is designed to produce chicken quickly and at a low cost. To accomplish this, the industry uses antibiotics to keep birds healthy and free of diseases (Gustafson and Bowen, 1997). Antibiotics are now used as growth promoters to improve the well being of birds, prevent diseases, and improve body weight and lower the feed conversion of birds when administered at low dosages (Gustafson and Bowen, 1997; Huyghebaert et al., 2011).

Concerns of Antibiotic Use

In recent years, there have been concerns from consumers that the use of antibiotics in poultry diets could be harmful to humans. A common misconception is that chicken meat contains antibiotic residues from the feed. It is believed that with the use of antibiotics in poultry feed, there is always a chance of antibiotic residue getting into poultry meat. However, if birds are withdrawn from antibiotic diets properly before processing, the chances of antibiotics being in the meat can be almost nonexistent. The

withdrawal period allows antibiotics that had been consumed by the birds to be flushed from the bird's system before processing (Gustafson and Bowen, 1997; Singer and Hofacre, 2006). The withdrawal period is there to protect humans from consuming meat that has antibiotic residue. If the poultry industry adheres to the correct withdrawal time for the antibiotic used, the chances of consumers eating contaminated meat is slim. However, it is still a concern of consumers, which puts pressure on the industry to limit the use of antibiotics used in diets.

Another concern raised by consumers is the emergence of antibiotic resistant bacteria due to the feeding of antibiotics to food animals. Antibiotic resistant bacteria have been seen worldwide and are a health risk. Poultry are given low therapeutic dosages of antibiotics in the diet which could cause the emerging of antibiotic resistant bacteria (Apata, 2009). This can be problematic if humans contract a pathogen in the farm to fork route. This is when pathogens that have become resistant to antibiotics on the farm most often enter the food chain and expose people to resistant bacteria which could cause a risk to human health (Singer and Hofacre, 2006). Thus, when humans receive antibiotics, they become no longer effective. Humans could also come into contact with antibiotic-resistant bacteria through the environment, such as through the water near poultry farms or used litter when it is recycled to gardens or other farms. This could cause a higher mortality rate in humans who come into contact with certain resistant bacteria, especially if a community is prone to a disease that has become resistant to antibiotic treatment (Singer and Hofacre, 2006; Apata, 2009). These concerns

have led governments of different countries to step in and establish regulations on the poultry industry with regards to antibiotic use.

The Banning of Antibiotics

Due to concerns from consumers, the European Union banned the use of antibiotics for growth promotion purposes in animal feed in 2006. A ban on anticoccidials was also established by the end of 2012 for reasons similar to those used for antibiotics (Huyghebaert et al., 2011). There is also a ban on antibiotics in animal feed in Denmark and Sweden. Since the ban, there has been a decrease in swine production in Sweden. In Denmark, there has been an increase in incidences of diseas outbreaks in swine and skeletal problems plague their poultry industry (Casewell et al., 2003). With the antibiotic ban, antibiotics prescribed by veterinarians have increased. This could still lead to human health problems because of emerging antibiotic resistant bacteria (Casewell et al., 2003). The United States has not currently issued a ban. However, some poultry integrators have voluntarily withdrawn antibiotics from the feed. Thus, alternatives need to be found to improve the health of the birds, assist in the defense against diseases, and improve the overall performance of poultry.

Effects of Antibiotic Diets on the Intestine

Before alternatives are to be considered, it is important to understand how the antibiotics can influence the intestines. Antibiotics can alter the microbiome within the intestine and play a beneficial role in protecting birds from disease (Dibner and Richards, 2005). Antibiotics, when supplemented to the diet, can influence the health of the

intestine by decreasing clostridia in the feces (Elam et al., 1952). Antibiotics can also delay the maturation of the microflora without compromising growth. The delay of microflora development without negatively affecting growth could be due to the ability of the antibiotics to reduce the pathogenic bacteria from colonizing the intestine (Gao et al., 2017).

Diseases such as coccidiosis and necrotic enteritis damage the intestine by causing lesions and disrupting the bacterial populations (Turk and Littlejohn, 1987). However, Brennan et al. (2003) conducted a study with birds that were supplemented with the antibiotic Bacitracin (55ppm), in their diet and exposed to necrotic enteritis (1×10^8 CFU *C. perfringens*/mL) through the feed from day 14 to 16 post hatch. Intestinal lesion scores were taken on birds that died on day 17 and throughout the study to day 41. In the study it was found that birds supplemented with Bacitracin had an overall decrease in mortality caused by necrotic enteritis, and reduced lesion scores, when compared to birds fed a nonmedicated diet and challenged with *C. perfringens* (Brennan et al., 2003).

Antibiotics are used mainly to control intestinal diseases, but each antibiotic can affect the intestine differently. Miles et al. (2006) conducted a study looking at the use of antibiotics as growth promoters on performance and intestine growth. Three diets were compared: a basal diet and two antibiotic diets, including Bacitracin methylene disalicylate (BMD) and Virginiamycin. The two antibiotic diets were different in the way they affected the gastrointestinal tract. In that study, they found that the intestinal morphology of the intestines was affected differently between the antibiotic diets, where the length and weights of the intestine decreased in birds fed Virginiamycin compared to

the birds fed BMD during weeks one and three. However, both antibiotics caused shorter intestinal lengths and lighter intestinal weights compared to the control throughout the trial. Birds fed Virginiamycin experienced an increase in villi numberd per unit in the duodenum, and in the ileum, birds had smaller villi heights and crypt depths compared to birds fed the BMD and control diets. Although the two antibiotic diets affected the intestines differently, they resulted in similar weights by 49 days (Miles et al., 2006) suggesting that antibiotics can promote growth in broilers compared to a conventional diet. However, the effect that antibiotics can have on the microflora of the birds can vary with the antibiotic type used.

Antibiotics can also affect the bacterial population within the intestine for the betterment of the host. When added to the poultry diet, Virginiamycin can reduce possible harmful antibiotics and allow bacteria such as lactobacilli to be able to multiply and improve intestinal health (Dumonceaux et al., 2006). Virginiamycin can also influence bacterial populations in different sections of the intestine. Dumonceaux et al. (2006) added Virginiamycin to poultry diets and analyzed the bacterial population on day 47 and compared it to birds fed a Virginiamycin-free diet. In that study, they found that Virginiamycin can increase bacterial populations in the upper intestine and then not change or decrease other populations in the lower intestine, while overall bacterial populations remain unchanged. It also increased bacterial populations, including *Lactobacillus* species, in the duodenum compared to birds fed a Virginiamycin-free diet. Antibiotics, such as Virginiamycin, can modify bacterial populations within the intestine

so as to allow beneficial bacteria to proliferate, while reducing bacteria that can be harmful (Dumonceaux et al., 2006).

Factors that Affect Intestinal Function

The intestines function to digest and provide the birds with nutrients from the feed. The small intestine of poultry is divided into three sections consisting of the duodenum, jejunum, and ileum. The duodenum is the main site of digestion, the jejunum is the main site of nutrient absorption, and the ileum is the site of mineral and water absorption. The ceca is located at the end of the ileum, and its primary function is to absorb electrolytes and water (Svihus, 2014). If something were to compromise intestinal function, the health of the birds would be at risk.

The day a chick hatches, bacteria begin to colonize the available space within the intestines. As the chick ages, it becomes harder for different species of bacteria to colonize the gut, due to the availability of colonizable areas within the intestine (Bedford, 2000). Thus, the bird's diet is crucial for intestinal health. It is important to realize that the diet provides nutrients not only to the birds, but also contributes to the microflora present in the intestine (Bedford, 2000).

The composition of the gut flora can be stabilized or manipulated, naturally or with effort, by factors such as age, the diet of the host, the oral administration of antibiotics, and the immune response of the host (Barrow, 1992). When the birds are placed in a poultry house, the new environment can be stressful and can cause the birds to be more susceptible to bacteria. When an animal is stressed, whether it is from the

environment or a change in diet, the intestines are negatively affected, allowing harmful bacteria to colonize (Tannock, 1983; Fuller, 1992).

Changes in the ingredients or the nutrients in a poultry diet can affect the intestine and its environment. Cereal grains can increase the viscosity within the intestine, which can cause problems for the birds' health and digestion (Yegani and Korver, 2008). Antibiotics can influence the intestine by minimizing changes in the microflora and can delay the maturation of the intestine (Bedford, 2000; Gao et al., 2017). Additives such as probiotics and enzymes can influence the microflora and improve the possibility of beneficial bacteria to colonize and limit the growth of pathogens (Bedford, 2000). A change in microbial communities can allow the birds to be more susceptible to bacteria, parasites, and toxins. Diseases such as necrotic enteritis and coccidiosis can cause damage to the intestine which in turn would cause a deterioration in health and performance (Yegani and Korver, 2008).

Turk and Littlejohn conducted a study in 1987 to determine the effects of coccidial species on the intestine of poultry. They used four-week-old White Leghorns fed a corn and soybean control diet. There were five treatments. One treatment group was not challenged and used as the control. The other four treatments were inoculated with one of the four *Eimeria* species: *Eimeria (E.) acervulina (*750,000 sporulated oocysts), *E. necatrix* (60,000 sporulated oocysts), *E. brunetti (*100,000 sporulated oocysts*),* and *E. tenella (*30,000 sporulated oocysts*).* These species were selected because of the location that they infect within the intestine. *E. acervulina* causes lesions in the duodenum, *E. necatrix* effects the jejunum, *E. brunetti* infects the ileum and ceca and *E. tenella* causes

lesions in the ceca. The fecal content was collected to measure bacterial number (Turk and Littlejohn, 1987).

Turk and Littlejohn (1987) reported that the distinct species of *Eimeria* affects the microflora present within the intestine at various times beginning on d 3 until d 14. They looked at fecal *lactobacilli* concentrations because of the benefit that it gives to the host. Fecal coliform counts were measured because they are microorganisms that are damaging to the host and migrate to the cardiovascular system, which further increases infection. When compared to the control from d 1 to d 21 post infection different concentrations of aerobic and anaerobic microflora, coliforms, and lactobacilli were seen to increase or decrease at various times due to different *Eimeria* species. Despite these changes, the aerobic and anaerobic microflora, fecal lactobacilli, and coliform concentrations were all similar to the control by d 21 post infection, however it was shown that when the birds were infected with coccidiosis, the ecosystem within the intestine was negatively disrupted and this imbalance within the intestines caused digestive issues which could negatively affect the growth of the birds (Turk and Littlejohn, 1987).

Coccidiosis

Benjamin Fantham was a zoologist and parasitologist from the United Kingdom who studied various microbial organisms. He was the first person to describe the life cycle of coccidia species that infect avian hosts. Despite coccidiosis being an important topic to the industry, there was little research conducted on the subject until the Bureau of Animal Industry was created in the United Kingdom in 1884. The purpose of the bureau was to study diseases that affect poultry, as well as to develop possible cures or

preventatives for those diseases. Before 1884, universities conducted research on these diseases. D. E. Salmon, a veterinarian, was the first chief appointed at the bureau. He wrote a book on poultry diseases where he included a chapter on coccidiosis. In this chapter, Salmon mentioned *Coccidium tenellum* and *Eimeria dubia*, describing the symptoms in the bird, as well as preventatives, such as sanitation of feeders and water troughs (Chapman et al., 2003).

Coccidiosis is problematic not only because of the cost of medication and treatments, but also because of the way this disease affects the birds, including increased mortality, decreased growth performance, lack of feed absorption, and lack of feed retention (Dalloul and Lillehoj, 2006). There are seven species of coccidiosis that affect chickens: *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox*, and *E. tenella*. The lesions caused by *Eimeria* species have visual differences that can help observers identify which species is infecting the birds. The locations, whether it be in the ceca or the small intestine, of the lesions are also an indicator of which species is infecting the birds (Allen and Fetterer, 2002).

The conditions for the growth and reproduction of coccidia need to be warm and humid to allow the oocysts to grow. This makes the litter inside a poultry house an ideal environment (Jamil et al., 2016). *Eimeria acervulina, E. maxima*, and *E. tenella* can survive in hot conditions. *Eimeria maxima*, under laboratory conditions, can survive longer in dry conditions compared to *E. acervulina* and *E. tenella*, which causes more opportunities for the birds to ingest the parasite and be further infected (Jenkins et al., 2013). *Eimeria* oocysts can survive in temperatures as low as -25˚ C and as high as 35.5˚

C (Koutz, 1950). Once coccidiosis is in a poultry house it is almost impossible to remove, thus it can only be managed through vaccines and anticoccidial programs (Chapman, 2007).

There are two ways to prevent an outbreak of coccidia in poultry, chemoprophylaxis and vaccination. Chemoprophylaxis is given to birds as an anticoccidial and is the most commonly used method to prevent and control coccidia. The problem with anticoccidials is the possibility of resistance, so anticoccidials are rotated periodically to reduce the chance of resistance. These chemicals are costly and for some, the period which they last can be short (Gussem, 2007).

These anticoccidials are given to the birds in three different programs. They can be administered in a single drug program, a shuttle program, or a rotation program. A single drug program is when birds are given only one drug continuously for multiple flocks, which will result in resistance with prolonged use. The shuttle program gives the birds two anticoccidials in a flock. The shuttle program gives a synthetic anticoccidial during the starter period and an ionophore anticoccidial in the growth period. This reduces the chance of resistance to the anticoccidials. The rotation program rotates the anticoccidials used in each flock. One flock will receive synthetic anticoccidials, and the next will receive an ionophore anticoccidial (Chapman, 2007). When using any one of these programs, it is essential to know the limitations of the anticoccidial used. Some anticoccidials cannot be used in certain seasons. For example, when nicarbazin is exposed to the summer heat, it will produce metabolic toxicity in the birds (Chapman, 2007).

Despite anticoccidial resistance, coccidiosis may still be manageable. This is possibly due to some of the parasites that are not anticoccidial resistant being ingested, allowing the birds to build up an immunity to coccidia (Peek and Landman, 2011). If anticoccidials were to be banned, the control of coccidiosis would become more difficult.

Antibiotic Alternatives

With the use of antibiotics in the poultry industry under scrutiny by consumers and the government, alternatives to antibiotics are being increasingly investigated. Even if an alternative meets the same growth promoting criteria as birds given antibiotics, it also needs to be able to combat the diseases that birds are exposed to in the environment. Alternatives need to be able to reduce the severity of infection, reduce microorganisms from taking the nutrients from the feed, increase nutrient absorption, and reduce gram-positive bacteria that would decrease the weights of the birds (Huyghebaert et al., 2011). There are multiple alternative options to antibiotics, with one of those options being probiotics (Fallah et al., 2013).

Probiotics

Probiotics are known as direct fed microbials (DFM) and are defined as 'live microbial feed supplements which beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 1992). Probiotics work by helping the ecosystem within the intestinal tract in one of three ways: 1) protecting the intestines by antagonistic action, 2) competitive exclusion, and 3) competition with other bacteria for the nutrients within the body (Patterson and Brukholder, 2003; Fallah et al., 2013). Probiotics were first discovered when Elie Metchnikoff realized that Bulgarian peasants who had ingested

large quantities of sour milk lived longer. Louden Douglas later supported his hypothesis in 1911 when he wrote a book on how *Bacillus,* a probiotic, improved life expectancy due to fermented milk (Fuller, 1992). Since then, there have been multiple studies on the benefit of *Bacillus* species on the performance of poultry (Cavazzoni et al., 1998; Jayaraman et al., 2017).

Mechanisms by which Probiotics Influence the Intestine

Probiotics work in multiple mechanisms in the body of the host by improving the epithelial barrier integrity within the intestine, by increasing a probiotic's ability to adhere to the intestine, competitively excluding pathogens, producing anti-microorganisms, and improving the immune system of the host (Bermudez-Brito et al., 2012).

The intestine is an important defense organ against pathogens. Probiotics may reinforce the intestinal barrier and even repair it. This provides protection from pathogens from attaching to the lining of the epithelial barrier (Bermundez-Brito et al., 2012). For a probiotic to survive for a long time in the host, it should be able to adhere to the intestine. If a probiotic cannot adhere to the intestine then it needs to be given continuously which may provide immunity benefits (Havenaar et al., 1992a).

Competitive exclusion is when a host's natural microflora competes against pathogenic bacteria from colonizing the same space (Jeffrey, 1999). This concept was brought about in poultry by a study done by Nurmi and Rantala in 1973 (Kabir, 2009). In this study, Nurmi and Rantala (1973) gave day old birds adult gut content to see if it would provide the bird an advantage when exposed to salmonella. They determined that

having the adult content provided the birds a defense against the salmonella compared to the birds that were not inoculated with ingesta from the older birds. The percentage of salmonella was below 20% in the crop, small intestine, and caeca, compared to those that were not treated which had a percentage of over 58% (Nurmi and Rantala, 1973).

There have been multiple studies showing that when birds are exposed to bacteria and parasites, giving probiotics assist in enhancing the immune system and reduce signs of infection (Lee et al., 2010; Abdelrahman et al., 2014; Lee et al., 2014). In addition, probiotics also improve the immunity of the host. Probiotics produce anti-microorganism substances to fight against the pathogenic microorganisms (Bermudez-Brito et al., 2012).

Due to probiotics beneficially affecting the intestine, they can subsequently improve growth. Baldwin et al. (2018) conducted a study to see how administering a probiotic mixture at hatch would affect the microbiota and growth of broilers. Birds were orally inoculated with either 1 ml of PBS (control) or a 1 ml of a probiotic mixture containing *Lactobacillus (L) ingluviei*, *L. agilis* and *L. reuteri* and fed an antibiotic and anticoccidial free diet. Birds were placed in floor pens and feces were collected on days 14 and 28 and cecal content was taken on d 28 for microbial analysis and individual birds were weighed daily. Results showed that although the first 14 days after hatch did not show differences in the overall microbiota between treatments only specific species were found in different amounts within the two treatment groups. Out of the three *Lactobacillus* species that were inoculated at hatch, *L. ingluvei* was more abundant in the orally inoculated probiotic birds compared to the control on day 14. By day 28, the two treatment groups continued to have differences in the population of different bacteria

species. The birds inoculated with the probiotic mixture had a higher body weight compared to the control birds by day 28 (Baldwin et al., 2018). The study showed that administering probiotics to birds at hatch can influence the bacterial population within the intestine by manipulating populations to improve growth compared to birds that did not receive probiotics.

Criteria for Probiotic Selection

The probiotic bacteria to be used should already be present in the gut, provide a beneficial effect on the host, be able to attach to the intestinal epithelium, be able to survive in the host environment, and be able to compete against other micro-organisms that are currently in the gut (Barrow, 1992; Fuller, 1992; Kabir, 2009). Also, the immune system of the host should not attack the probiotic. The probiotic also needs to survive through long term storage. If the probiotic cannot meet these needs, then the probiotic will not be activated in the intestines and will not be beneficial to the host (Havenaar et al., 1992b).

When the method by which the probiotic will be administered is determined, it is crucial that the microorganisms can survive the environment in to which it will be introduced. If the probiotic were to be delivered by an oral route, the organisms would have to be able to survive the enzymes, pH, bile, mucus, and pancreatic juices that it will come into contact with (Havenaar et al., 1992b). The microflora will change as the host ages. The diet also changes as the host ages thus possibly changing the gut flora at these times. Therefore, the probiotic microorganisms need to survive through these changing conditions (Barrow et al., 1992).

A specific amount of a viable microorganism is used when administering probiotics. When determining the amount administered, shelf life is important. Even if the microorganisms are labeled as live, it is not always going to contain the exact amount that was originally added because the species will begin to die as the viability of the microorganism decreases over time. Thus, quality control and the viability of the microorganisms are important to consider if they are to be used for probiotics in conjunction with the method used to administer the probiotic (Havenaar et al., 1992a; Fuller, 1992).

Probiotics can be administered in chickens through a spray, *in ovo* injection, in the feed, through the water, and through oral gavage. The strain of microorganisms chosen needs to be processed in specific ways to administer the product by these methods. Each microorganism has limitations and is affected by different things. Some cannot withstand centrifuge, while others cannot withstand pelleting. Also, chemical compounds in the feed or the body of the host that the probiotic could come in contact with could reduce the viability of the microorganisms or even kill them completely (Havenaar et al., 1992a).

The Effects of Probiotics in Response to a Disease

There have been multiple documented successes using probiotics to benefit poultry immune system and intestinal health. With the improved intestinal health and the beneficial effect that probiotics can have on the immune system, studies have been conducted to determine if probiotics can benefit birds during bacterial and parasitic infections that commonly affect poultry.

Ritzi et al. (2014) studied how various species of probiotics interact with the birds' growth and development of intestinal lesions caused by *Eimeria species*. There were six treatments. The first was a control group that received a nonmedicated control diet was not challenged. The remaining birds were all challenged with *Eimeria*: 2) an anticoccidial (salinomycin at the rate of 0.01%), 3) a positive control (nonmedicated control diet), 4) probiotics administered through water at a high dosage (5×10^{12} per kg at 20mg/day) , a 5) probiotic given at a low dosage through the water (5×10^{12} per kg at 2mg/day) and 6) probiotics supplemented through the feed (1×10^{8} CFU per kg of feed). Bird given probiotics through the water at a high dose received the probiotic on the first three days after hatch, the day before the challenge, once a week, the day of the challenge, and the day after a feed change (d 15, 21,35, and 42). The probiotic mixture contained *Bifidobacterium animalis* subspecies *animalis* DSM 16284, *Lactobacillus salivarius* subspecies *salivarius* DSM 16351, and *Enterococcus faecium* DSM 21913. Ritzi et al. (2014) orally gavaged all the birds in each treatment group, except the control group, with a 1 ml dose of *E. acervulina* (50,000 oocysts), *E. maxima (*10,000 oocysts*)*, and *E. tenella* (2,500 oocysts) on day fifteen. Ritzi (2014) found that on day 21, the birds given the anticoccidials had a higher body weight than the three probiotic treatment groups and the positive control group. However, birds given probiotics in the water at a high dosage and through the feed had similar lesion scores in the duodenum compared to the positive control on day 21. Birds administered the probiotics in the water at high and low dosages had lower lesion scores in the jejunum compared to the positive control but similar to that of the anticoccidial. The anticoccidial had similar lesion scores in the

jejunum compared to the positive control on day 21. It was deduced that the probiotics did not help improve growth performance during peak infection on day 21 like the anticoccidial. However, despite the early damage to the intestine on day 21, administering probiotics did support the birds overall in order to maintain similar growth to the anticoccidial group with similar body weights at the end of 42 days (Ritzi et al., 2014). Intestinal lesions can be caused by multiple such as coccidiosis and necrotic enteritis (Johnson and Reid., 1970; M'Sadeq et al., 2015). *E. acervulina, E. maxima,* and *E. tenella* are considered the most pathogenic of the seven coccidial species that infect poultry (Gussem, 2007). Ritzi et al. (2014) showed that providing probiotics in poultry feed and water could benefit birds during an infection of the three most pathogenic species and lessen lesions caused by them, thus promoting intestinal health.

Giannenas et al. (2014) conducted a trial to determine if probiotics could reduce poor performances of birds infected with *E. acervulina* (5×10^4), *E. maxima* (2×10^4), and *E. tenella* (2×10^4) on day fourteen and administered the probiotics in the feed and water. Giannenas et al. (2014) had eight treatment groups including 1) an unchallenged control nonmedicated diet. The remaining seven treatments were challenged with *Eimeria* species: 2) a positive unmedicated control diet group, 3) an anticoccidial group (Lasalocid 75mg/kg), 4) a probiotic mix delivered through drinking water at 2.5×10^7, 5) a probiotics mix delivered through drinking water at 5.0×10^7, and 6) a probiotic mix delivered through drinking water at 2.5×10^8 CFU/liter of water, 7) a probiotic mix administered through feed (5.0×10^8 CFU/kg), and 8) a probiotic mix coated by the feed (5.0×10^8 CFU/kg). The probiotics used were a mixture given in a ratio of 6:3:1 of

Enterococcus faecium #589, *Bifidobacterium animalis* #503 and *Lactobacillus salivarius* #505. In this study, they found that birds fed probiotics in the feed had continuously higher body weights compared to the positive control while being similar to birds fed the anticoccidial on days 21, 28, 35, and 42. Birds fed probiotics in the feed had similar body weights to birds fed anticoccidial and the control on day 35. By day 35 birds fed the positive control had higher feed conversion ratio compared to all other treatments. By day 42 birds administered probiotics through the feed at any level had similar body weights compared the anticoccidial group but also similar to the positive control. Birds fed probiotics through the feed had lower ileal lesion scores compared to birds fed the positive control on day 21. Giannenas et al. (2014) concluded that probiotics *Enterococcus faecium* #589, *Bifidobacterium animalis* #503 and *Lactobacillus salivarius* improved growth performance when compared to the infected control and similar to birds fed the anticoccidial (Giannenas et al., 2014). Administering probiotics through the feed allowed the birds to recover quicker by having lower lesion scores when compared to the positive control and bringing body weights similar to the anticoccidial group a week after challenge and throughout the trial by day 42. While birds that were challenged and received a control diet were not able to recover and had lower body weights compared to birds fed anticoccidials by day 42 (Giannenas et al., 2014).

Lee et al. (2010) demonstrated that probiotics within the same species of bacteria can be beneficial to poultry by supplementing one of eight different types of *Bacillus subtilis* strains resulting in eight probiotic treatments. The strains were Bs2084, LSSAO1, 3AP4, Bs18, 15AP4, 22CP1, Bs27, Bs278 and a multi-strain probiotic that containing

Bs2084, LSSAO1, and 15AP4. There was also a control group (unmedicated diet and non challenged) and positive control (unmedicated control diet and challenged). Birds in all treatment groups, except for the uninfected control group, were challenged with *E. maxima* (5.0×10^3) on day 21. Birds were weighed on day 21 and day 27. Out of the eight probiotic groups, 15AP4 and Bs27 had a higher body weight gain compared to the challenged control group by day 27. Birds fed the probiotic 15AP4, Bs27, or Bs278 resulted in lower intestinal lesion scores compared to the positive control group on day 27 (Lee et al., 2010). The study showed that supplementing *Bacillus* strains can improve growth and reduce the signs of an *Eimeria maxima* challenge within a week after challenge.

Giannenas et al. (2012) did a study on probiotics and the effects on birds when they are infected with *Eimeria tenella*. There were ten treatments: a control that was the only unchallenged treatment, a challenged control, and an anticoccidial treatment (60 mg of Lasalocid /kg of feed). The rest of the treatments were basal diets supplemented with different probiotic species: *Enterococcus faecium* at low (5×10^8 CFU/kg) and high (5×10^9 CFU/kg) inclusion rates, *Bifidobacterium animalis* (5×10^8 CFU/kg), *Bifidobacterium reuteri* (5×10^8 CFU/kg), *Bacillus s.* (5×10^8 CFU/kg), and a multispecies probiotic at high (5×10^9 CFU/kg) and low (5×10^8 CFU/kg) inclusion rates. All treatment groups, except for the uninfected control group, were challenged with *E. tenella* (2×10^4 sporulated oocysts) on day 14 by oral gavage. When comparing body weights to the anticoccidial group, birds fed *Bifidobacterium animalis* had a lower body weight compared to the anticoccidial group by day 28. All other treatments were similar to the

anticoccidial group, by day 28. By day 35, birds fed the anticoccidial had a higher body weight compared to birds in the infected control group. By day 42, all groups were similar to the anticoccidial and uninfected control group except the challenged control. Birds fed *Enterococcus faecium* at high and low inclusion, *Bifidobacterium animalis, Bifidobacterium reuteri* and *B. subtilis* had similar body weights to the infected control by day 42. *Bifidobacterium animalis* and the uninfected control had similar crypt depth and villous height in the ileum, whereas *B. subtilis* had similar crypt depth but higher villous height compared to the uninfected control (Giannenas et al., 2012). *E. tenella* can infect the host again around seven days after initial infection (Davies et al., 1963). At day 7, 8, and 9 post infection, Giannenas et al. (2012) saw that oocyst output was lower in the *B. subtilis* and *Lactobacillus reuteri* groups compared to anticoccidial and infected control treatment. This study shows that there are some probiotic species that can be used to improve bird health and growth during *E. tenella* exposure.

Similar to Ritzi et al. (2014) and Giannenas et al. (2014), Lee et al. (2014) did a study on the effects of *B. subtilis* and the anticoccidial salinomycin on birds that were raised on used litter which contained *Eimeria* or *Clostridium* species. There were three treatments: control, *B. subtilis* (1.5×10^5 CFU/g of *Bacillus subtilis*), and salinomycin premix (60 mg/kg of salinomycin) diet. Birds were placed on at least ten flock old used litter that contained *Eimeria* or *Clostridium* species. The birds receiving *B. subtilis* had a higher body weight than the birds receiving salinomycin but were similar to the control on day 28. There were no signs of disease or lesions during the study, but there were antibodies for *Eimeria* and *C. perfringens* in the birds. Antibodies for *C. perfringens* were

not different among treatments. Birds fed *B. subtilis* and salinomycin showed a decrease in the *Eimeria* serum antibody level compared to control on day 28 (Lee et al., 2014). These results suggest that *B. subtilis* can provide an immune response similar to that of salinomycin if birds are exposed to coccidiosis on used litter, which is beneficial if antibiotics are to be taken out of the poultry diet.

Abudabos et al. (2013) did a study with a *B. subtilis* and its effects on birds exposed to *Clostridium perfringens.* The birds were raised in a four decked cages that were heated by electric brooders. There were four treatments: 1) control group (unmedicated and uninfected control group, 2) positive control group (unmedicated control diet and challenged), 3) antibiotic group (Enramycin 0.1g/kg of feed), and 4) the last was a probiotic group (0.05% Clostat with *Bacillus subtilis* PB6). All of the birds, except for the control, were challenged on day 16 with a tenfold dose of the anticoccidial vaccine and a *C. perfringens* inoculation (4×10^8 CFU) on days 18 and 20. There were no significant variations in intestinal morphology or histology on day 16; however, the probiotic treatment had higher intestinal weight than the control birds on day 30. Birds that received the probiotic treatment and the positive control group had more extended villi in the jejunum than the control and antibiotic treatment on day 30. Also, the ileum villus height for those that received probiotics was similar to the positive control group but higher than the control group on day 30. On day 30 those that received the probiotic showed lower *C. perfringens* count than other treatments. They concluded that the probiotic was able to control the *C. perfringens* and improve the health of the small intestines (Abudabos et al., 2013) which if birds were in a more stressful condition and

exposed to *C. perfringe*ns in a commercial setting, the supplementation of probiotics could improve the intestinal health.

Cao et al. (2012) conducted a trial to test *C. perfringens* effects on gut health and intestinal lesions. They used the probiotic *Lactobacillus fermentum* 1.2029 to determine if it would help against lesions caused by the bacterium. There were three treatments: 1) an uninfected control group, 2) a challenged control group and 3) a challenged probiotic *Lactobacillus fermentum* (10^8 CFU/mL) group. Birds were challenged with *C.perfringens* by gavage on d 1 (0.5 mL/chicken) and challenged again with *C. perfringens* on d 14 and 21 (1.0ml/chicken). The birds given *Lactobacillus fermentum* was administered by gavage daily. They observed that there was a higher percentage of lesions ranging from 2 to 4 in the challenged control compared to birds that were challenged and received *Lactobacillus fermentum* 1.2029, whereas the control group did not show sign of lesions on d 28. The addition of the probiotic could help intestinal health by reducing the severity of lesions that *C. perfringens* induced (Cao et al., 2012).

Bacillus species are becoming popular in the poultry industry and has proved beneficial when added in diets. *Bacillus* species, such as *B.s subtilis*, can be added to the poultry diet because they can resist the heat of pelleting and are able to germinate in the gastrointestinal tract (Shivaramaiah et al., 2011). Although *Bacillus* species cannot colonize the intestine, they are able, once in the intestine, to stimulate the immune system by reducing the ability of pathogens from colonizing the intestine by producing antimicrobials (Casula and Cutting., 2002; Cartman et al., 2008; Amin et al., 2015).

Conclusion

There are many benefits of using antibiotics in poultry diets. Antibiotics help the birds fight against common diseases that poultry come in contact with in a poultry house and promote growth. With the voluntary ban in the United States on antibiotics in the poultry diet, the poultry industry is looking for alternatives. One of the alternatives being studied is the probiotic *B. subtilis* which has shown to benefit the growth performance and intestinal health of broilers. Despite the benefits that *B. subtilis* had on broiler growth and health, there have not been studies shown on when to remove the antibiotics from the broiler diet and supplement with *B. subtilis*. Studies have also not been done to show how *B. subtilis* will affect broiler growth and intestinal health when antibiotics are removed at different stages of the grow out period and supplemented with *B. subtilis*. By removing antibiotics earlier, it could reduce the possibility of antibiotic residue in the meat, reduce antibiotic resistant bacteria from entering the food chain and appease consumers concerns.

References

Abudabos, A. M., Alyemni, A. H. and Al Marshad, M. B. A. 2013. *Bacillus subtilis* PB6 Based-Probiotic (Clostat) Improve Intestinal Morphological and Microbiological Status of Broiler Chickens under *Clostridium Perfringens* Challenge. International Journal of Agriculture & Biology 15(5):973-982.

Abdelrahman, W., Mohnl,M., Teichmann,K., Doupovec,B., Schatzmayr, G., Lumpkins,B., and Mathis, G. 2014. Comparative Evaluation of Probiotic and Salinomycin Effects on Performance and Coccidiosis Control in Broiler Chickens. Poultry Science. 93(12):3002-3008.

Allen, P.C and Fetterer, R. H. 2002. Recent Advances in Biology and Immunobiology of *Eimeria* Species and in Diagnosis and Control of Infection with these Coccidian Parasites of Poultry. American Society for Microbiology. 15(1):58-65.

Amin, M., Rakhisi, Z., and Ahmandy, A.Z. 2015. Isolation and Identification of *Bacillus* Species from Soil and Evaluation of Their Antimicrobial Properties. Avicenna Journal of Clinical Microbiology Infection. 2(1):232-233.

Apata, D. F. 2009. Antibiotic Resistance in Poultry. International Journal of Poultry Science. 8(4):404-408.

Baldwin, S. Hughes, R. J., Hao Van, T. T., Moore, R. J., and Stanley, D. 2018. At-Hatch Administration of Probiotic to Chickens can Introduce Beneficial Changes in Gut Microbiota. Public Library of Science One. 13(3). 1-14.

Barrow, P. A. 1992. Probiotics for Chickens. In Scientific Basis of the Probiotic Use, ed. R. Fuller. Chapman and Hall, London, UK. 225-257.

Bedford, M. 2000. Removal of Antibiotic Growth Promoters from Poultry Diets: Implications and Strategies to Minimise Subsequent Problems. World's Poultry Science Journal. 56(4):347-365.

Bermudez-Brito, M., Plaza-Diaz, J., Munoz-Quezada, S., Gomes-Llorente, C., and Gil, A. 2012. Probiotic Mechanisms of Action. Annals of Nutrition and Metabolism. 61(2):160-174.

Brennan, J., Skinner, J., Barnum, D. A., and Wilson, J. 2003. The Efficacy of Bacitracin Methylene Disalicylate when Fed in Combination with Narasin in the Management of Necrotic Enteritis in Broiler Chickens. Poultry Science Journal. 82 (3):360-363.

Cao, L., Yang. X.J., Li, Z.J., Sun, F.F., Wu, X.H., and Yao, J.H. 2012. Reduced Lesions in Chickens with *Clostridium Perfringens*-Induced Necrotic Enteritis by *Lactobacillus fermentum* 1.2029. Poultry Science. 91(12):3065-3071.

Cartman, S. T., La Ragione, R. M., and Woodward, M. J. 2008. *Bacillus sub*tilis Spores Germinate in the Chicken Gastrointestinal Tract. Applied and Environmental Microbiology. 74(16):5254-5258.

Casewell, M, Friis, C, Marco, E, McMullin, P, and Phillips, I. 2003. The European Ban on Growth-Promoting Antibiotics and Emerging Consequences for Human and Animal Health. Journal of Antimicrobial Chemotherapy. 52(2):159-161.

Casula, G., and Cutting, S. M. 2002. Probiotics: Spore Germination in the Gastrointestinal Tract. Applied and Environmental Microbiology. 68(5), 2344–2352.

Cavazzoni, V, Adami, A, and Castrovilli, C. 1998. Performance of Broiler Chickens Supplemented with *Bacillus Coagulans* as Probiotic. British Poultry Science. 39(4):526-259.

Chapman, H. D., Cherry, T. E., Danforth, H. D., Richards, G., Shirley, M. W., and Williams, R. B. 2002. Sustainable Coccidiosis Control in Poultry Production: The Role of Live Vaccines. International Journal of Parasitology. 32(5):617-629.

Chapman, H. D. 2003. Origins of Coccidiosis Research in the Fowl-The First Fifty Years. Avian Diseases. 47(1):1-20.

Chapman, H. D. 2007. Rotation Programmes for Coccidiosis Control. International Poultry Production. 15(1):7-9.

Conway, D., and McKenzie, M. 2007. Poultry Coccidiosis: Diagnostic and Testing Procedures: Third Edition, Blackwell Publishing.

Dalloul, R. A., and Lillehoj, H. S. 2006. Poultry Coccidiosis: Recent Advancements in Control Measures and Vaccine Development. Expert Review of Vaccines. 5(1):143-163.

Davies, S. 1963. Coccidiosis. Edinburgh: Oliver and Boyd.

Dibner, J. J. and Richards, J. D. 2005. Antibiotic Growth Promoters in Agriculture: History and Mode of Action. Poultry Science. 84(4):634-643.

Donoghue, D. J. 2003. Antibiotic Residues in Poultry Tissues and Eggs: Human Health Concerns? Poultry Science. 82(4):618-621.

Dumonceaux, T. J., Hill, J. E., Hemmingsen, S. M., and Van Kessel, A. G. 2006.Characterization of Intestinal Microbiota and Response to Dietary Virginiamycin Supplementation in the Broiler Chicken. Applied and Environmental Microbiology. 71(4):2815-2823.

Elam, J. F., Jacobs, R. L., Tidwell, W. L., Gee, L. L., and Couch, J. R. 1952. Possible Mechanisms involved in the Growth-Promoting Response Obtained from Antibiotics. The Journal of Nutrition. 49(2): 307-317

Fallah, R., Kiani, A., and Azarfar, A. A. 2013. Review of the Role of Five Kinds of Alternatives to In-Feed Antibiotics in Broiler Production. Journal of Veterinary Medicine and Animal Health. 5(11):317-321.

Fuller, R., 1992. History and Development of Probiotics. In: Fuller, R. (Ed.), Probiotics – The Scientific Basis. Chapman and Hall, London. 1-7.

Gao, P., Ma, C., Sun, Z., Wang, L., Huang, S., Su, X., Xu, J., and Zhang, H. 2017. Feed-additive Probiotics Accelerate yet Antibiotics Delay Intestinal Microbiota Maturation in Broiler Chicken. Microbiome. 5(1):91.

Giannenas, I., Papadopoulos, E., Tsalie, E., Triantafillou, E., Henikl, S., Teichmann, K., and Tontis, D. 2012. Assessment of Dietary Supplementation with Probiotics on Performance, Intestinal Morphology and Microflora of Chickens Infected with *Eimeria tenella.* Veterinary Parasitology. 188(1-2):31-40.

Giannenas, I., Tsalie, E., Triantafillou, E., Hessenberger, S., Teichmann, K., Mohnl, M., and Tontis, D. 2014. Assessment of Probiotics Supplementation via Feed or Water on the Growth Performance, Intestinal Morphology, and Microflora of Chickens after Experimental Infection with *Eimeria acervulina, Eimeria maxima, and Eimeria tenella*. Avian Pathology. 43(3):209-216.

Gussem, M. 2007. Coccidiosis in Poultry: Review on Diagnosis, Control, Prevention and Interaction with Overall Gut Health. World Poultry Science Association, Proceedings of the 16Th European Symposium on Poultry Nutrition, Strasbourg, France, 26-30. 253-261.

Gustafson, R. H., and Bowen, R. E. 1997. Antibiotic Use in Animal Agriculture. Journal of Applied Microbiology. 83(5):531-541.

Havenaar, R., Brink, B.T., Huis, in'T., and Veld, J.H.J., 1992a. Selection of Strains for Probiotic Use. In: Fuller, R. (Ed.), Probiotics: The Scientific Basis, ch. 2. Chapman & Hall, London. 209–224.

Havenaar R, Huis In't Veld MJH. 1992b. Probiotics: A General View. In: Lactic Acid Bacteria in Health and Disease. Vol 1. Amsterdam: Elsevier Applied Science Publishers. 151-170.

Huyghebaert, G., Ducatelle, R., and Immerseel, F. V. 2011. Review: An Update on Alternatives to Antimicrobial Growth Promoters for Broilers. The Veterinary Journal. 187(2): 182-188.

Hrncar, C., Gasparovic, M., Weis, J., Arpasova, H., Pistova, V., Fik, M., and Bujko, J. 2016. Effect of Three-strains Probiotic on Productive Performance and Carcass Characteristics of Broiler Chickens. Scientific Papers Animal Science and Biotechnologies. 49(2): 149-154.

Jamil, M., Mansoor, M., Khan, A., Haq, R., and Anwar, F. 2016. Control of Avian Coccidiosis: Present and Future Strategies for Natural Alternatives of Therapeutic. Pakistan Journal of Science. 60(1):49-62.

Jayaraman S., Das P., Saini P., Roy B., and Chatterjee P. 2017. Use of *Bacillus Subtilis* PB6 as a Potential Antibiotic Growth Promoter Replacement in Improving Performance of Broiler Birds. Poultry Science. 96(8):2614-2622.

Jeffrey, J. S. 1999. Use of Competitive Exclusion Products for Poultry. University of California Cooperative Extension.

Jenkins M. C., Parker, C., O'Brian, C., Miska, K., Fetterer, R. 2013. Differing Susceptibilities of *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* Oocysts to Desiccation. Journal of Parasitology. 99(5):899-902.

Jones, F. T. and Ricke, S. C. 2003. Observations on the History of the Development of Antimicrobials and Their Use in Poultry Feeds. Poultry Science. 82(4): 613-617.

Johnson, J. and Reid, M. 1970. Anticoccidial Drugs: Lesion Scoring Techniques in Battery and Floor-Pen Experiments in Chickens. Experimental Parasitology. 28(1):30-36.

Kabir S. M. 2009. The Role of Probiotics in the Poultry Industry. International Journal of Molecular Sciences. 10(8):3531-3546.

Koutz, F. R. 1950. The Survival of Oocysts of Avian Coccidia in the Soil. The Speculum. 3(3):18.

Lee, K., Lillehoj, H. S., Jang, S. I., Li, G., Lee, S., Lillehoj, E. P., and Siragussa, G. R. 2010. Effect of *Bacillus*-Based Direct-Fed Microbials on *Eimeria Maxima* Infection in Broiler Chickens. Comparative Immunology, Microbiology and Infectious Diseases. 33(6):105-110.

Lee, K., Lillehoj, H. S., Jang, S. I., and Lee, S. 2014. Effects of Salinomycin and *Bacillus Subtilis* on Growth Performance and Immune Responses in Broiler Chickens. Research in Veterinary Science. 97(2): 304-308.

Miles, R.D. Butcher, G.D., Henry, P. R., Littell, R. C. 2006. Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters, and quantitative morphology. Poultry Science. 85(3):476-485.

M'Sadeq, S. A., Wu, S., Swick, R. A., and Choct, M. 2015. Towards the Control of Necrotic Enteritis in Broiler Chickens with In-Feed Antibiotics Phasing-Out Worldwide. Animal Nutrition. 1(1):1-11.

Nurmi, E. and Rantala, M. 1973. New Aspects of Salmonella Infection in Broiler Production. Nature. 241: 210-211.

Patterson, J. A. and Burkholder, K. M. 2003. Application of Prebiotics and Probiotics in Poultry Production. Poultry Science. 82(4):627-631.

Peek, H. W., and Landman, W. J. M. 2011. Coccidiosis in Poultry: Anticoccidial Products, Vaccines and other Prevention Strategies. Veterinary Quarterly. 31(3):143-161.

Ritzi, M. M., Adbelrahman, W., Mohnl, M., and Dalloul, R. A. 2014. Effects of Probiotics Application Methods on Performance and Response of Broiler Chickens to an *Eimeria* Challenge. Poultry Science. 93(11):2772-2778.

Shivaramaiah, S., Pumford, N. R., Morgan, M. J., Wolfenden, R. E., Wolfenden, A. D., Torres-Rodriguez, A., Hargis, B. M., Tellez, G. 2011. Evaluation of *Bacillus* Species as Potential Candidates for Direct-Fed Microbials in Commercial Poultry. Poultry Science. 90 :1574-1580.

Singer, R. S. and Hofacre, C. L. 2006. Potential Impacts of Antibiotic Use in Poultry Production. Avian Diseases. 50(2):161-172.

Svihus, B. 2014. Function of the Digestive System. Journal of Applied Poultry Research. 23(2):306-314.

Tannock, G. W. 1983. Effects of Dietary and Environmental Stress on the Gastrointestinal Microbiota. In Human Intestinal Microflora in Health and Disease, Vol 1. Ed. Hentges, D.J. Academic Press. New York. 226-257.

Turk, D. E., and Littlejohn, V. P. 1987. Coccidial Infections and Gut Microflora. Poultry Science. 66(9):1466-1469.

Van Boeckel, Thomas P., Brower, C., Gilbert, M., Frenfell, B. T., Levin, S.A., Robinson, T. P., Teillant, A., and Laxminarayan, R. 2015. Global Trends in Antimicrobial Use in Food Animals." Proceedings of the National Academy of Sciences of the United States of America. 112(18):5649-5654.

Yegani, M., and Korver, D. R. 2008. Factors Affecting Intestinal Health in Poultry. Poultry Science. 87(10): 2052-2063.

CHAPTER III

EFFECTS OF REPLACING DIETARY ANTIMICROBIALS WITH *BACILLUS SUBTILIS* ON GROWTH PERFORMANCE AND PROCESSING YIELDS OF MALE BROILERS

Abstract

The study was conducted to determine an optimal time to withdraw antimicrobials (antibiotics and anticoccidials) and replace them with probiotics in broiler diets without adverse effects on growth performance. A total of 1,536 male Ross × Ross 708 broilers were divided into 12 treatments with 8 replications each. Birds were fed in 6 phases: 0-14, 14-21, 21-28, 28-35, 35-46, and 46-56 d. Birds were fed either a control basal diet, *Bacillus subtilis* probiotic diet (1.1×10^5 CFU/g of feed), or an antimicrobial diet (antibiotic only from d 0-14) (50g bacitracin/ton of feed, 79.2g narasin/ton of feed) during each feeding phase. On d 14, all the birds were challenged by oral gavage of a 10 × dose of the commercial coccidial vaccine including live *Eimeria (E.) acervulina, E. maxima, E. maxima MFP, E. mivati,* and *E. tenella*. Groups fed antimicrobials until the withdrawal phase was considered as the practical control (PC). One-way ANOVA was used to analyze the data using Proc GLM of SAS 9.4. Within a week of *Eimeria* challenge, the removal of antimicrobials from the diets decreased BW on d 21. The removal of dietary antimicrobials on d 21 decreased BW on d 28, 35, and 47. However,

supplementation of probiotics to the basal diet brought the BW back close to that of antimicrobial groups. Birds that had antimicrobials taken out on d 28 showed a significantly lower BW on d 35, 47 and 55. Birds supplemented with probiotics starting on d 28 exhibited similar BW as those fed antimicrobial on d 35, 47, and 55. Withdrawal of antimicrobials or supplementation of probiotics on d 35 did not affect BW on d 47 or 55. Supplementation of probiotics in the last feeding phase (d 47 to 55) did not affect BW on d 55. Birds that had antimicrobials removed on d 21 or 35 and supplemented with either probiotic or basal diet had a body, carcass, wing and breast weights that were similar to PC. Removal of antimicrobials on d 28 lowered body, carcass, wing, and breast weights as compared to PC. However, removal of antimicrobials on d 28 and supplemented with probiotics resulted in the body, carcass, wing, breast weights that were similar to PC. In conclusion, the results suggest that supplementing probiotics may alleviate the adverse effects of coccidiosis on growth performance of broilers fed diets with antimicrobial taken out on d 21 or 28.

Keywords: Antibiotic, *Bacillus subtilis*, Broiler, Carcass, Coccidiosis

Introduction

Antibiotics have been used in the poultry industry for over 50 years (Feighner and Dashkevicz., 1986). Antibiotic use in the feed has been shown to improve growth performance, decrease mortality and improve immunity in broiler chickens (Gustafson and Bowern, 1997, Gadde et al., 2016). Despite these benefits, there has been growing concern about antibiotic residue getting into the meat, although few violations have been

reported (Donoghue, 2003). Studies have, however, shown that antibiotics in the poultry diet have caused bacteria resistance to antibiotics, causing concern for consumers (Singer and Hofacre, 2006).

A probiotic, also known as direct fed microbial, is defined as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1992). *B. subtilis* is a spore-forming probiotic and can survive during pelleting (Amin et al., 2015). It also has antimicrobial properties which can reduce the negative effects on hosts caused by pathogenic diseases (Amin et al., 2015). Studies have shown that probiotics have been positively effective when chickens are infected with *Eimeria* species (Giannenas et al., 2014; Abdelrahman et al., 2014). When birds are gavaged with a high dosage of *Eimeria acervulina* and given a *Lactobacillus*-based probiotic through the feed, the probiotic provided some protection against the disease (Dalloul et al., 2005).

Coccidiosis, caused by *Eimeria* infection, is a costly disease due to medication and treatment costs, increase in mortality, impaired growth, and reduction of nutrient absorption in the small intestine (Dalloul and Lillehoj, 2006). There are vaccines for coccidiosis available as an alternative to medications, but they have limited effectiveness and are costly to produce (Dalloul and Lillehoj, 2005). Coccidiosis is horizontally transmitted through the droppings of infected birds (Li et al., 2005), making it easy to spread throughout a house.

Growing concerns over antibiotic use in the poultry diet have led to alternatives such as probiotics to be studied. Despite knowing the benefits of using probiotics in

poultry diets, it is yet to be determined the opportune time to introduce them into the diet. Thus, the purposes of this study were to determine the optimal time to withdraw in-feed antimicrobials with or without probiotics and to determine if the probiotic *B. subtilis* can alleviate the negative effects of antimicrobial withdrawal on growth performance and processing yields during a coccidial challenge.

Materials and Methods

Bird Management and Treatment Outline

All bird husbandry, handling methods, and experimental procedures were approved by the Institutional Animal Care and Use Committee of Mississippi State University.

A total of 1,536 male Ross × Ross 708 broilers were hatched at a commercial hatchery and randomly assigned into 96 pens with 16 birds per pen at a density of 0.75ft^2 . Stocking density for day 21, 28, 35, 46 and 55 was 0.80 ft^2, 0.86 ft^2, 0.93 ft^2, 1.09 ft^2 and 1.2 ft^2 respectively. The 96 pens were randomly assigned to 8 blocks with 12 treatments in each block. The birds were spray vaccinated with a commercial coccidial vaccine at the hatchery. The birds were raised under industry like conditions and were given access to feed and water ad libitum. The environment was consistent throughout the house to commercial standards for broilers. The birds were placed on used litter top dressed with fresh pine shavings. Birds were checked daily and any mortalities were taken out and weighed.

The birds received light for 24 hours with no darkness (L24:D0) for the first 7 d and L20:D4 from d 8-10 at full intensity of 30 lux. After that, they received L20:D4 with the lights being dimmed a little each d from d 11-17 until they reached a light intensity of 2.7 lux. From d 18-55 L20:D4 the light intensity was kept at 2.7 lux.

On the d 14, every bird was orally gavaged with a 10 x dose of commercial coccidial vaccine which included live oocysts of *Eimeria acervulina, E. maxima, E. maxima MFP, E. mivati,* and *E. tenella.*

The birds were fed in six phases: Starter (d 0-14), Grower 1 (14-21), Grower 2 (21-28), Finisher 1 (28-35), Finisher 2 (35-46), and Withdrawal (46-56). Birds were fed one of the 3 diets including basal, antimicrobial (50g bacitracin/ton of feed, 79.2g narasin/ton of feed), and probiotics (1.1×10^5 CFU of *Bacillus subtilis*/g of feed) at each feeding phase. For the starter phase, birds were only fed antibiotics (50g/ bacitracin/ ton of feed). Treatments were designed to remove antimicrobials at different d and replace with either a basal diet or supplement with the probiotic *B. subtilis*. The layout of the treatments is shown in Table 3.1. Each letter of the abbreviations represents the diet fed at each phase.

Growth Performance, Carcass Yield, and Mortality

Group pen body weights and feed weights were taken on d 0, 14, 21, 28, 35, 47, and 55. Body weight gain, feed intake (FI), feed conversion ratio (FCR) and mortality were determined at each feeding phase. Mortality was recorded daily and FCR was calculated by taking into account the mortality weights of birds in each feeding phase. At d 55, five birds were randomly selected from each pen and tagged for processing and

deboning. After birds were processed on d 56, the carcasses and abdominal fat pads were weighed. Carcasses were submerged in ice water for four hours before deboning. The carcasses were cut into breasts, legs, wings, thighs, and tenders, and each part was weighed and recorded.

Experiment Design and Data Analysis

A randomized complete block design was used, with all 12 dietary treatments being equally represented in each of the 8 blocks, which served as 8 replications. There were 96 pens and each pen served as an experimental unit. All parameters were analyzed using one-way ANOVA using Proc GLM of SAS version 9.4 (SAS Institute, 2012) to determine the significance between dietary treatments. If there was a significant difference among treatments, Fisher's least significant difference test was conducted to separate the means. Significant level was set at $P \leq 0.05$.

Results

Body Weight

Dietary treatments did not affect BW for the first 14 d before the coccidial challenge ($P = 0.553$) (Table 3.1). By d 21 birds fed the AA (A = antimicrobial diet) diet had a higher BW compared to birds fed the AN (N = control basal diet), NN, AP (P = *B. subtilis* probiotic), and PP diets ($P < 0.0001$) (Table 3.2). Birds fed the AAA diet and AAP diet exhibited a higher body weight by d 28 compared to the birds fed all the other diets ($P < 0.0001$) (Table 3.3). By d 35 birds fed the AAAA exhibited a higher BW compared to birds fed the AAAN, AANN, ANNN, APPP, NNNN, and PPPP ($P < 0.0001$) (Table 3.4). Birds in the dietary treatment groups AAAP and AAPP had higher

BW compared to birds in the dietary treatment groups ANNN, APPP, NNNN, and PPPP by d 35 ($P < 0.0001$). Birds fed the AAAN had higher BW in comparison to birds fed the ANNN, APPP, NNNN, and PPPP diets by d 35 ($P < 0.0001$). Birds belonging to the dietary treatment group AANN had higher body weights by d 35 compared to birds in the dietary treatment groups APPP, NNNN, and PPPP ($P < 0.0001$). By d 47 birds fed the AAAAA, AAAAN, and AAAAP had higher body weights compared to fed the AAANN, AANNN, ANNNN, APPPP, NNNNN, and PPPPP diets ($P < 0.0001$) (Table 3.5). Birds fed the AAPPP had higher body weights by d 47 compared to birds fed the AANNN, NNNNN, ANNNN, APPPP, and PPPPP diets ($P < 0.0001$). Birds in the dietary treatment group AAAPP had a higher body weight on d 47 compared to birds in the dietary treatment groups ANNNN, APPPP, NNNNN, and PPPPP ($P < .0001$). By d 55 birds fed the AAAANN had a higher body weight compared to birds fed the ANNNNN, NNNNNN, PPPPPP, AAANNN, and APPPPP diets ($P = 0.001$) (Table 3.6). Birds fed the AAAAAN, AAAPPP, AANNNN, and AAPPPP had a higher body weight compared to birds fed the NNNNNN, PPPPPP, AAANNN, and APPPPP diets by d 55 ($P= 0.001$). By d 55 birds fed the AAAAAP and AAAAPP had a higher body weight compared to birds fed the AAANNN and APPPPP diets ($P = 0.001$).

Feed Intake

Feed intake was not affected by the dietary treatments by d 14 ($P = 0.321$) (Table 3.1). During d 15-21 birds fed the AA diet consumed more than birds fed the AN, AP, NN, and PP diets ($P = 0.0006$) diets. Overall by d 21, birds fed the AA diet had a higher feed intake than birds fed the PP diet ($P = 0.014$) (Table 3.2). Between d 22-28, birds fed

the AAA and AAP diets had higher feed intake compared to birds fed the AAN, ANN, APP, NNN, and PPP diets (P = 0.002) diets. Overall by d 28, birds fed AAN diet had a higher feed intake compared to birds fed ANN, NNN and PPP diets (P = 0.0001) (Table 3.3). Birds in the dietary treatment groups AAA and AAP had a higher feed intake by d 28 compared to birds in the dietary treatment group PPP (P = 0.0001). Birds fed the AAAA and AAPP diets had a higher feed intake between d 29-35 in comparison to birds fed the AAAP, ANNN, APPP, NNNN, and PPPP diets (P = 0.001) (Table 3.4). Birds fed the AAAN had a higher feed intake between d 29-35 compared to birds fed the ANNN diet (P = 0.001). Overall by d 35, birds in the dietary treatment groups AAAA, AANN, and AAPP had a higher feed intake compared to birds in the dietary treatment groups ANNN and PPPP (P < 0.0001). By d 35, birds fed the AAAP and APPP diet had a higher feed intake compared to birds fed the PPPP diet (P < 0.0001). There were no differences between treatments in feed intake during d 36-47 (P = 0.930) (Table 3.5), overall feed intake by d 47 (P = 0.317), during the week of d 48-55 (P = 0.345) (Table 3.6), or overall feed intake by d 55 (P = 0.254).

Feed Conversion Ratio

There were no differences in FCR for the first 14 d between dietary treatments (P = 0.241) (Table 3.1). Between d 15 to 21, birds fed the AA diet had the lowest FCR compared all other dietary treatments (P < 0.0001). Overall by d 21, birds fed NN had the highest feed conversion ratio compared to all other treatments (P = 0.0001) (Table 3.2). During d 22-28, birds fed the AAA diet had a lower FCR compared to birds fed AAP, APP, NNN, and PPP diets (P < 0.0001) diet. Overall by d 28, birds fed the AAA diet had

a lower FCR compared to birds fed ANN, APP, NNN, and PPP diets (P = 0.0001) diets (Table 3.3). Birds in the dietary treatment group AAAA had a lower FCR during d 29-35 compared to birds in the dietary treatment groups AAAN, AAAP, AAPP, NNNN, and PPPP (P < 0.0001). Birds fed the AANN diet had a lower FCR compared to birds fed the AAAN and PPPP diets during d 29-35 (P < 0.0001). Overall by d 35, birds fed the AAAA diet had a lower FCR compared to birds fed the AAAN, AANN, AAPP, ANNN, APPP, NNNN, and PPPP diets (P < 0.0001). Overall by d 35, birds fed the AAAP diet had a lower FCR compared to birds fed the APPP, NNNN, and PPPP diets (P < 0.0001). Overall by d35, birds fed the AANN diet had a lower FCR than birds fed the NNNN and PPPP diets (P < 0.0001). By d 35, birds in the dietary treatment groups AAAN, AAPP, and ANNN had a lower FCR compared to birds in the dietary treatment group NNNN (P < 0.0001) (Table 3.4). There were no differences seen in FCR between d36-47 or overall by d 47 between dietary treatments (P = 0.824, 0.057) (Table 3.5). Birds fed the PPPPPP and APPPPP diets had a lower FCR compared to birds fed the AAANNN, AAAAPP, AAAAAP, and AAAAAN diets during the d of 48-55 (P = 0.041). Birds fed the ANNNNN diet had a lower FCR compared to birds fed the AAAAPP, AAAAAP, and AAAAAN diets during the d of 48-55 (P = 0.041). Birds fed the AAAPPP diet had a lower FCR compared to birds fed the AAAAAN and AAAAPP diets during the d of 48-55 (P= 0.041). There were no differences in FCR overall by d 55 between dietary treatments (P = 0.547) (Table 3.6).

Body Weight Gain

There were no differences between dietary treatments for BWG between d 0-14 ($P = 0.513$) (Table 3.1). Birds fed the AA diet had a higher BWG compared to birds fed the AN, AP, NN, and PP diets between d 15-21 and overall by d 21 ($P < 0.0001$, < 0.0001) (Table 3.2). Birds fed the AAA diet had a higher BWG between d 22-28 compared to all other dietary treatments ($P < 0.0001$). Birds fed the AAP diet had a higher BWG between d 22-28 compared to birds fed the AAN, APP, and PPP ($P < 0.0001$). Overall by d 28, birds fed the AAA diet had a higher BWG compared to birds fed the AAN, ANN, APP, NNN and PPP diets ($P < 0.0001$). Birds in the dietary treatment group AAP had a higher BWG compared to birds in the dietary treatment groups ANN, APP, and PPP overall by d 28 ($P < 0.001$) (Table 3.3). The body weight gain of birds fed the AAAA diet was highest compared to the birds fed the AAAN, AAAP, AANN, AAPP, ANNN, APPP, NNNN, and PPPP diets during d 29-35 ($P < 0.0001$). The BWG from d 29-35 was higher for birds fed the AAAA diet compared to all other dietary treatments ($P < 0.0001$). The BWG was higher for birds fed the AAPP diet compared to birds fed the AAAN, APPP NNNN, and PPPP diets from d 29-35 ($P < 0.0001$). Birds in the dietary treatment group AANN had a higher BWG between d 29-35 compared to birds in the dietary treatment groups APPP and PPPP ($P < 0.001$). Overall by d 35, birds fed the AAAA diet had a higher BWG compared to birds fed the AAAN, AANN, AAPP, ANNN, APPP, NNNN, and PPPP diets ($P < 0.0001$). By d 35, birds fed the AAAP diet had a higher BWG compared to those fed the AANN, ANNN, APPP, NNNN, and PPPP diets ($P < 0.0001$). Birds fed the AAAN and AAPP diets had a higher BWG by d 35 compared to birds fed the ANNN, APPP, NNNN, and PPPP diets ($P <$

0.0001) (Table 3.4). BWG from d 36-47 was similar between dietary treatments (P = 0.705). Overall by d 47, birds fed the AAAAA, AAAAN, AAAAP and AAAPP diets had a higher BWG compared to birds fed AAANN, AANNN, ANNNN, APPPP, NNNNN, and PPPPP diets (P < 0.0001). Birds in the dietary treatment group AAPPP had a higher BWG overall by d 47 compared to those in the ANNNN, APPPP, NNNNN, and PPPPP dietary treatment groups (P < 0.0001). Birds fed the diet AANNNN had a higher BWG from d 48-55 compared to birds fed the AAAAAN, AAAAAP, AAAAPP, AAANNN, AAPPPP, and NNNNNN diets (P = 0.014) (Table 3.5). From d 48-55, birds fed the ANNNNN and PPPPPP diets had a higher BWG compared to birds fed the AAAAAP, AAAAPP, and AAANNN diets (P = 0.0014). Overall by d 55, birds fed the AAAAAN and AAAANN diets had a higher BWG compared to birds fed the AAANNN, ANNNNN, APPPPP, NNNNNN, PPPPPP diets (P = 0.001). Birds fed the AAAPPP, AAPPPP, and AANNNN diets had a higher BWG by d55 compared to birds fed the AAANNN, APPPPP, NNNNNN, and PPPPPP diets (P = 0.001). Birds fed the AAAAAP and AAAAPP had a higher BWG by d55 compared to birds fed the AAANNN and APPPPP diets (P = 0.001) (Table 3.6).

Processing: carcasses and abdominal fat pads

Birds fed the AAAANN diet had higher individual body weights compared to birds fed the AAAAAP, ANNNNN, NNNNNN, PPPPPP, AAANNN, and APPPPP diets (P = 0.0002) on day 56. Birds fed the AAAAAN diet had a higher individual BW compared to birds fed the ANNNNN, NNNNNN, PPPPPP, AAANNN, and APPPPP diets (P = 0.0002). Birds fed the AAAAPP, AAPPPP, and AANNNN diets had higher

individual BW compared to birds fed the PPPPPP, AAANNN, and APPPPP diets (P = 0.0002). Birds in the dietary treatment group AAAPPP had higher individual BW compared to birds in the APPPPP dietary treatment group (P = 0.0002). There were no differences between dietary treatments for abdominal fat pad weight (P = 0.247). Birds fed the AAAANN diet had higher carcass weights compared to birds fed the AAAAAP, NNNNNN, AAANNN, ANNNNN, PPPPPP, and APPPPP diets (P = 0.0004). Birds fed the AAAAAN diets had heavier carcass weights compared to birds fed the AAANNN, NNNNNN, ANNNNN, PPPPPP, and APPPPP diets (P = 0.0004). Feeding the AAAAPP diet increased the carcass weight compared to birds fed the AAANNN, ANNNNN, PPPPPP, and APPPPP diets (P = 0.0004). Birds fed the AAPPPP diet had higher carcass weights compared to birds fed the ANNNNN, PPPPPP, and APPPPP diets (P = 0.0004) diets. Birds fed the AAAPPP and AANNNN diets had higher carcass weights compared to birds fed the APPPPP diet (P = 0.0004) (Table 3.7).

Processing: cut-up parts

Birds fed the AAAAPP diet had higher wing weights compared to birds fed the AAAPPP, AAANNN, NNNNNN, PPPPPP, APPPPP, and ANNNNN diets (P = 0.0008). Birds fed the AAAAAN diet had higher wing weights compared to birds fed the AAANNN, NNNNNN, PPPPPP, APPPPP, and ANNNNN diets (P = 0.0008) diets. Feeding the AAPPPP and AAAANN diets had improved wing weights compared to birds fed the NNNNNN, PPPPPP, APPPPP, and ANNNNN diets (P = 0.0008). Birds fed the AAAANN diet had improved breast weight compared to birds fed the AAPPPP, AAAAAP, AANNNN, NNNNNN, AAANNN, ANNNNN, PPPPPP, and APPPPP diets

(P = 0.012). Feeding the AAAAAN diet improves breast weight compared to birds fed the AAANNN, ANNNNN, PPPPPP, and APPPPP diets (P= 0.012). Feeding the AAAPPP diet improves breast weight compared to birds fed the APPPPP diet (P = 0.012). There were no differences in tender weigh between dietary treatments (P = 0.427). Feeding the AAPPPP diet improved thigh weights compared to birds fed the ANNNNN, NNNNNN, PPPPPP, and APPPPP diets (P = 0.024). Feeding the AAAANN and AAAAPP diets improved thigh weights compared to feeding the APPPPP and PPPPPP diets (P = 0.024). Birds fed the AAAAAN, AAAAAP, AANNNN, and AAAPPP diets improved thigh weights compared to birds fed the APPPPP diet (P = 0.024). Birds fed the AAAAPP diet had improved drumstick weights compared to birds fed the AAANNN, AAAAAP, NNNNNN, PPPPPP, ANNNNN, and APPPPP diets (P = 0.001). Birds fed the AAAAAN and AAAANN diet had higher drumstick weights compared to birds fed the NNNNNN, PPPPPP, ANNNNN, and APPPPP diets (P = 0.001). Birds fed the AAAPPP and AAPPPP diets had higher drumstick weights compared to birds fed the ANNNNN and APPPPP diets (P = 0.001). Birds fed the AANNNN diet had higher drumstick weights compared to birds fed the APPPPP diet (P = 0.001) (Table 3.7). There were no differences between dietary treatments for processing yields relative to the carcass (Table 3.8).

Mortality

There were no differences in mortality between dietary treatments by d 14 (P = 0.457), between d15-21 (P = 0.688), or d 21 (P = 0.433). Between d 22-28, birds fed the APP diet had a higher mortality compared to birds fed the AAA, AAN, AAP, PPP and

NNN diets (P = 0.002). Birds fed the ANN diet had a higher mortality, between d 22-28, compared to birds fed the AAA, AAN, and NNN diets (P = 0.002). There were no differences in mortality between dietary treatments overall by d28 (P = 0.760), between d29-35 (P = 0.413), overall by d 35 (P = 0.732), between d 36-46 (P = 0.385) or overall by d 46 (P = 0.798). Birds fed the AANNNN diet, between d 47-56, had a higher mortality compared to birds fed the AAAANN, AAAAPP, AAANNN, AAAPPP, AAPPPP, APPPPP, NNNNNN, and PPPPPP diets (P = 0.035). Overall by d 55, no differences were seen in mortality between dietary treatments (P = 0.866) (Table 3.9).

Discussion

Due to concerns by consumers about antibiotic residue in poultry meat and antibiotic resistance, alternatives to antibiotics are being studied (Donoghue, 2003, Singer and Hofacre, 2006). The purpose of this study is to determine an opportune time to remove antimicrobials from the broiler diet by replacing them with the probiotic *B. subtilis*, and to determine if the probiotic *B. subtilis* can reduce negative effects of antimicrobial removal on growth performance and processing yields of broilers under coccidial challenge.

One week after coccidial challenge, birds fed dietary antimicrobials started exhibiting higher BW as compared to birds fed all other diets. Removing antimicrobials from the diets earlier than d 14 lowered BW at all ages, increased FCR d 15-21 and d 22-28, and lowered overall meat yield on d 56 including the carcass, wing, breast, thigh, and drumstick weights, no matter if *B. subtilis* was supplemented or not. In addition, the

mortality from d 22-28 (8-14 d after challenge) increased by removing antimicrobials on d 14. The antimicrobials fed to the birds include both antibiotic (bacitracin) and anticoccidial (narasin). In the current study, the birds were challenged with 10x coccidial vaccine on d 14. The vaccine includes live oocysts of *Eimeria acervulina, E. maxima, E. maxima MFP, E. mivati, and E. tenella* which caused lesions in various parts of the intestine leading to reduce nutrient digestions and absorptions (Williams, 2005). In addition, being the major organ for nutrient digestion and absorption, the intestine is also an important organ for disease defense. Necrotic enteritis may occur if coccidiosis is not under control, and if the bacterium *C. perfringens* is present in sufficient quantity. Ionophorus anticoccidial narasin used in the current study has shown effectiveness in control of coccidia including all the species in the coccidial vaccine used in this study (Ruff et al., 1979). Improved weight gains and feed conversion ratios, and reduced intestinal lesions and mortality were observed in birds fed narasin (Ruff et al., 1979). Bacitracin used in the current study mainly inhibits the synthesis of the cell wall of Gram-positive bacteria. Bacitracin also shows inhibitive effects on Gram-negative cocci and spirochetes (Cheng et al., 2014). Research has shown that bacitracin can inhibit *C. perfringens* in the chicken intestine and improve BW gain and feed efficiency (Stutz et al., 1983). In our current study, after the birds were challenged with cocci on d 14, they started developing lesions. Without the protection of anticoccidial or antibiotics, the growth performance was compromised in those birds fed antimicrobial free diets. The supplementation of *B. subtilis* did not provide comparable protection to gut health or growth promoting effects for the broilers challenged with cocci as the antimicrobials did in this study. Similar to

our study, Abdelrahman et al., (2014) performed a study using a probiotic mixture and salinomycin (an antibacterial and anticoccidial ionophore drug) diets while challenging with coccidia and found that birds challenged and given the control diet or probiotic mixture had lower body weights compared to birds fed salinomycin. Contradictory to our results, Jayaraman et al. (2017) found that birds fed bacitracin diet and *B. subtilis* PB6 diet had higher BW compared to the control. The difference in results could be due to different breeds used. Jayaraman et al. used VenCobb 400 and were not challenged (Jayaraman et al., 2017) while in our study we used Ross 708 and challenged with coccidiosis.

The removal of antimicrobials with or without adding *B. subtilis* on d 14 can leave birds exposed to infection. Two weeks after infection, mortality increased for birds that had antibiotics removed or *B.s subtilis* supplemented on d14 compared to birds continuously fed antibiotics between d 22-28. Lin et al. (2015) conducted a study to test immunity against *E. tenella* using control diets and two recombinant *B. subtilis* probiotic strains. Birds were vaccinated on d 7 and challenged on d 17 with *E. tenella*. By d 24 only one of the two probiotic strains were able to assist the birds against the challenge. The birds in our study were challenged with a 10× dose of a commercial vaccine on d 14. The peak of mortality occured 8-14 d after the oral challenge could be due to the life cycle stage of coccidia oocysts. Within one week after an initial infection, fecal coliforms increase (Turk and Littlejohn, 1987). The oocysts exit the body and upon reinfection, the sporulated oocysts will cause a more severe infection (Davies et al., 1963). With this

increase of fecal coliforms, it could allow for reinfection and an increase in mortality after initial infection.

The removal of dietary antimicrobials on d 21 decreased BW on d 28, 35, and 47. However, supplementation of *B. subtilis* brought the BW back similar to that of birds fed antimicrobials. Similarly, the removal of dietary antimicrobials on d 28 also lowered BW on d 35, 47 and 55. Supplementation of *B. subtilis* brought the BW back similar to that of birds fed antimicrobials on d 35, 47, and 55. The antimicrobial in the diet may have given the birds a benefit of improved growth by converting energy obtained from the diet for use of growth instead of tissue maintenance (Miles et al., 2006). Antimicrobials may assist the birds by helping normal bacteria colonizing the gut and reducing adhesion of other bacteria and pathogens and promoting intestinal microflora balance (Havenaar et al., 1992; Bedford, 2000; McEwen and Fedorka-Cray, 2002). As well as reduce the competition for nutrients between pathogenic bacteria and the host (Dibner and Richards, 2005). The removal of dietary antimicrobials may have exposed the birds, especially their intestines, to more pathogen challenge and resulted in damages, which further compromised their nutrient and energy utilization efficiency. Also, removing antimicrobials can cause the microflora in the bird to become stressed because they must adjust to their new environment (Tannock, 1983). The addition of the probiotic *B. subtilis* may have helped lessen the impact of the antimicrobial removal by positively stimulating the microflora. The benefit of *B. subtilis* can potentially be due to its ability to produce antimicrobials, reduce colonization of pathogens, promote colonization of beneficial bacteria in the gut, or enhance the epithelial barrier (Bermudez-Brito et al., 2012; Ahmed

et al., 2014; Amin et al., 2015). The removal of dietary antimicrobials on d 21 did not affect processing meat yield, no matter if dietary probiotic was added or not. However, the removal of antimicrobials on d 28 lowered carcass, wing, and breast weights. Supplementation of probiotic *B. subtilis* brought their weights back close to that of the antimicrobials. The different responses on BW and meat yields between d 21 and d 28 may be due to different recovery time. The birds who had the antimicrobials removed on d 28 might not have had enough time to recover from the negative effects caused by the antimicrobial removal.

Birds fed diets with antimicrobials removed on d 35 or later, with or without replacing with *B. subtilis*, showed similar body weights, feed conversion, and d 56 processing yields compared to positive control. The most accepted modes of action exhibited by probiotics are competitive exclusion and antagonism (Kabir, 2009). Competitive exclusion and antagonism work more efficiently when birds are young, and their intestinal bacteria loads are low, especially when these animals have not developed a stable intestinal microflora. (Schneitz, 2005). The lack of response to *B. subtilis* added to the feed of broilers at a later age may be because the older birds have already established stable healthy microflora in their gastrointestinal tract.

In conclusion, the results suggest that removing antimicrobials from poultry diets too early in the growth phase, or not including them at all, could negatively affect growth performance. Supplementation of probiotics may alleviate the adverse effects of coccidiosis on growth performance of broilers fed diets with antimicrobial removed on d 21 or 28. The negative effects of antimicrobial removal on d 28 on growth and meat yield

may due to an insufficient time for birds to recover from coccidiosis. However, supplementing probiotics on d 28 alleviated the negative effects on growth and overall meat yields. In addition, the removal of antimicrobials impacted processing yields of various body parts differently, especially the front and back halves of the carcasses.

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Table 3.0 Dietary treatment outline

Treatment	D 0-14	D 14-21	D 21-28	D 28-35	D 35-46	D 46-56
1	A	A	A	A	A	N
2	A	A	A	A	A	P
3	A	A	A	A	N	N
4	A	A	A	A	P	P
5	A	A	A	N	N	N
6	A	A	A	P	P	P
7	A	A	N	N	N	N
8	A	A	P	P	P	P
9	A	N	N	N	N	N
10	A	P	P	P	P	P
11	N	N	N	N	N	N
12	P	P	P	P	P	P

Each letter represents the diet fed at each feeding phase.
A = Antimicrobial diet (Antibiotic for d 0-14; Antibiotic and Anticoccidial for d 14-56)
N = Negative Control basal diet
P = Probiotic *Bacillus subtilis* diet

Table 3.1 D 0-14 Bird Performance

Additive	BW D0	BW D14	BWG D0-14	FCR D0-14	FI D0-14
A	39.7	252.0	212.4	1.211	257.2
N	39.7	247.3	207.6	1.249	259.6
P	40.1	248.7	208.5	1.184	246.4
SEM	0.26	3.77	3.73	0.0214	5.50
P-Value	0.493	0.553	0.513	0.241	0.321

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)
N = Negative control basal diet
P = Probiotic *Bacillus subtilis* diet
Body weights (g), BW gain (BWG, g), feed conversion ratio (FCR, feed intake/BW gain), and feed intake (FI, g) for D 0 to 14, before coccidial challenge.

Table 3.2 Growth Performance for D 21 and D 15-21

Additive	BW D21	BWG D0-21	BWG D15-21	FCR D0-21	FCR D15-21	FI D0-21	FI D15-21
AA	665.4[a]	625.8[a]	414.5[a]	1.251[b]	1.266[b]	802.2[a]	533.4[a]
AN	619.3[b]	579.2[b]	359.7[b]	1.281[b]	1.351[a]	758.8[ab]	500.6[b]
AP	607.6[b]	567.6[b]	354.0[b]	1.270[b]	1.316[a]	754.5[ab]	499.4[b]
NN	602.7[b]	563.0[b]	355.4[b]	1.350[a]	1.345[a]	769.2[ab]	502.2[b]
PP	605.0[b]	564.9[b]	356.3[b]	1.261[b]	1.310[a]	735.0[b]	488.6[b]
SEM	9.10	9.10	6.89	0.0161	0.0140	19.6	11.05
P-Value	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	0.014	0.0006

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21.
A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)
N = Negative control basal diet
P = Probiotic *Bacillus subtilis* diet
Body weights (g), BW gain (BWG, g), feed conversion ratio (FCR, feed intake/BW gain), and feed intake (FI, g) till D 21
[a-c] Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 3.3 Growth Performance for D 28 and D 22-28

Additive	BW D28	BWG D0-28	BWG D22-28	FCR D0-28	FCR D22-28	FI D0-28	FI D22-28
AAA	1.247[a]	1.208[a]	0.582[a]	1.341[b]	1.449[b]	1.679[ab]	0.882[a]
AAN	1.176[b]	1.137[bc]	0.522[c]	1.366[ab]	1.483[ab]	1.759[a]	0.843[b]
AAP	1.230[a]	1.184[ab]	0.551[b]	1.366[ab]	1.508[a]	1.696[ab]	0.892[a]
ANN	1.149[b]	1.109[c]	0.529[bc]	1.380[a]	1.475[ab]	1.636[bc]	0.867[b]
APP	1.130[b]	1.090[c]	0.522[c]	1.390[a]	1.517[a]	1.674[abc]	0.867[b]
NNN	1.130[b]	1.090[c]	0.527[bc]	1.398[a]	1.485[a]	1.616[bc]	0.837[b]
PPP	1.127[b]	1.087[c]	0.522[c]	1.379[a]	1.520[a]	1.589[c]	0.831[b]
SEM	0.0166	0.0168	0.0102	0.0118	0.0147	0.0152	0.0131
P-Value	<.0001	<.0001	<.0001	0.0001	<.0001	0.0001	0.0023

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

Body weights (kg), BW gain (BWG, kg), feed conversion ratio (FCR, feed intake/BW gain), and feed intake (FI, kg) till D 28

[a-c] Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 3.4 Growth Performance for D 35 and D 29-35

Additive	BW D35	BWG D0-35	BWG D29-35	FCR D0-35	FCR D29-35	FI D0-35	FI D29-35
AAAA	2.015^{a}	1.975^{a}	0.767^{a}	1.411^{e}	1.534^{d}	2.939^{a}	1.244^{a}
AAAN	1.927^{b}	1.887^{bc}	0.692^{cd}	1.442^{bcd}	1.618^{ab}	2.837^{abc}	1.211^{ab}
AAAP	1.966^{ab}	1.930^{ab}	0.713^{bcd}	1.427^{de}	1.603^{abc}	2.876^{ab}	1.187^{bc}
AANN	1.895^{bc}	1.856^{cd}	0.719^{bc}	1.434^{cd}	1.556^{cd}	2.911^{a}	1.197^{abc}
AAPP	1.964^{ab}	1.910^{bc}	0.733^{b}	1.447^{bcd}	1.602^{abc}	3.951^{a}	1.254^{a}
ANNN	1.850^{cd}	1.810^{de}	0.702^{bcd}	1.449^{bcd}	1.564^{bcd}	2.748^{bc}	1.142^{c}
APPP	1.806^{d}	1.766^{e}	0.676^{d}	1.455^{bc}	1.576^{bcd}	2.855^{ab}	1.171^{bc}
NNNN	1.815^{d}	1.775^{e}	0.685^{cd}	1.484^{a}	1.600^{abc}	2.795^{abc}	1.179^{bc}
PPPP	1.801^{d}	1.786^{de}	0.675^{d}	1.469^{ab}	1.642^{a}	2.710^{c}	1.161^{bc}
SEM	0.0239	0.0236	0.0135	0.0097	0.0190	0.0359	0.0488
P-Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0013

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

Body weights (kg), BW gain (BWG, kg), feed conversion ratio (FCR, feed intake/BW gain), and feed intake (FI, kg) till D 35

$^{a-e}$ Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 3.5 Growth Performance for D 47 and D 36-47

Additive	BW D47	BWG D0-47	BWG D36-47	FCR D0-47	FCR D36-47	FI D0-47	FI D36-47
AAAAA	3.306[a]	3.266[a]	1.305	1.587	1.915	5.618	2.703
AAAAN	3.318[a]	3.278[a]	1.293	1.599	1.959	5.714	2.773
AAAAP	3.300[a]	3.261[a]	1.266	1.594	1.943	5.621	2.662
AAANN	3.165[bcd]	3.125[bc]	1.238	1.623	1.978	5.476	2.636
AAAPP	3.246[abc]	3.238[a]	1.280	1.597	1.917	5.540	2.651
AANNN	3.152[cd]	3.113[bc]	1.257	1.653	2.012	5.640	2.765
AAPPP	3.270[ab]	3.218[ab]	1.306	1.615	1.917	5.699	2.754
ANNNN	3.111[d]	3.071[c]	1.261	1.645	2.021	5.376	2.807
APPPP	3.082[d]	3.042[c]	1.276	1.637	1.973	5.674	2.729
NNNNN	3.114[d]	3.074[c]	1.259	1.644	1.933	5.403	2.669
PPPPP	3.062[d]	3.022[c]	1.260	1.650	1.983	5.462	2.802
SEM	0.0382	0.0379	0.0272	0.0192	0.0516	0.1092	0.0932
P-Value	<0.0001	<0.0001	0.705	0.057	0.824	0.317	0.930

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35; fifth d 36-47

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

Body weights (kg), BW gain (BWG, kg), feed conversion ratio (FCR, feed intake/BW gain), and feed intake (FI, kg) till D 47

[a-d] Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 3.6 Growth Performance for D 55 and D 48-55

Additives	BW D55	BWG D0-55	BWG D48-55	FCR D0-55	FCR D48-55	FI D0-55	FI D48-55
AAAAAN	4.255^{ab}	4.215^{a}	0.934^{bcd}	1.642	2.016^{a}	7.556	1.937
AAAAAP	4.190^{abc}	4.150^{abc}	0.899^{cd}	1.664	1.976^{ab}	7.369	1.872
AAAANN	4.279^{a}	4.240^{a}	0.962^{abcd}	1.663	1.924^{abcd}	7.740	2.025
AAAAPP	4.191^{abc}	4.151^{abc}	0.891^{d}	1.663	1.995^{a}	7.522	1.908
AAANNN	4.055^{d}	4.015^{d}	0.890^{d}	1.675	1.970^{abc}	7.416	1.916
AAAPPP	4.228^{ab}	4.188^{ab}	0.982^{abc}	1.645	1.854^{bcd}	7.556	1.982
AANNNN	4.200^{ab}	4.161^{ab}	1.048^{a}	1.696	1.912^{abcd}	7.546	1.950
AAPPPP	4.211^{ab}	4.171^{ab}	0.941^{bcd}	1.674	1.900^{abcd}	7.758	2.019
ANNNNN	4.114^{bcd}	4.073^{bcd}	1.003^{ab}	1.678	1.836^{cd}	7.303	1.964
APPPPP	4.052^{d}	4.012^{d}	0.970^{abcd}	1.672	1.819^{d}	7.678	1.965
NNNNNN	4.065^{cd}	4.025^{cd}	0.951^{bcd}	1.690	1.891^{abcd}	7.330	1.949
PPPPPP	4.064^{cd}	4.024^{cd}	1.002^{ab}	1.686	1.821^{d}	7.389	1.992
SEM	0.0463	0.0462	0.0317	0.0178	0.0491	0.1378	0.041
P-Value	0.001	0.001	0.014	0.547	0.041	0.254	0.345

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35; fifth d 36-47; sixth d 48-55

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

Body weights (kg), BW gain (BWG, kg), feed conversion ratio (FCR, feed intake/BW gain), and feed intake (FI, kg) till D 55

[a-d] Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 3.7 Processing weights D 56

Additive	Live body	Carcass	Fat pads	Breast	Wings	Thighs	Drumsticks	Tenders
AAAAAN	4.306^{ab}	3.156^{ab}	46.0	934.7^{ab}	331.4^{ab}	493.8^{abc}	387.9^{ab}	178.8
AAAAAP	4.166^{bcde}	3.057^{bcdef}	43.8	898.5^{bcd}	323.9^{abcd}	490.3^{abc}	377.1^{bcde}	173.2
AAAANN	4.340^{a}	3.175^{a}	41.9	950.2^{a}	329.2^{abc}	504.1^{ab}	391.6^{ab}	181.7
AAAAPP	4.255^{abc}	3.136^{abc}	46.9	901.1^{abcd}	333.3^{a}	496.1^{ab}	396.1^{a}	175.2
AAANNN	4.074^{de}	3.007^{def}	38.1	877.4^{cd}	317.9^{cd}	484.9^{abcd}	377.6^{bcde}	172.8
AAAPPP	4.204^{abcd}	3.082^{abcde}	44.4	922.2^{abc}	319.2^{bcd}	491.4^{abc}	382.5^{abc}	181.4
AANNNN	4.234^{abc}	3.078^{abcde}	43.1	892.4^{bcd}	321.3^{abcd}	489.3^{abc}	381.7^{abcd}	173.5
AAPPPP	4.252^{abc}	3.113^{abcd}	43.8	899.2^{bcd}	330.0^{abc}	512.0^{a}	386.0^{abc}	174.1
ANNNNN	4.114^{cde}	2.979^{ef}	41.8	876.0^{cd}	311.0^{d}	482.8^{bcd}	365.5^{de}	174.6
APPPPP	4.056^{e}	2.960^{f}	40.6	852.9^{d}	310.8^{d}	459.8^{d}	364.2^{e}	167.7
NNNNNN	4.109^{cde}	3.022^{cdef}	42.6	889.1^{bcd}	314.6^{d}	481.6^{bcd}	371.0^{cde}	168.6
PPPPPP	4.081^{de}	2.977^{ef}	38.5	874.1^{d}	313.6^{d}	467.7^{cd}	369.6^{cde}	176.7
SEM	0.0531	0.0412	2.39	18.37	4.72	10.04	6.01	4.31
P-Value	0.0002	0.0004	0.247	0.012	0.0008	0.024	0.001	0.427

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35; fifth d 36-47; sixth d 48-55
A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)
N = Negative control basal diet
P = Probiotic *Bacillus subtilis* diet
Body, carcass weights (kg), abdominal fat pad, breast, wing, thigh, drumstick, and tender weights (g) on D 56.
$^{a-f}$ Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 3.8 Processing Weights Relative to the Carcass (%)

Additive	Breast	Wing	Thigh	Drumstick	Tender
AAAAAN	29.6	10.5	15.6	12.3	5.67
AAAAAP	29.3	10.6	16.0	12.4	5.65
AAAANN	29.9	10.4	15.9	12.4	5.71
AAAAPP	28.8	10.6	15.8	12.6	5.60
AAANNN	29.1	10.6	16.1	12.6	5.75
AAAPPP	29.9	10.4	16.0	12.4	5.89
AANNNN	28.9	10.5	15.9	12.4	5.63
AAPPPP	28.8	10.6	16.5	12.4	5.61
ANNNNN	29.2	10.4	16.1	12.2	5.87
APPPPP	28.8	10.5	15.5	12.3	5.67
NNNNNN	29.4	10.4	15.9	12.3	5.60
PPPPPP	29.3	10.6	15.7	12.4	5.93
SEM	0.3757	0.1293	0.2438	0.1403	0.1241
P-Value	0.352	0.873	0.398	0.757	0.516

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35; fifth d 36-47; sixth d 48-55

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

Table 3.9 Weekly and overall mortality (%)

Additive	D 0-14	Additive	D 15-21	D 0-21	Additive	D 22-28	D 0-28	Additive	D 29-35	D 0-35	Additive	D 36-46	D 0-46	Additive	D 47-56	D 0-56
A	4.06	**AA**	0.98	5.66	**AAA**	0.26^{c}	5.60	**AAAA**	0	5.86	**AAAAA**	0	4.30	**AAAAAN**	1.56^{ab}	5.47
N	1.56	**AN**	2.34	4.69	**AAN**	0^{c}	6.25	**AAAN**	0	3.91	**AAAAN**	0	7.81	**AAAAAP**	1.56^{ab}	6.25
P	3.13	**AP**	1.56	2.34	**AAP**	0.78^{bc}	7.81	**AAAP**	1.56	7.81	**AAAAP**	0	7.03	**AAAANN**	0^{b}	7.81
SEM	1.521	**NN**	0.78	2.34	**ANN**	3.13^{ab}	7.81	**AANN**	1.56	7.81	**AAANN**	0.78	4.69	**AAAAPP**	0^{b}	7.03
P-Value	0.457	**PP**	1.56	4.69	**APP**	3.91^{a}	6.25	**AAPP**	0.78	8.59	**AAAPP**	0.78	8.59	**AAANNN**	0^{b}	4.69
		SEM	0.846	1.87	**NNN**	0^{c}	2.34	**ANNN**	0.78	8.59	**AANNN**	0.78	8.59	**AAAPPP**	0^{b}	8.59
		P-Value	0.688	0.433	**PPP**	0.78^{bc}	5.47	**APPP**	0.78	7.03	**AAPPP**	0	8.59	**AANNNN**	2.34^{a}	10.94
					SEM	0.817	2.276	**NNNN**	0.78	3.13	**ANNNN**	0.78	9.38	**AAPPPP**	0^{b}	8.59
					P-Value	0.002	0.760	**PPPP**	1.56	7.03	**APPPP**	1.56	8.59	**ANNNNN**	0.78^{ab}	10.16
								SEM	0.708	2.358	**NNNNN**	1.56	4.69	**APPPPP**	0^{b}	8.59
								P-Value	0.413	0.732	**PPPPP**	0.0	7.03	**NNNNNN**	0^{b}	4.69
											SEM	0.597	2.540	**PPPPPP**	0^{b}	7.03
											P-Value	0.385	0.798	**SEM**	0.586	2.723
														P-Value	0.035	0.866

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35; fifth d 36-47; sixth d 48-55

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

$^{a-c}$ Means in a column not sharing a common superscript are different ($P \leq 0.05$)

References

Abdelrahman, W., Mohnl, M., Teichmann, K., Doupovec, B., Schatzmayr, G., Lumpkins, B., Mathis, G. 2014. Comparative Evaluation of Probiotic and Salinomycin Effects on Performance and Coccidiosis Control in Broiler Chickens. Poultry Science. 93 (12): 3002-3008

Ahmed, S. T., Islam, M., Mun, H. S., Sim, H. J., Kim Y. J. and Yang, C. J. 2014. Effects of *Bacillus Amyloliquefaciens* as a Probiotic Strain on Growth Performance, Cecal Microflora, and Fecal Noxious Gas Emissions of Broiler Chickens. Poultry science. 93(8):1963-1971.

Amin, M., Rakhisi, Z., and Ahmandy, A. Z. 2015. Isolation and Identification of *Bacillus* Species from Soil and Evaluation of their Antimicrobial Properties. Avicenna Journal of Clinical Microbiology and Infection. 2(1):10-13.

Bedford, M. 2000. Removal of Antibiotic Growth Promoters from Poultry Diets: Implications and Strategies to Minimise Subsequent Problems. World's Poultry Science Journal. 56(4):347-365.

Bermudez-Brito, M., Plaza-Diaz, J., Munoz-Quezada, S., Gomes-Llorente, C., and Gil, A. 2012. Probiotic Mechanisms of Action. Annals of Nutrition and Metabolism. 61(2):160-174.

Cheng, G., Hao, H., Xie, S., Wang, X., Dai, M., Huang, L., and Yuan, Z. 2014. Antibiotic Alternatives: The Substitution of Antibiotics in Animal husbandry? Frontiers in Microbiology. 13(5): 217.

Dalloul, R. A., and Lillehoj, H. S. 2006. Poultry Coccidiosis: Recent Advancements in Control Measures and Vaccine Development. Expert Review of Vaccines. 5(1):143-163.

Dalloul, R. A., Lillehoj, H. S., Tamim, N. M., Shellem, T. A., and Doerr, J. A. 2005. Induction of Local Protective Immunity to *Eimeria acervulina* by a *Lactobacillus*-based Probiotic. Comparative Immunology, Microbiology and Infectious Diseases. 28:351-361.

Davies, S. 1963. Coccidiosis. Edinburgh: Oliver and Boyd.

Dibner, J. J., and Richards, J. D. 2005. Antibiotic Growth Promoters in Agriculture: History and Mode of Action. Poultry Science. 84(4):634-643

Donoghue, D. J. 2003. Antibiotic Residues in Poultry Tissues and Eggs: Human Health Concerns? Poultry Science. 82(4):618-621.

Feighner, S. D. and Dashkevicz, M. P. 1986. Subtherapeutic Levels of Antibiotics in Poultry Feeds and their Effects on Weight Gain, Feed Efficiency, and Bacterial Cholyltaurine Hydrolase Activity. Applied and Environmental Microbiology. 53(2):331-336.

Fuller, R., 1992. History and Development of Probiotics. In: Fuller, R. (Ed.), Probiotics – The Scientific Basis. Chapman and Hall, London. 1-7.

Gadde, U., Kim, W. H., Oh, S. T., and Lillehoj, H. S. 2016. Alternatives to Antibiotics for Maximizing Growth Performance and Feed Efficiency in Poultry: A Review. Animal Health Research Reviews. 18(1): 26-45.

Giannenas, I., Tsalie, E., Triantafillou, E., Hessenberger, S., Teichmann, K., Mohnl, M., and Tontis, D. 2014. Assessment of Probiotics Supplementation via Feed or Water on the Growth Performance, Intestinal Morphology, and Microflora of Chickens after Experimental Infection with *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella*. Avian Pathology. 43(3):209-216.

Gustafson, R. H. and R. E. Bowen. 1997. Antibiotic Use in Animal Agriculture. Journal of Applied Microbiology. 83(5):531-541.

Havenaar, R., and Huis in'tVeld, J. H. J. 1992. Probiotics: A General View. In The Lactic Acid Bacteria in Health and Disease vol 1, ed. B. J. B. Wood. Elsevier applied science. New York and London Hall. 151-170.

Jayaraman, S., Das, P., Saini, P., Roy, B., and Chatterjee, P. 2017. Use of *Bacillus Subtilis* PB6 as a Potential Antibiotic Growth Promoter Replacement in Improving Performance of Broiler birds. Poultry Science. 96(8):2614-2622.

Kabir, S. L. 2009. The Role of Probiotics in the Poultry Industry. International Journal of Molecular Sciences. 10(8):3531-3546.

Li, G. Q., Kanu, S., Xiao, S.M., and Xiang, F. Y. 2005. Responses of Chickens Vaccinated with a Live Attenuated Multi-valent Ionophore-tolerant *Eimeria* vaccine. Veterinary Parasitology 129:179-186.

Lin, Z., Shi, Y., Deng, B., Mao, X., Yu, D., Li, W. 2015. Protective Immunity Against *Eimeria tenella* Infection in Chickens following Oral Immunization with *Bacillus subtilis* Expressing *Eimeria tenella* 3-1E protein. Parasitology Research. 114(9):3229-3236.

McEwen, S. A, and Fedorka-Cray, P. J. 2002. Antimicrobial Use and Resistance in Animals. Clinical Infectious Diseases. 34: 93-106.

Miles, R. D., Butcher, G. D., Henry, P. R., Littell, R. C. 2006. Effect of Antibiotic Growth Promoters on Broiler Performance, Intestinal Growth Parameters, and Quantitative Morphology. Poultry Science. 85(3):476-485

Ruff, M. D., Reid, W. M., Johnson, J. K., and Anderson, W. A. 1979. Anticoccidial Activity of Narasin in Battery Raised Broiler Chickens, Poultry Science, 58 (2): 298–303.

SAS Institute. 2012. SAS Proprietary software 9.4 SAS inst. Inc., Cary, NC, USA.

Schneitz C. 2005. Competitive exclusion in poultry–-30 years of research. Food Control. 16:657–667.

Singer, R. S. and Hofacre, C. L. 2006. Potential Impacts of Antibiotic Use in Poultry Production. Avian Diseases. 50(2):161-172.

Stutz, M. W., Johnson, S. L., and Judith, F. R. 1983. Effects of Diet and Bacitracin on Growth, Feed Efficiency, and Populations of Clostridium perfringens in the Intestine of Broiler Chicks. Poultry Science. 62(8): 1619–1625

Tannock, G.W. Effects of Dietary and Environmental Stress on the Gastrointestinal Microbiota. 1983. In Human Intestinal Microflora in Health and Disease, Vol 1. Ed. Hentges, D.J. Academic Press. New York. 226-257.

Turk, D. E. and Littlejohn V. P. 1987. Coccidial Infections and Gut Microflora. Poultry Science. 66(9):1466-1469.

Williams, R. B. 2005. Intercurrent Coccidiosis and Necrotic Enteritis of Chickens: Rational, Integrated Disease Management by Maintenance of Gut Integrity. Avian Pathology. 34:159-180.

CHAPTER IV

EFFECTS OF REPLACING DIETARY ANTIMICROBIALS WITH *BACILLUS SUBTILIS* ON THE INTESTINAL HEALTH OF MALE BROILERS

Abstract

The purpose of this study was to determine an optimal time to remove antimicrobials, including antibiotics and anticoccidials, and to replace them with probiotics without imposing a subsequent negative effect on intestinal health. One thousand five hundred and thirty six Ross × Ross 708 male broilers were randomly placed into each of 96 pens (16/pen), according to a randomized complete block design comprised of 8 replicate pens with 12 treatment groups. Birds were fed in 6 feeding phases: 0-14, 14-21, 21-28, 28-35, 35-46, 46-56 d. The 3 diets employed were: 1) a basal diet containing no antibiotic or anticoccidial, 2) an antimicrobial diet (antibiotic only from d 0-14) (50 g bacitracin/ton of feed, 79.2 g narasin/ton of feed), and 3) a *Bacillus subtilis* probiotic diet (1.1×10^5 CFU/g of feed). At each feeding phase, antimicrobials were removed with or without supplementation of the probiotic, *B. subtilis*. Birds were challenged by oral gavage on d 14 with a 10 × dose of a commercial coccidial vaccine including live *Eimeria (E.) acervulina, E. maxima, E. maxima* MFP, *E. mivati*, and *E. tenella*. A week after challenge, birds experienced lesions throughout the intestines. Birds fed antibiotics until d 14 and then supplemented with *B. subtilis* on d 14 and birds fed *B. subtilis* continuously had a higher incidence of lesions in the jejunum compared to birds

fed antimicrobials on d 20. By d 27, birds fed *B. subtilis* had reduced lesion scores in their duodenum in comparison to birds continuously fed the control diet. By d 34, birds that were fed diets in which antimicrobials were removed and which received supplementary *B. subtilis* on d 21 or 28, or which had antimicrobials removed on d 28 or were continuously fed *B. subtilis* had jejunum lesion scores that were similar to those fed the antimicrobial diet. In conclusion, supplementary *B. subtilis* reduced lesion severity in the intestine on d 27 and 34. The supplementary *B. subtilis* on d 21 and 28 after antimicrobial removal from the diet reduced the severity of intestinal damage by coccidial species when compared to birds fed antimicrobials.

Key Words: Antibiotic, *Bacillus subtilis*, Broiler, Coccidiosis, Intestine

Introduction

Antibiotics have been used to promote growth and to assist in disease prevention in the poultry industry. The amount of antibiotics consumed globally by farm animals in 2010 was estimated to be 63,151 tons. By 2030 that number will rise by 67% (Van Boeckel et al., 2015). Recently the industry has been pressured to remove antibiotics from poultry diets due to increases in antibiotic residues and drug resistant bacteria (Van Boeckel et al., 2015). With that growing concern, alternatives are needed that can replace antibiotics in the poultry diet without having a negative effect on bird health. An alternative that has provided positive results amd that demonstrates potential efficacy as a replacement for antibiotics are probiotics (Patterson and Burkholder, 2003).

Probiotics are a live microbial feed supplement that are beneficial to the intestine of the host (Fuller, 1992). Probiotics have been shown to improve the epithelial barrier of

the intestine, to reduce pathogens from binding sites in the intestine, and to repair intestinal damage previously incurred by disease (Bermudez-Brito et al., 2012). The use of probiotics have been shown to improve intestinal health that was compromised by coccidiosis vaccination (Ritzi et al., 2016), and to stimulate an immune response against challenge infections (Bermudez-Brito et al., 2012). The probiotic *B. subtilis,* has been shown to reduce the incidence of Necrotic enteritis, which has been induced by *Eimeria* and *C. perfringens* (gram positive pathogenic bacterium) challenges (Jayaraman et al., 2013). *B. subtilis* may likewise relieve the negative effects of an *E. maxima* challenge (Lee et al., 2010).

Coccidiosis is a severe disease that impacts the intestines of poultry. It is caused by a protozoan parasite that infects the intestine of poultry and other species. Coccidiosis causes malabsorption, blood loss, intestinal inflammation, and necrosis of the intestinal mucosal layer (Johnson and Reid, 1970). Birds exposed to coccidia oocysts develop lesions in the small intestines and ceca. The type of coccidial species determines where the lesions will form and how the birds will be affected (Johnson and Reid, 1970). The parasite can infect the birds at any age, however, most infections occur during the first couple of weeks after hatch (Jorden and Pattison, 1996). Coccidia causes the poultry industry about 3 billion dollars loss a year worldwide (Dalloul and Lillehoj, 2006).

Although positive results have been reported concerning the use of probiotics in the diet of poultry, there is no information regarding the effects of antimicrobial (antibiotics or anticoccidials) removal from the diet and its replacement with probiotics on the heath of broilers subjected to coccidial challenge. Therefore, the purpose of this study was to determine the effects of antimicrobial removal and supplementation of *B.*

subtilis on footpad lesions scores and on gut performance, morphology, and lesions incidence and severity.

Materials and Methods

Bird Management and Treatment Outline

All bird husbandry, handling methods, and experimental procedures were approved by the Institutional Animal Care and Use Committee of Mississippi State University. Ross × Ross 708 male broilers were purchased from a local hatchery and randomly assigned to 96 pens with 16 birds per pen. Stocking density for day 0 and 14 was 0.75ft^2 and for day 21, 28, 35, 46 and 55 stocking density was 0.75ft^2, 0.80 ft^2, 0.86 ft^2, 0.93 ft^2, 1.09 ft^2 and 1.2 ft^2 respectively. Each pen was randomly assigned to 8 replicate blocks within each of 12 treatment groups. All birds received a spray coccidial vaccination at the hatchery. Birds had ad libitum access to feed and water using nipple drinkers and gravity feed feeders. On d of hatch, birds were placed on used litter top dressed with new pine shavings. Birds were monitored daily and dead birds were removed and weighed. The birds received light for 24 hours with no dark period (L24:D0) for the first 7 d. Following d 7, birds received L20:D4 at a full 30 lux intensity from d 8-10. From d 11-17, they received L20:D4 with the lights slightly dimed each d. From d 18 until the end of the trial at d 55, birds received L20:D4 with a light intensity of 2.7 lux. On d 14, all of the birds were orally gavaged with a 10× dose of the commercial coccidial vaccine including *Eimeria acervulina, E. maxima, E. maxima* MFP*, E. mivati,* and *E. tenella.* The birds were fed in six phases: 0-14, 14-21, 21-28, 28-35, 35-46, and 46-56 d. The birds were fed 1 of the 3 diets: basal; antimicrobial supplemented (50 g antibiotic bacitracin/ton of feed, 79.2g ionophore anticoccidial narasin/ton of feed); or

probiotic supplemented (1.1×10^5 CFU of *Bacillus subtilis*/g of feed). From d 0-14, the feed included only antibiotics and did not contain anticoccidials, because the coccidial vaccine was applied at d of hatch. As biweekly phases progressed, the microbial in every other treatment group was successfully removed or replaced by the probiotic. This allowed for the formation of all 12 treatment groups. An outline of the treatments is shown in Table 4.0.

Sampling

On d 27, one bird from each pen from treatments AAA, AAN, AAP, ANN, APP, NNN, and PPP were sampled. On d 34, one bird from each pen from treatments AAAA, AANN, AAPP, ANNN, APPP, NNNN, and PPPP were sampled. On d 46, one bird from each pen from treatments AAAAA, AAAAN, AAAAP, AAANN, AAAPP, AANNN, AAPPP, ANNNN, APPPP, NNNNN, and PPPPP were randomly selected and sampled. Birds were euthanized by CO_2 asphyxiation and then weighed. The proventriculus, gizzard, spleen, pancreas, and bursal, were extracted and weighed. Intestinal lengths were recorded and then contents were removed before weighing. The pH of the ileum content and gizzard were recorded and then the contents were taken out before weighing. Two centimeter lengths of the mid-sections of the duodenum, jejunum, and ileum were collected and placed in a vial containing 10% formalin for histology. Duodenum samples were then cut into 5µm sections and put on glass slides and stained with Alcian blue. Ileum content were also collected for viscosity measurements. Intestinal lesions were scored for *Eimeria acervulina*, *E. maxima*, and *E. tenella* colonization.

Histology

Images were taken using a light microscope (Lexco SeBa, Bothell, WA). Pictures were taken with a magnification of 10× for villi length, crypt depth, and muscle thickness. The length of the villi and thickness of the crypt was measured using ImageJ software (National Institutes of Health). Villus to crypt depth ratio was obtained by dividing the villi length by crypt depth.

Viscosity

Ileum contents obtained from each bird were put on ice. The samples were then centrifuged at 4,500 × g for 10 min. The liquid samples were then analyzed using a Brookfield Viscosity Test Machine (Middleboro, MA), and centipoise (cP) readings from each sample were recorded.

Lesions

On d 20, one bird from each of the AA, AN, AP, NN and PP treatment groups was randomly selected for necropsy and for *Eimeria* lesion examination. On d 27, one bird from each of the AAA, AAN, AAP, ANN, APP, NNN, and PPP treatment groups was randomly selected for necropsy an for subsequent *Eimeria* lesion examination. On d 34, one bird from all pens in the AAAA, AAAN, AAAP, AANN, AAPP, ANNN, APPP, NNNN, and PPPP treatment groups was randomly selected and later examined and scored for *Eimeria* lesions. Lesions were scored according to the methods of Johnson and Reid (1970), without the scorer being knowledgeable of the treatments. The diameter of the lesions on the foot pads were measured and recorded on d 50 and 56. All birds were scored on day 50 and five birds per pen were scored on day 56. Birds were scored from 0

to 3. Zero signified no lesions, 0.1-5 mm was scored 1, those with lesions 6-14.9 mm were scored a 2 and those with lesions of 15mm or higher given a score of 3.

Experiment Design and Data Analysis

In a randomized complete block experimental design there were 96 pens with 12 dietary treatment groups. Each pen represented an experimental unit. All parameters were analyzed using one-way ANOVA using Proc GLM of SAS version 9.4 (SAS Institute, 2012) to determine the significance of responses to dietary treatments. Differences were considered significant at $P \leq 0.05$.

Results

Body and Organ weights, and Intestinal Lesions

On d 27, birds fed the AAA diet had a lower gizzard weight relative to the body weight compared to birds fed the AAN, ANN, APP, NNN and PPP diets (P= 0.001) (Table 4.2). Birds fed AAP diet had a lower gizzard weight relative to the body weight compared to those fed the ANN, APP, and NNN diets on d 27 (P = 0.001). Birds fed an AAA diet had a lower duodenum weight relative to the body weight compared to birds fed AAN, APP, and NNN diets (P = 0.021). Birds belonging to the AAP, ANN, and PPP treatment groups had lower duodenum weights on d 27 compared to birds in the APP treatment group (P = 0.021). On d 34, birds fed the AAAA diet had lower ileum weights compared to birds fed AANN, AAPP, and PPPP diets (P = 0.006) (Table 4.3). Birds fed AAAN, AAAP, ANNN, APPP, and NNNN had a lower ileum weight on d 34 compared to birds fed the AAPP diet (P = 0.006). On d 34, birds fed an AAAA diet had a lower ileum weight relative to the body weight compared to birds fed AANN, AAPP, APPP,

NNNN, and PPPP diets (P = 0.024). Birds fed an AAAP diet had a lower ileum weight relative to the body weight on d 34 compared to birds fed an PPPP diet (P = 0.024) (Table 4.4), and birds fed an AAAAA diet had a lower jejunum weight relative to the body weight on d 46 compared to birds fed AAANN, AAAPP, AANNN, AAPPP, ANNNN, APPPP, NNNNN, and PPPPP diets (P= 0.013).Birds fed the AAAAP diet had a lower jejunum weight relative to the body weight on d 46 compared to birds fed AANNN and ANNNN diets (P = 0.013), and birds belonging to the AAAAN treatment group had a lower jejunum weight relative to the body weight compared to birds belonging to the AANNN treatment group (P = 0.013) (Table 4.6). There were no significant treatment differences in total body or organ relative organ weights at necropsy on d 54 (Table 4.7 and Table 4.8). On d 20, a higher percentage of birds that lacked jejunum lesions were observed in the AA and AN dietary treatment groups when compared to those in the AP and PP treatment groups (P = 0.023) (Table 4.10). A higher percentage of birds that received a jejunum lesion score of 1occured in those belonging to the AA and AN treatment groups when compared to those belonging to the AP and PP treatment groups (P = 0.028).

On d 27, a higher percentage of birds lacking duodenal lesions occurred in those belonging to the PP treatment groups compared to those belonging to the AAA, AAP, APP, and NNN treatment groups (P = 0.023) (Table 4.11).

On d 34, a lower percentage of an intestinal lesion score of 2 were observed in the AAAP, AAPP, and PPPP treatment groups in comparison to birds belonging to the AANN and APPP treatment groups (P = 0.036) (Table 4.12).

Histology

There were no significant differences between treatments for villus length or villus length to crypt depth ratio on d 27 (P = 0.246, 0.172). On d 27, birds fed an ANN diet had a thicker crypt depth in the duodenum compared to all the other treatments (P = 0.001). On d 27, birds fed an ANN diet also had a thicker duodenal muscle layer compared to all other treatments (P = 0.004). However, on d 27, birds fed an APP diet also had a thicker duodenal muscle layer compared those fed an NNN diet (P = 0.004) (Table 4.9).

Foot Pad Lesions

On d 50, there were differences observed in foot pad lesions incidences between treatments. In the AAAAAP dietary treatment group there was a higher percentage of birds that lacked foot pad lesions compared to those in the AAAAAN, AAAPPP, AAPPPP, AANNNN, ANNNNN, NNNNNN, AAAANN, APPPPP, and PPPPPP dietary treatment groups (P = 0.003). In the AAANNN and AAAAPP dietary treatment groups on d 50 there was a higher percentage of birds lacking foot pad lesions compared those in the APPPPP and PPPPPP dietary treatment groups (P = 0.003). Feeding AAAAAN, AAAPPP, AAPPPP and ANNNNN diets increased the percentage of birds lacking footpad lesions on d 50 when compared to birds fed the PPPPPP diet (P = 0.003).

On d 50, the percentage of birds having a lesion score of three was higher in the group fed NNNNNN in comparison to those in the AAAAPP, AAANNN, AAPPPP, AAAAAN, AAAAAP, AAAANN, and AAAPPP dietary groups (P = 0.021).

Birds fed the ANNNNN and PPPPPP diets increased the percentage of scores of 3 when compared to birds fed the AAPPPP, AAAAAN, AAAAAP, AAAANN, and AAAPPP diets on d 50 (P = 0.021).

By d 50, birds in the dietary treatment group PPPPPP had a higher percentage of scores of two and three compared to birds the AAAAPP, AAAAAN, AAPPPP, AAANNN, and AAAAAP dietary treatment groups (P = 0.018). By d 50, birds fed the NNNNNN, APPPPP, AANNNN, and ANNNNN diets increased the higher percentage of lesions with scores of two and three when compared to birds fed AAANNN and AAAAAP diets (P = 0.018). On d 50, birds in the dietary treatment groups AAAPPP and AAAANN increased the percentage of birds scoring a two and three compared to birds in the AAAAAP dietary treatment group (P = 0.018). Foot Pad lesions for d 50 are on Table 4.13. There were no significant differences in foot lesions on d 56 between dietary treatments (Table 4.14)

Discussion

Lesion scoring determines the extent of the damage caused by coccidiosis. In this study, it was found that a week after challenge, birds fed *B. subtilis* experienced an increase in intestinal lesions when compared to birds fed antibiotics until d 14 or that were continuously fed antibiotics. However, two weeks after challenge, birds continuously fed *B. subtilis* had a lower incidence of lesions in the duodenum compared to birds continuously fed the control and antibiotics diets. Similar to this, Lee et al. (2010) studied the effects of *Bacillus* strains in birds and found that birds fed *Bacillus* species had lower intestinal lesion scores by d27 compared to the birds fed the control diet and that were later challenged with *E. maxima* (5.0×10^3 sporulated oocysts).

Jayaraman et al. (2013) induced Necrotic Enteritis in birds by infecting them with *Eimeria* species on d 14, and *C. perfringens* on d 19 through 21. It was discovered that by d 28, birds fed *B. subtilis* had lower Necrotic Enteritis lesion scores compared to birds fed control diets without *B. subtilis* and challenged. In this study, birds continuously fed *B. subtilis* had reduced *Eimeria* lesions in the duodenum within two weeks of challenge. This was indicated by their having a lower percentage of lesions in the duodenum compared to birds continuously fed antimicrobials. This improved duodenal health could be attributed to the ability of the probiotics to reduce pathogen colonization in the intestine and limiting the time birds are infected (Kabir, 2009).

By d 34, all birds had almost recovered from the coccidial challenge. However, birds fed antimicrobials until d 21 experienced a higher incidence of severe lesions in the jejunum compared to birds continuously fed antimicrobials. Birds which received diets supplemented with *B. subtilis* continuously, had *B. subtilis* supplemented on d 21 or d 28 had lesion scores that were similar to those continuously fed antimicrobials. Probiotics have been shown to reduce the signs of intestinal lesions caused by *Eimeria* species and to reduce oocyst shedding (Dalloul et al., 2005; Giannenas et al., 2012; Ritzi et al., 2014). Thus, removing antimicrobials without employing *B. subtilis* supplementation resulted in birds having more severe lesions compared to birds that received *B. subtilis* supplementation on day 21. This suggests that including the probiotic, *B. subtilis*, in the diet protected the intestine of birds from being severely damaged by lesions caused by *Eimeria* species.

Intestinal weights were altered by having antimicrobials in the diet. Birds that received the antimicrobial diet resulted in lighter intestinal weighs relative to the body

weight in the duodenum on d 27 compared to the birds fed the control, and although not significant, a similar trend was seen in the jejunum on d 27. Birds fed dietary antimicrobials by d 34 exhibited a lower relative ileum weight compared to the control, and although not significant a similar trend was seen for the duodenum and jejunum relative weights on d 34. Birds fed antimicrobials by d 34 also had a lower duodenum and ileum weights relative to the body weight and although not significant the same trend was seen for the jejunum relative weight. The trend continued to d 46 where the jejunum relative weight was lower for birds fed antimicrobials. Although not significant the trend was seen in the relative duodenum and ileum weights on d 46. The continuous use of antimicrobials in the diet lowered the weights of the intestine. Miles et al. (2006) observed that when feeding antibiotics in the diet the weights of the intestine decreased as compared to the control and despite the decrease in weight the intestine was not weaker (Miles et al., 2006).

In this study, there was an increase in duodenal crypt depth when antibiotics were removed on d 14. However, birds fed antibiotics until day 14 and that later received diets supplemented with *B. subtilis* had a lower crypt depth that was similar to that of birds fed antimicrobials by d 27. At the same time, it has been previously demonstrated that the administration of antibiotics can delay microbial development, while probiotic administration allows the microbiota to develop and mature as well as promoting beneficial bacterial species within the intestine (Gao et al., 2017; Baldwin et al., 2018). Thicker crypt depths indicate that the birds are possibly responding to a toxin and that the crypt is trying to replace villi that have been lost (Yason et al., 1987; Xia et al., 2004). For these reasons, it is believed that the removal of antibiotics influenced the bird's

ability to defend itself against *Eimeria* challenge. This is in contrast to birds that had antibiotics removed from their diets but were then provided protection by the dietary supplementation of *B. subtilis* on d 14 or of antimicrobials by d 27.

Foot pad dermatitis are lesions on the paw of the chicken, leading to economic losses due to processing condemnations. In addition, paw lesions are also a welfare issue (Shepherd and Fairchild, 2010). By d 50, birds fed antimicrobials until d 46 with subsequent supplementation with *B. subtilis* had a lower incidence of paw lesions compared to birds fed antimicrobials until d 46. Birds continuously fed *B. subtilis* had a higher incidence of paw lesions compared to birds fed antimicrobials until d 46, and had a higher frequency of lesion scores that were 2 or 3 on d 50 in comparison to birds fed antimicrobials until d 46. Along with footpad lesions, there remained signs of *Eimeria* lesions in the intestines of birds in all treatments by d 34. When birds are exposed to *Eimeria* species, birds can suffer from diarrhea or wet droppings which can cause an increase of moisture in the litter which has been previously shown to be the main cause of footpad dermatitis (Conway and McKenzie, 2007; Shepherd and Fairchild, 2010). It is possible that the birds that experienced more intestinal damage caused by lesions also had a higher incidence of wet droppings, which would lead to a higher incidence of foot pad lesions by d 50.

In conclusion, the results of this study suggest that the dietary supplementation of *B. subtilis* assisted in reducing *Eimeria* infection on different post hatch days, and supplementing the diet of broilers on d 21 and 28 with *B. subtilis* may provide protection against a subsequent *Eimeria* species infection.

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Tables

Table 4.0 Dietary treatment outline

Treatment	D 0-14	D 14-21	D 21-28	D 28-35	D 35-46	D 46-56
1	A	A	A	A	A	N
2	A	A	A	A	A	P
3	A	A	A	A	N	N
4	A	A	A	A	P	P
5	A	A	A	N	N	N
6	A	A	A	P	P	P
7	A	A	N	N	N	N
8	A	A	P	P	P	P
9	A	N	N	N	N	N
10	A	P	P	P	P	P
11	N	N	N	N	N	N
12	P	P	P	P	P	P

Each letter represents the diet fed at each feeding phase.
A = Antimicrobial diet (Antibiotic for d 0-14; Antibiotic and Anticoccidial for d 14-56)
N = Negative Control basal diet
P = Probiotic *Bacillus subtilis* diet

Table 4.1 Organ Weights, Lengths, and content pH, and Viscosity on D 27

	Weights (g)									Lengths (cm)			pH		
Additive	**BW**	**Proventriculus**	**Gizzard**	**Spleen**	**Pancreas**	**Bursa**	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**	**Ileum**	**Gizzard**	**Ileum Viscosity (cP)**
AAA	1,210	5.27	26.3	1.26	4.17	2.54	11.3	22.4	15.4	27.0	63.2	59.8	6.05	2.78	2.26
AAN	1,120	5.05	27.1	1.48	3.84	2.27	12.4	22.3	13.6	28.4	61	57.4	5.88	2.58	2.48
AAP	1,200	4.94	27.5	1.37	3.39	2.65	11.9	22.7	14.9	26.7	61.3	59.1	5.64	2.91	2.51
ANN	1,130	5.53	29.5	1.64	3.78	2.34	11.3	22.6	16.5	27.9	59.5	60.9	5.50	2.71	2.34
APP	1,090	4.93	27.6	1.39	3.62	2.30	13.1	23.6	15.3	28.9	57.8	56.9	5.69	2.72	2.33
NNN	1,100	4.96	27.9	1.33	3.64	2.42	12.3	24.9	15.7	28.9	64.5	60.8	5.76	2.67	2.61
PPP	1,080	4.79	26.7	1.39	3.67	2.11	11.1	21.8	15.3	27.9	59.3	56.5	5.80	2.56	2.38
SEM	37.0	0.235	0.992	0.112	0.194	0.247	0.681	1.309	0.828	0.935	2.605	2.43	0.144	0.162	0.1884
P-Value	0.076	0.469	0.516	0.444	0.161	0.770	0.268	0.658	0.401	0.567	0.520	0.758	0.251	0.770	0.828

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

Table 4.2 Relative Weights to Body Weights on D 27 (%)

Additive	Proventriculus	Gizzard	Spleen	Pancreas	Bursa	Duodenum	Jejunum	Ileum
AAA	0.437	2.19^{c}	0.105	0.346	0.210	0.87^{c}	1.80	1.23
AAN	0.451	2.42^{ab}	0.132	0.342	0.200	1.03^{ab}	1.92	1.18
AAP	0.41	2.30^{bc}	0.112	0.282	0.220	0.93^{bc}	1.84	1.21
ANN	0.49	2.62^{a}	0.146	0.336	0.210	0.93^{bc}	1.93	1.41
APP	0.452	2.53^{a}	0.127	0.333	0.210	1.12^{a}	2.07	1.36
NNN	0.448	2.53^{a}	0.120	0.329	0.220	1.04^{ab}	2.18	1.37
PPP	0.445	2.46^{ab}	0.129	0.341	0.200	0.95^{bc}	1.94	1.37
SEM	0.019	0.065	0.010	0.014	0.021	0.051	0.093	0.007
P-Value	0.331	0.001	0.181	0.060	0.983	0.021	0.068	0.133

Each letter represents the diet fed at each feeding phase.
The first letter represents the diet fed from d 0-14; second
d 15-21; third d 22-28
A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)
N = Negative control basal diet
P = Probiotic *Bacillus subtilis* diet
$^{a-c}$ Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 4.3 Organ Weights, Lengths, and content pH, and Viscosity on D 34

	Weights (g)									Lengths (cm)			pH		
Additive	**BW**	**Proventriculus**	**Gizzard**	**Spleen**	**Pancreas**	**Bursa**	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**	**Ileum**	**Gizzard**	**Ileum Viscosity (cP)**
AAAA	1,990	6.11	32.0	1.91	4.78	3.79	14.6	26.0	17.0^{c}	30.0	64.5	66.2	6.30	2.90	2.38
AAAN	1,860	6.20	30.7	1.87	4.69	3.57	15.1	28.2	18.2^{bc}	30.2	72.1	71.3	5.82	2.99	2.59
AAAP	1,930	6.66	30.8	2.60	4.97	3.14	15.4	27.9	18.7^{bc}	30.7	64.5	60.9	6.13	2.91	2.90
AANN	1,930	6.93	31.9	2.21	4.68	3.05	16.4	31.3	20.1^{ab}	32.5	74.4	75.4	5.08	2.84	2.54
AAPP	1,980	6.95	33.0	2.12	5.18	3.50	17.9	32.0	21.6^{a}	33.1	67.5	72.5	5.67	3.01	2.71
ANNN	1,790	6.61	31.3	2.29	4.68	3.84	15.8	28.6	17.7^{bc}	32.7	64.6	61.6	6.21	3.08	2.82
APPP	1,810	6.51	34.3	2.30	4.61	3.35	15.2	27.6	18.3^{bc}	31.0	69.9	69.8	5.90	2.85	2.66
NNNN	1,850	6.72	33.4	1.95	5.00	3.81	16.8	28.8	19.3^{bc}	39.8	67.6	64.9	5.92	2.75	2.51
PPPP	1,800	6.73	30.6	2.00	4.73	3.69	16.2	29.7	19.8^{ab}	32.1	66.3	69.4	5.68	2.82	3.04
SEM	59.0	0.340	1.49	0.199	0.286	0.381	0.863	1.636	0.809	2.636	2.843	3.727	0.292	0.189	0.173
P-Value	0.0954	0.673	0.561	0.292	0.856	0.823	0.180	0.268	0.006	0.280	0.249	0.152	0.250	0.942	0.314

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

$^{a-c}$ Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 4.4 Relative weights to body weights for D 34 (%)

Additive	Pancreas	Gizzard	Proventriculus	Spleen	Bursa	Duodenum	Jejunum	ileum
AAAA	0.239	1.621	0.306	0.096	0.190	0.727^{b}	1.30	0.854^{c}
AAAN	0.251	1.656	0.332	0.099	0.189	0.812^{ab}	1.51	0.988^{abc}
AAAP	0.258	1.590	0.345	0.134	0.163	0.792^{ab}	1.44	0.963^{bc}
AANN	0.243	1.646	0.359	0.114	0.156	0.852^{ab}	1.62	1.04^{ab}
AAPP	0.263	1.667	0.351	0.107	0.178	0.905^{a}	1.62	1.093^{ab}
ANNN	0.261	1.753	0.369	0.128	0.214	0.882^{a}	1.60	0.991^{abc}
APPP	0.254	1.886	0.358	0.126	0.184	0.835^{ab}	1.52	1.007^{ab}
NNNN	0.270	1.803	0.363	0.105	0.206	0.906^{a}	1.55	1.04^{ab}
PPPP	0.265	1.697	0.377	0.112	0.205	0.909^{a}	1.66	1.114^{a}
SEM	0.0160	0.0697	0.0168	0.0092	0.0190	0.0435	0.086	0.0505
P-Value	0.917	0.102	0.1195	0.078	0.449	0.052	0.117	0.024

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

$^{a-c}$ Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 4.5 Organ Weights, Lengths, and content pH, and Viscosity on D 46

Additive	Weights (g)									Lengths (cm)			pH		
	BW	Proventriculus	Gizzard	Spleen	Pancreas	Bursa	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Ileum	Gizzard	Ileum Viscosity (cP)
AAAAA	3,320[a]	9.59	41.7	3.14	5.96	5.18	16.5	29.0	21.7	33.9	65.3	63.6	7.14	3.26	3.02
AAAAN	3,250[ab]	9.36	40.2	3.38	5.96	5.77	18.7	31.9	23.0	34.9	67.4	68.4	6.60	3.14	2.58
AAAAP	3,280[ab]	8.93	38.7	3.51	6.21	4.80	18.0	31.6	22.1	34.0	62.7	64.7	6.73	3.39	2.97
AAANN	3,040[b]	8.16	37.0	2.85	5.42	5.87	17.0	31.1	21.3	34.4	63.4	61.8	6.54	3.14	3.36
AAAPP	3,200[ab]	8.46	38.3	3.49	5.86	5.60	19.0	32.3	21.6	37.3	66.1	64.8	7.12	3.49	2.82
AANNN	3,020[b]	8.24	39.0	3.54	5.97	4.99	18.6	33.8	23.1	36.3	65.5	66.0	6.70	2.77	3.14
AAPPP	3,320[a]	8.73	36.3	3.31	5.76	6.04	19.6	36.1	22.4	36.3	66.7	68.5	6.54	3.46	2.36
ANNNN	3,010[b]	8.08	37.2	2.91	6.04	5.71	18.5	32.8	21.5	33.4	67.6	60.7	6.64	3.22	2.66
APPPP	3,090[ab]	8.32	34.5	3.50	5.38	5.84	18.5	33.3	20.2	38.5	63.5	62.2	6.74	3.15	3.03
NNNNN	3,020[b]	9.08	39.5	3.16	5.45	6.33	18.7	32.7	21.4	35.0	68.1	66.1	6.64	3.03	3.07
PPPPP	3,010[b]	8.56	34.6	3.54	5.35	4.82	17.3	31.8	20.1	33.2	66.7	71.9	6.54	3.19	2.55
SEM	96.3	0.386	1.707	0.304	0.324	0.546	0.879	1.666	1.032	1.162	2.570	2.617	0.1957	0.1662	0.263
P-Value	0.0484	0.092	0.127	0.731	0.584	0.545	0.379	0.361	0.623	0.061	0.866	0.159	0.239	0.132	0.279

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35; fifth d 36-47

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

[a-b] Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 4.6 Relative Organ Weight to Body Weight on D 46 (%)

Additive	Proventriculus	Gizzard	Spleen	Pancreas	Bursa	Duodenum	Jejunum	Ileum
AAAAA	0.288	1.25	0.094	0.179	0.155	0.498	0.874^{d}	0.64
AAAAN	0.288	1.24	0.104	0.183	0.179	0.577	0.982^{bcd}	0.708
AAAAP	0.271	1.18	0.108	0.188	0.148	0.549	0.961^{cd}	0.671
AAANN	0.270	1.22	0.094	0.178	0.194	0.565	1.03^{abc}	0.701
AAAPP	0.264	1.20	0.109	0.183	0.175	0.592	1.01^{abc}	0.674
AANNN	0.272	1.29	0.117	0.199	0.162	0.619	1.11^{a}	0.737
AAPPP	0.263	1.10	0.099	0.174	0.183	0.591	1.09^{abc}	0.676
ANNNN	0.271	1.25	0.097	0.202	0.191	0.616	1.10^{ab}	0.714
APPPP	0.270	1.12	0.114	0.174	0.191	0.600	1.08^{abc}	0.660
NNNNN	0.302	1.31	0.105	0.181	0.210	0.619	1.08^{abc}	0.707
PPPPP	0.285	1.15	0.118	0.179	0.161	0.577	1.06^{abc}	0.671
SEM	0.0109	0.058	0.0102	0.0099	0.018	0.028	0.046	0.026
P-Value	0.235	0.222	0.721	0.544	0.367	0.107	0.0127	0.329

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35; fifth d 36-47

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

$^{a-d}$ Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 4.7 Organ Weights, Lengths, and content pH, and Viscosity on D 54

	Weights (g)									Lengths (cm)			pH		
Additive	BW	Proventriculus	Gizzard	Spleen	Pancreas	Bursa	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Ileum	Gizzard	Ileum Viscosity (cP)
AAAAAN	4,160	9.69	3.17	4.30	6.72	5.30	15.7	32.3	22.6	31.0	62.4	57.9	6.82	3.17	2.39
AAAAAP	4,170	9.30	3.45	4.17	6.44	5.93	17.1	32.4	22.8	29.6	63.7	64.3	6.49	3.45	2.50
AAAANN	4,130	9.47	3.50	4.97	6.14	5.72	17.9	32.0	22.7	31.6	63.1	57.9	6.75	3.50	2.71
AAAAPP	4,130	8.07	3.68	3.78	5.26	6.09	16.6	29.6	21.2	33.2	60.6	62.8	6.45	3.68	2.39
AAANNN	3,910	8.61	3.45	4.74	5.86	5.43	15.8	28.7	20.5	28.8	60.2	56.7	6.69	3.45	2.73
AAAPPP	4,130	9.56	3.35	5.20	5.76	5.79	16.1	30.8	21.8	32.9	66.9	61.8	6.96	3.35	2.62
AANNNN	4,200	9.27	3.22	5.04	6.22	6.81	17.1	32.0	22.1	32.2	66.7	58.8	6.68	3.22	2.69
AAPPPP	4,210	9.56	3.58	4.47	7.00	6.18	17.0	32.5	23.3	32.5	67.9	66.8	6.03	3.58	2.71
ANNNNN	3,920	9.27	3.22	5.11	6.73	6.35	16.2	31.0	20.8	31.1	66.3	69.3	6.79	3.217	3.33
APPPPP	3,890	9.24	3.72	4.09	6.10	5.95	17.1	30.3	21.9	30.2	64.8	63.4	6.43	3.72	2.32
NNNNNN	3,820	8.58	3.34	3.56	6.08	5.90	17.0	30.2	18.7	31.2	59.0	53.2	6.42	3.34	2.29
PPPPPP	3,910	9.29	3.18	4.40	6.33	4.53	15.4	32.1	22.2	30.3	59.7	57.0	6.38	3.18	2.55
SEM	115.8	0.410	0.163	0.599	0.473	0.704	1.147	1.717	1.326	1.682	3.381	3.438	0.269	0.163	0.273
P-Value	0.117	0.313	0.260	0.664	0.565	0.756	0.941	0.854	0.457	0.785	0.595	0.065	0.552	0.260	0.439

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35; fifth d 36-47; sixth d 48-55

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

Table 4.8 Relative Organ Weights to Body Weight D 54 (%)

Additive	Proventriculus	Gizzard	Spleen	Pancreas	Bursa	Duodenum	Jejunum	Ileum
AAAAAN	0.233	1.03	0.104	0.161	0.128	0.384	0.778	0.543
AAAAAP	0.223	1.16	0.099	0.154	0.142	0.409	0.774	0.544
AAAANN	0.230	1.16	0.120	0.148	0.142	0.434	0.776	0.546
AAAAPP	0.196	0.993	0.091	0.127	0.148	0.405	0.718	0.516
AAANNN	0.219	1.21	0.122	0.150	0.139	0.401	0.735	0.526
AAAPPP	0.232	1.15	0.126	0.140	0.142	0.392	0.748	0.528
AANNNN	0.221	1.21	0.120	0.149	0.162	0.407	0.759	0.526
AAPPPP	0.228	1.13	0.107	0.166	0.147	0.401	0.769	0.550
ANNNNN	0.237	1.30	0.129	0.172	0.162	0.413	0.792	0.530
APPPPP	0.238	1.13	0.106	0.157	0.153	0.443	0.782	0.565
NNNNNN	0.225	1.29	0.093	0.159	0.155	0.444	0.789	0.490
PPPPPP	0.230	1.09	0.114	0.161	0.115	0.379	0.788	0.547
SEM	0.0105	0.0668	0.0150	0.0109	0.0180	0.0285	0.0382	0.0291
P-Value	0.543	0.103	0.751	0.427	0.846	0.838	0.977	0.905

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35; fifth d 36-47; sixth d 48-55

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

Table 4.9 Histology Measurements for Duodenum (mm) on D 27

Additives	Villus Length	Crypt Depth	Muscle Thickness	Villus length/Crypt Depth ratio
AAA	2.18	0.226^{b}	0.189^{bc}	9.79
ANN	2.32	0.317^{a}	0.232^{a}	7.50
APP	2.33	0.249^{b}	0.207^{b}	9.64
NNN	2.25	0.247^{b}	0.181^{c}	9.20
PPP	2.15	0.227^{b}	0.196^{bc}	9.60
SEM	0.069	0.0121	0.0079	0.612
P-Value	0.246	0.001	0.004	0.172

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28;
A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)
N = Negative control basal diet
P = Probiotic *Bacillus subtilis* diet
$^{a-c}$ Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 4.10 Intestinal Lesions D 20 (%)

Additive	Duodenum				Jejunum				Ceca		
	Lesion Score 0	Lesion Score 1	Lesion Score 2	Lesion Score 3	Lesion Score 0	Lesion Score 1	Lesion Score 2	Lesion Score 0	Lesion Score 1	Lesion Score 2	Lesion Score 3
AA	50.0	50.0	0.0	0.0	66.7[a]	33.3[b]	0.0	50.0	33.3	0.0	16.7
AN	0.0	0.0	85.7	14.3	57.1[a]	28.6[b]	14.3	57.1	28.6	0.0	14.3
AP	0.0	28.6	71.4	0.0	0.0[b]	85.7[a]	14.3	42.9	28.6	0.0	28.6
NN	0.0	25.0	75.0	0.0	25.0[ab]	50.0[ab]	25.0	50.0	25.0	25.0	0.0
PP	16.7	33.3	50.0	0.0	16.7[b]	83.3[a]	0.0	50.0	16.7	16.7	16.7
SEM	13.94	20.72	19.51	7.990	14.08	14.41	13.01	22.73	19.43	11.31	11.07
P-Value	0.093	0.613	0.056	0.494	0.023	0.028	0.548	0.965	0.985	0.601	0.293

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21.

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

[a-b] Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 4.11 Intestinal Lesions D 27 (%)

Additive	Duodenum				Jejunum			Ileum		Ceca		
	Lesion Score 0	Lesion Score 1	Lesion Score 2	Lesion Score 3	Lesion Score 0	Lesion Score 1	Lesion Score 2	Lesion Score 0	Lesion Score 1	Lesion Score 0	Lesion Score 1	Lesion Score 3
AAA	12.5[b]	37.5	50.0	0.0	37.5	50.0	12.5	75.0	25.0	87.5	12.5	0.0
AAN	37.5[ab]	12.5	37.5	12.5	37.5	37.5	25.0	87.5	12.5	87.5	0.0	12.5
AAP	14.3[b]	42.9	42.9	0.0	57.1	14.3	28.6	100	0.0	85.7	14.3	0.0
ANN	40.0[ab]	40.0	20.0	0.0	60.0	20.0	20.0	80	20.0	100	0.0	0.0
APP	25.0[b]	37.5	25.0	12.5	25.0	37.5	37.5	87.5	12.5	100	0.0	0.0
NNN	0.0[b]	50.0	50.0	0.0	50.0	50.0	0.0	87.5	12.5	100	0.0	0.0
PPP	75.0[a]	25.0	0.0	0.0	75.0	25.0	0.0	100	0.0	100	0.0	0.0
SEM	14.92	18.20	17.52	7.426	13.47	15.62	13.95	11.13	11.13	9.164	7.337	5.196
P-Value	0.023	0.779	0.372	0.594	0.160	0.689	0.361	0.553	0.553	0.774	0.615	0.488

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic Bacillus subtilis diet

a-b Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 4.12 Intestinal Lesions D 34 (%)

Additive	Duodenum			Jejunum			Ileum			Ceca		
	Lesion Score 0	Lesion Score 1	Lesion Score 2	Lesion Score 0	Lesion Score 1	Lesion Score 2	Lesion Score 0	Lesion Score 1	Lesion Score 2	Lesion Score 0	Lesion Score 1	Lesion Score 2
AAAA	100	0.0	0.0	50.0	33.3	16.7^{bc}	83.3	0.0	16.7	100	0.0	0.0
AAAN	100	0.0	0.0	33.3	50.0	16.7^{bc}	83.3	16.7	0.0	66.7	33.3	0.0
AAAP	100	0.0	0.0	60.0	40.0	0.0^{c}	100	0.0	0.0	80.0	20.0	0.0
AANN	75.0	25.0	0.0	25.0	0.0	75.0^{a}	75.0	25.0	0.0	75.0	25.0	0.0
AAPP	100	0.0	0.0	42.9	57.1	0.0^{c}	71.4	28.6	0.0	100	0.0	0.0
ANNN	71.4	28.6	0.0	42.9	42.9	14.3^{bc}	42.9	57.1	0.0	85.7	14.3	0.0
APPP	66.7	33.3	0.0	33.3	16.7	50.0^{ab}	66.7	33.3	0.0	83.3	16.7	0.0
NNNN	66.7	16.7	16.7	33.3	33.3	33.3^{abc}	33.3	50.0	16.7	66.7	33.3	0.0
PPPP	100	0.0	0.0	25.0	75.0	0.0^{c}	100	0.0	0.0	75.0	12.5	12.5
SEM	13.77	12.63	5.589	20.42	19.40	15.18	17.53	16.40	7.75	16.75	16.10	5.581
P-Value	0.268	0.260	0.445	0.907	0.435	0.036	0.065	0.066	0.459	0.690	0.647	0.651

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

[a-c] Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 4.13 Foot Pad Lesions D 50 (%)

Additive	Zero	One	Two	Three	Two and Three
AAAAAN	58.8^{bc}	19.3	20.5	1.39^{c}	21.9^{bcd}
AAAAAP	82.6^{a}	10.8	5.31	1.25^{c}	6.56^{d}
AAAANN	47.8^{bcd}	24.1	26.8	1.25^{c}	28.1^{abc}
AAAAPP	65.4^{ab}	12.0	19.1	3.52^{bc}	22.6^{bcd}
AAANNN	69.9^{ab}	19.8	7.83	2.53^{bc}	10.4^{cd}
AAAPPP	55.6^{bc}	15.7	28.1	0.501^{c}	28.6^{abc}
AANNNN	53.6^{bcd}	14.6	23.8	7.98^{abc}	31.8^{ab}
AAPPPP	53.6^{bc}	25.4	18.7	2.27^{c}	21.0^{bcd}
ANNNNN	59.6^{bc}	9.7	20.2	10.5^{ab}	30.6^{ab}
APPPPP	38.4^{cd}	27.4	28.3	5.86^{abc}	34.1^{ab}
NNNNNN	52.4^{bcd}	11.4	23.6	12.64^{a}	36.2^{ab}
PPPPPP	31.4^{d}	23.6	34.6	10.37^{ab}	45.0^{a}
SEM	8.000	4.950	6.370	2.884	7.177
P-Value	0.003	0.105	0.081	0.021	0.018

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35; fifth d 36-47; sixth d 48-55
A = Antimicrobial diet (Antibiotic for d 0-14; Antibiotic and Anticoccidial for d 14-56)
N = Negative Control basal diet
P = Probiotic *Bacillus subtilis* diet
Zero = no lesions
One = 0.1-5 mm lesion
Two = 6-14.9 mm lesion
Three = lesion ≥ 15 mm
[a-d] Means in a column not sharing a common superscript are different ($P \leq 0.05$).

Table 4.14 Foot Pad Lesions after Scalding on D 56 (%)

Additive	Zero	One	Two	With Lesions
AAAAAN	50.0	45.0	5.0	50.0
AAAAAP	60.0	40.0	0.0	40.0
AAAANN	27.5	62.5	10.0	72.5
AAAAPP	38.6	48.6	12.8	61.4
AAANNN	45.0	55.0	0.0	55.0
AAAPPP	45.0	50.0	5.0	55.0
AANNNN	37.5	50.0	12.5	62.5
AAPPPP	40.0	45.0	15.0	60.0
ANNNNN	55.0	35.0	10.0	45.0
APPPPP	45.0	42.5	12.5	55.0
NNNNNN	40.0	40	20.0	60.0
PPPPPP	26.3	66.0	7.70	73.7
SEM	7.757	7.928	4.497	7.757
P-Value	0.091	0.200	0.055	0.091

Each letter represents the diet fed at each feeding phase. The first letter represents the diet fed from d 0-14; second d 15-21; third d 22-28; fourth d 29-35; fifth d 36-47; sixth d 48-55

A = Antimicrobial diet (antibiotic for d 0-14; antibiotic and anticoccidial for d 14-56)

N = Negative control basal diet

P = Probiotic *Bacillus subtilis* diet

Zero = no lesions

One = 0.1-5 mm lesion

Two = 6-14.9 mm lesion

[a-b] Means in a column not sharing a common superscript are different ($P \leq 0.05$).

References

Baldwin, S., Hughes, R.J., Van T. T. H., and Moore, D. S. 2018. At-Hatch Administration of Probiotic to Chicken can Introduce Beneficial Changes in Gut Microbiota. Public Library of Science ONE. 13(3):1-14.

Bermudez-Brito, M., Plaza-Díaz, J., Muñoz-Quezada, S., Gómez-Llorente, C., and Gil, A. 2012. Probiotic Mechanisms of Action. Annals of Nutrition and Metabolism. Annals of Nutrition and Metabolism. 61(2):160-174.

Conway, D. P. and McKenzie, M. E. 2007. Poultry Coccidiosis: Diagnostic and Testing Procedures. Ames, Iowa: Blackwell Pub.

Dalloul, R. A., Lillehoj, H. S., Tamim, N. M., Shellem, T. A., and Doerr, J. A. 2005. Induction of Local Protective Immunity to Eimeria acervulina by a Lactobacillus-based Probiotic. Comparative Immunology, Microbiology and Infectious Diseases. 28:351-361.

Dalloul, R. A., and Lillehoj, H. S. 2006. Poultry Coccidiosis: Recent Advancements in Control Measures and Vaccine Development. Expert Rev Vaccines. 5:143–163.

Fuller, R. 1992. History and Development of Probiotics. In: Fuller, R. Probiotics – The Scientific Basis. Chapman and Hall, London.

Giannenas, I., Papadopoulos, E., Tsalie, E., Triantafillou, E., Henikl, S., Teichmann, K., and Tontis, D. 2012. Assessment of Dietary Supplementation with Probiotics on Performance, Intestinal Morphology and Microflora of Chickens Infected with Eimeria tenella. Veterinary Parasitology. 188(1-2):31-40.

Gao, P., Ma, C., Sun, Z., Wang, L., Huang, S., Su, X., Xu, J., and Zhang, H. 2017. Feed-additive Probiotics Accelerate yet Antibiotics Delay Intestinal Microbiota Maturation in Broiler Chicken. Microbiome. 5(1):91-104.

Jayaraman, S., Thangavel, G., Kurian, H., Mani, R., Mukkalil, R., and Chirakkal, H. 2013. *Bacillus subtilis* PB6 Improves Intestinal Health of Broiler Chickens Challenged with *Clostridium perfringens*-induced Necrotic Enteritis. Poultry Science. 92(2):370-374.

Johnson, J., and Reid, M. 1970. Anticoccidial Drugs: Lesion Scoring Techniques in Battery and Floor-Pen Experiments with Chickens. Experimental Parasitology. 28(1):30-36.

Jorden, F. W. and Pattison, M. 1996. Poultry Diseases Edited by F. T. W. Jorden, M. Pattison. London; Philadelphia: Saunders, Agricola.

Kabir S. M. 2009. The Role of Probiotics in the Poultry Industry. International Journal of Molecular Sciences. 10(8):3531-3546.

Lee, K. W., Hyun, S., Lillehoj, H. S., Janga, S. I., Li, G., Lee, S.H., Lillehoj, E. P., and Siragusac, G. R. 2010. Effect of *Bacillus*-based direct-fed Microbials on *Eimeria* Infection in Broiler Chickens. Comparative Immunology, Microbiology and Infectious disease. 33(6):105-110.

Miles, R. D., Butcher, G. D., Henry, P. R., Littell, R. C. 2006. Effect of Antibiotic Growth Promoters on Broiler Performance, Intestinal Growth Parameters, and Quantitative Morphology. Poultry Science. 85(3):476-485

Patterson, J. A., and Burkholder K. M. 2003. Application of Prebiotics and Probiotics in the Poultry Production. Poultry Science. 82(4):627-631.

Ritzi, M. M., Abdelrahman, W., Mohnl, M., and Dalloul, R. A. 2014. Effects of Probiotics and Application Methods on Performance and Response of Broiler Chickens to an *Eimeria* Challenge. Poultry Science. 93(11):2772-2780.

Ritzi, M. M, Abdelrahman, W., Van-Heerden K., Mohnl, M., Barrett, N. W. and. Dalloul, R. A. 2016. Combination of Probiotics and Coccidiosis Vaccine Enhances Protection against an *Eimeria* Challenge. Veterinary Research. 47(1):111-118.

SAS Institute. 2012. SAS Proprietary software 9.4 SAS inst. Inc., Cary, NC, USA.

Shepherd, E. M., and Fairchild, B. D. 2010. Footpad Dermatitis in Poultry. Poultry Science. 89(10):2043-2051.

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., Teillant, A., and Laxminarayan, R. 2015. Global Trends in Antimicrobial Use in Food Animals. Proceedings of the National Academy of Sciences of the United States of America. 112(18):5649-5654.

Xia, M. S., Hu, C. H., Xu, Z. R. 2004. Effects of copper-bearing montmorillonite on growth performance, digestive enzyme activities, and intestine microflora and morphology of male broilers. Poultry Science. 83(11):1868–1875.

Yason, C. V., Summers, B. A., and Schat, K. A. 1987. Pathogenesis of Rotavirus Infection in Various Age Groups of Chickens and Turkeys: Pathology. American Journal of Veterinarian Research. 48(6):927-938.

CHAPTER V

CONCLUSION

With all the concerns on antibiotic resistance and antibiotic residue in poultry meat consumers have pressured the poultry industry to look for antibiotic alternatives such as the probiotic *B. subtilis*. In the current study, the removal of antimicrobials with probiotic supplementation resulted in promising results to be able to reduce the amount of antibiotics used in the broiler diet to d 28 or even d 21. If antimicrobials were to be removed on d 28, based on our study the birds would not be able to recover compared to birds fed antimicrobials until d 46 unless *B. subtilis* was supplemented on day 28. Removing antibiotics on d 21 would be possible due to our study showing the birds had similar body weights compared to birds fed antimicrobials until withdrawal by day 56, however, it took longer for the birds to recover from antimicrobial removal compared to supplementing *B. subtilis*. Feed conversion was affected at different growth periods, however, overall feed conversion was not affected. Processing yields also showed that supplementing *B. subtilis* into broiler diets at antimicrobial expense at d 28 would result in similar yields compared to birds fed antimicrobials until d 46. The birds that had antimicrobials removed on d 21 and supplemented with *B. subtilis* had lower lesion scores in the jejunum compared to birds that had antimicrobials removed on d 21. This study gives the industry an idea of what days antimicrobials can be removed without

compromising growth performance and overall health of the birds under coccidial challenge.