

The use of *Bacillus subtilis* as a direct-fed microbial and its effects on production and colonization of *Salmonella* Enteritidis and *Clostridium perfringens* in production broilers

by

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A Thesis Submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
December 12, 2015

Keywords: direct-fed microbials, *Bacillus subtilis*, *Salmonella*,
Clostridium perfringens, antibiotic resistance

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Abstract

The growing emergence of antimicrobial resistant bacteria has given rise to consumer fear that the use antibiotics in the meat industry can lead to more antimicrobial resistant strains. Because of this fear, the poultry industry is moving away from using antibiotics and antibiotic growth promoters (APG) in production birds. However, removing antibiotic therapy in treating disease can lead to an increase in pathogenic bacteria. Alternative methods to antibiotic therapy are currently being explored.

One alternative method being research is the use of direct-fed microbials (DFM), also known as probiotics. Probiotics have been in use for several decades but their exact modes of action have yet to be identified. DFMs in the diet of production birds are thought to be production enhancers as they are capable of promoting beneficial bacteria within the microflora. The use of probiotics in commercial broilers has been shown to provide an intestinal ecosystem that benefits the bird by inhibiting the growth and colonization of pathogenic bacteria, such as *Salmonella* and *Clostridium perfringens*. It has been suggested that the use *Bacillus subtilis*, a common soil bacterium, could improve growth performance as well as improve feed conversions in broilers. One important characteristic of *Bacillus subtilis* that makes it a viable prospect as a DFM is the ability to produce endospores. These endospores

are capable of withstanding inhospitable environments meaning *Bacillus subtilis* endospores are capable of surviving the feed-milling procedure.

The objective of this study was to determine the viability of seven isolates of soil-origin *Bacillus subtilis* as a direct-fed microbial in production broilers and determine their ability to inhibit the growth of the pathogenic bacteria *Salmonella* Enteritidis and *Clostridium perfringens*. The isolates used were designated AP71, AB01, AP302, AP183, AP185, AP206 and AP294. Isolates AP71 and AB01 were found to be inconclusive in inhibiting the colonization of *Salmonella* Enteritidis in male broilers. A screening trial provided insight into the ability of the seven isolates of *Bacillus subtilis* in inhibiting the growth of *Clostridium perfringens*. AP302, AB01, AP206 and AP183 were found to be able to significantly inhibit the growth of *Clostridium perfringens*. These isolates were utilized in a bird trial and were found to not be able to significantly increase the performance of the birds or significantly inhibit the growth of *Clostridium perfringens*. More research should be done in order to determine the efficacy of these isolates as a DFM.

Acknowledgments

I would like to thank all of those who have helped me thus far in the pursuit of my academic goals. First and foremost I want to thank my family for all of their encouragement, prayers and guidance. Without them I would not be where I am today. I would especially like to thank my mother and father for their support throughout my academic career. Secondly, thank you Dr. Macklin granting me the opportunity to work in your lab and giving me the chance to obtain my academic dreams. Your guidance and mentoring have been highly beneficial to success. Lastly I want to thank everyone in the lab that helped me in any way. James Krehling, thank you for all you have done for me and for putting up with me on my worst days. A special thank you to Amnuay Segrest for being the workhorse of the lab and providing me countless hours of technical lab work. To Laci Mackay, Katrina English and Elle Chadwick, thank you for being there to help me through the long days of lab and field work and for being my friends. Thank you to the undergraduate workers Victoria Holland, Brad Barretto and Kayleen McCafferty for all of the hard labor and hours you all put in. Lastly but certainly not the least, a huge thank you to the farm workers at the Auburn poultry unit. I cannot thank you enough for the hard work on any project I was a part of. Thank you all and War Eagle!

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Chapter 1:

Introduction

With the growing concern about the emergence of antimicrobial resistance, the use of antibiotic therapy is being waned. Consumer pressure coupled with the fact that certain bacteria have adopted the ability to be impermeable to antibiotic treatment, the poultry industry has begun researching alternative ways to treat bacterial infection. One such way is the use of direct-fed microbials (DFM)—probiotics. Probiotics are live bacteria that are given to a host to enhance the gastrointestinal health and microflora within the gut. Direct-fed microbials are currently being used within the poultry industry and are considered production enhancers. They are given to broiler flocks to enhance broiler growth performance as well as to convey beneficial properties to the microflora environment within the gut. Moreover, direct-fed microbials are thought to be able to inhibit the growth of pathogenic bacteria.

Bacillus subtilis, a common Gram-positive soil bacterium, has been shown to be a possible candidate as a direct-fed microbial. The major characteristic of *Bacillus subtilis* that makes it a viable candidate for being a direct-fed microbial is its ability to produce endospores. Most known probiotics are not capable of producing these endospores. *Bacillus subtilis* has been shown to produce spores capable of withstanding high temperatures when being processed in the feed-milling procedure as its spores are metabolically dormant and are highly resistant to extreme pH levels and extreme temperatures (Nicholson, 2002).

Several studies have shown the ability of *Bacillus subtilis* to be able to reduce the colonization of certain important pathogenic bacteria to the poultry industry including *Salmonella enterica* and *Clostridium perfringens* (La Ragione & Woodward, 2003; Baltzley *et al.*, 2010; Knap *et al.*, 2011; Allaart *et al.*, 2013; Barbosa *et al.*, 2005; Klose *et al.*, 2010; Teo *et al.*, 2005). The exact mode of action that allows for the spores of *Bacillus subtilis* to proliferate and induce health beneficial properties is unknown. However, studies have been done to try and solve this mystery; most studies indicate that *Bacillus subtilis* has the ability to enhance the already present microflora environment (Hoa *et al.*, 2000; Jeong and Kim, 2014; Wu *et al.*, 2011). Other studies have indicated that there may be a more indirect route for the improved gut health associated with direct-fed microbial usage. These studies show that the spores of *Bacillus subtilis* are capable of initiating an innate immune response, providing the host with the ability to fight bacterial infection (Rhee *et al.*, 2004; Latorre *et al.*, 2014). These studies have shown various degrees of success and further research is needed.

The purpose of the current research presented was to determine the ability of seven strains of soil-based *Bacillus subtilis* to enhance the production of broiler flocks as well as to determine the ability of the *Bacillus* strains to inhibit the growth of the pathogenic bacteria *Salmonella enterica* and *Clostridium perfringens*. The goal of this study was to determine the viability of *Bacillus subtilis* isolates AB01, AP71, AP206, AP294, AP302, AP183 and AP185 of being direct-fed microbials and their efficacy to replace traditional antibiotic therapy.

Chapter 2:

Literature Review

Introduction

The poultry industry is growing at a high rate world wide as well as in the United States. In 2010, it was approximated that the value of the U.S. broiler industry had a net worth of \$45 billion. Meat production from the broiler industry totaled at 36.9 billion pounds in 2010. Not only is the broiler industry valuable in the U.S. market, the export of broiler meat brought in \$3.1 billion in 2010 of revenue (USDA Economic Research Service, 2012).

With the growth of the broiler industry and the poultry industry as a whole, there is an inevitable growth of food related diseases associated with bacteria found within the animals. For the poultry industry, two of the more economically important bacterial pathogens are *Salmonella* and *Clostridium perfringens*. Human illness from foodborne related pathogens is a major concern for the poultry industry; in 2010, 11% of all foodborne related illnesses were due to poultry and poultry products (Centers for Disease Control and Prevention, 2013). *Salmonella enterica* was responsible for 30% of all confirmed, single-etiological outbreaks in the United States in 2010. *Salmonella* was also responsible for 583 outbreak-related hospitalizations in the same year—that's nearly half (49%) of all outbreak-related hospital cases. *Clostridium perfringens* is a lesser pathogen to humans but still accounted for 11% of foodborne related illness (Centers for Disease Control and Prevention, 2013). The illnesses associated with *Clostridium perfringens* are more detrimental to chickens as they cause severe diseases such as necrotic enteritis and

gangrenous dermatitis. Necrotic enteritis is estimated to cost the poultry industry nearly \$2 billion annually due to mortality and morbidity associated with the disease (Martin & Smyth, 2009). Moreover, the lesions caused by necrotic enteritis and gangrenous dermatitis can cause condemnations at slaughter costing the industry more money for disposal as well as the economic loss at the market.

The economic impact is also observed through the treatment and prevention of these diseases in the animals. The primary technique of treatment is the use of antibiotics or in-feed antibiotics; however, with the use of antibiotics comes the rise of antimicrobial resistance (Knap *et al.*, 2011). Unfortunately, without the use of antibiotics pathogenic bacteria are not kept under control. Because of the fear of antimicrobial resistant strains of bacteria such as *Salmonella* entering the food supply, many countries have banned the use of antibiotic growth promoters (AGP). By doing so, the prevalence of *Salmonella* in commercial poultry flocks has risen (EFSA, 2007; Knap *et al.* 2011). Methods such as vaccination are in use but have many issues that are less than desirable for the industry—the main issue being consumer pressure on the industry. New techniques for pathogenic microbial control are currently being explored including the use of prebiotics and probiotics.

One such technique is the use of direct-fed microbial diets, which are diets that contain probiotics. Probiotics have been in use for several decades but the exact mechanisms as to how they work are not yet established for all cases. However, it has been shown that the use of certain probiotic bacteria, such as *Bacillus subtilis*, can reduce the colonization and prevalence of pathogenic bacteria in the gastrointestinal tract of the host animal. The poultry industry is currently

researching the possibility of using probiotic-based diets to combat the colonization of pathogenic bacteria in broilers.

Probiotics

Probiotics are live bacteria or yeast that are believed to improve the health of the gastrointestinal tract. Ilya Mechnikov is regarded as the father of modern probiotics (Fuller, 1992). Mechnikov's research was based on the observations of a Bulgarian microbiologist—Stamen Grigorov—in which Grigorov documented the health benefits of yogurt as well as identified *Lactobacillus bulagricus* as the active organism responsible for these benefits (Hume, 2011). The term “probiotika” is accredited to Werner Kollath who defined the term as “active substances that are essential for a healthy development of life” (Guarner *et al.*, 2005). Lilly and Stillwell (1965) first used the word probiotic to describe “a substance released by a particular microorganism that benefits the growth of another” (Fuller *et al.*, 1992). In 1974, Parker used the term probiotic to describe “organisms or substances that can contribute to the balance of the intestinal microbiota”. As of today, the most recent definition, which is ever-changing as the knowledge on the subject continues to grow, is credited to Reid *et al.* (2003) who defined probiotics as “live microorganisms which when administered in the proper amount can benefit the health of the host recipient”. The USDA in 1989 however, provided that manufacturers use the term “direct-fed microbials” (DFM) when describing products that included any source of living, naturally occurring microorganism (Hume, 2011).

Probiotics in Poultry

Probiotics in poultry are being used as an alternative to antibiotic growth promoters and in-feed antibiotics (Chen *et al.*, 2013). Viewed as production enhancers, probiotics are currently being used to promote health beneficial properties in the intestinal microflora to promote broiler performance as well as to protect the host from colonization by pathogenic bacteria (Hume, 2011). The utilization of probiotics is accredited to several desirable characteristics including the organism being non-pathogenic; the ability of the organism to adhere to the intestinal epithelial cells; their ability to colonize and reproduce and survive the acidity of stomach acid and bile; the capability of being host specific; and their ability to produce certain chemicals that inhibit or kill potentially pathogenic bacteria (Smith, 2014). It has also been established that probiotics benefit the host animal by enhancing important vitamin synthesis, providing digestive enzymes, and increasing nutrient uptake in the GI tract (Fuller 2001).

The use of probiotics in commercial broilers has been shown to provide a beneficial intestinal ecosystem by inhibiting the colonization of pathogenic bacteria as well as reinforcing the natural microbiota (Chen *et al.*, 2013; Fuller, 2001; Higgins *et al.*, 2008). Moreover, a probiotic infused diet has also been demonstrated to enhance the concentration of lymphocytes as well as increase the relative weight of the spleen and bursa to body weight; therefore, because the spleen and bursa are secondary and primary lymphoid organs in broilers, it could be concluded that the inclusion of a probiotic diet could enhance the broiler immune system (Chen *et al.*, 2013; Teo & Tan, 2007; Willis *et al.*, 2007).

Bacillus subtilis

Bacillus subtilis is a common soil bacterium that is characterized as being a Gram-positive, obligate aerobe rod capable of endospore formation (Bergey's Manual 9th edition, 1994). An important characteristic of *Bacillus subtilis* that makes it a prospect as a probiotic is its ability to produce endospores. Certain bacteria produce endospores when environmental phenomena such as high temperature or humidity decrease the nutrients needed for growth allowing the organism to go dormant until conditions are viable for growth. The spores will then go into a vegetative phase of growth and begin proliferating. Most known probiotics are not capable of producing these endospores. *Bacillus subtilis* has been shown to produce spores capable of withstanding high temperatures when being processed in the feed-milling procedure as its spores are metabolically dormant and are highly resistant to extreme pH levels and extreme temperatures (Nicholson, 2002).

It has been suggested that the use of *Bacillus subtilis* as a probiotic in poultry could increase the growth performance and improve feed conversion ratio in broilers by increasing the secretion of protease, amylase and lipase (Anjum *et al.*, 2005; Chen *et al.*, 2013). However, the exact mode of action that enables the organism to provide such an environment within the intestinal tract of broilers is still unknown. For one strain of *Bacillus subtilis*, designated C-3102, it has been suggested that the spores produced by the bacteria can favor the environment of *Lactobacilli* and other lactic acid producing bacteria by creating a positive anaerobic environment due to the rapid use of oxygen that occurs through the vegetation of the spores (Hoa *et al.*, 2000; Jeong and Kim, 2014). Other research seems to validate

the notion that *Lactobacilli* are enhanced in the presence of *Bacillus subtilis*. Wu *et al.* (2011) showed that a *Bacillus subtilis* strain designated KD1, which was isolated from intestinal microflora of healthy broilers, was capable of increasing intestinal *Lactobacilli* while subsequently reducing the colonization of *Escherichia coli*. Knarreborg *et al.* (2008) also suggested that the intestinal microbiota is enhanced with the inclusion of a *Bacillus subtilis* direct fed diet as the probiotic based diet favors the growth of lactic acid bacteria, which incurs health-beneficial properties.

Several studies have indicated a more indirect route for promoting beneficial properties to the bird. It has been indicated that *Bacillus* spores are involved in the rapid activation of innate immune cells (Rhee *et al.*, 2004; Latorre *et al.*, 2014). It has also been demonstrated that the spores of several *Bacillus* species are capable of producing exogenous enzymes, which could help the digestion of complex feed particles, diminish intestinal viscosity in non-starch polysaccharide diets and decrease the amount of substrates available for growth and proliferation of pathogenic bacteria (Latorre *et al.*, 2014).

There are certain pitfalls that must be addressed when considering the use of direct-fed microbial diets. Nurmi and Rantala (1973) presented the “gold standard” probiotics as being an undefined cecal or fecal preparation. Unfortunately, the potential for the introduction of a number of pathogenic organisms is too great for undefined cultures in poultry and poultry food products; moreover, undefined probiotic cultures would be unreliable batch to batch as materials obtained for each new batch would come from a different source each time a new inoculum was prepared (Hume 2011). Despite a few of the downfalls of probiotic use, there is

more potential upside to using them in commercial poultry for the maintenance of broiler health.

Salmonella enterica

Salmonella enterica is an important Gram-negative, enteric pathogen for both human health and the poultry industry. Theobald Smith and Daniel E. Salmon are credited with discovering *Salmonella enterica* during the early 1880's (Schultz, 2008). Formerly known as *Salmonella choleraesuis*, *Salmonella enterica* was mistaken as hog cholera at first by Smith and Salmon (Schultz, 2008).

Commercial poultry production is expanding at a rapid pace in the animal agriculture industry. In the United States, 9 billion broilers are raised and processed annually while more than 77 billion eggs were produced and processed in 2009 alone (Foley *et al.* 2011). With this increase in industry growth, an unwarranted side effect is the increase in some pathogenic bacteria such as *Salmonella*. There are several factors that can contribute to the colonization of *Salmonella* in poultry. Some of these factors include bird age, genetic susceptibility, stress level, infection dose of *Salmonella* as well as the serotype of *Salmonella*, host gut microbiota and host immunity (Bailey, 1988; Foley *et al.* 2011). Bailey (1988) explains that researchers Milner & Shaffer in 1952 concluded that day-old chicks were more susceptible to *Salmonella* infections at relatively low doses.

The species of *Salmonella enterica* is quite diverse. However, there are four serovars that have a primary host-species adaptation for chickens. These serovars are *Salmonella* Enteritidis (SE), *Salmonella* Heidelberg, *Salmonella* Kentucky, *Salmonella* Newport and *Salmonella* Typhimurium (Foley *et al.*, 2013; Foley *et al.*,

2011). *Salmonella* Typhimurium was formerly the serovar most associated with human illness; however, recent evidence from the CDC and USDA confirm that SE has become the leading cause of Salmonellosis in humans (Centers for Disease Control and Prevention, 2011). Furthermore, data from the USDA's National Veterinary Services Laboratory was compared to that of other studies for the occurrence of *Salmonella* serovars and the results showed that SE was the most commonly associated with chickens and eggs (Centers for Disease Control and Prevention, 2011).

Salmonellosis—whose causative agent is *Salmonella enterica*—is a severe public health concern worldwide as well as in the United States. There are an estimated 1.028 million cases of Salmonellosis in the United States annually with 19,000 cases resulting hospitalizations and approximately 400 deaths (Scallen *et al.*, 2011). Salmonellosis has an economic impact as it costs the United States approximately \$3.1 billion due to medical related expenditures; in addition, *Salmonella enterica* contaminated food recalls and poultry flock decontamination practices increase the financial impact (Foley *et al.*, 2013; Kassem *et al.*, 2012). Although *Salmonella* have adapted the ability to survive in a wide range of hosts, human cases of Salmonellosis are more often than not associated with consuming contaminated foods such as processed meat and eggs (Foley *et al.*, 2011).

Poultry associated products are widely acknowledged as one of the most important vectors of *Salmonella* transmission to humans. *Salmonella* have the capability of not only acting as pathogens but can also survive in animals and poultry as transient bacteria in the gastrointestinal (GI) tract without causing

disease, as well as not negatively affecting body weight gains or production; therefore, there is greater likelihood of transmission to humans (Carter *et al.*, 2009). As the consumption of poultry products has increased six and half fold since 1910, the potential risk of human exposure to *Salmonella* through contaminated poultry products has also risen (Foley *et al.*, 2011).

Management and Treatment of Salmonella

Control of *Salmonella* is a priority within the poultry industry due to the foodsafety concerns surrounding the bacteria. There have been several efforts made to limit the spread of the bacteria and the contamination that follows. Several of these practices include rearing and management techniques, antimicrobial use, vaccination and the use of prebiotics and probiotics. Data from both nonconventional poultry farms (i.e., free-range, organic) and conventional production farms indicates that the prevalence of *Salmonella* is dependent upon the individual farm and not the type of farming system (Bailey & Cosby, 2005; Cui *et al.*, 2005; Van Overbeke *et al.*, 2006). It was shown that the *Salmonella* serovars that dominated contamination of conventional farms also dominated the nonconventional farming systems regardless of the overall level of *Salmonella* contamination (Lestari *et al.*, 2009; Melendez *et al.*, 2010; Van Overbeke *et al.*, 2006; Foley *et al.*, 2011). While the use of good farm practices such as biosecurity, litter treatment and sanitation techniques, can reduce the overall number of *Salmonella* in the flock, these practices cannot reduce individual serotypes regardless of the farming system.

The use of antibiotics and antibiotic growth promoters (AGP) has been shown to reduce the colonization of *Salmonella* (Knap *et al.*, 2011; Dibner & Richards, 2005; Barza, 2002; Bywater, 2005); however, there is a growing concern for the continued use of AGP's. Because of the usage of antibiotics, there is a rising unease for the increase of antimicrobial resistant bacteria including that of *Salmonella*. The main concerns are concentrated on resistant bacteria associated with food products; the recognition of a multidrug-resistant *Salmonella* has initiated wide spread distress regarding the safety of the food supply directly from the resistant *Salmonella* strains as well as indirectly through genetic exchange of antimicrobial genes between intestinal bacteria (Callaway *et al.*, 2008).

Vaccination use within the poultry industry is currently increasing (Gast, 2007; Vandeplass *et al.*, 2010; Foley *et al.*, 2011). As of now, Currently, a live-attenuated vaccine and an inactive vaccine are available for the poultry industry (breeder and layer flocks). The targets of most *Salmonella* vaccines are serovars *Salmonella* Enteritidis and *Salmonella* Typhimurium (Foley *et al.*, 2011). Unfortunately, the effectiveness of the vaccines is dependent on certain variables that include the targeted serovar, host species, and whether the intention of the vaccine is reduction or eradication (Knap *et al.*, 2011; Doyle & Erickson, 2006). Total protection and cross-protection against all-serogroups is not provided with either type of vaccine currently available (Chambers & Lu, 2002; Gast, 2007). There has been evidence that inactivated vaccines have the potential to reduce fecal shedding and decrease the colonization of the intestine; however, inconveniences such as the need for individual application hinder the usage of inactivated vaccines (Freitas-

Neto *et al.*, 2008). Live attenuated vaccines are thought to better protect the birds as compared to the inactivated vaccines as live vaccines can induce both cell-mediated and humoral immune responses (Arnold *et al.*, 2014; Gantois *et al.*, 2006; Galis *et al.*, 2013). However, the effectiveness of these vaccines is again dependent on target serovar, host species and the intention of the vaccine. With the growing concerns of antimicrobial resistant *Salmonella* as well as the consumer pressure to move towards organic products, other methods of control are being explored (Jacob *et al.*, 2008).

Because the prevalence of *Salmonella* extends from the poultry farm all the way to the processing unit, it is thought that reducing the contamination levels before slaughter could produce effective control techniques (Kassem *et al.*, 2012). One way of doing this is the use of in-feed additives. The use of feed additives is another alternative to antibiotic therapy being researched. These include probiotics and prebiotics. As mentioned above, probiotics are live cultures of bacteria considered beneficial to the host organism that are given to the host through feed or water. Prebiotics are defined as indigestible substrates that are capable of selecting for a specific population of bacteria (Grizard & Barthom, 1999). Prebiotic usage has seen variable and limited degrees of success for many different reasons (Rehman *et al.*, 2009). Feed additives such as sodium bisulfate (NaHSO₄), an acid salt, have not showed promising results in the inhibition of *Salmonella* (Kassem *et al.*, 2012). This could be due to additives such as sodium bisulfate not being specific to *Salmonella* and inhibiting other bacteria that would normally compete with *Salmonella* for colonization space within the host bird (Kassem *et al.*, 2012).

Probiotic usage has also shown various degrees of success. It has been revealed that *Bacillus subtilis* and its spores could be an effective competitive agent against several potential pathogenic organisms (La Ragione & Woodward, 2003). Studies have revealed that there is a reduction in *Salmonella* fecal shedding when birds are treated with a *Bacillus*-infused direct-fed microbial diet when compared to untreated birds (La Ragione & Woodward, 2003). These findings have been backed by studies done by Baltzley *et al.* (2010) and Knap *et al.* (2011) who found a significant reduction in the presence of *Salmonella* from drag swabs from *Bacillus subtilis* treated birds. Furthermore, *Salmonella* load has been shown to be significantly reduced in the intestinal tract of *Bacillus*-treated broilers (Knap *et al.*, 2011).

Clostridium perfringens

Since the late 19th century, *Clostridium perfringens* has been identifiable as it was then shown to be the causative agent of gas gangrene in humans (Niilo, 1980). *Clostridium perfringens*—previously recognized as *Clostridium welchii*—was shown in 1935 to be the etiological agent of certain ovine enteric diseases (Bosworth and Glover, 1935). Enterotoxin (CPE)-mediated food poisoning is considered one the most common foodborne illnesses worldwide (Li and McClane, 2006; Lindström *et al.*, 2011). The disease, first described in the 1940's, is characterized by the consumption of contaminated food—such as poultry—and is diagnosed in humans by the presence of diarrhea and abdominal cramping (Bailey *et al.*, 2013).

Clostridium perfringens is an important enteric disease of both humans and animals. The genus *Clostridium* is a large and genetically diverse group with over

150 species. Of the 150, 35 species are considered pathogenic. *Clostridia* are Gram-positive, anaerobic bacilli that have the capability to form endospores. *Clostridium perfringens* species are also known for their toxin production as they produce around 18% of all known bacterial toxins (Bailey *et al*, 2013). There are five types of *Clostridium perfringens* (A, B, C, D, E) and they are classified based on the production of alpha, beta, epsilon and iota toxins (Bailey *et al*, 2013). Of the toxins produced by *Clostridium perfringens*, NetB has been demonstrated to be the major toxin produced by *Clostridium perfringens* (Keyburn *et al.*, 2008).

Necrotic enteritis is a major enteric disease of poultry caused by *Clostridium perfringens*; it is estimated that the total world cost of necrotic enteritis due to mortality and morbidity is nearly \$2 billion per year (Martin and Smyth, 2009; Opengart, 2008a). Lesions primarily in the small intestine of the bird characterize necrotic enteritis. Moreover, *Clostridium perfringens* is also the causative agent of gangrenous dermatitis, another important disease of poultry. *Clostridium perfringens* infections in humans are mostly associated with foodborne illness; there are approximately 965,000 human cases of *Clostridia* related diseases in the U.S. each year (Scallan *et al.*, 2011).

Clostridium perfringens type A is the causative agent of gangrenous dermatitis in poultry and gas gangrene in humans. Also known as myonecrosis, it is characterized by the necrosis of healthy soft tissue that was left undamaged by previous trauma; an untreated gangrenous dermatitis infection will spread from the initial point of infection and lead to shock and death (MacLennan, 1962; Bunting *et al.*, 1997). Like necrotic enteritis, lesions on the diseased bird characterize

gangrenous dermatitis; however, unlike necrotic enteritis the lesions caused by gangrenous dermatitis are found on soft tissue such as on the breast, abdomen, or wings. The occurrence of gangrenous dermatitis outbreaks usually follow viral infections such as Infectious Bursal Disease Virus (IBDV) or Chicken Infectious Anemia Virus (CIAV), both of which are immunosuppressive and reduce the host's resistance to other infectious pathogens (Ficken & Wages, 1997; Rosenberger & Cloud, 1998). Gangrenous dermatitis is mostly associated with older birds and is known to cause sudden mortality and can lead to substantial financial and economic losses. Due to the worldwide production losses, gangrenous dermatitis is considered to be a disease of great importance by the United States Animal Health Association's Committee on Transmissible Diseases of Poultry and other Avian Species (Opengart, 2008b; Li *et al.*, 2010a,b).

There are several management practices that can lead to the decrease of *Clostridium perfringens* diseases. Several of these practices coincide in the reduction of other economically and pathogenically important bacteria such as *Salmonella*. Proper animal welfare, dietary maintenance and timely identification of necrotic enteritis can be helpful in the management of the flock. Unfortunately, treatment of *Clostridium perfringens* has fallen almost predominantly to antibiotic therapy. While antibiotic treatment is effective in the reduction of the disease, the poultry industry is rapidly moving away from antibiotic treatment due to the increased numbers of antimicrobial resistant strains of bacteria. Moreover, the consumer pressure to move away from antibiotic therapy is greatly due to the consumer fear of antibiotic resistant strains of bacteria. Vaccination against *Clostridia perfringens* related

diseases has shown promise and is widely used. However, there is currently only one vaccine effective against necrotic enteritis—Netvax—that is available for commercial use in the poultry industry (Keyburn *et al.*, 2013a). Other alternative methods, such as the implication of probiotics and prebiotics in the feed are being explored for the replacement of antibiotics.

Necrotic Enteritis

Clostridium perfringens type A is the causative agent of necrotic enteritis in the chicken GI tract. The disease may be characterized as either clinical or subclinical. The occurrence of subclinical necrotic enteritis has been shown to lead to a 12% reduction in body weight while increasing the feed conversion ratio by 10.9% when compared to healthy birds (Van der Sluis, 2000; Skinner *et al.*, 2010). The hallmark of the disease is the necrosis of the small intestine, particularly the ileum and jejunum (Bailey *et al.*, 2013, Mot *et al.*, 2014). Clinical signs of infection are less than helpful in identification of the disease as necrotic enteritis is characterized by sudden onset mortality without any premonitory symptoms and require postmortem necropsies to determine if diseased birds are diagnosable with necrotic enteritis (Antonissen *et al.*, 2014). Lesions in the small intestine can vary in severity ranging from thickened mucosa and multifocal ulceration in less severe cases to more severe cases, which see birds with a greenish or yellowish pseudo-membrane in extensive mucosal inflammation as well as necrosis (Bailey *et al.*, 2013).

The causation of necrotic enteritis from *Clostridium perfringens* has been credited to the NetB toxin produced by the bacteria. The NetB toxin is released

continuously throughout the vegetative phase of the cell (Moran, 2014). The NetB gene has been recently identified to reside on a plasmid-encoded pathogenicity locus that is only found in necrotic enteritis causing strains of *Clostridium perfringens* (Engström *et al.*, 2012). Moreover, this plasmid can be transferred to other *Clostridium perfringens* species making them more pathogenic in nature and causing an increased prevalence in NetB toxicity (Uzal *et al.*, 2014; Moran, 2014).

Because *CP* is part of the normal microflora, there are predisposing factors that occur that cause the incidence of disease in the bird to increase. One of these predisposing factors includes high stocking density. Stocking density is a management practice that is defined as the number of birds or the total live weight of birds in a production house at the same time (Tsiouris *et al.*, 2015). Excess high stocking density has been shown to lead to a decrease in growth rate and an increase in downgrading at processing as well as an increase in the bird's susceptibility to disease; high stocking has also been shown to predispose the bird to necrotic enteritis. In a study done by Tsiouris *et al.* (2015), it was shown that birds in high stocked pens not challenged with *Clostridium perfringens* had similar incidences of gross lesions in the intestine and liver and lowered intestinal viscosities when compared to birds in a normal stocked pen that received a *Clostridium perfringens* challenge, indicating that high stocking had an increased predisposing affect on necrotic enteritis incidence.

Other stressful conditions that affect animal welfare can also predispose birds to proliferating numbers of *Clostridium perfringens*. One of the most important management practices in a production house is the temperature of the house. It has

been reported that long-term heat stress can lead to decreased performance and immunity as well as an increase in intestinal damage (Quinteiro-Filho et al., 2012b). Heat stress has also been linked to a decrease in intestinal immunity and an increase in impaired intestinal morphology; these factors separate or together can increase the prevalence of pathogenic bacteria (Calefi *et al.*, 2014). The effects of heat stress on the development of necrotic enteritis was evaluated by Calefi *et al.*, (2014); from the experiment it was found that experimentally induced heat-stress did not allow for the onset of necrotic enteritis from *Clostridium perfringens* type A. However, Calefi hypothesizes that under field conditions, heat stress could be a predisposing factor for the development of necrotic enteritis.

Another important predisposing factor for necrotic enteritis is the presence of mycotoxins within the feed of the production flock. One of the more common poultry feed contaminants is the mycotoxin deoxynivalenol (DON); deoxynivalenol, produced by the fungi *Fusarium graminearum* and *Fusarium culmorum*, was shown to be present in 59% of 5,819 feed samples taken worldwide (Antonissen *et al.*, 2014). While poultry is considered rather tolerant of DON, the mycotoxin has been shown to negatively affect the small intestine (Antonissen *et al.*, 2014). A study was done by Antonissen *et al.*, (2014) which demonstrated birds challenged with *Clostridium perfringens* and fed a DON-infused diet had significantly higher numbers of birds with necrotic enteritis. Therefore, while poultry are tolerant to the effects of the mycotoxin deoxynivalenol, the ability of the mycotoxin to negatively affect the GI tract of the bird, particularly the small intestine, leads to an increased predisposition for developing necrotic enteritis.

Of all the predisposing factors that lead to the proliferation of type A *Clostridium perfringens*, coccidiosis is the most important. Coccidiosis is the most important disease in poultry and is caused by the protozoal species *Eimeria*. *Eimeria* infections coincide with the increased presence of NetB strains of *Clostridium perfringens* and vice versa. The excretion of oocysts and the extent of the lesions typically seen with *Eimeria* infection were supported remarkably by a following *C. perfringens* challenge in both infection models compared with groups infected with either coccidia or *Clostridium perfringens* alone in a study done by Alnassan *et al.* (2014). The overall consensus is that parasites, such as *Eimeria*, and *Clostridium perfringens* work in tandem and show a mutualistic interaction as they enable one another to increase their numbers in the GI tract as well as enhance their abilities to cause disease (Alnassan *et al.*, 2014).

Moreover, it has been demonstrated that certain dietary events in association with *Eimeria* infections can lead to the increase in necrotic enteritis development. It has been established that feeding birds diets high in fishmeal in association with *Eimeria* infections lead to the increase in *Clostridium perfringens* multiplication and proliferation (Fernandes da Costa *et al.*, 2013; Williams *et al.*, 2003; Wu *et al.*, 2010; Wu *et al.*, 2014). It has been reported that diets containing fishmeal can increase the abundance of *Clostridium perfringens*, which could be due to the high protein and nutritional components of the fishmeal diet leading to higher proliferation of *Clostridium perfringens* and thus, a higher incidence of disease (Drew *et al.*, 2004; Kocher, 2003; Wu *et al.*, 2014).

Management and Treatment of Necrotic Enteritis

Proper and adequate management of a poultry flock is essential to prevent disease outbreak. Common sense biosecurity practices are also vital to flock health. Preventing necrotic enteritis outbreaks can be done through proper animal welfare practices as well as maintaining homeostatic conditions within the poultry house. As mentioned before, stocking density can be crucial in maintaining proper bird health in the flock. Over stocking or high stock density can lead to stressed birds resulting in disease outbreaks including necrotic enteritis (Tsiouris *et al.*, 2015). Other management practices include maintaining temperature levels in the poultry house. Heat stress has been shown as mentioned above to affect intestinal immunity and could lead to an increase in pathogenic bacteria presence including *Clostridium perfringens* (Calefi *et al.*, 2014).

Another important management practice is the use of litter in the poultry house. It is a common economic practice within the poultry industry to reuse litter from previous grow-outs to raise new broiler flocks; however, this practice could possibly be detrimental to flock health. Used litter is a complex mixture consisting of bedding materials, unconsumed feed, shed feathers and a plethora of bacteria (Lee *et al.*, 2011). The practice of reusing litter could be harmful to the health of the bird as ingestion of litter-born pathogenic bacteria such as *Clostridium* could lead to the disruption of the normal homeostatic gut microbiota, which would result in poor bird health and performance (Lee *et al.*, 2011). While it might be a bit more costly, using fresh bedding on broiler flocks could result in better bird health and performance, which could lead to increased profit margins.

Treatment of flocks with necrotic enteritis or treatment of chicks to prevent the proliferation of *Clostridium perfringens* has primarily been done with the use of ionophore anticoccidials or in-feed antibiotics (Van Immerseel *et al.*, 2009). Anticoccidials are used to eliminate the presence of *Eimeria* species, the causation of coccidiosis; this is an indirect method to treatment of necrotic enteritis. Because of the growing fear of antimicrobial resistant bacteria, the European Union has banned the use of antibiotic growth promoters or in-feed antibiotics; unfortunately, banning these practices without a viable replacement has led to an increase in disease occurrence in European broiler flocks (Shojadoost *et al.*, 2012; Van Immerseel *et al.*, 2004; Van der Sluis, 2000). Due to these variables, there is a rising interest in developing new approaches to treat and prevent *Clostridium perfringens* based diseases in broiler flocks.

Vaccination against *Clostridium perfringens* is an alternative approach to replacement of antimicrobial drugs and antibiotics. While vaccines against other *Clostridia* diseases are widely used and distributed for other production animals, necrotic enteritis is a notable exception; there is currently only one commercially available vaccine that is effective against necrotic enteritis—Netvax (Keyburn *et al.*, 2013ab). There have been recent studies that have shown experimental vaccines could be viable techniques for control of *Clostridium perfringens*. Attenuated anticoccidial vaccination has been shown to reduce the severity of necrotic enteritis lesions in the intestine (Tsiouris *et al.*, 2012; Williams *et al.*, 2003). As mentioned above, this vaccine is effective against *Eimeria* spp.; coccidiosis is the most common predisposing factor for the onset of necrotic enteritis. Reducing coccidiosis infection

will effectively reduce necrotic enteritis infection. Utilizing the toxin NetB as a single subunit vaccine antigen has shown some protective potential; however, it has also been shown that recombinant NetB (rNetB) when combined with other cellular or secreted antigens has far greater protective efficacy (Keyburn *et al.*, 2013b). It has also been demonstrated that vaccination using rNetB followed by maternal immunity passed on to their progeny has the ability to significantly increase protection against necrotic enteritis (Keyburn *et al.*, 2013a). However, these vaccines are still highly experimental and other alternatives such as prebiotics and probiotics should continue to be explored while viable vaccines are developed.

One other alternative method of treatment of *Clostridium perfringens* is the use of prebiotics. Prebiotics are defined as non-digestible food ingredients, such as non-digestible carbohydrates, that promote the growth of beneficial bacteria, such as *Lactobacillae* and *Bifidobacteria*, improving the gut health of the bird (Allaart *et al.*, 2013). Stimulating the growth of these beneficial bacteria could possibly diminish the growth and toxin production of *Clostridium perfringens* thereby reducing the incidence of necrotic enteritis. Several prebiotic candidates include fructo-oligosaccharides, inulin, lactose, mannan-oligosaccharides (MOS) and synthetic prebiotics such as galacto-oligosaccharides (Allaart *et al.*, 2013). There has been a lack of consistent evidence in regards to the prevention of *Clostridium perfringens*, which is possibly due to the inconsistency between the studies performed examining prebiotics; however, there is sufficient evidence to hypothesize that prebiotics could be a viable alternative to the treatment of necrotic enteritis.

The use of probiotics against the colonization of *Clostridium perfringens* is also being explored. *Bacillus subtilis* has been shown to be able to inhibit the growth of *Clostridium perfringens*; however, the inhibition of *Clostridium perfringens* is variable between the strains of *Bacillus subtilis* that were used or between the individual studies (Barbosa *et al.*, 2005; Klose *et al.*, 2010; Teo *et al.*, 2005; La Ragione & Woodward, 2003). *In vivo* studies in mice, pigs and chickens have all revealed that several probiotic strains including *Bacillus subtilis* are capable of colonizing the host GI tract after oral inoculation and reducing the colonization and persistence ability of *Clostridium perfringens* and mortality associated with *Clostridium perfringens* diseases (Allaart *et al.*, 2013). Furthermore, toxin production by *Clostridium perfringens* has also been shown to be reduced in probiotic treated hosts, which decreases the prevalence of diseases such as necrotic enteritis (Allaart *et al.*, 2013). The use of probiotics is an exciting proposition due to the many health-promoting properties exhibited by species such as *Bacillus subtilis*.

Conclusion

Because of the ever-growing concern of antimicrobial resistance in pathogenic bacteria, there is a need to move away from antibiotic therapy and in-feed antibiotics in the commercial broiler industry. The risk of potentially contaminating poultry food products due to antibiotic resistance is a direct result from the evolutionary, genetic modifications pathogens are making in response to antibiotic usage. While the mechanisms as to how probiotics produce the properties and intestinal environment that are beneficial to the host are not yet clear in all

cases, there is growing evidence that probiotic direct-fed microbial diets could be a viable technique for the treatment and prevention of pathogenic bacteria such as *Salmonella* and *Clostridium perfringens*. More research into the possibilities of probiotics is necessary. In general however, the poultry industry is readily embraced the development, production and commercialization of probiotics as a possible pathogen intervention practice (Hume, 2011).

Chapter 3:

Effect of two *Bacillus subtilis* strains on production and *Salmonella* colonization in male broilers

Abstract

With the growing concern about antibiotic resistant bacteria in commercial poultry, there is need for a new approach. Previous research indicates a direct-fed microbial (DFM) diet can help reduce *Salmonella* in the chicken gastrointestinal (GI) tract. A commonly used probiotic is the bacterium *Bacillus subtilis* (BaS). An experiment was conducted to test the effects of a DFM diet with two BaS strains (AB01 and AP71) on broiler production and the effects of the diet against *Salmonella* Enteritidis (SE). Six hundred day-old chicks were placed into 24 pens (25 chicks/pen) that were then randomly assigned one of four treatments. The four treatments (trt) consisted of a BaS treated feed and challenged group (trt1); a BaS treated feed and non-challenged group (trt2); a non-BaS containing feed and challenged group (trt3); and a non BaS-feed and non-challenged group (trt4). From day 0 to day 14 the chicks were fed a starter ration that either contained the BaS at 1.5×10^5 CFU/g (trt1 and 2) or had no BaS added (trt3 and 4). At day 14, challenge pens were inoculated with SE and pens in trt1 and trt2 were fed a BaS treated grower (1.8×10^5 CFU/g). Trt3 and trt4 pens were placed on non-treated grower. At day 28, trt1 and trt2 pens were placed on a finisher containing BaS (1.8×10^5 CFU/g), while trt3 and trt4 pens were fed a standard finisher ration. Liver, spleen and ceca samples were taken from five birds per pen at necropsy on days 14, 28 and 35. Bird

and feed weights were also assessed. Feed conversions; anaerobic and aerobic bacteria counts; and SE counts from ceca, liver and spleen samples were analyzed using generalized linear model (GLM) with means separated using Tukeys honest significant difference (HSD). Analysis of the data revealed no significant difference in feed conversion ($P > 0.05$) or in anaerobic or aerobic bacteria for all sampling dates. Day 28 showed a significant increase in *Salmonella* in ceca, spleen and liver samples in the challenged treatments (1 and 3). There was no significant difference in the AFCR seen between the treatments during this experiment. After analysis of all data, the AB01& AP71 BaS DFM diet had no effect on broiler production or *Salmonella* colonization.

Introduction

Salmonella enterica is a serogroup of pathogenic bacteria important to both the general public and the poultry industry. Salmonellosis is major health concern as it is estimated cause of 1.028 million cases of foodborne illness, 19,000 hospitalizations and 400 fatalities in the United States each year (Scallen *et al.*, 2011). The poultry industry is rapidly growing unfortunately, the risk of *Salmonella* contamination has increased with the growth of the industry. Poultry meat and eggs are considered to be a very important vehicle of transmission of *Salmonella* to humans. *Salmonella enterica* is most commonly associated with poultry and poultry products.

Salmonella enterica is a Gram-negative, enteric pathogen that has adapted the ability to use animals as vectors of transmission to other species, including humans (Carter *et al.*, 2009; Knap *et al.*, 2011). Control of *Salmonella* includes the

use of in-feed antibiotics in commercial poultry and other meat-animal processing markets. One concern of antibiotic use is antimicrobial resistant strains of pathogenic bacteria entering the food supply. Moreover, the overuse of antibiotics not only in human health but also in food production animals has allowed for the evolution of antimicrobial resistant strains of pathogenic bacteria. Recent studies have revealed a multi-drug resistant strain of *Salmonella* Heidelberg from poultry processing plants (Rothrock Jr. *et al.*, 2015). Antibiotic growth promoters (APG) and in-feed antibiotics have been shown to reduce the colonization of *Salmonella*, however, the use of antibiotics is causing a rise in the prevalence of antimicrobial resistant strains (Knap *et al.*, 2011). Vaccines against *Salmonella* do exist as either a live-attenuated vaccine or an inactive vaccine. Broiler breeder and egg laying flocks are often given *Salmonella* vaccination, but the efficacy of the vaccine is dependent upon assorted variables including the targeted serovar of *Salmonella*, the host species the vaccine is administered to, and whether or not the vaccine's intent is to control or eradicate the disease (Doyle & Erickson, 2006). Unfortunately, these vaccines do not eliminate the colonization of the mucosa; therefore, it is difficult to completely rid the bird and subsequently the flock entirely of *Salmonella* (Knap *et al.*, 2011; La Ragione & Woodward, 2003; Callaway *et al.*, 2008). Thus, it is important to explore alternative methods of control and prevention of *Salmonella* in production birds.

One such method is the use of direct-fed microbial diets (DFM) that contain probiotics. Probiotics are viewed as production enhancers and are currently being used and further assessed in the poultry industry as a potential alternative to the

use of APG's and in-feed antibiotics (Chen *et al.*, 2013). Probiotics have several favorable characteristics that render them capable of being utilized as a technique for control and prevention of *Salmonella* in the poultry industry: the organism is non-pathogenic to the host; it has the capability to adhere to intestinal epithelial cells; the organism can colonize, survive and reproduce even in the presence of harmful stomach acids and bile; and probiotic organisms have the ability to inhibit the growth of pathogenic bacteria or possibly kill them directly (Smith, 2014). Several studies have indicated that the common soil bacterium, *Bacillus subtilis*, has the capability to act as a probiotic. Knap *et al.*, (2011) showed that *Bacillus subtilis* DSM17299 when used as a DFM was able to significantly reduce the colonization of *Salmonella* Heidelberg in production broilers; moreover, drag swabs taken from the experimental houses showed significantly less (42%) *Salmonella* shedding than the positive control (100%).

Furthermore, *in vitro* studies performed by Williams (2012) provided insight into the ability of several strains of *Bacillus subtilis* to inhibit the growth of *Salmonella*. Two strains that were shown to be effective in those trials were AP71 and AB01. Utilizing these strains of *Bacillus subtilis*, a study was conducted to test the capability of AP71 and AB01 as an in-feed probiotic. The purpose of the study was to test the effects of *Bacillus subtilis* strains AB01 and AP71 on broiler production and the effects of the diet against *Salmonella* Enteritidis colonization.

Materials and Methods

Preparation of Diets

The *Bacillus subtilis* strains AP71 and AB01 were obtained in spore-form from the laboratory of Dr. Mark Liles. The sporulated *Bacillus subtilis* isolates were mixed into a mash feed at the Auburn University Poultry Research Feed Mill. All three (starter, grower and finisher) diets were in mash form and the composition of these diets can be found in Table 1. *Bacillus* strains were mixed at 1.5×10^5 CFU/g of *Bacillus* spores into the starter ration and 1.8×10^5 CFU/g of *Bacillus* spores were mixed into the grower and finisher rations. The birds received the starter diet between day 0 and day 14, the grower diet between day 14 and day 28 and the finisher diet between day 28 until trial termination at day 35.

Experimental Design and Statistical Analysis

Six hundred mixed sex day-old chicks were placed into 24 pens—25 birds per pen—and were randomly assigned 1 of 4 treatments: treatment 1, a *Bacillus* treated diet with a *Salmonella* Enteritidis (SE) challenge; treatment 2, a *Bacillus* treated diet with no SE challenge; treatment 3, a non-treated diet with an SE challenge; or treatment 4, a non-treated diet with no SE challenge. The challenge groups were given a 1 mL SE challenge via oral gavage at day 14. The *Salmonella* Enteritidis challenge given has been used extensively in the lab and contains antibiotic markers to novobiocin and naladixic acid. Table 2 displays the treatments and their make-up.

Necropsies were performed at days 14, 28 and 35. A total of 5 birds were randomly taken from each pen and were pooled. The ceca samples were plated on plate count agar (PCA) for aerobic bacterial counts and also plated on anaerobic agar (Ana) for anaerobic bacterial counts. The PCA plates were incubated

aerobically at 37°C, while the Ana plates were incubated anaerobically (90%N₂, 5%H₂ and 5% CO₂) at 40°C overnight. For day 14, liver and spleen samples were enriched in Tetrathionate Broth (TTB) overnight and then plated on Xylose Lysine Tergitol-4 agar (XLT-4) with out added antibiotics to determine if *Salmonella* was present in the birds.. At days 28 and 35, ceca samples were plated on XLT-4 with novobiocin, for *Salmonella* quantification. Liver and spleen samples were also plated on XLT-4 agar with novobiocin for plus/minus *Salmonella* counts at days 28 and 35 after overnight enrichment in TTB. Bird and feed weights were assessed on necropsy dates. Statistical analysis was performed using GLM with means separated using Tukey's HSD with a significance at p-value ≤ 0.05 . Analysis was performed on *Salmonella*, total aerobic and anaerobic counts within the ceca. It was also performed on the results from the spleen and liver for the presence or absence of *Salmonella*. Analysis was also conducted on the amount feed consumed, mortality, bird weights and feed conversion.

Results

Assessing Feed Conversions

To assess the effects of the direct-fed microbial diet on performance, the adjusted feed conversion ratio (AFCR) was evaluated for each treatment on the feed weigh dates. The AFCR is a ratio of the birds weight for each pen in comparison to the amount of feed consumed by the birds in that pen. What makes it adjusted is that this ratio takes into account any mortality or culls that may have happened in the pen. Table 3 displays the average mortality for each treatment group from the time periods that the birds were weighed as well as cumulative mortality. Table 4

shows the average body weight for each treatment at each weigh in. There was no significant difference seen in mortality or body weight when comparing any of the treatments. As seen in Table 5, there was no significant difference found with the AFCR for any of the treatment dates for the duration of the experiment. There was also no significant difference between the treatments at each sampling date. A few experimental errors due to the feed could have affected the AFCR. For example, the feed was observed bridging within the gravity feeder—meaning that the feed was not available for access by the birds. This was due to the make-up of the feed itself as it was in mash form.

Bacterial Analysis

At day 14, a baseline assessment of the bacterial counts revealed that there was no significant difference in the aerobic and anaerobic bacterial counts in the normal microflora of the birds as shown in Figure 1. However, in the pooled liver and spleen samples for one of the pens there was found to be a positive *Salmonella* sample. This implied that some of the birds were contaminated with *Salmonella* prior to the experimental challenge. Table 6 displays the plus/minus percentages seen for each treatment for *Salmonella* presence in the spleen and liver for all sampling dates.

For day 28 samplings, a significant increase in the *Salmonella* incidence among the liver and spleen samples were found in both of the SE challenge groups compared to the non-SE challenged groups; this can be seen in Table 6. There was a significant difference in the ceca *Salmonella* challenged birds compared to the non-challenged group (Figure 2). This significance was more than likely due to the

Salmonella challenge that occurred prior to day 28 at day 14. There was no significant difference found between the anaerobic counts and aerobic counts for the treatment groups.

At day 35, a significant difference between treatment 1 when compared to treatments 2 and 4 (Figure 3). The difference seen showed that treatment 1 had a higher incidence of *Salmonella* when compared to the other treatments. There was also a decrease in the total incidence of *Salmonella*. There was a higher percentage of *Salmonella* in the spleen and liver of treatments 1 and 3 when compared to treatments 2 and 4 (Table 6). However, this could be due to the *Salmonella* challenge.

Discussion

The *Bacillus* treated feed did not have any negative effects on the growth of the birds; however, it did not have a positive effect on the growth of the birds either. Studies have revealed that a direct fed microbial diet infused with *Bacillus subtilis* can improve the growth of broilers because of the improved growth of lactic acid bacteria in the normal microflora of the birds (Knarreborg *et al.*, 2008). There was no significant difference for any of the treatments when comparing the AFCR for each treatment at or between sampling dates (Table 5). This could be due to the form of feed, which was mash or because of the bridging of the feed in the gravity feeders. The feed bridging interfered with the birds having free access to the feed continuously throughout the day, thus affecting the FCR. Neither the *Salmonella* challenge nor the *Bacillus* diet had an affect on the FCR.

The bacterial counts for each sampling date revealed that the *Bacillus subtilis* cocktail utilized for this experiment had no significant effect on reducing *Salmonella* in the birds. Other studies conducted, which utilized a *Bacillus subtilis* isolate DSM 17299 infused diet, saw a reduction in the *Salmonella* load in fecal shedding and in the gastrointestinal tract of the challenged birds (Knap *et al.*, 2011; Baltzley *et al.* 2010; La Ragione & Woodward, 2003). The inconsistency seen between the current study and past findings could be due to several factors including the *Bacillus* strains working better *in vitro* than *in vivo*; the inability of the birds to have free and total access to the feed due to its mash form and the bridging of the feed in the gravity feeders; the experimental flock being contaminated with an unknown serotype of *Salmonella* prior to inoculation with *Salmonella* Enteritidis. All may have contributed either individually or together to prevent the *Bacillus* cocktail from affecting the *Salmonella* colonization and levels in the birds. Perhaps this prior contamination with *Salmonella* within the bird was such as to render the *Bacillus* treatment ineffective. The *Bacillus subtilis* isolates used in this experiment are soil-origin strains unlike the strains used in the work cited above. However the strains utilized herein showed promise in inhibiting *Salmonella in vivo* in a previous study (Stough, 2013).

The current study revealed that the *Bacillus subtilis* strains used had no effect on the colonization of *Salmonella* Enteritidis in challenged birds and had no affect on bird growth. One possible solution to this issue could simply be increasing the load of *Bacillus* put into the feed. Knap *et al.*, (2011) utilized 8.0×10^5 CFU/g in their direct fed microbial diet; the current study only used 1.5×10^5 CFU/g for the grower diet

and 1.8×10^5 CFU/g for the finisher diet, which is 20.6% more than what was utilized in this study. Another remedy could be to include more *Bacillus subtilis* strains in the cocktail or utilize an entire new set of isolates. While the present study did not reveal any significant beneficial effects when using *Bacillus subtilis* strains AP71 and AB01 as a direct-fed microbial, there is an abundance of evidence that implies that direct-fed microbials can reduce the colonization of *Salmonella* as well as improving bird health and growth.

Table 3.1: Ingredient composition for each diet phase used in the experiment

Ingredient	Amount in Starter (%)	Amount in Grower (%)	Amount in Finisher (%)
Corn	60.778	63.84	71.173
Soybean Meal	29.526	27.291	20.915
Poultry Meal	4	3	3
Poultry Oil	1.533	2.291	1.669
Dicalcium-Phosphate	1.405	1.348	1.12
Limestone	1.124	1.011	0.9367
Vitamin Premix	0.5	0.25	0.25
Salt	0.4041	0.4173	0.4191
DL-Methionine	0.279	0.208	0.2139
TM Premix	0.25	0.25	0.25
L-Lysine	0.15	0.045	0.0522
Coban 90	0.05	0.05	NA

Table 3.2. Treatment groups as composed of *Bacillus* treated/untreated and *Salmonella* challenged/unchallenged

Treatment	Diet/Challenge
1	BaS Treated + SE Challenge
2	BaS Treated + no SE
3	non-BaS Treated + SE Challenge
4	non-BaS Treated + no SE

Table 3.3: Mortality numbers and weight in kilograms (kg) for each treatment on the listed days

Treatment	Day 0-14 Avg. Mortality	Day 14-28 Avg. Mortality	Day 28-35 Avg. Mortality	Overall Avg. Mortality
1	0.33	0.5	0	0.28
2	0	0.17	0.17	0.11
3	0.67	0.5	0	0.39
4	0.5	0.17	0	0.22

p-value < 0.05

Table 3.4. The average body weight in kilograms (kg) for each treatment that was assessed for each treatment date as well as overall average body weight

Treatment	Day 14 Avg. Body Weight	Day 28 Avg. Body Weight	Day 35 Avg. Body Weight
1	0.348	1.217	1.822
2	0.357	1.150	1.829
3	0.353	1.197	1.741
4	0.363	1.182	1.887
SE	0.225	0.917	1.312

p-value < 0.05

Table 3.5. Adjusted feed conversion ratios analyzed for all treatments at and in between the experimental dates

Treatment	0-14	14-28	0-28	28-35	14-35	0-35
Trt 1	1.36	1.56	1.46	1.75	1.62	1.54
Trt 2	1.30	1.51	1.43	1.79	1.60	1.52
Trt 3	1.44	1.53	1.43	1.74	1.59	1.50
Trt 4	1.37	1.53	1.44	1.73	1.59	1.51
Std. Err.	0.105	0.030	0.022	0.059	0.028	0.019

p-value < 0.05

Table 3.6. The percentage of *Salmonella* incidence within the spleen and liver for each treatment across the experimental time frame.

Treatment^β	Day 14 Spleen	Day 14 Liver	Day 28 Spleen	Day 28 Liver	Day 35 Spleen	Day 35 Liver
1	20 ^α	20 ^α	43.3	16.7	56.7	20
2	0	0	0	0	3.3	0
3	0	0	26.7	20	60	33.3
4	0	0	3.3	3.3	0	13.3

α. The positive result for *Salmonella* at day 14 occurred prior to the *Salmonella* Enteritidis challenge given following the day 14 necropsy. The birds were contaminated in the hatchery prior to pen placement.

β. All samples taken were plated on XLT-4 agar with an added antibiotics (novobiocin and naladixic acid) after being enriched overnight in TTB.

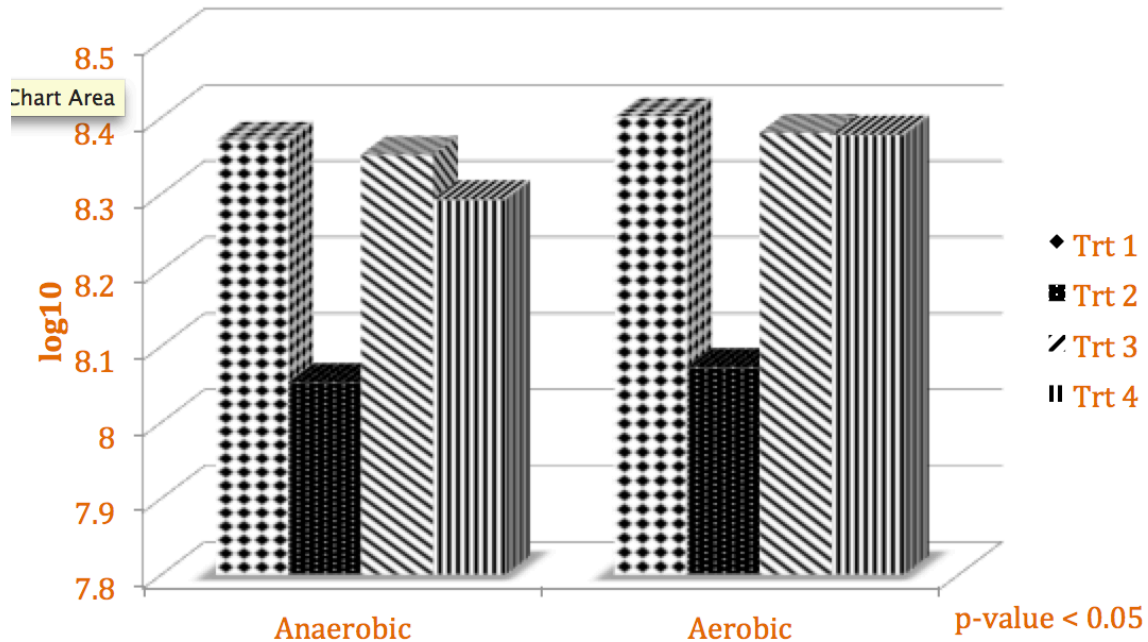


Figure 3.1: Day 14 bacterial counts taken for analysis of bird microflora before *Salmonella* challenge. Trt 1 consists of a *Bacillus* treated diet with a SE challenge; trt 2 consists of a *Bacillus* treated diet without a SE challenge; trt 3 is the untreated diet with a SE challenge group; and trt 4 is the untreated and unchallenged control group. Anaerobic bacterial counts were obtained using ANA and aerobic counts using PCA. Baseline analysis showed there was no significant difference in the anaerobic and aerobic bacterial counts.

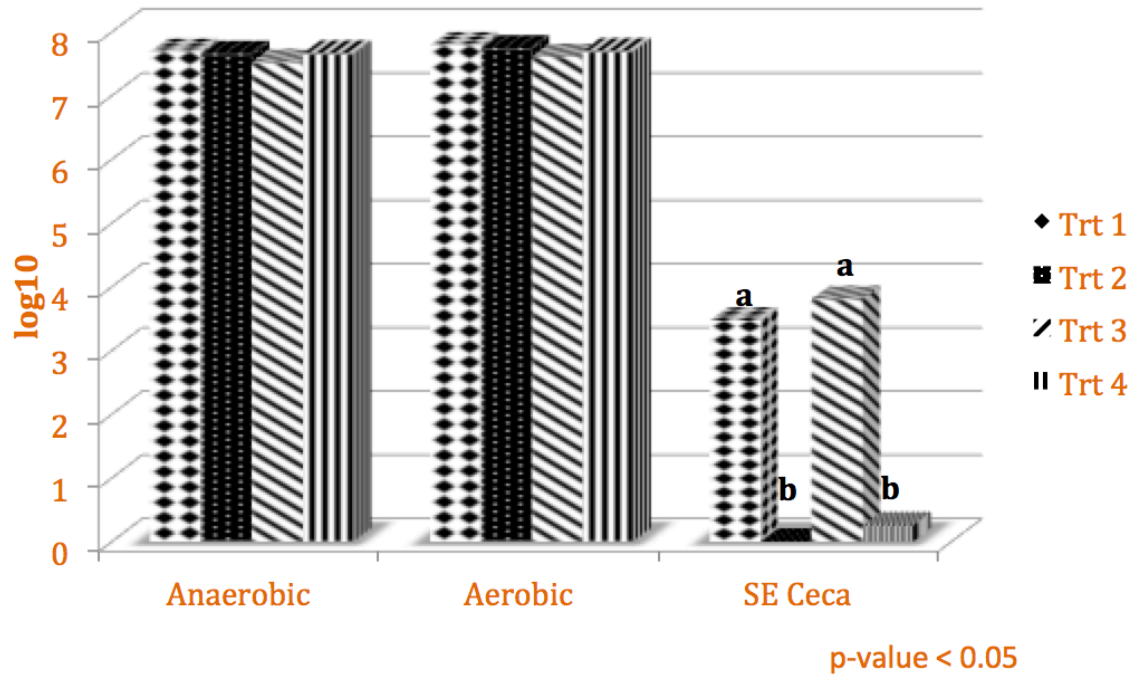
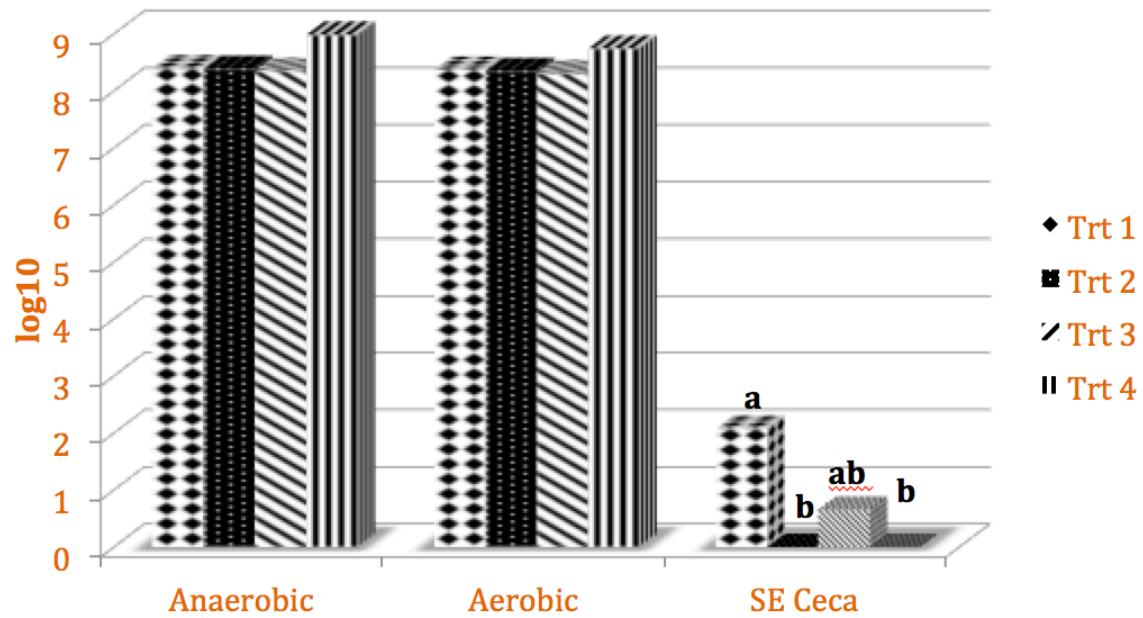


Figure 3.2. Day 28 bacterial counts for the analysis of the bacterial community post *Salmonella* challenge. Anaerobic bacterial counts were obtained using Ana, aerobic counts using PCA and XLT-4 with antibiotics was used for *Salmonella* isolation. Significant difference was seen within each sample (marked by letter designation); however, no significant difference was seen overall between the samples for all treatments.



p-value < 0.05

Figure 3.3. Day 35 bacterial counts for analysis of bird microflora at trial termination. Anaerobic bacterial counts were obtained using Ana, aerobic counts using PCA and XLT-4 with antibiotics was used for Salmonella isolation As seen at day 35, significant difference was seen within each sample (marked by letter designation); however, no significant difference was seen overall between the samples for all treatments.

Chapter 4:

In-vitro* inhibition of *Clostridium perfringens* by seven strains of *Bacillus subtilis

Abstract

Necrotic enteritis (NE) is an economically important bacterial disease caused by *Clostridium perfringens* (CP) it is estimated to cost the U.S. poultry industry \$2 billion annually. Currently, antibiotics are used to control NE; however, due to concerns about antimicrobial resistance, the use of subtherapeutic levels of antibiotics is being reduced. Moreover, there are limited vaccines available for NE control. One possible solution in controlling NE is the application of a direct-fed microbial (DFM) diet infused with *Bacillus subtilis* (BaS). An *in vivo* experiment was conducted to test the ability of seven strains of BaS to inhibit the growth of CP. The CP isolates tested came from one of three sources - environmental samples, gangrenous dermatitis (GD) cases or came from NE cases. Cultures of BaS and CP were grown on trypticase soy agar (TSA) with 5% sheep blood overnight: BaS aerobically at 37°C and CP anaerobically at 40°C. These cultures were then inoculated into brain-heart infusion (BHI) broth and incubated in their respective environment overnight at 37°C. Standard TSA plates were taken and 3, 1-mm cores holes were cored into the agar. These plates are referred to as “cored plates”. Ten µL of the BHI-BaS solution were pipetted onto cored plates (1 BaS isolate per plate) and allowed to incubate for 3-4 hours. Following the BaS incubation period, the cored plates were overlaid with a solution consisting of 1 mL of the overnight BHI-CP

broth and 7 mL of molten TSA (1 BaS strain per CP strain; 7 total plates per CP isolate). The overlay plates were then incubated at 37°C in an anaerobic environment overnight. After which the zone of inhibition (ZI) created by BaS were measured. A significant ZI was considered to be ≥ 5 mm. From this test, four BaS strains were found to cause significant growth inhibition amongst the environmental, GD and NE CP isolates. These BaS strains are designated AB01, AP206, AP183 and AP302. Future bird pen trials will be conducted in order to test the efficacy of these strains as probiotics for the treatment of NE in poultry.

Introduction

Clostridium perfringens is an important enteric disease of both humans and animals. The genus *Clostridium* is a large and genetically diverse group with over 150 species. Of the 150, 35 species are considered pathogenic. *Clostridia* are Gram-positive, anaerobic bacilli that have the capability to form endospores. *Clostridium* species are also known for their toxin production as they produce around 18% of all known bacterial toxins (Popoff & Stiles, 2005; Bailey, 2013). There are five types of *Clostridium perfringens* (A, B, C, D, E) and they are classified based on the presence or absence of one of the four major toxins - alpha, beta, epsilon and iota (Bailey, 2013). Of the toxins produced by *Clostridium perfringens*, NetB has been demonstrated to be a major contributor for causing necrotic enteritis in chickens (Keyburn *et al.*, 2008).

Necrotic enteritis is a major enteric disease of poultry caused by type A and C *Clostridium perfringens*; it is estimated that the total world cost of necrotic enteritis due to mortality and morbidity is nearly \$2 billion per year (Martin and Smyth,

2009; Opengart, 2008). Lesions primarily in the small intestine of the bird characterize necrotic enteritis. Moreover, *Clostridium perfringens* is also the causative agent of gangrenous dermatitis, another important disease of poultry. *Clostridia* infections in humans are primarily associated with foodborne illness; there are approximately 965,000 human cases of *Clostridium perfringens* related diseases in the U.S. each year (Scallan *et al.*, 2011).

There are several management practices that can lead to the decrease of the disease. Several of these practices coincide in the reduction of other economically and pathogenically important bacteria such as *Salmonella*. Proper animal welfare, dietary maintenance and timely identification of necrotic enteritis can be helpful in the management of the flock. Effective treatment of *Clostridium perfringens* has fallen predominantly to antibiotic therapy. While antibiotic treatment is effective in the reduction of the disease, the poultry industry is decreasing usage of antibiotic treatment. Vaccination against *Clostridium perfringens* related diseases has shown promise and is being used. However, there is currently only one vaccine effective in controlling necrotic enteritis—Netvax—that is available for commercial use in the poultry industry (Keyburn *et al.*, 2013a). Other alternative methods, such as prebiotics and probiotics are being explored for the replacement of antibiotics.

One alternative method for the prevention and treatment of necrotic enteritis that is being explored is the use of probiotics—direct-fed microbials (DFM). Probiotics in poultry are being used as an alternative to antibiotic growth promoters and in-feed antibiotics (Chen *et al.*, 2013). Viewed as production enhancers, probiotics are currently being used to promote health beneficial

properties in the intestinal microflora to promote broiler performance as well as to protect the host from colonization by pathogenic bacteria (Hume, 2011).

Bacillus subtilis, a spore-forming, Gram-positive bacteria has been shown to be able to inhibit the growth of *Clostridium perfringens* when used as a direct-fed microbial; however, the inhibition of *Clostridium perfringens* is variable between the strains of *Bacillus subtilis* that have been used as well as between the individual studies investigating the organisms viability of being used as a DFM (Barbosa *et al.*, 2005; Klose *et al.*, 2010; Teo and Tan, 2005; La Ragione & Woodward, 2003). *In vivo* studies in mice, pigs and chickens have all revealed that several probiotic strains including *Bacillus subtilis* are capable of colonizing the host gastrointestinal tract after oral inoculation and reducing the colonization and persistence ability of *Clostridium perfringens* and mortality associated with *Clostridium perfringens* diseases (Allaart *et al.*, 2013). Furthermore, it has been demonstrated that toxin production by *Clostridium perfringens* can be reduced in probiotic treated hosts, which decreases the prevalence of diseases such as necrotic enteritis (Allaart *et al.*, 2013).

Past studies have indicated the ability of several strains of *Bacillus subtilis* to inhibit the growth of the pathogenic bacteria *Salmonella* both *in vivo* and *in vitro* (Williams, 2012; Knap *et al.*, 2011). The current study was conducted to examine the viability of seven strains of *Bacillus subtilis* in inhibiting the *in vitro* growth of *Clostridium perfringens*.

Materials and Methods

Zone of Inhibition Plating Preparation

Seven strains of *Bacillus subtilis* were received from the laboratory of Dr. Mark Liles. Previous work performed in his lab indicated that these seven strains had promise as a potential inhibitor of pathogenic bacteria including *Salmonella* and *Clostridium perfringens*. The seven strains of *Bacillus subtilis* were labeled AB01, AP71, AP302, AP206, AP294, AP183 and AP185. There were 75 *Clostridium perfringens* isolates tested and they came from one of three sources—environmental samples (36), gangrenous dermatitis (GD) cases (24) or from necrotic enteritis (NE) cases (15). Glycerol stock cultures of *B. subtilis* and *C. perfringens* were grown on trypticase soy agar (TSA) with 5% sheep blood overnight. The *Bacillus subtilis* TSA plates were grown at 37°C in an aerobic environment, while the *Clostridium perfringens* TSA plates were incubated at 40°C in an anaerobic (90% N₂, 5% H₂ and 5% CO₂) environment. This was performed to allow optimal growth for each organism. These cultures were then inoculated into brain-heart infusion (BHI) broth and incubated overnight at 37°C in their respective environments. Fresh TSA plates were utilized to create 3, 1mm cores into the agar of each plate. These plates are referred to as “cored plates”. Ten µL of the BHI-*B. subtilis* solution were pipetted into the cores of the cored plates; each plate was inoculated with only one *B. subtilis* isolate. The inoculated, cored plates were then allowed to incubate for 3-4 hours at 37°C in an aerobic environment. Following the *B. subtilis* incubation period, the cored plates were overlayed with a solution consisting of 1 mL of the overnight BHI-*C. perfringens* broth and 7 mL of molten TSA (1 *B. subtilis* isolate per *C. perfringens* isolate; seven total plates per *C. perfringens* isolate). The overlay plates were

incubated anaerobically; however, because *Bacillus subtilis* is an aerobic organism the plates were incubated at the optimal temperature for growth of the *Bacillus* isolates, which was 37°C. *Bacillus subtilis* is thought to be a facultative anaerobe as it can grow under strict anaerobic conditions by using nitrate as an electron acceptor (Hoffmann *et al.*, 1995). Following the incubation period, the zones of inhibition (ZI) were measured in millimeters (mm). A significant ZI was considered to be ≥ 5 mm. The zone was measured from the edge of the core to the edge of noticeable inhibition of *C. perfringens* growth. A zone that is considered significant shows that the *Bacillus* isolate being tested has the propensity to inhibit *Clostridium perfringens*. Significance was taken for importance from the NE, GD and environmental samples with NE samples being of greatest importance. Figure 1 displays an example of a successful inhibition of *Clostridium perfringens* by *Bacillus subtilis*.

Results

. The average ZI was taken from the 3-hole core plate for all of the samples. The levels of inhibition for the NE samples are displayed in Table 1. Table 2 shows the average levels of inhibition for the GD samples and Table 3 displays the average zones for the environmental les. The *Bacillus* strains AB01, AP206, AP183 and AP302 showed greater inhibition potential against all samples. Because the environmental samples did not contain the *netB* gene, which is considered important for the development of necrotic enteritis, the ZI for these samples were not considered important when determining the strains suitable for direct-fed microbial use. However, it should be noted that the strains chosen showed greater

inhibition of the environmental samples when compared to the remaining the samples.

Discussion

The NE and GD ZI from Tables 1 and 2 indicate that strains AB01, AP206, AP183 and AP302 inhibited these CP isolates more than the other three strains of *Bacillus subtilis*. Moreover, as can be observed in Table 3, these strains showed greater success in inhibiting growth on the environmental samples as well. Based on the results presented, these *Bacillus subtilis* strains could potentially inhibit the growth of type A *Clostridium perfringens*, within the gastrointestinal tract of broiler chickens. Previous work indicates that the use of *Bacillus subtilis* as a probiotic could prove beneficial; however, due to inconsistencies in the data, more research is warranted (Barbosa *et al.*, 2005; Klose *et al.*, 2010; Teo and Tan, 2005; La Ragione & Woodward, 2003).

The current study showed that four of the seven tested *Bacillus subtilis* isolates were capable of providing significant zones of inhibition against *Clostridium perfringens*. These four strains of *Bacillus subtilis* could be viable as a direct-fed microbial. While the inhibition study provided valuable insight into the inhibition ability of *Bacillus subtilis* against *Clostridium perfringens*, a bird trial is needed to test the efficacy of the strains as a direct-fed microbial in production poultry.

Table 4.1. Average zone of inhibition in millimeters (mm) of NE samples from the 3-hole core TSA-BaS plate

CP Sample	<i>Bacillus subtilis</i> Serotype						
	AB01	AP183	AP185	AP206	AP294	AP302	AP71
C103-79	4.13	2.83	0.00	0.00	4.80	4.40	0.00
C103-80	1.70	2.03	0.00	0.00	4.87	5.57	0.00
C103-81	4.10	0.00	4.33	0.00	6.20	0.00	0.00
C103-82	1.27	0.00	4.77	3.07	1.60	5.17	0.00
C103-83	4.20	4.00	0.00	0.00	2.57	5.67	4.97
C103-84	0.00	3.47	0.00	0.00	2.93	0.00	6.03
C103-86	5.83	7.63	2.73	8.40	6.77	10.03	0.00
C103-87	4.53	5.37	1.93	7.97	9.77	4.00	0.00
C103-88	3.00	4.27	0.00	0.00	2.30	2.33	1.00
C103-91	3.93	1.87	0.00	0.00	4.53	6.00	3.93
C103-94	0.00	0.00	0.00	5.40	0.00	0.00	0.00
C103-95	4.07	0.00	0.00	5.33	4.70	4.63	0.00
C103-96	0.00	5.00	6.37	0.00	0.00	6.23	3.87
C103-99	0.00	0.00	0.00	0.00	0.93	2.50	0.00
C103-100	3.67	5.60	6.13	0.00	2.13	1.60	1.20

Table 4.2. Average zone of inhibition in millimeters (mm) of GD samples from the 3-hole core TSA-BaS plate

<i>Bacillus subtilis</i> Serotype							
CP Sample	AB01	AP183	AP185	AP206	AP294	AP302	AP71
C103-46	7.23	0	0	11.5	1.03	0	7.63
C103-48	6.47	5.53	5.2	0	4.2	0	2.47
C103-49	5.87	4.87	2.4	11.03	8.17	5.53	0
C103-51	13.63	0	2.13	0	0.7	2.87	4.73
C103-54	11.9	9.1	9.77	11.57	0	14.1	1.8
C103-56	11.8	9.6	7.07	10.2	11.83	10.9	1.93
C103-57	3.8	10.47	8.57	0	1.9	10.2	1.9
C103-58	10.3	9.5	8.63	10.83	11.87	15.17	2.4
C103-59	15.2	7.83	9.3	16.53	0	10.77	0.6
C103-60	4.93	3.4	4.03	6.13	1.6	4.43	2.87
C103-61	6.6	3.47	5.8	6.73	5.03	4.47	2.03
C103-62	0	0	5.37	0	5.43	0	7.4
C103-63	10	6.9	7.63	6.93	0	13.27	1.4
C103-64	0	0	0	0	0	0	0
C103-65	9.4	3.3	2	3.87	3.33	5.4	3.4
C103-66	2.87	0	6.33	0	0	6.77	6.83
C103-67	7	4.03	0	0	4.1	7.83	0.4
C103-68	7.43	0	6.47	0	4.1	12.07	0.83
C103-69	2.3	3.83	0	0	4.3	3.97	0

C103-70	0	0	0	0	4.3	0	6.4
C103-71	11.73	0	4.6	5.83	13.23	9.43	1.23
C103-72	2.57	4.83	0	0	0.9	0	4.53
C103-73	9.33	7.4	8.27	9.2	11	0	1.7
C103-74	6.67	6.23	3.23	0	13.73	4.5	5

Table 4.3. Average zone of inhibition in millimeters (mm) of Environmental samples from the 3-hole core TSA-BaS plate

<i>Bacillus subtilis</i> Serotype							
CP Sample	AB01	AP183	AP185	AP206	AP294	AP302	AP71
C103-6	14.6	13.83	11.63	12.63	0	12.63	10.13
C103-7	18.33	12.47	13.53	13.1	0	13.1	13.37
C103-8	13.3	12.2	11.87	12.4	0	13	12.17
C103-9	10.13	10.97	12.7	14	0	13.17	13.3
C103-10	12.7	10.53	11.93	13.17	0	12.17	12.33
C103-11	1.1	2.97	0.63	0	0	5.67	0
C103-12	13.03	12.4	10.5	0	0	14.1	0
C103-13	0	10.63	0	0	0	13.77	0
C103-14	0	11.2	10.43	0	0	12.9	13.03
C103-15	0	3.07	0	0.83	0	8.83	0
C103-16	0.83	2.63	0	1.03	0	8	0
C103-17	1.17	2.47	0	0	0	4.83	0
C103-18	1.23	2.23	0	0	0	7.8	0
C103-19	0.8	2.4	0	1.23	0	7.53	0
C103-20	2.27	0	10.3	0	8.27	0	0
C103-21	1.2	0	9.17	0.67	10.07	9.4	0
C103-22	1.27	0.8	8.8	0.87	9.6	0	0
C103-23	0.93	0.73	8.33	0.5	10.9	0	12.6
C103-24	0.73	0.47	8.6	0.2	9.5	9.87	11.07

C103-25	0.37	0.73	8.47	0	9.77	8.93	0
C103-26	1.17	0.93	9.4	0	9.9	9.33	10.97
C103-27	0	0	0	0	12	7.13	9.8
C103-28	1.1	0.4	8.8	0	8.77	9.13	11.3
C103-29	0	0	9.8	0	9.8	9.43	11.7
C103-30	1.4	0	9.2	0	9.33	9.13	10.73
C103-31	1.03	0	9.23	1	0	8.63	0
C103-32	3.27	0	0	0	10.1	0	0
C103-33	0	2	0	0	0	8.13	0
C103-36	0	0.93	1.2	0	0	7.37	0
C103-37	4.53	0.8	0	0.73	0	0	0
C103-38	0.97	0	9	0.47	9.5	9.33	0
C103-39	1.67	1.47	1.07	0	0	7.57	0
C103-41	10.47	9.3	0	9.33	1.03	12	0
C103-43	1.23	2.53	0	0	0	5.77	0
C103-44	10.53	4.57	0	0	1.6	0	0
C103-45	10.67	0	0	10.3	0	0	0

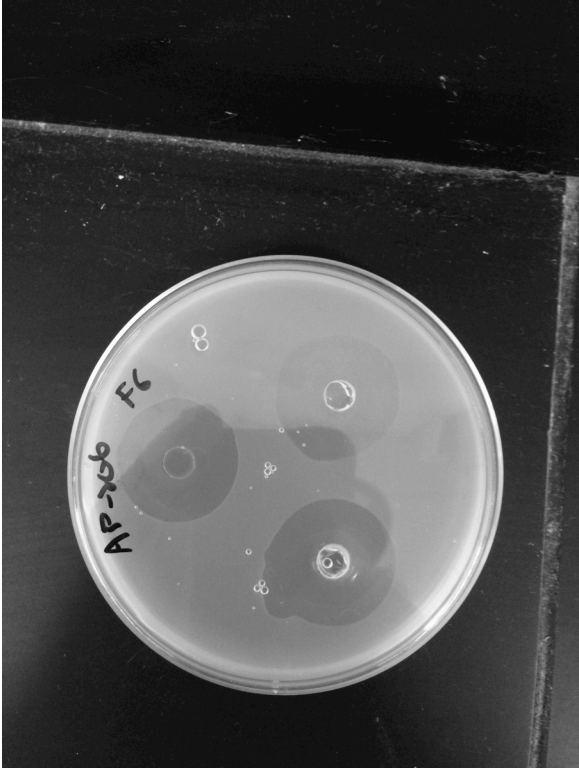


Figure 4.1. Example of a successful zone of inhibition; inhibition of three *Clostridium perfringens* isolates by *Bacillus subtilis* isolate AP-206

Chapter 5:

Effect of *Bacillus subtilis* strains AB01, AP302, AP183 and AP206 on production and inhibiting the colonization of *Clostridium perfringens* in production broilers

Abstract

Due to the growing concern amongst consumers about antimicrobial resistance, the poultry industry is searching for new techniques to replace antibiotics. Previous research indicates that the use of direct-fed microbial (DFM) diets could be a viable option in reducing the numbers of the pathogen type A *Clostridium perfringens* (CP). A commonly used DFM is *Bacillus subtilis* (BaS). Utilizing results from previous *in vitro* studies, an experiment was conducted using four isolates of *Bacillus subtilis* designated AB01, AP302, AP183 and AP206 to test their efficacy in inhibiting CP as well as their effect on broiler production. Nine hundred and sixty day-of-hatch female Ross x Ross broilers were randomly placed into 32 pens (30 birds per pen). There were four treatment (trt) groups: trt 1—a BaS treated diet without a CP challenge; trt 2—a BaS treated diet with a CP challenge; trt 3—an untreated diet with a CP challenge; and trt 4—an untreated diet and unchallenged group. A starter diet was given to the birds at day 0 through day 14 with the treated groups receiving a 2×10^8 spores/gram concentration of the BaS cocktail (trts 1 and 2) in the starter diet. A baseline necropsy was performed at day 14 with necrotic enteritis (NE) lesion scores assessed from the small intestines and CP bacterial analysis completed via 5 ceca samples from 5 randomly selected

individual birds per pen. Feed and pen weight were also assessed for each pen at this time. Also on day 14 the birds diet was switched from starter to a standard grower diet with treated groups (trt 1 and trt 2) receiving BaS concentrations of 2×10^5 spores/gram. On days 15, 16 and 17 the birds were gavaged with CP at 4.5×10^8 , 2.0×10^8 and 1.3×10^7 cfu/ml, respectively. At day 28 a second necropsy was performed following the same manner as day 14 with feed and bird weight also being assessed. Birds were placed on a standard finisher diet at this time interval with treated groups receiving a BaS concentration of 2×10^5 spores/gram. The trial was terminated at day 35 with a necropsy that obtained NE lesion scores from all remaining birds; however, ceca samples were taken from 5 randomly selected birds per pen, as previously performed at days 14 and 28. Analysis was done using generalized linear model (GLM) with means separated using Tukeys honest significant difference (HSD) with significance at p-value ≤ 0.05 . No significant difference was found for lesion score, bacterial count or bird production between the treatments for any of the experimental data.

Introduction

Necrotic enteritis is an important disease to the poultry industry. The enteric disease causes severe financial loss that is estimated to be \$2 billion annually due to mortality and morbidity caused by the disease (Martin and Smyth, 2009; Opengart, 2008). *Clostridium perfringens* type A is the primary causative agent of necrotic enteritis in the chicken small intestine. The disease may be characterized as either clinical or subclinical. The occurrence of subclinical necrotic enteritis has been shown to lead to a 12% reduction in body weight while increasing the feed

conversion ratio by 10.9% when compared to healthy birds (Van der Sluis, 2000; Skinner *et al.*, 2010). The hallmark of the disease is the necrosis of the small intestine, particularly the ileum and jejunum (Bailey *et al.*, 2013, Mot *et al.*, 2014). Clinical signs of infection are less than helpful in identification of the disease as necrotic enteritis is characterized by sudden onset mortality without any premonitory symptoms and require postmortem necropsies to determine if diseased birds are diagnosable with necrotic enteritis (Antonissen *et al.*, 2014). Lesions in the small intestine can vary in severity ranging from thickened mucosa and multifocal ulceration in less severe cases to more severe cases, which birds will have a greenish or yellowish pseudo-membrane and extensive mucosal inflammation as well as necrosis (Bailey *et al.*, 2013).

The causation of necrotic enteritis from *Clostridium perfringens* has been credited to the NetB toxin produced by the bacteria (Keyburn *et al.*, 2008). The netB gene is carried only by necrotic enteritis *Clostridium perfringens* type A strains (Keyburn *et al.*, 2008). The NetB toxin is released continuously throughout the vegetative phase of the cell (Moran, 2014). The *netB* gene has been recently identified to reside on a plasmid-encoded pathogenicity locus that is found in necrotic enteritis causing strains of *Clostridium perfringens* (Engström *et al.*, 2012). Moreover, this plasmid can be transferred to other *Clostridium perfringens* making them more pathogenic in nature and causing an increased prevalence in NetB toxicity (Moran, 2014; Uzal *et al.*, 2014).

Control of necrotic enteritis has been primarily done through the use of ionophore anticoccidials or in-feed antibiotics (Van Immerseel *et al.*, 2009).

Anticoccidials are capable of reducing the incidence of necrotic enteritis by inhibiting the growth of *Eimeria* spp., the causative agent of coccidiosis. Coccidiosis infections are common in poultry and predispose birds to necrotic enteritis infections. The use of in-feed antibiotics is decreasing in the poultry industry due to consumer concerns over antimicrobial resistant strains of bacteria. Because of antimicrobial resistant bacteria, the European Union has banned the use of antibiotic growth promoters or in-feed antibiotics; unfortunately, banning these practices without a viable replacement has led to an increase in enteric disease occurrence in European broiler flocks (Shojadoost *et al.*, 2012; Van Immerseel *et al.*, 2004; Van der Sluis, 2000).

Research into a viable replacement for antibiotic therapy is currently a high priority within the poultry industry. One possible alternative that has shown promise is the use of direct-fed microbials (DFMs) or probiotics. The use of probiotics in commercial broilers has been shown to provide a beneficial intestinal ecosystem by inhibiting the colonization of pathogenic bacteria as well as reinforcing the natural microbiota (Chen *et al.*, 2013; Fuller, 2001; Higgins *et al.*, 2008). Moreover, a probiotic infused diet has also been demonstrated to enhance the concentration of lymphocytes as well as increase the relative weight of the spleen and bursa to body weight; therefore, it could be concluded that the inclusion of a probiotic diet could enhance the broiler immune system (Chen *et al.*, 2013; Teo & Tan, 2007; Willis *et al.*, 2007).

A primary candidate for use as a direct-fed microbial is *Bacillus subtilis*. *Bacillus subtilis* is a common soil bacterium that is characterized as being a Gram-

positive, obligate aerobe rod capable of endospore formation (Bergey's Manual 9th edition, 1994). An important characteristic of *Bacillus subtilis* that makes it a stimulating prospect as a probiotic is its ability to produce endospores. Certain bacteria produce endospores when environmental phenomena such as high temperature or humidity exasperate the nutrients needed for growth allowing the organism to go dormant until conditions are viable for growth. The spores will then go into a vegetative phase of growth and begin proliferating. Most known probiotics are not capable of producing these endospores. *Bacillus subtilis* has been shown to produce spores capable of withstanding high temperatures when being processed in the feed-milling procedure as its spores are metabolically dormant and are highly resistant to extreme pH levels and extreme temperatures (Nicholson, 2002).

Bacillus subtilis strains AB01, AP302, AP183 and AP206 were previously shown to be able to effectively inhibit the growth of *Clostridium perfringens* in a laboratory environment (Frazier, 2015). These four strains are capable of producing endospores, which could be used as a viable direct-fed microbial. An experiment was conducted to test the efficacy of these strains of *Bacillus subtilis* as a direct-fed microbial on enhancing performance of production broilers as well as their ability to inhibit the growth of type A *Clostridium perfringens*.

Materials and Methods

Bird Source and Diet

Nine hundred and sixty, day-of-hatch, female Ross x Ross broiler chicks were obtained and randomly placed into 32 pens. The birds were allowed *ad libitum* access to water and their assigned diet. The birds were fed a standard three feed

diet that consisted of a starter, a grower and a finisher. There were two diets one that consisted of only standard feed and the other was standard feed that had *Bacillus* spores added.

Direct-fed Microbial and Spore Preparation

Bacillus subtilis strains AB01, AP302, AP183 and AP206 were supplied from the laboratory of Dr. Mark Liles and a glycerol stock solution was made for each individual strain. These four strains were chosen based on their ability to consistently inhibit the growth of *Clostridium perfringens* (Frazier, 2015). The spores of the four strains of *B. subtilis* were prepared using a spore preparation media (Table 1).

One bead from the stock solution of each strain of *B. subtilis* was individually plated onto a tryptic soy agar with 5% sheep's blood (TSA) plate and allowed to incubate aerobically overnight at 37°C to provide evidence of non-contaminated growth. An inoculate-loop full of each individual strain was taken and placed into brain-heart infusion (BHI) broth and allowed to grow aerobically overnight at 37°C, which allowed for optimal growth of the *Bacillus subtilis* strains. A cotton swab applicator was then used to plate each individual *Bacillus*-BHI solution onto 10 spore preparation agar plates; this yielded at least 1×10^9 or 1×10^8 total spore concentration. These plates were then allowed to incubate for 5 days at 28°C. The spores were harvested by pipetting 5 mL of sterile water onto each plate and using a plastic, disposable inoculating loop to scrape the cells free. This process was repeated for each plate to maximize the spore yield. Each spore solution was then heat shocked at 80°C for 20 minutes to kill any remaining vegetative cell. The spore

solutions were then subjected to 100-fold serial dilutions to determine the spore concentrations. One hundred μL of the stock solution was taken and pipetted into 9.9 mL of sterile water; 100 μL of the subsequent dilution were taken and placed into 9.9 mL of sterile water again until a total of 8, 100-fold dilutions were completed. Following the serial dilution process, 50 μL of each dilution were plated onto TSA in a duplicate manner (i.e., 2 plates per dilution) and allowed to incubate at 28°C for 36-48 hours. These plates were grown at 28°C instead of 37°C in order to maintain homeostatic conditions throughout the spore process. Following the incubation period, the plates were counted in order to determine the amount needed for each strain to be added to the treated feed diets at a concentration of 2×10^8 spores/gram of feed. A concentration of 2×10^8 spores/gram of feed was deemed adequate to allow the best chance for each *Bacillus subtilis* strain to inhabit the intestinal tract of the bird. The *Bacillus* strains were combined to form a cocktail and were added to the feed. Each treatment group received 50 lbs. of the starter diet.

The sporulation process was repeated to obtain more stock solution for the grower and finisher treated diets. The same process as detailed above was followed except a total of 15 sporulation preparation agar plates per isolate were used to obtain a greater quantity of spores. Because the total amount of each stock solution per strain was too great to be properly heat shocked, the stock solutions were aliquotted out into 50 mL centrifuge vials—20 mL of the stock solution per vial. These vials were individually labeled and stored in a laboratory refrigerator until needed. At the time appropriate for mixing the treated-grower feed, two, 20 mL

vials for each of the stock solutions per strain were taken and subjected to heat shock at 80°C. To determine the concentration, 1 vial of each heat-shocked solution was taken and subjected to a 10-fold serial dilution—100 µL of each stock solution were individually taken and diluted into 900 µL of water. This was repeated until a total of 9 dilutions were completed. To determine the concentration, 100 µL of each dilution per heat-treated stock solution were plated onto TSA plates and allowed to incubate in the same manner as described above for determining counts for the grower diet. An additional 20 mL vial of the stock solutions for each strain were heat-treated, serially diluted and plated in the same manner to determine the counts for the treated-finisher diet. The grower and finisher diets were mixed twice due to poor spore yields, which inhibited 50 lbs. bags from being utilized per treated-diet. Instead, 2, 30 lbs. bags were mixed per diet. The spores were mixed into the grower and finisher feeds at a concentration of $\sim 2 \times 10^5$ spores/gram of feed. The amount of spores was reduced due to the lack of availability of the spores from each stock solution.

Experimental Design

The experiment was conducted using a 35-day grow-out period for each treatment. Each treatment group consisted of 8 pens with each pen containing 30 birds (240 birds per treatment; 960 birds total). A total of 4 treatments groups were designed. Treatment 1 consisted of a *Bacillus subtilis* treated diet without a *Clostridium perfringens* challenge. Treatment 2 consisted of a *Bacillus subtilis* diet with a *Clostridium perfringens* challenge. Treatment 3 was given a non-treated diet

and was challenged with *Clostridium perfringens* Treatment 4 was the control group (non-treated diet/no challenge). Table 2 further illustrates the treatment make-ups.

The birds were placed on their perspective starter diet (either treated or untreated based on treatment group; refer to Table 2) at day 0. All birds were given 50 lbs. of starter feed regardless of it being treated or untreated. After day 14, the grower diet was added to the bird's feeders. Treated birds were given 30 lbs. of feed, while untreated birds were given 50 lbs. of feed. Treated birds only received 30 lbs. due to the lack of availability of the *Bacillus* spores. An additional 30 lbs. was given when more spores were available. Untreated birds were given additional feed on an as-needed basis. At day 28, the birds were placed on a finisher diet in the same manner as described for the grower diet. The finisher diet was given in the same manner as the grower diet. Treated birds were given 30 lbs. of feed, while the untreated birds were given 50 lbs. of feed. Throughout the trial, each diet was placed on top of the previous feed. In other words, the feed was not removed before placing the next diet phase.

A baseline necropsy was taken at day 14 to evaluate the presence of *Clostridium perfringens* before the necrotic enteritis challenge. Five birds from each pen were randomly selected, weighed, scored for NE lesions and had their ceca removed for bacterial analysis. The birds were scored for NE lesions on a scale of 0-4 as described by Prescott *et al.* (1978) with a score of 0 being no lesions present and a score of 4 being a highly necrotized intestinal tract. Ceca samples from each bird were weighed and the appropriate amount of phosphate-buffered saline (PBS) was added to obtain a 10-fold dilution. The samples were then subjected to a serial

dilution in a 10-fold manner to obtain the -2 and -3 dilutions. The -2 and -3 dilutions were then plated in a triplicate onto tryptose sulphite cycloserine (TSC) agar—this is a selective agar that is utilized for the growth and observation of *Clostridium perfringens*. For day 35, a -4 dilution was also plated. The TSC plates were then allowed to incubate in an anaerobic (5% H₂, 5% CO₂, 90% N₂) environment for 48 hours at 40°C. Colony counts were then taken to determine the *Clostridium perfringens* concentration. Pen weight and feed weight were both assessed at the necropsy date.

Following the baseline necropsy at day 14, treatment groups 2 and 3 were subjected to a *Clostridium perfringens* challenge. The challenge model consisted of a 3-day challenge at days 15, 16 and 17. To obtain the challenge solution, a bead from a glycerol stock solution of *Clostridium perfringens* designated C103-99 was plated onto TSA and allowed to incubate overnight at 40°C in an anaerobic environment. An inoculation-loop full of C103-99 was taken from the TSA plate and placed into BHI and allowed to grow overnight at 40°C in an anaerobic environment. One mL of the C103-99/BHI solution was then taken and pipetted into 500 mL of BHI and allowed to grow overnight within the same environmental parameters as described above. To determine the concentration of the *Clostridium perfringens* challenge, 1 mL of the C103-99/ BHI solution was taken and diluted into 9 mL of PBS to obtain a 10-fold dilution. This was repeated until a total of 9 dilutions were obtained. The dilutions were then plated onto TSA in a duplicate manner and allowed to incubate overnight in an anaerobic environment at 40°C. Colony counts were taken and the concentrations were determined. The concentrations for each NE challenge at each

challenge date are detailed in Table 3. An unknown contaminant was observed when determining the concentrations for day 16 and day 17. The contaminant concentrations are listed below Table 3. The role the contaminant played throughout the experiment is unclear.

At day 28, a second necropsy was performed following the same protocol as the baseline necropsy. Pen and feed weights were also assessed at this time. Following feed assessment, the bird's feeders were filled with their respective finisher diets. As mentioned above, treatments 1 and 2 were given a *Bacillus*-treated, finisher diet and treatments 3 and 4 were given a non-treated finisher diet. This diet was administered to the birds until trial termination at day 35. A final necropsy was performed at day 35 following a modified protocol of the previous necropsy dates. All birds were necropsied and were assessed for NE lesions, while only 5 ceca samples were taken from 5 randomly selected birds from each pen (total of 160 samples). As before pen and feed weights were assessed at this time.

Statistical Analysis

Statistical analysis was performed using the generalized linear model (GLM) with means separated via Tukey's HSD with a significance at p-value ≤ 0.05 . Analysis of total *Clostridium perfringens* concentrations was done as well as the incidence of NE lesions within the birds. Feed and bird weights as well as mortality weights by treatment group were also assessed. One of the pens data had to be excluded from analysis as the feed weight; mortality numbers and mortality weight data was lost when the pen sheet was destroyed in the pen before the final necropsy.

Results

Feed Conversion Analysis

Mortality results are presented in Table 4. As can be observed there was no significant difference in mortality when comparing the untreated diets with the *Bacillus* treated diets at days 14 and 35; however there was an increase in mortality on day 28 in treatment groups 2 and 3 compared to treatment groups 1 and 4. This is due to the CP challenge that was administered at days 15-17 and that the probiotic did not offer any protective advantage against mortality. The average bird body weight and analysis for each treatment is shown in Table 5. There was no significant difference in average bird body weight between the treatments at any of the measured times. The results for the AFCR are listed in Table 6. There was no significant difference seen when comparing all of the treatment groups across the experimental timeline. However, at time interval days 14-28, there was a significant difference seen between treatment groups 1 and 3. Unfortunately, comparing treatment 1 (*Bacillus*-diet without a *Clostridium perfringens* challenge) to treatment 3 (untreated diet with a *Clostridium perfringens* challenge) tells us little about the viability of the *Bacillus* isolates as a direct-fed microbial.

Bacterial Analysis and Necrotic Enteritis Lesion Score Analysis

The *Clostridium perfringens* counts obtained for each treatment on days 14 and 35 are shown in Figure 1. For the day 28 samplings, no growth appeared on a majority of the plates across the 2 dilutions tested. Because of the minimal growth on the dilutions, a plus/minus test done on TSC plates from the -1 dilution was performed. All samples were positive for *Clostridium perfringens*, which meant the

bacterium was present. There are several reasons why there was no growth at the higher dilutions. This includes the possibility that the -2 and -3 dilutions were not the correct dilutions and the addition of additional dilutions could have remedied this error. Because of this error the data for day 28 was not included in Figure 1. As can be seen in Figure 1 there was no significant difference in the number of *Clostridium perfringens* across day 14. There was a significant difference in bacterial count when treatment 1 was compared to treatment 4 for day 35. NE lesion score analysis is displayed in Figure 2. There was no significant difference between the treatments and lesion scores at each necropsy date. Moreover, while the present experiment did not incorporate a cocci challenge, there were pens that had the presence of *Eimeria maxima*, which was more than likely in the litter before the arrival of the birds, as the birds were placed on used litter.

Discussion

Necrotic enteritis caused by the *netB* gene in type A *Clostridium perfringens* is a leading cause for concern within the poultry industry. In addition, the concern about antimicrobial resistant bacteria amongst consumers has pushed the industry to look for alternatives to antibiotics. One possible alternative currently being investigated is the use of direct-fed microbial diets utilizing *Bacillus subtilis*. The present study investigated the affect of four isolates of soil origin *Bacillus subtilis* used as a direct-fed microbial on the development and inhibition of type A *Clostridium perfringens* as well as the affect on bird production.

Analysis of the four *Bacillus subtilis* effects on bird production was performed by comparing the adjusted feed conversion (AFCR), mortality and pen weight across

the treatment groups. The results displayed in Table 6 show that there was no significant difference between the treatments for AFCR when comparing the treatments overall. There was a significant difference seen between treatment groups 1 and 3 at time interval day 14-28. However, comparing a treated diet without a necrotic enteritis challenge to a group with an untreated diet with a necrotic enteritis challenge tells little about the viability of the *Bacillus* isolates to in fact inhibit the growth of *Clostridium perfringens*. The increase in necrotic lesions seen between days 14 and 28 was due to the inoculation of the challenged birds with *Clostridium perfringens*. This would explain the significance seen between treatments 1 and 3 at this time interval. There was a 4-point increase in the AFCR for treatment 1 (1.45) when compared to treatment 4 (1.49) for the overall experiment (day 0-35). Comparing a *Bacillus*-treated diet (treatment 1) to the control group (treatment 4) shows that the *Bacillus subtilis* cocktail used in this experiment could have a positive affect on the feed conversions of treated birds. However, this difference seen was not significant overall. It could be possible that increasing the amount of the *Bacillus* cocktail would improve the feed conversion. It could also be used in tandem with anticoccidials to reduce necrotic lesions thereby, increasing feed conversions. Moreover, table 5 reveals that the average bird weight was not significantly different when the treatments were compared. The mortality seen for each treatment was not conclusive that the treated diets enhanced the bird's ability to fight infection (Table 4). Both of the challenged groups (treatments 2 and 3) had marked increases in mortality when compared to the unchallenged groups (treatments 1 and 4). Previous studies conducted have concluded that *B.*

subtilis when used as a direct-fed microbial can improve the production and growth of broilers by improving the normal microbiota environment, which can increase beneficial bacterial numbers such as *Lactobacilli* (Knarreborg *et al.*, 2008). However, many of these *B. subtilis* strains are isolated from the normal microbiota of healthy birds. The present study utilizes four strains of *B. subtilis* that were isolated from a soil environment. The isolates used in this study could have been identified as problematic by the host and eliminated by immune responses; or, these isolates could have been unable to compete for colonization space within the gut of the bird.

There was no significant difference in the bacterial counts for *Clostridium perfringens* for day 14 (see Figure 1). There was a significant difference seen for day 35 between treatments 1 and 4 in *Clostridium perfringens* bacterial counts. Treatment 1 had lower counts when compared to treatment 4. Treatment 1 was given a *Bacillus* treated diet without a *Clostridium perfringens* challenge, while treatment 4 was the untreated and unchallenged control. It is possible that the *Bacillus subtilis* infused diet was able to reduce *Clostridium perfringens* numbers when given to birds that are not artificially challenged with *Clostridium perfringens*. *In vivo* studies in mice, pigs and chickens have all revealed that several probiotic bacteria including *Bacillus subtilis* are capable of colonizing the host gastrointestinal (GI) tract after oral inoculation. Colonization by the probiotic was shown to reduce the colonization and persistence ability of *Clostridium perfringens* and mortality associated with this bacterium (Allaart *et al.*, 2013). It is possible as mentioned above that the *B. subtilis* strains used were unable to compete for colonization space due to the strains being of soil origin. It is also possible that the concentration of the

B. subtilis cocktail was too low to have an effect on *Clostridium perfringens* colony counts. Moreover, experimental error in the plating process could have interfered with the analysis.

The hallmark of necrotic enteritis is the causation of lesions within the small intestine. When analyzing lesion scores in the present study, there was no significant difference or decrease in lesion scores when *B. subtilis* treated groups were compared to untreated groups (see Figure 2). While several studies have showed that *B. subtilis* treated diets can reduce the prevalence of *Clostridium perfringens* in the GI tract of production birds, these results have varied between the isolates of *B. subtilis* used in individual studies (Barbosa *et al.*, 2005; Klose *et al.*, 2010; Teo & Tan, 2005; La Ragione & Woodward, 2003). Moreover, a few of the birds were positive for *Eimeria maxima*, which could have led to an increase in the prevalence of *Clostridium perfringens* in those particular birds and pens. Of all the predisposing factors that lead to the proliferation of type A *Clostridium perfringens*, coccidiosis is the most important. Coccidiosis is an important and common disease in poultry and is caused by the protozoal species *Eimeria*. *Eimeria* infections could coincide with the increased presence of NetB strains of *Clostridium perfringens* and vice versa (Alnassan *et al.*, 2014). The excretion of oocysts and the extent of the lesions typically seen with *Eimeria* infection were supported remarkably by a following *C. perfringens* challenge in both infection models compared with groups infected with either coccidia or *Clostridium perfringens* alone in a study done by Alnassan *et al.* (2014). The overall consensus is that parasites, such as *Eimeria*, and *Clostridium perfringens* work in tandem and show a mutualistic interaction as they

enable one another to increase their numbers in the GI tract as well as enhance their abilities to cause disease (Alnassan *et al.*, 2014). The birds were placed on recycled litter for this trial. The litter used was more than likely contaminated with *Eimeria* before the birds were placed. It might be possible to better test the efficacy of the *Bacillus* cocktail against the colonization of *Clostridium perfringens* if the birds were placed on clean litter that was not contaminated with *Eimeria*.

For the present study, there was no observable effect on production or inhibition of *Clostridium perfringens* by the four isolates of *Bacillus subtilis* used. While there was a significant decrease in bacterial counts between treatment 1 and treatment 4 at trial termination (day 35), there was no significant difference between treatment 2 and treatment 3. It is possible that the concentration of the *B. subtilis* was not high enough to effectively inhabit the GI tract of the birds. The concentration of *Clostridium perfringens* in the challenge groups could have been greater than that of the *B. subtilis* diet and could have out competed the *Bacillus* for colonization space. Increasing the concentration of the *Bacillus* could potentially enhance their colonization ability. However, it could be because these strains of *Bacillus subtilis* are of soil origin and not from the bird's normal microbiota. This might mean that they are not viable as a direct-fed microbial. Future studies could investigate the use of a cocci model and look at the viability of these *Bacillus* strains as a direct-fed microbial against the colonization of cocci species. While this study is not conclusive on the use of *Bacillus subtilis* strains AB01, AP302, AP183 and AP206 as a direct-fed microbial against the inhibition of *Clostridium perfringens*, future research should be done, as other avenues of viability could still be obtained.

Table 5.1. Sporulation preparation media recipe

Item	Amount per Liter
Peptone	3.3 g
Beef Extract Powder	1.0 g
Sodium Chloride (NaCl)	5.0 g
Potassium Phosphate (K ₂ HPO ₄)	2.0 g
Potassium Chloride (KCl)	1.0 g
Magnesium Sulfate Heptahydrate (MgSO ₄ •7H ₂ O)	0.25 g
Manganese Sulfate (MnSO ₄)	0.01 g
Lactose	5.0 g
Agar	18.0 g

Table 5.2. Treatment groups used for duration of experiment

Treatment	Diet/Challenge
Treatment 1	<i>Bacillus</i> -treated diet + non-challenge
Treatment 2	<i>Bacillus</i> -treated diet + <i>Clostridium perfringens</i> challenge
Treatment 3	Non-treated diet + <i>Clostridium perfringens</i> challenge
Treatment 4	CONTROL: non-treated diet + non-challenge

Table 5.3. *Clostridium perfringens* challenge concentration used for the necrotic enteritis 3-day challenge model

Challenge Date	CP103-99 Concentration
Day 15	4.5x10 ⁸
Day 16	2.0x10 ⁸ α
Day 17	1.3x10 ⁷ β

α —unknown contaminant observed at a concentration of 2.28x10⁹

β —unknown contaminant observed at a concentration of 2.24x10⁸

Table 5.4. Average mortality for each treatment across the experiment

Treatment	Day 14 Avg. Mortality	Day 28 Avg. Mortality	Day 35 Avg. Mortality	Overall Avg. Mortality
1	1.140	0.140b	0.000	0.427
2	0.625	4.500a	0.000	1.708
3	0.750	4.000a	0.125	1.625
4	0.875	0.125b	0.125	0.375
Std. Err.	1.004	1.842	0.65	

p ≤ 0.05

ab For days 14-28, there was a significant increase seen when treatments 2 and 3 (designated by the letter “a”) were compared to treatments 1 and 4 (designated by the letter “b”). However, there was no significance for the *Bacillus* treated diet versus that of the untreated diet.

Table 5.5. The average bird weight in kilograms (kg) for each treatment that was assessed for each treatment date as well as overall body weight

Treatment	Day 14 Avg. Body Weight	Day 28 Avg. Body Weight	Day 35 Avg. Body Weight	Overall Average Body Weight
1	0.403	1.463	2.000	1.289
2	0.399	1.451	1.980	1.277
3	0.403	1.374	2.016	1.264
4	0.395	1.389	1.958	1.247
<i>Std. Err.</i>	0.001	0.007	0.009	

p-value < 0.05

Table 5.6. Adjusted feed conversion ratios analyzed for all treatments at and in between the experimental dates

Treatment	0-14	14-28^α	14-35	28-35	0-28	0-35
1	1.25	1.45a	1.56	1.81	1.34	1.45
2	1.23	1.50ab	1.58	1.79	1.35	1.45
3	1.24	1.52b	1.60	1.77	1.38	1.47
4	1.17	1.49ab	1.61	1.87	1.37	1.49
Std. Err.	0.009	0.005	0.003	0.029	0.001	0.002

p-value < 0.05

^α The AFCR for days 14-28 were not significantly different when comparing all treatment groups. However, treatments 1 and 3 were significantly different in between this time interval. Treatment 1 consisted of a *Bacillus* treated diet without a challenge and treatment 3 was an untreated diet group with a *Clostridium* challenge; therefore, the significance between these two groups does not have an overall importance.

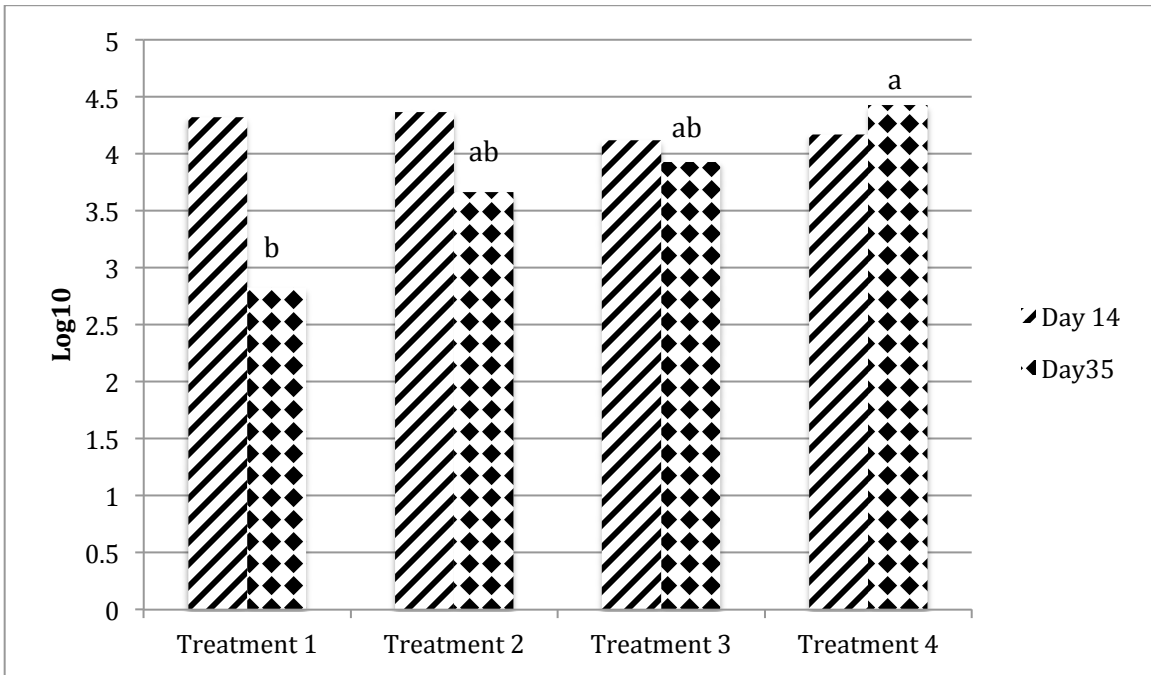


Figure 5.1. *Clostridium perfringens* counts obtained for each treatment on days 14 and 35. Significant differences ($P \leq 0.05$) by day are differentiated by letter differences.

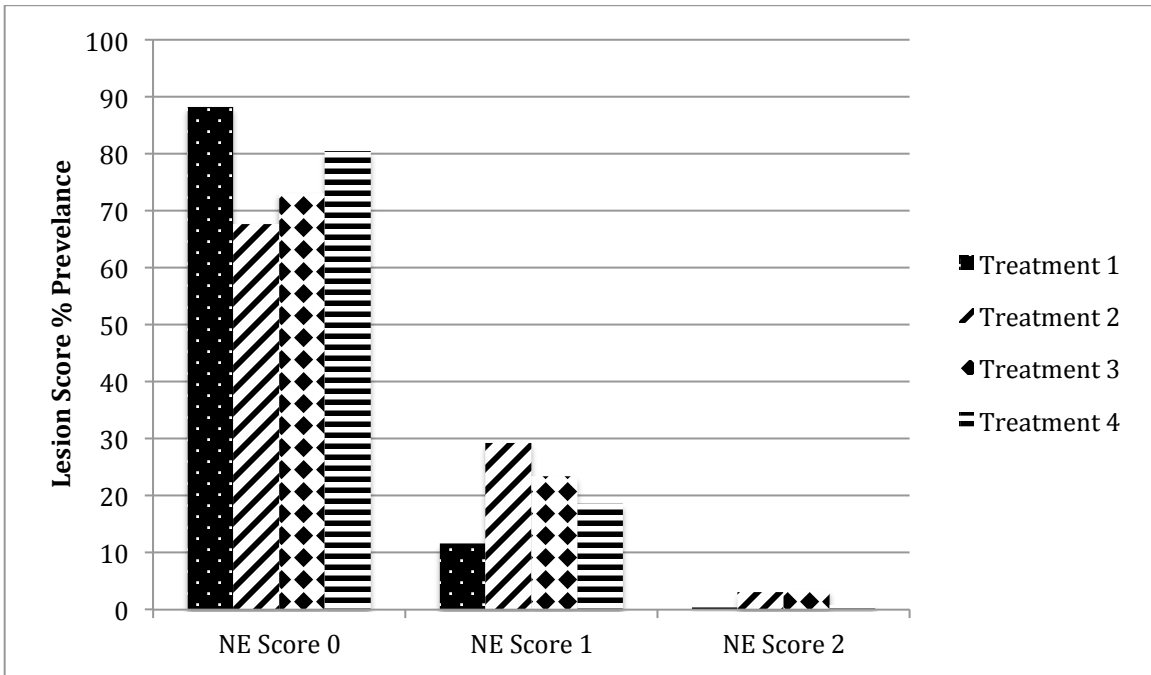


Figure 5.2. Total percentage of NE lesion score by treatment date. There was no difference between the lesion scores for the treatments across the experimental timeline.

Chapter 6:

General Discussion and Conclusions

The data obtained from the current work presented insight into the ability of *Bacillus subtilis* as being a possible candidate for use as direct-fed microbial in production broilers. Direct-fed microbials are thought to be production enhancers utilized to increase the production of broiler flocks as well as to inhibit the growth of pathogenic bacteria. The current study utilized seven isolates of *B. subtilis* designated AB01, AP71, AP206, AP294, AP302, AP183 and AP185. Previous studies have indicated the ability of strains AB01 and AP71 to inhibit the growth of *Salmonella* Enteritidis *in-vitro* (Stough, 2013); the current study also showed the ability of strains AB01, AP302, AP183 and AP206 to inhibit the *in-vitro* growth of *Clostridium perfringens*.

Utilizing this information, bird trials were ran to test the efficacy of the strains of *Bacillus subtilis* as in feed direct-fed microbials. One trial tested the ability of isolates AB01 and AP71 to inhibit the growth of *Salmonella*, while the other tested the ability of strains AB01, AP302, AP183 and AP206 to inhibit the growth of *Clostridium perfringens*. Overall, the treated diets in each trial were unable to significantly enhance production and were also unable to reduce the growth of *Salmonella* Enteritidis and *Clostridium perfringens* in the gastrointestinal tract of broiler flocks. Several errors could account for the *Bacillus* isolates not being able to work effectively as a direct-fed microbial. Most importantly, the spores were more than likely given at a concentration that was too low to allow for successful colonization of the host gastrointestinal tract. It could also be that because the

Bacillus isolates used in this study were of soil origin they were unable to colonize the host intestine.

For the *Clostridium perfringens* trial, it is also possible that the observation of *Eimeria* lesions within several of the birds proliferated the growth of type A *Clostridium perfringens*. Coccidiosis infections coincide with the increased prevalence of necrotic enteritis within the bird intestinal tract. The excretion of oocysts and the extent of the lesions typically seen with *Eimeria* infection were supported remarkably by a following *C perfringens* challenge in both infection models compared with groups infected with either coccidia or *Clostridium perfringens* alone in a study done by Alnassan *et al.* (2014). The birds used in this trial were placed on recycled litter, which was more than likely contaminated with *Eimeria* before the birds were placed. To reduce the incidence of coccidiosis in the birds, the experiment should have utilized clean litter. The ability of the isolates used to decrease the incidence of necrotic enteritis could have been skewed because of the presence of coccidiosis.

In order to remedy the problem of *Bacillus* concentration, it could be possible to alter the sporulation process to better enable the ability to obtain higher spore counts than used in this study. The process used in the current study obtained spore counts that were lower than previously used in other studies. Knap *et al.* (2011) utilized spore counts of 8.0×10^5 CFU/g, while the current study utilized counts of 1.5×10^5 CFU/g and 1.8×10^5 CFU/g for the *Salmonella* study and 2×10^5 spores/gram of feed were mixed into the feed for the *Clostridium perfringens* trial. The sporulation process used in the present study could have also obtained vegetative

cells instead of the needed endospores. Adjusting the protocol of obtaining spores could yield a better quality product; however, it could also reduce the quantity of spores.

The research presented within this thesis provides knowledge about the efficacy of seven strains of *Bacillus subtilis* as direct-fed microbials. While the evidence acquired through these studies offered little support for their use as a probiotic, there is an abundance of support that details the ability of *Bacillus subtilis* to effectively act as a direct-fed microbial. Utilizing the *Bacillus* isolates in tandem with other alternate techniques to antibiotic therapy could prove beneficial to the success of these seven strains as direct-fed microbials. Future research should focus on maximizing the concentrations of spores given in the treated diets all the while controlling other bacterial or parasitic infections.

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