EFFICACY OF DIRECT FED MICROBIAL COMPONENTS COMPARED TO TRADITIONAL FEEDING PROGRAMS AGAINST SALMONELLOSIS AND PERFORMANCE OF TURKEY POULTS

A Thesis

by

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ABSTRACT

The research evaluated in this thesis focuses on *Salmonella* infection in turkeys. In both experiments, poults were randomly placed into pens of four experimental treatment groups. These four treatments groups consisted of a traditional feeding program inclusive of an AGP (positive control, PC), a basal diet (absent of AGP) (negative control, NC), and two additional basal diets consisting of two different brands of commercially available DFMs (both *Bacillus Subtilis* strains) (DFM1 and DFM2, respectively).

The initial experiment focused on examining an alternative inoculation methodology in order to better represent *Salmonella* transmission and introduction into a commercially produced flock. Simultaneously, effects and possible relationship of inoculation period and administration of feed treatments were evaluated. Experiment 1 focused solely on the first six weeks, which is a typical brooding period for commercial turkeys. Experiment 2 focused more heavily on the various feed treatments and their effects on turkey hen performance and impact on *Salmonella* colonization. This second experiment focused on the entire lifespan typical of commercially reared turkey hens, which is approximately 12 weeks.

Data collected included weekly body weights, environmental overshoe gauze booties taken from day of placement through trial terminations, liver and ceca plate counts from 7 through 42 days, jejunum measurements at 12 weeks, feed conversion and mortality. Over the course of both experiments, data collected from litter booties, ceca

colonization, and liver invasion demonstrated evidence of the alternative inoculation model effectiveness. In Experiment 1, significant differences were only observed across treatments in mean body weights and livability. The negative control exhibited significantly lower adjusted livability at 0 to 42 days. In Experiment 2, jejunum morphology samples further indicated successful *Salmonella* colonization and comparable treatment efficacies by increased mucosal barrier protection in PC, DFM1 and DFM2. Mean body weights at 12 weeks in Experiment 2 also indicate a similar relationship with no significant difference in PC and DFM2. These data indicate that the alternative inoculation methodology could be utilized as a means to mimic a field challenge and DFMs can be effective as a potential alternative to AGP in commercial turkey operations.

DEDICATION

I dedicate my work to my husband, Barry Koppen. You are my sounding board, coffee maker, and cheer leader. Thank you for waiting up and making me meals throughout all the late nights and early mornings. I appreciate your inability to say no when asking to read the nth research paper. Thank you for always pushing me to never give up on my dreams and stay the course. Your unwavering love and support has given me the ability to navigate through all the ups and downs with patience and unfailing determination. Thank you; I love you.

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CHAPTER I

INTRODUCTION

There are three main challenges currently facing the poultry industry. These include the need to satisfy market demand, create solutions to reduce human exposure to *Salmonella* foodborne pathogens, and the need to find alternatives for antibiotic growth promoters. According to the National Chicken Council (2016), per capita consumption of poultry has increased from 1960 to 2015 by approximately double. Meanwhile there has also been a change in consumer preferences which desire the identification of commercial rearing practices of products. This new awareness focuses on types of facilities birds are raised in, nutrition fed, and use of antibiotics. Focus has also been directed on the need to reduce Salmonella prevalence in poultry. This comes at a time where many food recalls concerning Salmonella contamination derive from poultry products. These include instances in 2010, where Jennie-O Turkey Store recalled about 55,000 pounds of turkey burgers; followed by 2011, where Cargill Solutions recalled approximately 36 million pounds of ground turkey and more recently, in 2015, Barber and Koch recalled over 2 million pounds of chicken products (USDA, 2016). Additionally, there has been banning and increased restrictive regulations by governments of antibiotic growth promoters in agriculture across the globe due to the increasing incidence of antibiotic resistance illness in humans and animals.

Paired with these challenges are the industry's production goals; to raise high performing birds with increased feed efficiency. A high performing bird is best

achieved by raising a healthy bird from start to finish. This entire process can take over 1 year. It begins with breeder operations where the eggs are produced, to the hatchery, to placement of day old birds, through moving a flock to the larger grower barn, and finally the last day where birds are loaded out and sent to the processing facility. Meeting these goals allow for production companies to yield larger quantities of product at a more economical price for the consumer. In order to accomplish said goals, best management practices, high quality nutrition, and preventative vaccination programs are employed. Management practices include comprehensive biosecurity programs, good husbandry, optimal ventilation, and a clean water supply. Management practices, including those focusing on litter quality can help aid in prevention of diseases and control of bacterial populations housed within. Wet litter can serve as an ideal environment for mold, *Salmonella,* and *Clostridia* bacteria (Larrison, 2009).

Approximately 2,500 serotypes of *Salmonella* have been identified to date (Revolledo et al., 2009). It is estimated that *Salmonella* is responsible for illnesses of 76 million Americans annually (Doyle and Erickson, 2006), or about 300,000 to 4,000,000 foodborne illnesses and approximately, 30.6% of associated deaths (Payne et al., 2007). The majority of foodborne illnesses are linked to meat products, specifically contaminated poultry meat and eggs (Cox et al., 2000). *Salmonella* is commonly found within the intestinal tract of birds. Sources of contamination are vast and vectors for transmission can include the rearing facility, breeder birds or their facility, hatchery, feed, farm workers, rodents, insects, and wild birds (Doyle and Erickson, 2006).

Contamination occurs and is spread via vertical transmission (shell contamination) or horizontal transmission through the fecal-oral route.

Biosecurity programs aim to mitigate the spread of disease or infection by both humans and animals. Rodent control programs, sanitation of barn environments, disposal of mortality, and facility traffic are addressed by biosecurity programs. Biosecurity programs often require personnel to wear specific gear which is changed before going into and out of barns. Footbaths or showers can also be used to provide further protection to birds. Some industry biosecurity practices observe an "all-in, all-out" operation. This is accomplished by limiting the age of birds on a farm site, where all birds at a location are raised to a certain age before all being moved to another facility or sent off to a processing plant before a new set of birds is brought in. In doing so, younger birds with naïve immune systems are offered greater protection by being reared separately from older birds, which are more disease resistant due to a more mature immune system (Larrison, 2009). In the event of illness or disease, and mitigation by management is unsuccessful and a veterinarian consultation results in a prescription, antibiotics will be utilized therapeutically.

Antibiotics have been administered in multiple ways aiding production. In general, there are four ways antibiotics are applied: therapeutic, prophylaxis, metaphylaxis, and sub-therapeutic. Antibiotics used for increasing feed efficiency, performance, and maintaining intestinal health are known as AGPs (antibiotic growth promoters) and are normally administered via poultry feed. Traditional feeding programs typically include AGPs. Early regulations regarding AGPs were set by

European countries (Hume, 2011). Inclusion of AGPs in traditional feeding programs has been standard practice in the United States since 1951, when AGPs were allowed by the FDA (Food and Drug Administration) to be administered via feed without veterinarian prescriptions (Bray et al., 2009). Studies have repeatedly demonstrated AGPs in poultry feed correlating primarily to aiding and maintaining the balance of the intestinal microbiome and secondary functions being increasing feed efficiency (Hume, 2011; Sugiharto, 2014). These are characteristics of AGPs are especially important in a time of increasing feed ingredients' costs and where 75% of production costs are feed.

Antibiotic resistant illnesses and deaths are at an all-time high in the human population. Likewise, antibiotic resistance in poultry has been occurring. Resistance to streptomycin was observed in turkeys shortly after AGPs were approved for use in poultry (Bray et al., 2009). In the 1950's, tetracycline resistance was observed in broilers (Bray et al., 2009). Both of which were used as AGPs in the past. Bacterial resistance to AGPs in poultry has stimulated major concern in the public and human medical communities of a potential zoonotic link of antibiotic resistant bacteria between poultry and humans (Hume, 2011). As a result consumers have put pressure on governments and poultry producers to eliminate use of antibiotics.

In January 2006, Europe completely banned the use of AGPs in poultry feed (Tellez et al., 2011). These restrictions were implemented because of export market restrictions and consumer preferences (Tellez et al., 2011). Restrictions were and continue to be fueled by public concern of increased appearance of antibiotic-resistant bacterial strains, particularly zoonotic pathogens. This controversial theory suggests that

AGPs in poultry are maintaining residual status, consumed by the public, and therefore contributing to the antibiotic resistance epidemic occurring in the human population. This potentiated link has not been successfully proven in any scientific capacity. It has been defended by food animal producers as results of unwarranted and overuse of antibiotics in human medicine; particularly those used as precautionary measures for bacterial infections and during inappropriate times of viral infections in people (Hume, 2011). Regardless, increasing pressure to find antibiotic alternatives for production purposes continues due to growing antibiotic resistant illnesses and deaths in the human population.

Consequently, the US still allows voluntary use of some major AGPs, though the list of approved antibiotics continues to decline. Most recently the US Federal Department of Agriculture (FDA) published the Veterinarian Feed Directive (VFD) which focuses on establishing framework for restriction of therapeutic use of "medically important" antibiotics as needed for specific animal health purposes (Clark and Bailey, 2013). The sub-therapeutic use of antibiotics as an aide to balancing microbiome bacterial populations and growth promotors is quickly disappearing from poultry production. As consumer demands for increased supply of poultry continue to grow, the industry is forced both legally and economically to make necessary adaptations.

Currently, much research is being done to investigate antibiotic alternatives. The need for such efforts is reinforced by requirements to meet the industry primary objectives of raising high performing birds with increased feed efficiency. By removing AGPs from production, the need to support basic gut performance becomes critical.

There are several alternative ingredients being investigated in the poultry industry. One alternative product includes probiotics or direct fed microbials (DFMs) (Wolfenden et al., 2011). DFMs are live beneficial microorganisms that can be supplied to poultry by spray application, drinker systems, or feed.

The purpose for research presented was to evaluate *Salmonella* infection in turkeys. In both experiments poults were randomly placed into four experimental groups consisting of a traditional feeding program inclusive of an AGP (positive control, PC), a basal diet (absent of AGP) (negative control, NC), and two additional basal diets consisting of two different brands of commercially available DFMs (both *Bacillus Subtilis* strains) (DFM1 and DFM2, respectively). The first experiment studied an alternative inoculation methodology in order to better represent *Salmonella* transmission and introduction into a commercially produced flock over the course of six weeks. The second experiment further evaluated these four feed treatments in order to determine their effects on turkey hen performance and impact on *Salmonella* colonization over an entire life-span (12 weeks).

CHAPTER II

REVIEW OF LITERATURE

Salmonella the Organism

Salmonellosis is a zoonotic gastrointestinal disease caused by microorganism genera *Enterobacteriacea*, genus *Salmonella*. Other pathogens in this family include *Escherichia* coli, *Klebsiella*, and *Shigella*. *Salmonella* is a gram-negative rod that is a non-sporeforming facultative anaerobe and is motile by peritrichous flagella (Larrison, 2009). It is a mesophilic microorganism which thrives under the body temperature of host organisms. *Salmonella* are commonly found and distributed within nature, with animals and humans being primary reservoirs. The genus *Salmonella* is categorized into two main species, *Salmonella bongori* and *Salmonella enterica* (Jay et al., 2005). There have been over 2,500 serotypes of *Salmonella* identified and classified (Revolledo et al., 2009). They are classified according to three antigenic categories including flagellar, capsular, and somatic (Larrison, 2009). Serotypes are further divided into five subspecies groups (Larrison, 2009).

For epidemiological purposes, *Salmonella* can be separated into three groups, host adapted serovars, unadapted serovars, and those infectious to humans only (Larrison, 2009). Host adapted serovars are serotypes which have adapted to specific animal hosts including *Salmonella dublin* in cattle, *Salmonella abortus-equi* in equine, and *Salmonella gallinarum* in poultry (Jay et al., 2005). Unadapted serovars have no host preference and as such are found host to a large variety of animal species. These

unadpated serovars are commonly referred to as paratyphoid *Salmonella*. These can be pathogenic for humans and animals, and include most foodborne serovars. Salmonellosis is caused by these unadapted serovars or *Salmonella enterica* serotypes. These are typically found within the intestinal tract of animals (Larrison, 2009). The intestinal tracts of birds, reptiles, farm animals, humans, and occasionally insects have been identified as the primary habitat of *Salmonella spp.* (Jay et al., 2005). Though *Salmonella* is commonly found within the intestinal tract it is often done so asymptomatically. *Salmonella* can be transmitted via two routes, vertically or horizontally. Vertical transmission occurs when *Salmonella* are passed from the mother to her progeny. Horizontal transmission occurs when *Salmonella* are shed in feces, transmitted via vectors, and consumed.

Salmonella grows best in neutral environments with ideal pH levels between 6.6 to 8.2 (Jay et al., 2005). Though it is able to withstand highly acidic environments, it does not proliferate within them. Environments maintaining a pH above 9.0 and below 4.0 can be lethal (Larrison, 2009). *Salmonella* are best suited to an environmental temperature around 37˚C, with 45˚C being the upper limit for growth. Though *Salmonella* can be maintained at a high temperature, they are not heat resistant as pasteurization temperatures are used as a means to eliminate the microorganism from eggs and milk products. *Salmonella* are not considered salinity tolerant, with research indicating brine solutions above 9% bactericidal (Jay et al., 2005).

Salmonellosis in Poultry

Unadapted serovars or paratyphoid *Salmonella* group reside in many animal species including poultry. However, the bacterium is not a native member of the microbiota of poultry (Revolledo et al., 2006). According to Bailey and associates (1988), several factors can affect the susceptibility of chickens to *Salmonella* colonization, including age, stress, general health, feed additives, the genetics of the bird, and others. *Salmonella* serotypes have differing abilities to invade, efficiently colonize the gastrointestinal tract (GIT), or to localize in organs of poultry (Revolledo et al., 2009). An example of this would be *Salmonella* serotype Montevideo which persists in the intestines and is shed in feces for a longer period compared to *Salmonella* serotype Typhimurium (Revolledo et al., 2009).

The paratyphoid group of *Salmonella* has been observed and attributed to illness within young poultry. Turkey poults hatched and reared in commercial production facilities have a reduced opportunity to rapidly develop a diverse and mature intestinal microbiome (Morris et al., 2015). Due to the intensive nature of cleaning and disinfection done in modern-day facilities, the poult is exposed to a limited array of bacteria within their environment. This ironic, modern day conundrum shows producers utilizing rigorous cleaning regimes to limit exposure to pathogenic bacteria, such as Salmonella, and ultimately eliminating exposure to some beneficial bacteria as well. Shortly after hatching, the microbial colonization of the GIT begins, stimulated by the poult eating (Revolledo et al., 2006). Though the GIT is relatively devoid of microbes, there are a small population initially present including facultative anaerobes and

clostridia originating from the parent (Collett, 2013). Bacteria present early on predominate the GIT (this can include pathogens) quickly and any undigested nutrients will fuel their growth in the lower intestine and ceca (Leeson, 2015). The microbial population can develop quickly and often the bacteria present in their surroundings and environment, such as the hatching tray, delivery, and first few days at the farm will dictate early colonization (Leeson, 2015; Collett, 2013).

The microbiome can take up to four weeks to reach maturity in turkeys and bacterial population continues to change as the bird ages (Revolledo et al., 2009). Newly hatched poults are vulnerable and highly susceptible to *Salmonella* colonization due to their naïve microbiome compared to older birds which have had time to develop and establish more of resistant microflora population (Morris et al., 2015; Larrison, 2010). Paratyphoid infections in young birds often result in systemic infections with high mortality (Morris et al., 2015). Clinical symptoms of paratyphoid infections in chickens are generally mild in nature compared to other host specific *Salmonella* or they may be entirely absent, which can lead to difficulty in diagnosing birds at on-set. The ceca have been identified as the primary site of *Salmonella* colonization in poultry (Revolledo et al., 2006). This is due to the equilibrious nature and abundance of nutrient availability.

Adult birds appear more resistant to paratyphoid infections. They can harbor *Salmonella* in soft tissues without showing clinical signs (Revelledo et al., 2009). *Salmonella* have the ability to persist in the host for a few weeks or during all of the rearing period (Revolledo et al., 2006). *Salmonella* become localized in the ceca and can

occur in the upper small intestine, jejunum, ileum, and duodenum, as well as the gizzard and proventriculus (Revolledo et al., 2009). Adult hens that are infected with *Salmonella* serotype Enteritidis (SE) will appear healthy and continue to shed SE in the feces (Larrison, 2009). These feces contaminate the barn environment and can be responsible for increasing the spread and number of infected birds (Revolledo et al., 2006).

Salmonella in poultry are transmitted vertically and horizontally. Paratyphoid *Salmonella* in poultry possess the ability to disseminate extra-intestinally and to invade numerous avian tissues (Revolledo et al., 2009). Horizontal transmission is also known as the fecal-oral route and occurs when *Salmonella* is distributed into the environment from contaminated feces via a vector (other birds, insects, or rodents) and ingested by a bird. *Salmonella* can infect tissues such as the hen's ovaries which can then be transmitted vertically to the progeny. Vertical transmission occurs during the egg laying process through shell contamination, or *Salmonella* may be deposited inside the egg and infect the embryo.

Intestinal Mucosa

The first line of defense against reducing *Salmonella* colonization is the intestinal barriers (Larrison, 2009). Barriers are composed of two layers, the mucus layer and the underlying epithelium. The mucus layer is composed of mucins (mucus) secreted by goblet cells. Both the mucus layer and the underlying epithelium are components of the intestinal mucosal innate immune system (Choct, 2009). Rapid early development of the intestinal epithelium is a prerequisite for normal digestion (Leeson, 2015). The presence

of pathogens can and will delay microvilli development and in return, delay nutrient uptake (Leeson, 2015). According to Yamauchi (2002), the morphological changes of the intestinal villi are dependent on the presence of digested nutrients in the small intestinal lumen. The intestinal epithelium serves as a barrier that prevents uncontrolled passage of partially digested food, bacteria, and bacterial products into the host and also regulates fluid and electrolyte absorption and secretion (Collett, 2013). As colonization occurs, microbes attach to one another and the epithelium to form a tightly adherent mat over the intestinal surface (Collett, 2013).

The small intestinal mucosa is arranged into two fundamental structures: villi and crypts. Villi are finger-like projections into the lumen covered predominantly with mature, absorptive enterocytes, along with occasional mucus-secreting goblet cells (Leeson, 2015). This mucus acts as an important barrier against pathogenic bacterial colonization (Leeson, 2015). Work done by Applegate and associates (1999) reported that mucosal development consists of the increase in the height and density of the villi, which corresponds to an increase in the number of epithelial cells (Larrison, 2009). Longer villi represent a bigger absorption area. Crypts are invaginations of the epithelium around the villi, lined largely with younger epithelial cells, which are involved primarily in secretion (Leeson, 2015). The lamina propria contains capillaries and a central lacteal (lymph vessel) in the small intestine, as well as lymphoid tissue (Rahimi et al., 2009). Lamina propria also contains glands with ducts opening on to the mucosal epithelium that secrete mucus and serous secretion (Revolledo et al., 2006).

It is widely known that feed efficiency is directly dependent on GIT function, specifically intestinal surface area and integrity of the epithelial lining (Collett, 2013). This is directly related to the small intestine, specifically the crypts and villi. These features of the absorptive epithelium are important in nutrient utilization and absorption. Villus height determines the surface area available for digestion and absorption (Laudaido et al., 2012). Reports of anatomical changes of the gut and alterations of villus shape in different species have been published. Intestinal development can be accessed via measurements of the crypt depth where new intestinal cells are formed. Often large crypt depths are indicative of fast tissue turnover and re-growth (Demir et al., 2005). Tissue turnover requires increased nutrients for maintenance. Demir and associates (2005) concluded reduced turnover rates enable epithelial cells utilization of nutrients for lean tissue mass synthesis rather than maintenance.

Studies have shown that improved feed efficiency and body weight gain correspond with increased villus height and surface area in the small intestine (Samanya and Yamauchi, 2002). Longer villi indicate increased surface area for nutrient absorption, and indicate overall gut health because longer villi suggest active cell mitosis and significant enterocyte turnover occurring (Samanya and Yamauchi, 2002). Salmonellosis negatively effects nutrient absorption in the small intestine causing epithelial cell sloughing. The loss of these absorptive enterocytes impairs growth and feed utilization (Laudadio et al., 2012).

Salmonella Interventions

In order to mitigate *Salmonella* contamination of poultry, identifying sources of contamination is crucial. This enables appropriate actions to be developed and implemented (Rigby et al., 1980). To achieve federal and processing plant pathogen-control standards, interest has centered around on-farm pathogen reduction programs to reduce contamination in and on birds entering the processing plant (Payne et al., 2007). This idea centers around the belief that reduced numbers of bacteria coming in the plant will allow fewer bacteria to leave the plant on the final product.

Cecal and intestinal contents have been pinpointed as the primary source(s) of *Salmonella* contamination. So mitigating potential areas of transmission have been targeted including litter, external surfaces and feathers, and processed carcasses after rupture of the intestinal tract during evisceration in processing facilities (Larrison, 2009). Paratyphiod *Salmonella* contamination of carcasses continues to be a potential problem for the poultry industry (Larrison, 2009). Cross contamination has been shown to increase with successive stages of processing such as tray pack or burgers (Larrison, 2009). Poultry integrators have and continue to develop on-farm strategies to control paratyphoid *Salmonella* with the desired result of significantly reducing or altogether eliminating initial populations of the organism from entering the processing environment.

The industry uses a variety of methods to target and control pathogen populations at the production level. Three elements heavily focused on in controlling pathogens are mitigating contamination, reducing microbial growth, and killing pathogens. This

approach is widely applied across the entire production group including feed mills, rearing facilities, hatcheries, live haul, and breeders. In order to mitigate contamination, one must first identify sources of contamination. There are a plethora of potential *Salmonella* transmission vehicles. Feed, water, bedding, pests (rodents, insects, and wild birds), equipment, housing, and personnel are among the top targeted.

Overall farm management is a major factor to help prevent contamination and control pathogen populations of farms. Bailey and colleagues (2001) data showed high recovery rates from boot swabs (12%) and the outside dirt (6.1%) near the entrance doors to the houses, showing how easily movement and cross-contamination can occur and point out the need for an effective foot-bath system. Various sanitation practices can help control contamination. Removing all manure between flocks, cleaning and disinfection of housing and all equipment between flocks, and disinfection of waterlines have become standard operating procedures in recent years. Additionally, designing and adhering to strict bio-security programs include having and continued maintenance of rodent, insect, and wild bird control measures in place. Observing vehicle and personnel sanitation stations at farms and in-between barns will aim to remove possibly contaminated organic materials, disinfect surfaces, and prevent recontamination. Killing of pathogens is best accomplished after the first two steps have been addressed. With sources of contamination identified and a plan in place to reduce microbial growth havens from perpetrating the rearing environment, a reduced pathogen load is left and can be more easily tackled. Though there are many tools which can be used to combat and kill the remaining pathogens, these are not fool proof. One of the tools used by live

production in feed mills include chemicals such as Formaldehyde-based treatments, acids, adding heat by way of pelleting or extruding feed, and irradiation (Doyle and Erickson, 2006).

A variety of intervention practices have been developed and applied to commercial productions to target *Salmonella* reduction at the farm level, including genetic selection of animals resistant to pathogen colonization, effective sanitation practices for the farm and transportation environments, feed or water amendments to reduce pathogen contamination, and animal treatments to reduce pathogen colonization (Doyle and Erickson, 2006). In determining if interventions and strategies are useful they must fulfill 3 criteria: be efficacious, be practical and cost effective, and be safe with no interference on animal growth or development (Doyle and Erickson, 2006). It is unlikely one lone product will be introduced to the market as a means to completely control *Salmonella*, as it a very complex microorganism. In order to combat it, a multi-prong approach is necessary.

Direct Fed Microbials

DFMs, or probiotics, have been studied and utilized for hundreds of years. They date back to the 1400's where reports of the benefits of yogurts were known and utilized by people in the Middle East and Asia (Hume, 2011). One such incidence references yogurt being prescribed to treat diarrhea (Hume, 2011). Bulgarian microbiologist, Stamen Grigorov, was documented touting and promoting yogurt and its' bacterial components contributing to longevity of Bulgarian peasants (Hume, 2011). The term probiotic is proposed to derive from the Greek word "probiotika" meaning "for life"

(Guarner, et al., 2005). Originally, probiotics were described as substances produced by one protozoan which were stimulated by another; and later described as animal feed supplements which had a beneficial effect on the host animal by affecting its' gut flora (Kabir, 2009).

The term probiotic has been redefined multiple times over the last several decades. In 1989, the US Food and Drug Administration (FDA) made the declaration requiring product manufacturers to use the term DFMs rather than probiotic(s) (Hume, 2011). The US National Food Ingredient Association presented DFMs as a source of live naturally occurring microorganisms inclusive of bacteria, fungi and yeast (Rahimi et al., 2009). Guillot defined DFMs as live microorganisms of non-pathogenic and non-toxic nature, which when administered through the digestive route, are favorable to the host's health (Kabir, 2009).

The most studied and frequently used DFMs include *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Enterococcus*, *Lactococcus*, *Streptococcus*, and *Saccharomyces cerevisae* (Salim, et al. 2013). Several studies with DFMs administered to poultry have demonstrated increased weight gain and feed efficiency as well as potential to reduce food safety pathogens (Fritts et al., 2000). In 2004, Hooge and colleagues conducted four trials evaluating effects of C-3102 spores (*Bacillus Subtilis*), for improving broiler performance as an alternative to AGPs. Trial diets included a basal diet with 0.05% inclusion of C-3102 (probiotic ingredient) with no AGP and basal diets as controls (absent of AGPs and probiotic). Trial diets with C-3102 exhibited significantly increased body weight with an average of 2.90% increase and a decreased

feed conversion in 2 of the 3 trials averaging -1.46% (Hooge et al., 2004). Studies have also shown that increased body weight and feed conversion rates demonstrated in birds supplemented with DFMs correlated to increased intestinal surface area. Research has shown that this improvement in performance can correspond to increased villus height, which in turn expands the surface area of the intestine thus enlarging the capacity for absorption of nutrients (Samanya and Yamauchi, 2002).

DFMs have demonstrated several modes of action including the abilities to modulate intestinal microbiota by reducing pH through acid fermentation including lactic acid, hydrogen peroxidase, and acetic acid (Rahimi et al., 2009). Probiotics compete against pathogens for mucosal attachment and nutrients. DFMs have also demonstrated abilities to stimulate the immune system associated with the intestinal tract, or immunomodulation (Kabir, 2009; Sadgehi et al., 2014). They produce bacteriocins, or natural antibodies which kill undesirable bacteria (Rahimi et al., 2009), increase production of short-chain fatty acids (Milbradt et al., 2014), and increase epithelial integrity (Salim et al., 2013). In increasing epithelial integrity, probiotics have demonstrated abilities to regulate mucus synthesis and secretion by goblet cells (Rahimi et al., 2009). Probiotics can also enhance integrity of tight junctions during times of inflammation (Rahimi et al., 2009). Many studies have claimed DFMs reduce epithelial cell apoptosis, and stimulate the intraepithelial lymphocytes (Salim, et al. 2013).

Competitive Exclusion

DFMs have demonstrated potential to compete with pathogens by way of competitive exclusion (CE), or the Nurmi concept (Jay et al., 2005). CE is used to

describe the protective effect of natural or native intestinal bacteria to limit colonization of pathogens (Revolledo et al., 2009). This concept was first demonstrated by Nurmi and Rantala in 1973 with their study inoculating young chicks with the undefined intestinal contents of adult birds (Schleifer, 2015). This process led to the increased resistance of chicks to *Salmonella* colonization (Revolledo, 2009). Nurmi and Rantala (1973) proposed three mechanisms for action of the protective nature of the contents fed to the chicks: physical obstruction of attachment sites, competition for essential nutrients, and production of volatile fatty acids that limit growth of *Salmonella* and other bacteria.

Research surrounding CE and use of undefined cultures continued throughout to the 1990's and expanded to include *Campylobacter* (Schleifer, 2015). Commercial products were even introduced into the poultry industry but later removed due to the Food Drug Association (FDA) refusing to grant label claim status due to the potential safety risks (Schleifer, 2015). This led to the next phase of discovery which focused on efficacious bacterial components of the undefined products. DFMs are often divided into two classes based on stability, heat labile and heat stabile. Heat labile products are often restricted to use in drinking water and spray application on newly hatched poults. These usually consist of *Lactobacillus, Bifidobacterium spp., Pediococcus, Enterococcus, and Proprionibacteria* (Schleifer, 2015). Heat stable DFMs are usually spore-formers and derived from *Bacillus spp*. These have been tested to demonstrate resistance to heat and pressure of feed manufacturing and predominantly consist of the *Bacillus subtilis* strain (Schleifer, 2015).

Several studies have reported reduction of *Salmonella* colonization in young poultry with oral inoculation of probiotic cultures (Grimes et al., 2008). Soerjadi and fellow researchers (1982) demonstrated that *Salmonella* most readily adhere to intestinal mucosa when there is an absence of other microflora (Schleifer, 2015). Rahimi et al. (2009) determined obstruction of physical attachment of pathogenic bacteria by production of increased amounts of mucoid glycocalyx and reduction of intestinal tissue by feeding *Bacillus subtilis* C-3102 to turkey poults (Schleifer, 2015). Research has also illustrated an increase in the amount of lactobacilli populations after feeding of C-3102 (Schleifer, 2015). It is noted that *Bacillus subtilis* similar to *Salmonella* is not an intestinal organism and strict aerobe (Maruta et al, 1995). As such, it is unable to proliferate or metabolize in the GIT and therefore must be continuously fed in order to persist (Maruta et al, 1995). Maruta and associates (1995) also indicate that by feeding *B. subtilis*, the organism is able to exist in the GIT and thereby influence existing conditions.

Conclusion

Salmonella is a highly complex microorganism. It has been the source of many recent poultry product recalls and continues to contribute to a high prevalence of illnesses within the human population. Originally *Salmonella* was targeted at the processing plant; focus has now expanded to include on-farm interventions. Researchers and industry personnel continue to seek out ways to combat Salmonella. A variety of intervention practices have been developed to target Salmonella reduction at the farm level, including genetic selection of animals resistant to pathogen colonization, effective

sanitation practices for farm and transportation environments, feed or water amendments to reduce pathogen contamination, and animal treatments to reduce pathogen colonization (Doyle and Erickson, 2006). Feed amendments, including probiotics is one area of study which particular emphasis has been focused. Researchers have demonstrated abilities of probiotics to modulate a host's gut flora and reduce food safety pathogens similar to AGPs. Additionally, there is also potential that probiotics like AGPs can minimize or counteract the negative effects of Salmonella which succeed in colonization. The research in this thesis evaluates an alternative inoculation methodology and the efficacy of two direct fed microbial components compared to a traditional AGP component against Salmonellosis and performance of turkey hens.

CHAPTER III

EVALUATION OF ALTERNATIVE *SALMONELLA* INOCULATION METHODOLOGY

Introduction

Food safety is rapidly becoming a major issue for the poultry industry. Each year, an estimated 76 million Americans become ill from consuming foods contaminated with pathogenic microbes and their toxins (Larrison, 2009). Poultry product contamination poses both human and financial risks (Hayes, 2000). The poultry industry is largely concerned with *Salmonella* as it is commonly found on raw poultry products and has been the source of major food recalls in recent years. Paratyphoid *Salmonella* is a pathogen of main concern due to the fact that isolations from humans in the U.S. have risen steadily since 1955 and a fourfold increase accrued in the 1990s (Center for Disease Control, 1992).

To successfully meet federal and processing plant pathogen-control standards, the need for implementation of on-farm pathogen reduction programs to reduce contamination loads in and on birds entering the processing plant has increased (Payne et al., 2007). The goal is to reduce bacterial numbers entering the processing plant, therefore eliminating the potential human risk of consumption of contaminated product. In order to determine what processes and products serve to reduce *Salmonella* colonization within turkeys, a need to identify an alternative challenge model is of upmost importance. Typical methodology consists of orally gavaging day old poults

with a *Salmonella* inoculum. While this has proven to successfully colonize birds, it does not closely mimic the fecal-oral transmission which typically occurs under normal field conditions. Turkeys are very curious and copraphagic animals. They will often peck at their environment as a means of familiarizing themselves with their surroundings. It is important to simulate as close to normal conditions in a challenge model thus enabling possible program efficacies in a commercial setting to be best determined. Very few studies have been conducted utilizing alternative challenge models.

By manipulating environmental conditions and relying on innate behavioral traits often associated with turkeys, a *Salmonella* challenge model was introduced to day-old poults via spraying down bedding with a *Salmonella* inoculum prior to poult placement in pens. The objective of this experiment was to study an alternative inoculation methodology in order to determine if a better representative model of *Salmonella* transmission and introduction into a commercially produced flock could be mimicked.

Materials and Methodology

Animals

Four thousand one-day-old commercial turkey hen poults (Hybrid Converters) were obtained from a private hatchery and housed in a private research facility. Poults underwent fourth toe removal, three toe microwave, and beak treatment services performed at the hatchery. To ensure poults were not contaminated with any *Salmonella spp*., 40 birds were euthanized using a carbon dioxide chamber upon arrival to farm site. The livers and ceca of these birds were aseptically removed, enriched with tetrithionate

broth (mixed with iodine and brilliant green), and incubated for 24 hours at 42˚C. Samples were streaked on to XLT4 agar and incubated overnight. No *Salmonella spp*. was recovered.

Poults were randomized and homogenized in boxes. Birds were weighed as a group by pen and weights documented. After, 50 poults from each pen were chosen at random to be tagged and weighed individually. Poults were brooded in floor pens (8 X 16 square feet) for six weeks. Husbandry practices were adapted according to commercial management guidelines provided by Hybrid (Hendrix Genetics). The birds were brooded following standard temperature regimes with room temperature being 32˚C to 34˚C the first week and gradually decreasing by approximately 2˚C each week.

Experimental Design and Treatments

A completely randomized design with four dietary treatments (3,960 birds total), 165 birds in each treatment was utilized, as follows: a traditional feeding program with an antibiotic growth promoter at 1lb/ton or 0.05% of the complete feed (positive control diet, PC), basal diet without antibiotic growth promoter (AGP) (negative control, NC), basal diet free of antibiotic growth promoter supplemented with *Bacillus Subtilis*, with an inclusion level of 1 lb/ton or 0.05% (DFM 1), and a basal diet free of AGP supplemented with a different commercial *Bacillus Subtilis* component at 1lb/ton or 0.05% (DFM 2). The AGP and DFM components were all included at levels recommended by their respective product manufacturers.

All the birds were fed a corn-soybean meal-based mash diet formulated to meet or exceed all of the National Research Council (1994) requirements for turkeys. Birds

were fed a multi-phase diet transitioning as they progressed in age, starter (0 to 3 weeks) and grower (4 to 6 weeks). All feed was manufactured at a local commercial feed mill. Water and feed were supplied *ad libitum*.

Biosecurity procedures were maintained between each treatment group. The pens were kept separated by a solid barrier while in the brood stage. A more intensive biosecurity procedure was followed including donning clean coveralls between each set of treatments. New plastic boots were to be worn on top of work boots as well as a new pair of Nitrile gloves and both changed upon entry into each pen.

Challenge Inoculum Preparation & Application

Salmonella used for these experiments was isolated from a poultry facility. It was serotyped as *Salmonella* ser. Schwarzengrund and prepared for use as a litter inoculum. It was selected for Nalidixic Acid (Nal) resistance through successive passage on XLT4 Agar containing various levels of Nal (1:100, 1:75, 1:50, 1:25, 1:10, 1:2, and 1:1). This process was adapted using the methodology presented in J. Bauer-Garland and associates work (2006). The challenge inoculum consisted of *Salmonella* cultures grown on Brain Heart Infusion Broth at 42˚C for 24 hours. Serial Dilutions in PBS (Peptone Buffered Solution) were used to determine the number of colony forming units (CFU). All bacterial counts were performed by plating 0.1mL of the culture and the serial dilutions (PBS) in duplicates on XLT4 (+Nal). These plates were incubated for 42˚C for 24 hours. The inoculum was transported to the research facility 15 minutes after removing it from the incubator. The inoculum (165 mL/pen) was sprayed directly on to fresh bagged pine shavings (used for bedding) upon arrival to research facility and

prior to placing poults into the pens, and containing a Nalixidic Acid resistant *Salmonella* Schwarzengrund with a concentration of 9.27 Log 10 CFU.

Facility & Equipment Monitoring

The facility and equipment were subjected to a vigorous cleaning and disinfecting regime. This regime consisted of scraping and removing of litter, sweeping of any leftover debris, blowing down any dust, pre-wash with a pressure sprayer, washing with commercial barn soap, disinfection with an aldehyde-based product, disinfection of waterlines with a peroxide-based product, and fumigation with a peroxide-based disinfectant. Before proceeding to a subsequent step in the cleaning process, sampling was conducted to confirm absence of *Salmonella spp*. If *Salmonella spp.* presence was confirmed, the previous cleaning step was repeated followed by additional sampling. Down-time between completion of washing and disinfection steps ranged between 12 to 48 hours, depending on moisture in the barn and sampling results. Simultaneously, each step of the cleaning process was accompanied by a similarly rigorous sampling protocol with 2 to 5 pre-moistened gauze swabs (BPW, Buffered Peptone Water) taken from each of the following areas of the barn walls, doors, fencing, scales, vents, ledges, drinkers, feeders, and floor. Swabs were used to vigorously wipe an area of 0.5 to 1.0 in^2 and then placed in sterile Whirl-pac bags filled with 225 mL BPW, kept on ice, and then transported to a private 3rd party lab within 6 to 12 hours. All results were negative for *Salmonella spp*.

Performance Monitoring

During the experimental period, feed intake was recorded per pen and during each feed phase. Individual BW (body weight) of 50 tagged birds was recorded weekly for six weeks to calculate daily BW gain (g/bird per day). Feed conversion ratio (FCR) was calculated on a per pen basis. Mortality was monitored, weighed, and documented daily along with being characterized by a licensed veterinarian.

Sample Collection

Each week booties samples were collected to analyze *Salmonella* litter contamination levels for each pen. Post-weighing, seven birds were randomly selected (non-tagged) from each pen and humanely euthanized by a carbon dioxide chamber (42 birds per treatment) weekly. Birds were necropsied and livers and ceca were aseptically removed. Due to the large volume of birds being collected, liver samples were pooled on a per pen basis as were ceca. Additionally, 100 gram feed samples were collected from each pen container weekly. These samples were composited and sent off for laboratory analysis in order to validate DFM presence and inclusion rates as well as formaldehyde treatment presence and inclusion rates. Additionally, *Salmonella spp.* presence was also checked and submitted for serotyping, if detected. No *Salmonella spp*. was detected in the feed.

Litter Bootie Samples

Litter booties collection was performed using an adaptation of the overshoe method, which was first described by Aho (1992). Litter bootie kits were made similar to those utilized and described by Aho (1992). Kits contained two 1.5 inch thick strips

of dry sterile surgical gauze overshoes contained in a sterile Whirl-pac sample bag, a pair of clean Nitrile gloves, and two clean plastic boots. One kit was utilized for each pen. Rubber boots were sanitized prior to entrance into barn followed by applying alcohol hand gel to hands. Once door to pen has been unlocked and in the open position, Nitrile gloves were donned. Upon entering into an individual pen, plastic boots were placed over rubber boots. Once inside the pen, overshoe booties were put on and worn over plastic boots. Litter booties were collected pre-inoculation and post-inoculation each experimental round. Booties were also collected weekly for 6 weeks. These booties were submitted to a third party contracted laboratory and underwent enrichment by using a combination of tetrithionate broth, brilliant green, and iodine. Following such, the liquid enrichment solution was utilized in order to run qPCR (real-time polymerase chain reaction). Procedures similar to those described by Chalghoumi and associates (2009) were followed. The PCR machine subsequently utilized these results to compute an estimated number of colony forming units via a pre-determined regression curve. Colony forming units per bootie were recorded on a per pen basis. The remaining enrichment solution and booties were incubated for 24 hours at 42˚ and retested for *Salmonella* presence. All positive samples were submitted for serotyping. All positive samples confirmed qPCR incidence of Salmonella. All serotypes were identified as *Salmonella* Schwarzengrund.

Liver Colony Counts

Livers were pooled on a per pen basis (7) and placed in sterile filtered Whirl-pac bags. Livers were weighed and diluted at a 1:10 with BPS. They were homogenized

and stomached for 30 seconds; followed by serial dilution. After, 0.1mL from each serial dilution vial was plated on XLT4 (+Nal) agar using spread plated methodology and incubated for 24 hours at 42˚C. Colonies were enumerated and recorded. Organ bags were then submitted to a third party laboratory for further testing of *Salmonella* presence and serotyped (if positive).

Cecal Colony Counts

Ceca were pooled on a per pen basis (7) and placed in sterile filtered Whirl-pac bags. Ceca were weighed and diluted at a 1:10 with BPS. They were then homogenized and stomached for 30 seconds; followed by serial dilution. After, 0.1mL from each serial dilution tube were plated on Nal Resistant XLT4 agar using spread plated methodology and incubated for 24 hours at 42˚C. Colonies were enumerated and recorded. Organ bags were then submitted to a third party laboratory for further testing of *Salmonella* presence and serotyped (if positive).

Statistics

Statistical analysis was performed using JMP software. Live performance data from dietary treatments was analyzed using randomized complete block design with 4 treatments and 6 replications per treatment. Feed Conversion Ratios were adjusted for normal mortality as well as mortality accrued euthanizing birds for sampling purposes. Livability was also adjusted to omit birds euthanized for sampling. All *Salmonella* counts were log transformed to base 10 logarithm before analysis for purposes of normalization. Analysis of variance was completed and means separated by Tukey's multiple comparison test where $p \leq 0.05$. Pen is the experimental unit.

Results

The litter bootie results for Experiment 1 (**TABLE 1**) showed an increasing trend in mean log 10 values from day 7 through 28 days for all treatments. After 28 days of age, there was a numerical drop or decreasing trend seen in mean log 10 values across all treatments through day 42. There were no statistically significant differences across any of the treatments throughout the entire 42 day experimental period.

Plate counts (**TABLE 2**) for mean log 10 *Salmonella* invasion of the liver were elevated during the first seven days with a graduation reduction or decreasing trend observed over the 42 day period across all treatments. However, PC and DFM1 exhibited a temporary numerical increase at 28 days. There were no statistically significant differences observed in liver invasion throughout the entire six week period.

Plate counts for ceca mean log 10 *Salmonell*a colonization (**TABLE 2**) were also elevated during the initial sampling at 7 days of age with a gradual reduction starting at 14 days of age. However, only NC exhibited a slight numerical increase in plate counts at 28 days of age. There were no statistical significant differences observed in ceca colonization or liver invasion levels of any treatments during the entire six week timeframe.

TABLE 1. Normalized mean log 10 colonization of litter booties 7 to 42 days of turkey hen poults environmentally challenged with *Salmonella* Schwarzengrund * inoculum and continuously fed diets supplemented with an antibiotic growth promotors, direct fed microbials, or neither starting at day of placement through 42 days.

	Mean Log 10/g Litter Booties Colony Plate Counts					
Treatment	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
PC[1]	1.85	3.07	3.42	5.3	3.41	2.85
NC[2]	1.51	3.53	3.72	5.25	3.58	3.31
DFM1[3]	2.42	2.75	2.83	5.23	4.21	2.95
DFM2[4]	0.93	2.11	2.29	5.25	4.08	2.15
n	24	24	24	24	24	24
Pooled SEM	0.78	0.35	0.52	0.27	0.26	0.59
P-value	0.6752	0.1013	0.2616	0.9983	0.1395	0.5997

1 Positive Control Diet formulated with a 0.05% inclusion of a standard AGP

2 Negative Control (Basal Diet)

3 Basal Diet formulated with a 0.05% inclusion of DFM 1 (*Bacillus Subtilis*)

4 Basal Diet formulation with a 0.05% inclusion rate of DFM 2 *(Bacillus Subtilis*)

* Salmonella inoculum was concentrated to 9.256 Log 10 CFU

TABLE 2. Normalized mean log 10 colonization of ceca and liver invasion 7 to 42 days of turkey hen poults environmentally challenged with *Salmonella* Schwarzengrund inoculum * and continuously fed diets supplemented with an antibiotic growth promoter, direct fed microbials, or neither starting at day of placement through 42 days.

		Mean Log 10/g Ceca and Liver Colony Plate Counts					
Treatment	Sample	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
PC[1]	Liver	1.274	0.378	0.257	0.381	0.000	0.000
	Ceca	4.386	2.681	1.390	0.551	0.667	0.167
NC[2]	Liver	1.466	0.000	0.000	0.000	0.000	0.000
	Ceca	3.617	2.184	0.566	1.143	0.167	0.000
DFM1[3]	Liver	2.432	1.262	0.000	0.000	0.000	0.000
	Ceca	3.691	3.185	1.008	0.400	0.360	0.167
DFM2[4]	Liver	2.641	0.411	0.000	0.340	0.000	0.000
	Ceca	3.590	2.750	1.336	0.887	0.167	0.167
n	Liver	168	168	168	168	168	168
	Ceca	168	168	168	168	168	168
Pooled SEM	Liver	0.790	0.360	0.260	0.180	0.000	0.000
	Ceca	0.290	0.480	0.420	0.340	0.900	0.130
P-value	Liver	0.693	0.230	0.413	0.581	1.000	1.000
	Ceca	0.221	0.626	0.524	0.505	0.426	0.801

[1] Positive Control Diet formulated with a 0.05% inclusion of a standard AGP

[2] Negative Control (Basal Diet)

[3] Basal Diet formulated with a 0.05% inclusion of DFM 1 (*Bacillus Subtilis* strain)

[4] Basal Diet formulation with a 0.05% inclusion rate of DFM 2 *(Bacillus Subtilis* strain)

* Salmonella inoculum was concentrated to 9.256 Log 10 CFU

Mean weekly weights exhibited a steady increase over the entire experimental period (**TABLE 3**). Days 0, 14, and 28 showed no significant differences in any of the treatments. On day 7, DFM1 exhibited the heaviest birds with a mean weight of 0.118 kg/bird. The DFM1 treatment was significantly heavier than PC, means were separated through Tukey's HSD with a p-value of 0.0322 exhibited. On day 21, PC and NC exhibited the highest weights of all treatments at 0.440 and 0.442 kg/bird respectively. Treatments PC and NC were significantly different than DFM2. Means were separated via Tukey's HSD with p-values of 0.0009 and 0.0003, respectively. On day 35, PC demonstrated significantly higher mean weights compared to DFM1 and DFM2. Means were compared by Tukey's HSD and resulted in p-values of 0.0224 and < 0.0001, respectively. The NC treatment also demonstrated significantly higher mean weights compared to DFM2. Mean comparison by Tukey's HSD showed a p-value of 0.0001. On day 42, PC, NC, and DFM2 were all significantly different from DFM1. Mean comparison via Tukey's HSD exhibited p-values of < 0.0001, 0.0340, and 0.0211, respectively. The DFM1 treatment had significantly lighter birds with mean weights of 1.792 kg/bird.

TABLE 3. Mean body weights in kilograms from initial placement to 42 days of turkey hen poults environmentally challenged with *Salmonella* Schwarzengrund inoculum * and continuously fed diets supplemented with an antibiotic growth promoter, direct fed microbials, or neither starting at day of placement through 42 days.

	Mean Body Weights (kg/bird)						
Treatment	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
PC[1]	0.056	0.114 ^{B}	0.233	0.440 ^{A}	0.753	1.220 ^{A}	1.842 ^{A}
NC[2]	0.057	0.115 ^{AB}	0.232	0.442 ^{A}	0.753	1.216 ^{AB}	1.792 ^{A}
DFM1[3]	0.062	0.118 ^{A}	0.235	0.432 ^{AB}	0.739	1.179 ^{BC}	1.733 ^{B}
DFM2[4]	0.058	0.115 ^{AB}	0.236	0.416 ^{B}	0.744	1.152 ^{C}	1.792 ^{A}
n	1200	1095	1081	1078	1075	1076	1067
Pooled SEM	0.003	0.003	0.023	0.010	0.016	0.023	0.033
P-value	0.3908	0.0355	0.6841	0.0001	0.5117	< 0.0001	< 0.0001

[1] Positive Control Diet formulated with 0.05% inclusion of a standard AGP

[2] Negative Control (Basal Diet)

[3] Basal Diet formulated with 0.05% inclusion of DFM 1 (*Bacillus Subtilis*)

[4] Basal Diet formulation with 0.05% inclusion rate of DFM 2 *(Bacillus Subtilis*)

A, AB, B, BC Means within a column and treatment group with different superscripts differ significantly ($P \leq 0.05$)

* Salmonella inoculum was concentrated to 9.256 Log 10 CFU

TABLE 4. Mean adjusted livability** for 0 to 42 days of turkey hen poults environmentally challenged with *Salmonella* Schwarzengrund inoculum * and continuously fed diets supplemented with antibiotic growth promoter, direct fed microbial, or neither starting at day of placement through 42 days.

	Adjusted Livability
Treatment	Days 0-42
PC[1]	94.39% [AB]
NC[2]	91.35% [B]
DFM1[3]	94.37% [AB]
DFM2[4]	95.42% [A]
n	24
Pooled SEM	0.64
P-value	0.016

[1]	Positive Control Diet formulated with 0.05% inclusion of a standard AGP
[2]	Negative Control (Basal Diet)
[3]	Basal Diet formulated with 0.05% inclusion of DFM 1 (*Bacillus Subtilis*)
[4]	Basal Diet formulation with 0.05% inclusion rate of DFM 2 *(Bacillus Subtilis*)
A, AB, B	Means within a column and treatment group with different superscripts differ significantly ($P \leq 0.05$)
*	Salmonella inoculum was concentrated to 9.256 Log 10 CFU
**	Adjusted for birds euthanized for sampling

Livability was adjusted to account for mortality from birds euthanized for sampling purposes (**TABLE 4**). For the total adjusted mortality for 0 to 42 days significant differences were only observed in NC and DFM2. Means were compared and separated with Tukey's HSD and a p-value of 0.0132 was calculated.

Feed Conversion Ratio (FCR) was adjusted on a per pen basis to account for mortality and birds euthanized for sampling purposes (**TABLE 5**). For 0 to 42 days

adjusted FCR, there were some slight numerical differences across treatments but nothing of significance.

TABLE 5. Mean adjusted feed conversion rates** 0 to 42 days of turkey hen poults environmentally challenged with *Salmonella* Schwarzengrund inoculum * and continuously fed diets supplemented with an antibiotic growth promoter, direct fed microbials, or neither starting at day of placement through 42 days.

	Adjusted feed conversion rates (FCR)
Treatment	Days 0-42
PC[1]	1.79
NC[2]	1.80
DFM1[3]	1.77
DFM2[4]	1.69
n	24
Pooled SEM	0.04
P-value	0.2638

[1] Positive Control Diet formulated with 0.05% inclusion of a standard AGP

[2] Negative Control (Basal Diet)

[3] Basal Diet formulated with a 0.05% inclusion of DFM 1 (*Bacillus Subtilis*)

[4] Basal Diet formulation with 0.05% inclusion rate of DFM 2 *(Bacillus Subtilis*)

[*] Salmonella inoculum was concentrated to 9.256 Log 10 CFU

[**] Adjusted for birds euthanized for sampling and normal mortality.

Discussion

There were significant differences observed in the adjusted livability metrics, with NC having the lowest livability, which could be due to lack of additives in this feed. The NC treatment was the only basal feed without any addition of either AGP or DFM components. This agrees with research conducted within the industry that the possible lack of protection offered by the addition of AGP and DFM products could impact the ability for birds to be able survive as well as the others, especially with the challenge being administered during such a vulnerable life stage.

Overall, the data provides significant evidence that the inoculation methodology applied and utilized for the challenge model was effective. Each treatment observed colonization within the key intestinal microbiome developmental window, approximately 0 to 4 weeks (Revolledo et al., 2009). Additionally, all *Salmonella* recovered and isolated from litter booties, liver invasion, and ceca colonization samples were confirmed as only being the strain utilized. Though the treatments did not appear to negatively impact any results of *Salmonella* colonization, it would be ideal to experiment with this inoculation model in a trial with a greater number of replications and only a positive and negative control used as feed treatments. Research by Milbradt and fellow scientists (2014) also suggests that ceca and internal organ colonization might not be affected by AGP or DFMs, if infection pressure is high.

Also, the separation of each bird's ceca and liver rather than collective pooling of each pen would better help to determine the individual nature of each bird. By separating the ceca and liver out, one would be able to determine the percentage or

incidence of the birds within a population that are actively infected as well as shedding. Another type of measurement which might assist in determining infection would be weighing the individual spleens of each euthanized bird. Research indicates that a heavier spleen can be associated with inflammation resulting from infection (Revolledo et al, 2009). Lastly, expanding the investigation by studying various individual and combinations of *Salmonella* serotypes would be of great value. Since different strains of *Salmonella* are noted to behavior differently and exhibit various growth patterns this would allow for observation of competition between beneficial bacteria and *Salmonella* as well as possible competition amongst the *Salmonella* strains as the bird ages. So while the alternative methodology data presented appears effective, more work needs to be done in further evaluating the effectiveness across various serotypes of *Salmonella*.

CHAPTER IV

EVALUATING EFFECTS OF DFM AND AGP ON SALMONELLA COLONIZATION AND TURKEY HEN PERFORMANCE

Introduction

Over the past several decades, AGPs have been used to improve performance in commercial poultry production by reducing the burden of pathogens in the gastrointestinal tract. Bacitracin Methylene Disalicylate (BMD) and Flavomycin (Flavo) are two AGPs which have been consistently used in poultry diets at sub-therapeutic levels since their approval for increased gain and reduced feed conversion. These antibiotics may also prevent the occurrence of the bacterial infection and necrotic enteritis.

Consumer pressure has forced the poultry industry worldwide to examine pathogen resistance from using AGP feed additives on a continuous basis for prophylactic prevention of disease and improved performance. Concerns derive from antimicrobial resistance to antibiotics used in animal feeds and the controversial hypothesized link to microbial resistance in human medicine. With the trend of antibiotic resistant disease outbreaks ever rising, this unproven hypothesis is becoming increasingly scrutinized. This has led to the ban of antibiotic growth promoters by the European Union in 2006. In addition to the continuous decrease of AGP availability for use at sub-therapeutic levels in the United States, which most recently was pushed further by the establishment of the VFD.

The objective of this experiment was to utilize the alternative challenge model tested in the first experiment and further evaluate use of traditional feeding programs inclusive of an AGP, a basal diet without any additives (absent of AGPs), and two additional basal diets each inclusive of individual DFM commercial components (both *Bacillus subtilis*) and determine their effects on turkey hen performance and impact on *Salmonella* colonization over their entire lifetime (12 weeks).

Materials and Methodology

Animals

Four thousand one-day-old commercial turkey hen poults (Hybrid Converters) were obtained from a private hatchery and housed in a private research facility. Poults had fourth toe removal and three toe microwave and beak treatment services performed at the hatchery. To ensure poults were not contaminated with any *Salmonella spp.,* 40 birds were euthanized using a Carbon Dioxide Chamber upon arrival to farm site. The livers and ceca of these birds were aseptically removed, enriched with tetrithionate broth (mixed with iodine & brilliant green), and incubated for 24 hours at 42˚C. Samples were streaked on to XLT4 agar and incubated overnight. No *Salmonella spp.* was recovered.

Poults were randomized and homogenized in boxes. Birds were weighed as a group by pen and weights documented. After fifty poults from each pen were chosen at random to be tagged and weighed individually. Poults were brooded in floor pens (8 X 16 square feet) for five weeks. Birds were moved to grower floor pens (16 x 16 square feet) at 35 days and remained there for seven weeks. Birds were reared on new wood pine shavings in both the brood and grow floor pens. Birds were reared on a 24 hour

lighting schedule. Husbandry practices were adapted according to commercial management guidelines provided by Hybrid (Hendrix Genetics). The birds were brooded following standard temperature regimes with room temperature being 32˚C to 34˚C the first week and gradually decreasing by approximately 2˚C each week.

Experimental Design and Treatments

A completely randomized design with four dietary treatments (4,000 birds total) 165 birds in each treatment was utilized, as follows: a traditional feeding program with an antibiotic growth promoter at 1lb/ton or 0.05% of the complete feed (positive control diet, PC), basal diet without antibiotic growth promoter (AGP) (negative control, NC), basal diet absent of AGP supplemented with a *Bacillus Subtilis* component with an inclusion level of 1 lb/ton or 0.05% (DFM1), and a basal diet absent of AGP supplemented with a different commercial *Bacillus Subtilis* component, with an inclusion of 1lb/ton or 0.05% (DFM2). The AGP and DFM components were all included at levels recommended by their respective product manufacturers.

All the birds were fed a corn-soybean meal-based mash diet formulated to meet or exceed all of the National Research Council (1994) requirements for turkeys. Birds were fed a multi-phase diet transitioning as they progressed in age, starter (0 to 3 weeks), grower (4 to 6 weeks), developer (7 to 9 weeks), and finisher (9 to 12 weeks). All feed was manufactured at a local commercial feed mill. Water and feed were supplied *ad libitum*.

Biosecurity procedures were maintained between each treatment group. The pens were kept separated by a solid barrier while in the brood stage. A more intensive

biosecurity procedure was followed including donning clean coveralls between each set of treatments. New plastic boots were to be worn on top of work boots as well as a new pair of Nitrile gloves and changed upon entry into each pen.

Challenge Inoculum Preparation & Application

The *Salmonella* strain used for these experiments was isolated from a poultry facility and prepared for use as a litter inoculum. It was serotyped as *Salmonella* ser. Schwarzengrund and prepared for use as a litter inoculum. It was selected for Nalidixic Acid (Nal) resistance through successive passage on XLT4 Agar containing various levels of Nal (1:100, 1:75, 1:50, 1:25, 1:10, 1:2, and 1:1). This process was adapted using the methodology presented in J. Bauer-Garland and associates work (2006). Serial Dilutions in PBS were used to determine the number of colony forming units (CFU). All bacterial counts were performed by plating 0.1mL of the culture and the serial dilutions (PBS) in duplicates on XLT4 (+Nal). These plates were incubated for 42˚C for 24 hours. The inoculum (165 mL/pen) was sprayed directly on new pine shavings (bedding) prior to placing poults into the pens with a concentration of 9.258 Log 10 CFU *Salmonella* ser. Schwarzengrund.

Facility & Equipment Monitoring

The facility and equipment were subjected to a vigorous cleaning and disinfecting regime. This regime consisted of scraping and removing of litter, sweeping of any leftover debris, blowing down any dust, pre-wash with a pressure sprayer, washing with commercial barn soap, disinfection with an aldehyde-based product, disinfection of waterlines with a peroxide-based product, and fumigation with a

peroxide-based disinfectant. Before proceeding to a subsequent step in the cleaning process, sampling was conducted to confirm absence of *Salmonella spp.*. If *Salmonella spp.* presence was confirmed, the previous cleaning step was repeated followed by additional sampling. Down-time between completion of washing and disinfection steps ranging between 12 to 48 hours, depending on moisture in the barn and sampling results. Simultaneously, each step of the cleaning process was accompanied by a similarly rigorous sampling protocol with 2 to 5 pre-moistened gauze swabs (BPW, Buffered Peptone Water) taken from each of the following areas of the barn walls, doors, fencing, scales, vents, ledges, drinkers, feeders, and floor. Swabs were used to vigorously wipe an area of 0.5 to 1.0 in^2 and then placed in sterile Whirl-pac bags filled with 225 mL BPW, kept on ice, and then transported to a private 3rd party lab within 6 to 12 hours. All results were negative for Salmonella spp.

Performance Monitoring

During the experimental period, individual body weights of 50 tagged birds was recorded weekly for the first six weeks in addition to days 63 and 84 to calculate daily BW gain (g/bird per day). Feed intake for each pen was documented in order to calculate feed conversion ratio (FCR). Feed intake continued to be recorded during each additional feed phase change after six weeks. Mortality was monitored, weighed, and recorded daily. All mortality was characterized by a licensed veterinarian.

Sample Collection

Each week booties samples were collected to analyze *Salmonella* litter contamination levels for each pen. Post-weighing, seven birds were randomly selected

(non-tagged) from each pen and humanely euthanized via a carbon dioxide chamber (42 birds per treatment) weekly. Birds were necropsied and livers and ceca aseptically removed. Due to the large volume of birds being collected, liver samples were pooled on a per pen basis as were ceca. Jejunum samples were collected from five birds per treatment at 12 weeks. Additionally, 100 gram feed samples were collected from each pen container weekly. These samples were then composited and sent off for laboratory analysis in order to validate DFM, formaldehyde treatment, and *Salmonella spp.* presence (if any) and levels.

Litter Bootie Samples

Litter bootie collection was performed using an adaptation of the overshoe method, which was first described by Aho (1992). Litter bootie kits were made similar to those utilized and described by Aho (1992). Kits contained two 1.5 inch thick strips of dry sterile surgical gauze overshoes contained in a sterile filtered Whirl-pac sample bag, a pair of clean Nitrile gloves, and two clean plastic boots. One kit was utilized for each pen. Rubber boots were sanitized prior to entrance into barn followed by applying alcohol hand gel to hands. Once door to pen has been unlocked and in the open position, Nitrile gloves were donned. Upon entering into an individual pen, plastic boots were placed over rubber boots. Once inside the pen, overshoe booties were put on and worn over plastic boots. Litter booties were collected pre-inoculation and post-inoculation each experimental round. Booties were also collected weekly for 12 weeks. These booties were submitted to a third party contracted laboratory and underwent enrichment by using a combination of tetrithionate broth, brilliant green, and iodine. Following

such, qPCR (real-time polymerase chain reaction) was performed. Procedures similar to those described by Chalghoumi and associates (2009) were followed. An estimated number of colony forming units was determined via a pre-programmed regression curve. Colony forming units per bootie were recorded on a per pen basis. The remaining enrichment solution and bootie material was incubated for 24 hours at 42˚ and tested for *Salmonella* presence confirmation. All positive samples were submitted for serotyping. All positive samples confirmed qPCR presence of Salmonella. All serotypes were identified as the utilized inoculum strain, *Salmonella* Schwarzengrund.

Liver Colony Counts

Livers were pooled on a per pen basis (7) and placed in sterile filtered Whirl-pac bags. Livers were weighed and diluted at a 1:10 with BPS. They were then homogenized and stomached for 30 seconds; followed by serial dilution. After, 0.1mL were plated on Nal Resistant XLT4 agar using spread plated methodology and incubated for 24 hours at 42˚C. Colonies were enumerated and recorded. Organ bags were then submitted to a third party laboratory for *Salmonella spp.* presence confirmation. All positive samples were submitted for serotyping. *Salmonella* presence was confirmed in all positive samples and identified as the utilized inoculum strain, *Salmonella* Schwarzengrund.

Cecal Colony Counts

Ceca were pooled on a per pen basis (7) and placed in sterile filtered Whirl-pac bags. Ceca were weighed and diluted at a 1:10 with BPS. They were homogenized and stomached for 30 seconds; followed by serial dilution. After, 0.1mL were plated on Nal

Resistant XLT4 agar using spread plated methodology and incubated for 24 hours at 42˚C. Colonies were enumerated and recorded. Organ bags were then submitted to a third party laboratory for further testing of *Salmonella spp.* presence. All positive samples were submitted for serotyping. *Salmonella* presence was confirmed in all positive samples and identified as the utilized inoculum strain, *Salmonella* ser. Schwarzengrund.

Morphology of Jejunum

The jejunum was aseptically removed at the Meckle's Diverticulum Junction at 12 weeks. Two one-inch sections were dissected from each bird. The jejunum was fixed in a sterile jar filled with 10% buffered formalin, labeled, sealed, and submitted to a 3rd party lab. The jejunum was processed, embedded in paraffin, cut into cross sections, and fixed to slides. Slides were then stained with Eosin and Hemotoxylin for further measurements under a light microscope. Images were acquired and measurements taken including: crypt depth and villus height.

Statistics

Statistical analysis was performed using JMP software. Live performance data from dietary treatments were analyzed using randomized complete block design with 4 treatments and 6 replications per treatment. Feed Conversion Ratios were adjusted for normal mortality as well as mortality accrued euthanizing birds for sampling purposes. Livability was adjusted to omit birds euthanized for sampling. All *Salmonella* counts were log transformed to base 10 logarithm before analysis for purposes of normalization.

Analysis of variance was completed and means separated by Tukey's multiple comparison test where $p \leq 0.05$. Pen is the experimental unit.

Results

Mean litter bootie log 10 counts for Experiment 2 (**TABLE 6**) exhibited a numerical increase or increasing trend from 7 through to 14 days. After 14 days a numerical decrease or decreasing trend was observed until 28 days across all treatments. At 35 days of age, there was a numerical increase (upward trend) in mean litter bootie log 10 counts. This was seen through 42 days, after which counts trended downward throughout most treatments until 56 days. The exception, NC, which showed an upward trend, with increasing mean log 10 counts at 49 days. Treatment DFM1 showed a similar increase at 63 days. By 77 days, an upward trend was observed across all treatments and continued this trend through 84 days. Only at 28 days, was a significant p-value (0.0385) detected via ANOVA across treatments. However, after means were separated and compared via Tukey's HSD, a p-value approaching significance 0.0540 was observed between PC and DFM2.

No plate counts for liver invasion were observed throughout the entire experiment (**TABLE 7**). Plate counts for mean log 10 ceca colonization showed high numerical values or an increasing trend at 7 days with a reduction (decreasing trend) across all treatments from 14 through 21. At 28 days, birds from PC and NC experienced elevated mean log 10 plate count values while a reduction continued in DFM1 and DFM2. At 35 days this role reversed with PC and NC experiencing a numerical drop while DFM1 and DFM2 increased numerically. At 42 days of age all

birds demonstrated a drop in mean log 10 plate counts. There were no statistical significant differences observed throughout the entire 42 day sampling window.

TABLE 6. Normalized mean log 10 colonization of litter booties 7 to 42 days of turkey hen poults environmentally challenged with *Salmonella* Schwarzengrund inoculum * and continuously fed diets supplemented with antibiotic growth promoter, direct fed microbials, or neither starting at day of placement through 84 days.

Mean Log 10 Litter/g Booties Colony Counts

Treatment	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63	Day 70	Day 77	Day 84
PC[1]	5.152	5.634	4.830	0.889	2.474	2.636	0.267	0.116	0.000	0.000	0.774	0.918
NC[2]	5.247	5.965	4.602	2.618	2.721	2.942	0.000	0.196	0.500	0.000	0.050	0.905
DFM1[3]	5.460	5.536	4.969	2.735	3.526	3.484	0.428	0.333	0.591	0.492	1.018	2.232
DFM2[4]	4.791	5.412	4.758	2.867	2.717	4.066	0.519	0.327	0.186	0.000	0.201	0.186
n	48	48	48	48	48	48	48	48	48	48	48	48
Pooled SEM	0.370	0.200	0.220	0.480	0.560	0.410	0.170	0.240	0.320	0.080	0.350	0.660
P-value	0.65	0.309	0.710	0.039	0.594	0.159	0.290	0.919	0.703	0.089	0.335	0.277

1 Positive Control Diet formulated with 0.05% inclusion of a standard AGP

2 Negative Control (Basal Diet)

3 Basal Diet formulated with 0.05% inclusion of DFM 1 (*Bacillus Subtilis*)

4 Basal Diet formulation with 0.05% inclusion rate of DFM 2 *(Bacillus Subtilis* strain)

* Salmonella inoculum was concentrated to 9.258 Log 10 CFU

TABLE 7. Normalized mean log 10 colonization of ceca colonization and liver invasion 7 to 42 days of turkey hen poults environmentally challenged with *Salmonella* Schwarzengrund inoculum * and continuously fed diets supplemented with antibiotic growth promoter, direct fed microbials, or neither starting at day of placement through 42 days.

		Mean Log 10/g Ceca and Liver Colony Plate Counts					
Treatment	Sample	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
PC[1]	Liver	0.00	0.00	0.00	0.00	0.00	0.00
	Ceca	4.72	2.17	0.59	0.71	0.17	0.00
NC[2]	Liver	0.00	0.00	0.00	0.00	0.00	0.00
	Ceca	4.6	2.00	0.43	0.64	0.00	0.00
DFM1[3]	Liver	0.00	0.00	0.00	0.00	0.00	0.00
	Ceca	4.81	2.65	1.47	0.41	0.9	0.52
DFM2[4]	Liver	0.00	0.00	0.00	0.00	0.00	0.00
	Ceca	4.83	2.27	0.89	0.26	0.43	0.00
n	Liver	168	168	168	168	168	168
	Ceca	168	168	168	168	168	168
Pooled SEM	Liver	0.00	0.00	0.00	0.00	0.00	0.00
	Ceca	0.18	0.42	0.38	0.36	0.27	0.13
P-value	Liver	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
	Ceca	0.8425	0.2760	0.8142	0.7522	0.3078	0.4133

[1] Positive Control Diet formulated with 0.05% inclusion of a standard AGP

[2] Negative Control (Basal Diet)

[3] Basal Diet formulated with 0.05% inclusion of DFM 1 (*Bacillus Subtilis*)

[4] Basal Diet formulation with 0.05% inclusion rate of DFM 2 *(Bacillus Subtilis* strain)

[*] Salmonella inoculum was concentrated to 9.258 Log 10 CFU

Weekly weights at 0, 35, 63, and 84 days observed no significant difference across any treatments (**TABLE 8**). At 7, 14, 21, 28, 42, 63, and 84 days significant differences were observed. At 7, 14, and 28 days, there was a significant difference between and among all treatments observed through Tukey's HSD p-values for each comparison. At 21 days, significance was detected in PC and DFM1 compared to NC and DFM2. Mean weights were separated by treatment via Tukey's HSD, which indicated a p-value of < 0.0001 for all comparisons. At 42 days, PC and NC were significantly different compared to DFM1 and DFM2. Tukey's HSD indicated p-values of < 0.0001 for all comparisons. Treatments DFM1 and DFM2 were also significantly different, with a Tukey's HSD p-value of .0002 detected. At 63 and 84 days, PC had the lowest weights. At 63 days, PC also demonstrated significant means with p-values of 0.0007, 0.0006, and 0.0003 compared to treatments NC, DFM1, and DFM2, respectively. At 84 days of age, a similar trend continued with PC having significantly lower mean weights compared to treatments NC, DFM1 and DFM2 with p-values of 0.0002 (NC and DFM1) and < 0.0001 (DFM2).

Livability was adjusted to account for mortality accrued from birds euthanized for sampling purposes (**TABLE 9**). At 42 days, PC was had significantly higher livability compared to all other treatments. Comparisons via Tukey's HSD indicated p-values of < 0.0001 with treatments NC and DFM1 and 0.0001 with DFM2. At 84 days, similar significance persisted. Treatment PC continued to have significantly higher livability compared to treatments NC, DFM1, and DFM2. Tukey's HSD showed p-values of < 0.0001, 0.0006, and 0.0008, respectively.

TABLE 8. Mean body weights in pounds from initial placement to 42 days, 63 days, and 84 days of turkey hen poults environmentally challenged with *Salmonella* Schwarzengrund inoculum * and continuously fed diets supplemented with AGP, DFMs, or neither starting at day of placement through 84 days.

	Mean Body Weights (kg/bird)								
Treatment	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35[E]	Day 42	Day 63	Day 84
PC[1]	0.064	0.120 [D]	0.332 [B]	0.594 [A]	0.839 [C]	1.352	1.914 [C]	3.570 [B]	5.892 [B]
NC[2]	0.064	0.139 [C]	0.362 [A]	0.499 [B]	0.957 [B]	1.334	1.941 [C]	4.500 [A]	7.711 [A]
DFM1[3]	0.063	0.150 [B]	0.295 [C]	0.590 [A]	0.812 [D]	1.324	2.091 [B]	4.500 [A]	7.697 [A]
DFM2[4]	0.063	0.155 [A]	0.269 [D]	0.513 [B]	0.984 [A]	1.334	2.168 [A]	4.577 [A]	7.874 [A]
n	1200	1100	1091	1089	1081	1074	1072	1068	1062
Pooled SEM	0.001	0.003	0.023	0.009	0.014	0.02	0.029	0.236	0.389
P-value **	0.103	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.2027	< 0.0001	0.0001	< 0.0001

[1] Positive Control (AGP)

[2] Negative Control (Basal Diet)

[3] Basal Diet w/ Probiotic 1

[4] Basal Diet w/ Probiotic 2

[A, B, C, D] Means within a column and treatment group with different superscripts differ significantly ($P \leq 0.05$)

[E] Moved to grower barn.

[*] Salmonella inoculum was concentrated to 9.258 Log 10 CFU

TABLE 9. Mean adjusted livability for 0 to 42 and 0 to 84 days of turkey hen poults environmentally challenged with *Salmonella* Schwarzengrund inoculum * and continuously fed diets supplemented with antibiotic growth promoters, direct fed microbials, or neither starting at day of placement through 84 days.

	Adjusted Livability	
Treatment	Days 0-42	Days 0-84
PC[1]	98.89% [A]	98.51% [A]
NC[2]	93.56% [B]	92.68% [B]
DFM1[3]	92.95% [B]	92.85% [B]
DFM2[4]	93.96% [B]	91.45% [B]
n	24	24
Pooled SEM	0.82	0.70
P-value	< 0.0001	< 0.0001

[1] Positive Control Diet formulated with 0.05% inclusion of a standard AGP

[2] Negative Control (Basal Diet)

[3] Basal Diet formulated with 0.05% inclusion of DFM 1 (*Bacillus Subtilis*)

[4] Basal Diet formulation with 0.05% inclusion rate of DFM 2 *(Bacillus Subtilis*)

[*] Salmonella inoculum was concentrated to 9.258 Log 10 CFU

[A, B] Means within a column and treatment group with different superscripts differ significantly ($P \leq 0.05$)

Feed Conversion Rates were also adjusted to account for normal mortality and mortality accrued from birds euthanized for sampling purposes (**TABLE 10**). At 42 days, PC and NC showed a significant difference compared to DFM1 and DFM2. Treatment PC demonstrated a significance of < 0.0001 and 0.0012 compared to DFM1 and DFM2, respectively. Treatment NC detected significance of < 0.0001 and 0.0012 compared to DFM1 and DFM2, respectively. At 84 days, no significant differences

were detected across any of the treatments.

Mean villus heights were logged and demonstrated a significant difference in PC compared to NC and DFM1 (**TABLE 11**). These values were separated via Tukey's HSD methods and showed p-values of 0.0202 and 0.0259 respectively. Crypt depths also demonstrated a significant difference in PC versus DFM1, with a p-value of 0.0080.

TABLE 10. Mean adjusted feed conversion rates 0 to 42 and 0 to 84 days of turkey hen poults environmentally challenged with *Salmonella* Schwarzengrund inoculum * and continuously fed diets supplemented with AGP, DFMs, or neither starting at day of placement through 84 days.

	Adjusted Feed Conversion Rates (FCR)	
Treatment	Days 0-42	Days 0-84
PC[1]	1.62 [A]	2.13
NC[2]	1.64 [A]	2.2
DFM1[3]	1.43 [B]	2.12
DFM2[4]	1.48 [B]	2.17
n	24	24
Pooled SEM	0.02	0.04
P-value	< 0.001	0.654

1 Positive Control Diet formulated with 0.05% inclusion of a standard AGP

2 Negative Control (Basal Diet)

3 Basal Diet formulated with 0.05% inclusion of DFM 1 (*Bacillus Subtilis*)

4 Basal Diet formulation with 0.05% inclusion rate of DFM 2 *(Bacillus Subtilis*)

* Salmonella inoculum was concentrated to 9.258 Log 10 CFU

A, B Means within a column and treatment group with different superscripts differ significantly ($P \leq 0.05$)

TABLE 11. Mean jejunum morphometric measurements of villus and crypts (um) of turkey hen poults environmentally challenged with *Salmonella* Schwarzengrund inoculum *and continuously fed diets supplemented with antibiotic growth promoters, direct fed microbials, or neither starting at day of placement through 84 days.

	Jejunum Measurements (um)		
Treatment	Villus Height	Crypt Depths	Villus Height : Crypt Depth
PC[1]	2187.50 [A]	195.00 [A]	11.60
NC[2]	1657.50 [B]	155.00 [AB]	10.89
DFM1[3]	1606.25 [B]	121.88 [B]	14.02
DFM2[4]	1842.50 [AB]	152.50 [AB]	13.52
n	38	38	38
Pooled SEM	107.50	14.50	1.20
P-value	0.01	0.01	0.27

[1] Positive Control Diet formulated with a 0.05% inclusion of a standard AGP

[2] Negative Control (Basal Diet)

[3] Basal Diet formulated with a 0.05% inclusion of DFM 1 (*Bacillus Subtilis*)

[4] Basal Diet formulation with a 0.05% inclusion rate of DFM 2 *(Bacillus Subtilis*)

* Salmonella inoculum was concentrated to 9.258 Log 10 CFU

A, AB, B Means within a column and treatment group with different superscripts differ significantly ($P \leq 0.05$)

Discussion

These collective results for Experiment 2 demonstrate a few critical points. There was a general increase experienced for mean log 10 litter booties at 35 days in a majority of treatments (the exception being DFM2), which is believed to be due to crowding of birds prior to move. Birds were restricted in these pens and had significant densities which would demonstrate a potential for increased stress. Similarly, Bailey and associates (1988) research concluded colonization of *Salmonella* can be more readily experienced during times of stress in a bird's life. This seems to be an accurate explanation as within a few weeks after move, *Salmonella* mean log 10 counts started to decrease. Weights can also be used to demonstrate this concept of induced or increased stress. The birds can be seen increasing in weight (upward trend) every week following day of placement with the exception of 35 days. The only main event which happened during this time period was the move from the brooder to the grower facility. It was at this moment that birds were put in a new environment with new pine shavings.

The data also show a dramatic decrease in mean log 10 counts of litter booties at approximately 28 days or the 4 week mark, with almost a 50% reduction compared to what was observed the previous week across all treatments. This could be due to the maturity of the intestinal mucosa and protection acquired by a more diverse intestinal microbiome population which could better fight off and defend again *Salmonella*. Research conducted by Revellodo and associates (2009) demonstrated colonization of Salmonella Typhimurium decreased as birds aged because resistance was acquired due to cecal colonization and organ invasion independent of treatment applied. These

findings agree with earlier research indicating younger birds are often more susceptible to Salmonella colonization as compared to older birds (Bailey et al., 1988) and that susceptibility to oral infection diminishes with age (Revellodo et al., 2009).

Later in life, around 63 to 77 days, there was another increase of mean log 10 *Salmonella* cecal colonization across all treatments, which could have been due to stress. As the birds aged they were increasing in size with limited death loss. The density of the birds within the pens was as a result also increasing, which is a potential stressor. The numerical differences as well as waves of increases and decreases in mean log counts could also be explained by the mortality experienced within treatments. A large percentage of the mortality occurred prior to 42 days of age. Upon necropsy of mortality, lesions indicated severe Salmonellosis. This can be further demonstrated by the large differences observed in Experiment 1 compared to Experiment 2, with 7 day mean log 10 litter bootie values ranging from1 to 2 compared to approximately 5 mean log 10 CFU respectively. Another potential reason why the increase across treatments occurred towards the end stages of life would be that the bird's intestinal microbiota changes as the bird ages. Perhaps, the new wave of bacteria that the intestine was composed of was less able to fight against the *Salmonella* and therefore it was more easily able to colonize than in previous weeks.

Prior to move, data including mean weights, litter booties, ceca colonization, and livability seem to numerically trend positively with regards to the PC. This does stand to reason as research would indicate the treatment of a traditional feeding program with an AGP component should have enabled birds to out-perform others not treated with such.

However, with livability far exceeding the other treatments, these birds were subjected to much more confined conditions with increased density. This also has a significant impact on the weight of these birds as indicated by the last two weigh dates compared to all other treatments. Another telling sign of the potential stress experienced by these birds is the increased mean log 10 litter booties colonization values. These values demonstrate the increase of *Salmonella* being shed in the environment by this treatment. This parallels with the crypt depth data. The crypt depths for PC are significantly larger than all other treatments. The crypt depths correlate to the amount of tissue turnover and new cell creation which is believed by researchers to be increased during successful *Salmonella* colonization (Laudadio, 2012). However, there is some data which contradicts this by stating that increased apoptosis of cells could be an induced differential regulation in order to reduce infection (Tellez et al, 2011). Additionally, there is also a reduced numerical villus height to crypt depth ratio in the PC treatment compared to all other treatments.

The DFM treatments did yield some promising results. Treatment DFM2 had increased villus height similar to PC, therefore having an increased absorptive area for better feed efficiency and increased weight. The feed efficiency while significantly reduced at 42 days did not hold true through to the termination of the trial. This correlated with DFM2 having significantly higher mortality, especially when compared to PC. Treatment DFM2 also demonstrated the highest numerical mean weight values at the end of the trial, which again is more than likely due to the increased mortality experienced within this particular treatment. Similarly, DFM1 displayed promising

results. These include having a significantly reduced crypt depth, numerically increased V : C, and significantly lower adjusted feed conversion rate at 42 days. Treatment DFM 1 and 2 displayed the highest numerical villus height to crypt depth ratio which can be used to indicate a well-balanced intestinal mucosal environment. Present studies agree with findings of Grimes et al. (2008) who reported supplementation of probiotics in poultry diets improved villus height and reduced crypt depths.

While the trial data signifies these DFM products have favorable results necessary of potential alternatives of AGPs, more information is needed to make a well informed decision. More replications of this experiment for the full 12 weeks would help determine the consistency of application. An expansion of ceca colonization measurements should be done in order to better observe what is happening internally in the birds towards the end of their life-time such as 63 and 84 days. In addition to the expansion of ceca colonization measurements, the separation of each bird's ceca and liver rather than collective pooling of each pen would better help to determine the individual nature of each bird. By separating the ceca out, one would be better able to determine the percentage or incidence of the birds within a population that are actively infected as well as shedding.

There are also some measurements not evaluated in this study that could be included such as fecal samples. It would be beneficial to be able to determine what the microbial populations of the birds are throughout the entire study. This could be accomplished by means of fecal sampling. One such microbe population which should be closely examined within the context of this particular sampling would be the

Lactobacilli which are said to be increased with feeding of *Bacillus subtilis* DFM components (Schleifer, 2015). Lastly, there is a possibility that the *Salmonella* colonization prevented or out-competed the DFMs. Research by Revellodo and associates (2009) demonstrated efficacy of competitive exclusion products against Salmonella could be demonstrated at a few days of age, confirming importance of age in application of CE products. This indicates the window for introduction of the probiotics is very critical for success. Similarly, Griggs and Jacob (2005) also concluded DFMs administered within 24 hours of a challenge would reduce colonization of pathogens. In order to try to test this hypothesis, one could utilize a probiotic in a spray application at the hatchery prior to poults coming to the farm site in addition to the feed additive at the farm. This would allow the probiotic the potential to have some lead time to start colonizing the intestine of the poult.

CHAPTER V

CONCLUSION

There are three main challenges currently facing the poultry industry. These include the need to satisfy market demand, create solutions to reduce human exposure to *Salmonella* foodborne pathogens, and the need to find alternatives for antibiotic growth promoters. These challenges are paired with the industry's continued production goals of maintaining high performing birds with increased feed efficiencies.

Recent years have exhibited many food recalls, thus prompting increased food safety awareness. Paratyphoid *Salmonella* continues to be a foodborne pathogen of concern and has been linked to the consumption of various contaminated poultry products. While poultry processing plants were initially targeted for *Salmonella* reduction, focus has shifted to reducing the overall pathogen load and thus emphasis being placed on all potential contamination points. Therefore, rearing facilities among many other facets of the live production spectrum are also being targeted. The objective being if microbial loads are reduced at the farm, fewer microbes would potentially enter the plant, therefore reducing the risk of human consumption of contaminated product.

The primary objective of this research was focused on evaluating *Salmonella* colonization in turkey hens. Both experiments evaluated an alternative inoculation methodology in order to better represent *Salmonella* transmission and introduction into a commercially produced flock; and the use of four feed treatments including: a traditional feeding program inclusive of AGPs, a basal diet without any additives (absent

of AGPs), and two additional basal diets each inclusive of individual commercial DFM products. The first experiment focused on studying an alternative inoculation methodology in order to determine if a better representative model of *Salmonella* transmission and introduction into a commercially produced flock could be mimicked in the first six weeks of production. Experiment 2 further evaluated potential effects of these feed treatments on turkey hen performance and impact on *Salmonella* colonization over the lifetime of a flock (12 weeks). The alternative challenge model showed great promise with potential to be improved with further testing inclusive of other *Salmonella* serovars and methodology amendments. Similarly, both DFM products also demonstrated potential as viable alternatives to AGPs. Further research on both experiments is necessary to make any further conclusions.

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