EVALUATION OF VACCINES ON THE PREVALENCE OF *SALMONELLA* AND/OR *CAMPYLOBACTER* IN LAYER AND BROILER CHICKENS

A Thesis

by

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ABSTRACT

The control of foodborne pathogens especially *Salmonella* and *Campylobacter* are of great concern to the commercial poultry industry. The control of these pathogens could be essential in the reduction of foodborne illness and deaths related to eggs and poultry meat. Previous studies have found that the presence or disappearance of *Salmonella* or *Campylobacter* is linked to various environmental and management-based factors, of which include vaccines used in the industry. Presently, we evaluated the effect of the infectious bronchitis virus (IBV) vaccine on the incidence of *Salmonella* or *Campylobacter* prevalence in broiler chicks. In the current study, a high vaccine dosage of IBV vaccine was associated with an increase the prevalence of *Campylobacter* during the first two weeks of age. Although in a previous study a high vaccine dose of IBV was linked in to increased prevalence of *Salmonella*, this was not seen in our study. In a subsequent trial, we also evaluated the potential cross-protection against three *Salmonella* serotypes of two-previously formulated vaccines when used in various dosage combinations. The combination vaccine was effective in reducing shedding of S. Enteritidis however reduction of S. Typhimurium and S. Hadar were not seen consistently. The vaccines were also shown to not significantly affect the body weights of the birds.

Vaccines have been an essential component in the control of diseases within flocks in the commercial poultry industry. Ensuring the uniform application of IBV vaccine could help prevent and/or reduce the prevalence of *Campylobacter* in broiler

flocks. The combination vaccine was effective against one serotype of *Salmonella* but further trials are needed to complete evaluate its potential as a vaccine that could be used in the poultry industry.

DEDICATION

I would like to dedicate this thesis to my mother, Julia Garcia. Thank you for being my mother, my best friend, my teacher and my greatest ally and supporter.

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NOMENCLATURE

BGA	Brilliant Green Agar
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CJ	*Campylobacter jejuni*
FDA	Food and Drug Administration
FSIS	Food Safety Inspection Service
SST	Serum Separator Tube
RV	Rappaport-Vassiliadis Enrichment Broth
NA	Nalidixic acid
N/N	Novobiocin and Nalidixic acid
NO	Novobiocin
ST	*Salmonella* Typhimurium
SH	*Salmonella* Hadar
SE	*Salmonella* Enteritidis
XLT4	Xylose-Lysine-Tergitol 4

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CHAPTER I

INTRODUCTION

Salmonella and *Campylobacter* are two of the most common causes of bacterial foodborne illness in the United States. Eggs and poultry meat have been identified as common sources for these two microorganisms. The control of these microorganisms is of great concern to the commercial poultry industry. This task is difficult for the poultry industry since the contamination of these products may occur at various stages of production. Current pre-harvest interventions included the use of antibiotics, probiotics and other feed additives to reduce the colonization of *Salmonella* and *Campylobacter*. Recent *Salmonella* outbreaks have increased consumer demands to improve the safety of food and reduce the use of antibiotics in animal feed. Recent research has evaluated environmental and management-based factor that may influence the prevalence of these microorganisms. It was found that vaccines commonly used to control diseases such as Marek's disease and infectious bronchitis virus in broilers have been linked to a higher incidence of detecting *Salmonella* at rearing and on carcasses. Conversely, the use of *Salmonella* vaccines has been shown to be effective in reducing *Salmonella* in flocks.

The concept of vaccination is to improve an organism's immunity to a particular disease. However, poor vaccine administration could lead to problems such as immunosuppression. With a weakened immune system, the chances of illness and infection may greatly increase. In the case of broilers, a weakened immune system may increase the possibility of *Salmonella* and/or *Campylobacter* colonization. In the

development of a vaccine, cross-protection is a very important factor that is to be considered. In the case of *Salmonella*, cross protection of different serotypes would be ideal for the commercial poultry industry. Vaccines are a powerful tool, which can be essential in the control of disease. Proper use of vaccine is required in order to obtain the desired benefit. The main goal of this manuscript is to evaluation the effect of the infectious bronchitis virus vaccine of *Salmonella* or *Campylobacter* prevalence and to determine the effect of two previously-formulated *Salmonella* vaccines when used in various dosage combinations.

CHAPTER II

LITERATURE REVIEW

Salmonella is a gram-negative, facultative anaerobic, rod-shaped bacterium, which may cause food borne illness in humans. *Campylobacter* is a gram-negative, microaerophilic, spiral-shaped microorganism and the second most frequently reported cause of bacterial foodborne illness in the United States (Weinack, et al., 1984; FSIS, 2012). Poultry products such as eggs and meats have been linked to *Salmonella* and *Campylobacter* infections in humans (Henzler, et al., 1994; Hassan and Curtiss, 1997; Van Immerseel, et al., 2005; Luber, et al., 2006). The Centers for Disease Control and Prevention (CDC) estimates that about 9.8 million persons are affect by food borne illnesses each year by known pathogens. Of the food borne illnesses reported in 2009-2010, twenty-seven percent of the illnesses were associated with eggs and ten percent with poultry meat. Recent major outbreaks of *Salmonella* in eggs and poultry meat have driven consumers to demand improvements in food safety (CDC, 2010). The federal government has been very focused on consumer fears and has reacted by improving regulations regarding the safety of food. The Food Safety and Inspection Service (FSIS) and the Food and Drug Administration (FDA) have put in place new regulations with the hopes of reducing *Salmonella* and *Campylobacter* infections related to poultry meat and eggs and improving food safety. These new guidelines put pressure on the commercial poultry industry to reduce *Salmonella* and *Campylobacter* in poultry products. Current pre-harvest intervention in the control of *Salmonella* and *Campylobacter* include the use

of antibiotics in feed, the use of probiotics, the use of vaccines, and disinfection of production houses. However, the use of antibiotics in animal feed has been under scrutiny by consumers in fear of it causing the emergence of antibiotic-resistant food borne pathogens (Zhang-Barber, et al., 1999). The poultry industry must look into different management practices such as the use of vaccines to order to control the prevalence of foodborne pathogens within flocks and factors that may cause these pathogens to become prevalent in flocks.

Outbreaks in Poultry

Since 1909, the consumption of poultry products has significantly increased, a 6-fold increase in chicken consumption and a 17-fold in turkey consumption has been seen (Foley, et al., 2008). Although the prevalence of *Salmonella* in poultry dates back to the 1930s, the serotypes of concern were different. *Salmonella pullorum* and *S. gallinarum* posed a big threat to the commercial industry in the 1930s. In poultry, *S, pullorum* causes a white diarrhea with significant mortality and *S. gallinarum* causes fowl typhoid which has high mortality in growing and older birds. The National Poultry Improvement Plan was developed in 1935 to help combat these pathogens and by the mid-1970s; both microorganisms were eliminated from commercial poultry flocks. It is believed that the elimination of *S. pullorum* and *S. gallinarum* from commercial poultry flocks may have helped *S. enteritidis* become prevalent (Bäumler, et al., 2000; Foley, et al., 2008). Reports of human infection of *S. enteritidis* have steadily increased since the 1960’s (Bäumler, et al., 2000).

It is estimated by the CDC that *Salmonella* causes approximately 1.4 million cases of human foodborne illness, which result in 17,000 hospitalizations and 585 deaths each year (Mead, et al., 1999; Kimura, et al., 2004; Voetsch, et al., 2004; Braden, 2006). In 2010, a major outbreak of *Salmonella* Enteritidis was linked to shell eggs and resulted in about 1,939 illnesses. In response, two egg companies recalled over half a billion eggs. FDA conducted an investigation on layer houses of the two companies and found that many of the locations in which samples were taken, *Salmonella* was present. In 2011, a foodborne illness outbreak occurred involving a mult-drug resistant strain of *Salmonella*, which had infected 136 persons from 34 states and about 35% of the affected individuals were hospitalized. The outbreak was linked to ground turkey. In response, the turkey company recalled 36 million pounds of ground turkey due to a possible *Salmonella* contamination and to prevent further infection, making it the third largest meat recall in United States history. These two major recalls caused uproar from consumers demanding more surveillance and regulation from the government. It also began a movement to demand the ban of antibiotics for animal-use in fear that their use may bring about drug-resistant bacteria to human populations. These outbreaks have put pressure on the commercial poultry industry to become more proactive in the control of *Salmonella* and consider possible alternatives if antibiotics for animal-use is to be banned.

Government Policy

Late in 2009, FDA announced its new regulation regarding the safety of eggs, "Prevention of *Salmonella* Enteritidis in Shell Eggs during Production, Storage, and

Transportation" (FDA, 2009). With this new regulation, FDA hopes to reduce over 79,000 cases of foodborne illnesses and 300 deaths related to the consumption of eggs. Under this regulation, producers with 3,000 or more laying hens, whose eggs are not processed with a treatment, are required to have a written *Salmonella* Enteritidis prevention plan. It also covers the refrigeration of stored and transported eggs, pasteurization, rodent control, cleanliness of layer houses (FDA, 2009). Sherri McGarry, emergency coordinator for the FDA's Center for Food Safety and Applied Nutrition, stated in a conference call that if this regulation was in place before the major outbreak of 2010, that the outbreak could have been prevented (Martin, 2010).

In 2010, FSIS announced a new regulation regarding performance standards in chicken and turkey production, New Performance Standards for *Salmonella* and *Campylobacter* in Young Chicken and Turkey Slaughter Establishments (FSIS, 2011). Under this new regulation, verification sample sets will be now analyzed for both *Salmonella* and *Campylobacter*. If no more than five positive samples in a 51-sample set for young chickens and no more than four positive samples in a 56-sample set for turkeys, the establishment will pass the updated *Salmonella* standards. If no more than eight positive samples in a 51-sample set for young chickens and no more than three positive samples in a 56-sample set for turkeys, the establishment will pass the new *Campylobacter* standards. If the establishment excides the positive limits, a follow-up sample set will be taken and analyzed for both organisms. Based on the percent of positive samples, the establishment will be placed into one of FSIS's four process control categories. The categories determine the frequency of the verification of sample set for

the establishment. If an establishment fails to meet the *Salmonella* standard in its follow-up set, the name of the establishment and its test results will be posted on the FSIS website.

Federal regulatory agencies have begun to take steps to improve the food safety of eggs and poultry meats. With these new regulations, the government hopes to reduce foodborne illness caused by *Salmonella* and *Campylobacter*. Although these microorganisms are highly susceptible to killing by normal cooking temperature the poultry industry has been given the task to reduce these pathogens in raw poultry products. It forces the poultry industry to look into its current pathogen reduction programs and make improvements in order to meet the new requirements.

Campylobacter

Campylobacter has been a troubling problem to the commercial poultry industry for decades. How it appears in commercial flocks is a mystery to the industry. Some studies have found that *Campylobacter* might be vertically transmitted from broiler breeders to their progeny. It was reported that *Campylobacter* was detected in the reproductive tracts of roosters and hens, which could contaminate the egg (Buhr, et al., 2002; Cox, et al., 2002b; Hiett, et al., 2002; Hiett, et al., 2003; Cox, et al., 2005). However, contaminated eggs did not give rise to *Campylobacter* infected chick as the chick would found to be negative for *Campylobacter* (Cox, et al., 2002a). In a later study, hatchery tray liners recovered from commercial hatcheries, were found to have low numbers of *Campylobacter* (Byrd, et al., 2007). These results give evidence that vertical transmission is possible and the chance that chicks actively shedding *Campylobacter*

could contaminate other chicks. The control of *Campylobacter* is essential to the poultry industry and further research is needed to improve detection of the microorganism.

***Salmonella* in Eggs**

Eggs have been linking to various outbreaks, usually caused by the consumption of raw or uncooked eggs. The most common strain associated with egg-related outbreaks is *Salmonella* Enteritidis, although other strains such as *S.* Typhimurium, *S.* Hadar and *S.* Heidelberg are also of concern. It is believed contamination of eggs may occur via two routes of transmission. The vertical transmission theory states that *Salmonella* originates from an infected hen, which in turn infects the egg. The horizontal transmission theory states that the egg is infected after it has been laid (Cox, et al., 2000).

Previous reports have shown that *Salmonella*-contaminated eggs can be produce by artificially inoculating the hen. Laying hens orally challenged with one million CFUs of *Salmonella* caused infection of the ovarian follicles and oviduct. However, hens show no signs of pathogenesis and *Salmonella* was not detected in all fecal samples (Timoney, et al., 1989). This may also demonstrate that infected hens may be present within flocks and spread the pathogen while not being detected. Keller and coworkers (1995) found that contamination of the lower oviduct was important in the production of infected eggs. They found that during egg development, eggs may become infected due to colonization in the hen’s infected ovarian follicles. As the eggs progresses through in the oviduct, the contamination could be lessened by the addition of the albumen, which contains antibacterial compounds. Upon entering the lower oviduct, the egg could become

recontaminated with the addition of the egg membranes prior to egg shell formation (Keller, et al., 1995).

Contamination of eggs may also occur in the nesting box or on floor after oviposition due to the presence of *Salmonella* in the environment of laying hens. The bacteria can be carried on the egg shell or penetrate through the egg shell. Various reports have shown that *Salmonella* is able to penetrate the cuticle and the outer and inner egg membranes and to grow within the contents of eggs (Schoeni, et al., 1995; Cox, et al., 2000). Egg quality is a major determinant in bacterial penetration. Objective measures can be used to determine egg quality such as conductance, specific gravity and shell strength. Conductance is a measure of eggshell porosity, which indicates the eggshell's ability to allow the passage of water vapor and other gases. Shell strength and thickness can also be used as measures of egg quality. Specificity gravity is used to determine shell thickness since it is positively correlated and does not require the loss of the egg. A study found that eggs with a low specific gravity had a higher chance of being penetrated by *Salmonella* (Sauter and Petersen, 1974). Once the bacterium penetrates the egg, it can further invade the egg contents or infect the developing embryo.

Contaminated eggs can serve as a threat to both consumers and producers. In eggs used for human consumption, foodborne illness could results if infected eggs are uncooked and ingested. The illness could be more severe for children, the elderly and immunocompromised individuals (Braden, 2006; CDC, 2010, 2013). In eggs used for hatching not only the newly hatched chick be infected with *Salmonella* but others within the same hatching cabinet could also become infected. These infected chicks could later

infect others at the grow-out farm. This scenario would be a dilemma for the processing plant, forcing it to take step to prevent contamination of other carcasses.

***Salmonella* in Broilers**

Although eggs are mainly associated with *Salmonella* outbreaks, human salmonellosis can also be linked to poultry meat (Kimura, et al., 2004; Voetsch, et al., 2004; Altekruse, et al., 2006; CDC, 2013). Various factors have been associated with *Salmonella* infection of broilers. Eggs contaminated with *Salmonella* can further cross contaminate other eggs at the hatchery. Studies have found that breeder and broiler hatcheries were highly contaminated with *Salmonella*. The bacterium was detected in 71% of egg fragments, 80% of chick conveyors belt swab samples and 74% of samples pads placed under newly hatched chicks to collect fecal samples (Cox, et al., 1990; Cox, et al., 1991; Bailey, et al., 1994). Cason and coworkers (1994) demonstrated that *Salmonella*-positive eggs could lead to the contamination of other eggs in the hatching cabinet. After hatching, *Salmonella* is dispersed throughout the hatching cabinet due to fan forced-air. They found that >80% of the chicks hatched above or below the inoculated eggs were positive for the *Salmonella* strain used to infection the eggs (Cason, et al., 1994). Contamination at the hatchery could lead to possible problems at the grow-out farm as infected chicks could spread the bacterium to others within the flock.

Bailey and coworkers (2001) found that the emergence or disappearance of *Salmonella* from commercial broiler flocks was influenced by a wide range of environmental and management-based factors. Some of these factors include temperature, humidity and pre and post-inventions. Significant rates of *Salmonella* were

recovered in the dirt at the entrance of the poultry houses and in the litter samples taken from boots swabs. During the fall season, higher recovery rates were seen (Bailey, et al., 2001). Feed, rodents, insects and direct contact between uninfected and infected birds have also been linked with *Salmonella* (Sander, et al., 2001; Liljebjelke, et al., 2005). In a previous study, it was found that feed samples, taken from a commercial broiler operation, had the highest frequency for recovered *Salmonella* (Morris, et al., 1969; Jarquin, et al., 2009). The contamination of feed can be caused by rodents, wild birds or other pests. It was also found that less than one *Salmonella* per g of feed was able to infect and colonize 1- to 7-day-old chicks, making feed an important source of *Salmonella* (Schleifer, et al., 1984). The controls of pests are necessary to prevent possible contamination of feed. Good biosecurity and management practices can be essential in preventing many of these factors and reducing the chances of *Salmonella* in flocks.

Vaccines commonly used in broiler vaccination programs have been shown to potentially increase the detection of *Salmonella* in broiler flocks. Volkova and coworkers (2010) reported that an increase dosage of Infectious bronchitis virus vaccine (IBV) applied via spray to 1-day- old birds was linked to a higher probability of detecting *Salmonella* in the flock during rearing and on the broiler carcasses at the pre-chilling and post-chilling at processing. The authors also reported a higher chance of detecting *Salmonella* was seen in flocks at time of delivery and at rearing in birds that were vaccinated for Marek's disease at day 1 versus bird's vaccinated for Marek's disease *in-ovo* (Volkova, et al., 2011a). The control of *Salmonella* at the hatchery is essential to

reducing *Salmonella* in flocks. However, other means of intervention at grow-out must be applied to further improve the reduction of *Salmonella*.

Infectious Bronchitis Virus

Infectious bronchitis virus (IBV) is an acute, highly contagious upper respiratory disease of poultry. It can be transmitted after inhalation or direct contact with contaminated poultry, litter, equipment or other fomites. This virus is of great interest to the commercial poultry industry because it may decrease in egg production and quality in layers. In broilers, it can cause poor feed conversion, reduce growth rate and increase condemnation of carcass at processing due to airsacculitis and other bacterial related conditions (Cavanagh, 2008). In a study, it was found that IBV and *Mycoplasma galliseptium,* which also affects the respiratory system of birds, significantly increased the shedding of *E.coli* and *S.* Typhimurium in chickens (Weinack, et al., 1984). The increase in theses microorganism could have been caused by a disruption in the intestinal microflora caused by respiratory stress. Broilers are usually vaccinated at the hatchery with a live infectious bronchitis virus vaccine and can be given a booster at 10-18 days of age at the grow-out farm. At the hatchery, the vaccine is given by mass administration due to its convenience however uniformity is a problem with the method of administration. Vaccinated birds may horizontally shed the virus to “missed” vaccinated individuals within the flock. Transmission of the virus via this route may be variable causing a variation in reaction, which is also known as a “rolling reaction”. This reaction may cause the birds to become immunocompromised, making them more susceptible to

Salmonella and *Campylobacter* colonization. The prevalence of these microorganisms could lead to increases in contamination at processing.

Processing of Broilers

Once birds reach market age, steps are taken to prepare the birds for processing. Feed is withdrawn 8-12 hours before the birds are taken to the processing plant. This is done to ensure the intestinal tracts of the birds are empty, which could reduce possible contamination at processing. However, studies have shown that increases the incidences of *Salmonella* and *Campylobacter* are seen in the crop, which is caused by increased litter pecking after feed withdrawal (Ramirez, et al., 1997; Byrd, et al., 1998a, b). The addition of lactic acid and other products to drinking water have been shown to help reduce *Salmonella* and *Campylobacter* contamination during feed withdrawal (Byrd, et al., 2001; Byrd, et al., 2003). After these birds have been caught and placed onto transportation coops, where infected birds can infect other birds (Rigby and Pettit, 1980). Transportation coops can serve as another source for *Salmonella* and *Campylobacter* since very few of these coops are cleaned before each use and broilers spend between 3 to 12 hours, between catching to holding at the processing plant, in transportation coops (Berrang and Northcutt, 2005; Berrang, et al., 2011). The addition of organic acids and other products into drinking water and the disinfection of coops could be essential in controlling and/or preventing contamination of birds before it reaches the processing plant.

In 1998, FSIS began the enforcing of Hazard Analyzes and Critical Control Points (HACCP) in meat and poultry processing plants in hopes of reducing

contamination of meat and poultry and decreasing incidences of foodborne illnesses (Dorea, et al., 2010). Under HACCP, processing plants must identify possible locations in the processing line where contamination, whether it is a biological, chemical or physical, could occur. After identifying these locations, the plant must design an intervention to prevent or reduce this contamination from occurring. *Salmonella* and *Campylobacter* are critical microorganisms that commercial poultry processors must control with their HACCP plans (Lister and Barrow, 2008). Control of these pathogens at other phases of production such as in breeders, at the hatchery and the grow-out farms could be critical in reducing hurdles at processing.

The automation of the processing plant has made the poultry industry very efficient in processing a large amount of birds in a short amount of time. However, contamination of equipment could lead to cross contamination issues. Microorganisms such as *Salmonella* and *Campylobacter* are prevalent in the intestines and ceca and a small cut to any of these organs could cause their contents to leak out and contaminate the equipment (Oosterom, et al., 1983; Hargis, et al., 1995). Taking into account the amount of birds being processed in a matter of minutes, cross contamination could occur very quickly. The crop has also been identified as source of contamination. Studies have found that both *Salmonella* and *Campylobacter* had higher rates of contamination than the ceca (Hargis, et al., 1995; Byrd, et al., 1998a, b; Corrier, et al., 1999). With the crop having a higher incidence of *Salmonella* and *Campylobacter* than the ceca, contamination of the surrounding muscle could occur. This was found to be true when a fluorescent marker was used to evaluate possible leakage contamination and found contamination

around the neck and breast muscle (Byrd, et al., 2002). In the chiller, contaminated carcasses could further contaminate other carcasses (Smith, et al., 2005). The use of chlorinated water in the chiller has been seen to be effective in controlling *Salmonella* and *Campylobacter* contamination (Berrang, et al., 2007; Northcutt, et al., 2008). The use of post-chill sprays have also been shown to be effective to controlling the contamination of these microorganisms (Oyarzabal, et al., 2004). Preventing leakage from the crop and/or intestinal tract can help reduce possible contamination of carcasses.

Vaccines

Vaccines have played an important role in controlling diseases that once plagued the commercial poultry industry such as Marek's disease and Newcastle disease (Fadly and Smith, 1991; Fussell, 1998). In previous studies, broiler breeder flocks which were vaccinated for these diseases were used to determine the benefits of passive immunity related to these diseases. They found that maternal antibodies from the mother's blood were transferred into the egg yolk. Immunoglobulin G (IgG) was the main antibody transferred to the yolk (Hassan and Curtiss, 1996; Hamal, et al., 2006; Lister and Barrow, 2008). Other studies have found that antibodies are secreted by the reproductive tracts of vaccinated birds. Once they are absorbed, the antibodies are diffused into the amniotic liquid and ingested by the developing embryo (Dohms, et al., 1978). Based on these findings, passive immunity of chicks to *Salmonella* due to *Salmonella vaccine* can occur via the same pathways.

Vaccination of Breeders

Newly hatched chicks have been shown to be highly susceptible to *Salmonella* infection and gut colonization (Bailey, 1988). In older birds, a well-developed intestinal microflora and a mature immune system make the birds more resistant to *Salmonella* infections (Gast and Holt, 1998). However in previous challenge studies, birds challenged at day 1 of age were shown to remain infected and continue to shed the pathogen until maturity (Gorham, et al., 1991). Inoue and coworkers (2008) examined passive immunity of progeny from broiler breeders vaccinated against *Salmonella* Enteritdis (SE). They found that lower cecal counts of SE were recovered from progeny of vaccinated broiler breeders. In addition, less SE was recovered from the liver and spleen. Vaccinated groups also showed a lower shedding rate, which reduces the chance of horizontal transmission and environmental contamination (Inoue, et al., 2008). Immunoglobulin A (IgA) plays a significant role the immunity against *Salmonella* in the intestinal tract because it inhibits bacterial adherence and colonization of the intestinal mucosa (McGhee, et al., 1992). Maternal antibodies play a crucial role in providing the progeny with immunity to early exposure to diseases at times when they are the most susceptible.

Killed and Live Vaccines

Vaccinations against host-specific *Salmonella* serotypes have been shown to induce a strong serotype-specific protective immunity against infection and disease. However, vaccinations against non-host specific *Salmonella* serotypes have had variable success rates. This is due to the fact host-specific *Salmonella* serotypes cause systemic

disease in which the immunity system of the bird reacts to the infection would does not occur in non-specific *Salmonella* serotypes (Van Immerseel, et al., 2005). Bacterins have been used in the commercial poultry industry in past years with varied results. The varied results might be caused by the unintentional destruction of liable antigens during the preparation of the vaccine (Barrow, et al., 2003). However, these vaccines have been shown to have some effect. In research trials, breeders vaccinated using killed *Salmonella* vaccine, had an increase in *Salmonella*-specific antibody titers. In the progeny of the vaccinated breeders, a decrease in *Salmonella* prevalence and loads was seen (Berghaus, et al., 2011). Field trials in Europe, the vaccination with bacterins were effective in decreasing shedding of *Salmonella* and increased the productivity of the broiler breeders (Feberwee, et al., 2000).

Live avirulent *Salmonella* vaccines which are given orally have been shown to replicate, colonize and invade both visceral and intestinal organs (Hassan and Curtiss, 1997; Mastroeni, et al., 2001). These vaccines have been shown to induce a strong immune response in the vaccinate chickens. In a previous study it was seen that a live vaccine was more effective in increasing lymphocyte proliferation to response to a *Salmonella* antigen and a killed vaccine (Babu, et al., 2003). Killed vaccines may be destroyed rapidly and eliminated from the host without inducing cytotoxic T cells (Barrow, 2007). When live vaccines are administered orally, additional protective effects could be seen such as competitive exclusion and stimulation of primed polymorphonuclear leukocytes in the gut (Van Immerseel, et al., 2005; Bailey, et al., 2007; Barrow, 2007). Adjuvants also play an effective role in induce a response to the

antigens present in the vaccine. Aluminum salt adjuvants help induce Th2 response and also act as immunomodulators. Emulsion adjuvants are able to induce antibody responses (Spickler and Roth, 2003). Both killed and live vaccines have been shown to be effective in the reduction of *Salmonella* shedding, which could reduce the spread of the pathogen to other birds. However, both differ in their abilities to stimulate the immune system. The development of vaccines must take this into account in order to produce a vaccine that would stimulate the immune system effective and produce a prolonged protection.

Summary

Salmonella and *Campylobacter* are two problem microorganisms in the poultry industry. These microorganisms have been shown to be able to vertically and horizontally contaminate table and hatching eggs have proven to be troubling to both the egg and broiler industries. The control of these microorganisms is essential to the poultry industry. The purpose of the following research to evaluated the influence of infectious bronchitis virus (IBV) vaccination on the prevalence of *Salmonella* or *Campylobacter* colonization in young broilers. We will also we evaluated the cross-protection of a combination *Salmonella* vaccine when used in various dosage combinations.

CHAPTER III

EVALUATION OF THE INFLUENCE OF INFECTIOUS BRONCHITIS VIRUS VACCINATION *SALMONELLA* OR *CAMPYLOBACTER* COLONIZATION IN YOUNG BROILER CHICKENS

Description of Problem

According to the Centers for Disease Control and Prevention (CDC), *Salmonella* and *Campylobacter* are the two leading causes of bacterial foodborne illness in the United States (CDC, 2013). Poultry meat has been found to be common source for these microorganisms since they are ubiquitous to the gastrointestinal tract of poultry. Given the opportunity, these microorganisms could proliferate and invade the intestinal tract and ceca of the bird. In broiler flocks the disappearance or reappearance of *Salmonella* and *Campylobacter* has been linked to various environmental and management based factors which include: temperature, humidity and pre- and post-harvest interventions (Bailey, et al., 2001). The control of these microorganisms is of great importance to the commercial poultry industry. Identifying factors that may influence the prevalence of these microorganisms is essential to reducing possible contamination issues at the processing plant.

Infectious bronchitis virus is a highly contagious respiratory disease of poultry. It can be transmitted after inhalation or direct contact with contaminated poultry, litter, equipment or other fomites. This disease is of great concern to the poultry industry because of the economic impact it can have by affecting production as well as egg and

meat quality. Vaccination for this disease occurs at the hatchery and is delivered to newly hatch chick via mass administration. This method can lead to problems in uniformity and cause severe reactions as vaccinated animals shed the virus to unvaccinated animals. This phenomenon is known as a "rolling reaction" and can leave the bird immunocompromised, which might cause the bird to be susceptible to *Salmonella* and/or *Campylobacter* colonization. A previous study reported a high dose of the infectious bronchitis virus vaccine could lead to higher detection of *Salmonella* prevalence in broilers at rear and on carcasses pre-chiller (Volkova, et al., 2011b). In the present study, we evaluated the influence of infectious bronchitis virus (IBV) vaccination on the prevalence of *Salmonella* or *Campylobacter* colonization in young broilers.

Materials and Methods

Animals

Three-hundred day-of-hatch commercial broiler chicks were obtained from a local commercial hatchery. Paper chick tray liners were taken for *Salmonella* and *Campylobacter* evaluation. Intervet MILDVAC-Ark® (Merck Animal Health, Summit, NJ) was sprayed (10x dose) onto a total of ten day-of-hatch chicks using a spray cabinet. Vaccine included a green dye to encourage uptake of the vaccine. After 30 minutes, chicks were individually indentified to indicate they had received the vaccine and placed into their respective pens. Both trials occurred of three disinfected, environment-controlled rooms with 1.8 x 2.8 m floor pens with new pine shavings. Heat lamps were used at the first few days to ensure chicks had adequate heat. All floor pens were equipped with nipple drinkers and feed trays, which were monitored daily. On Day 17,

non-vaccinated chicks (n=10 in trial 1 and n=5 in trial 2) were selected for a booster vaccination, which was administered using the intraocular method and marked with paint to indicate they had received a booster. All birds were given a non-medicated corn-soybean meal starter diet obtained from Texas A&M University Poultry Research Center. All diets were formulated to meet or exceed National Research Council guidelines. Chicks were provided feed and water *ad libitum* from time of placement until termination of the trial.

Tray Liner Evaluation

Using disposable gloves, each individual tray liner was placed into a gallon size bags (S.C. Johnson& Johnson, Racine, WI) and 150 mL of Buffered Peptone Water (BPW) was added into each bag. Bags were then massaged for 60 secs to ensure proper contact between tray liner and BPW. For each peptone-tray-liner sample, 10 mL of soultion was transferred in 10 mL of 2x Bolton broth (Lab M, Bury, Lancashire, UK) and incubated at 42° for 24 hours in a microaerophilic environment (85% N_2, 10% CO_2, and 5% O_2). The rest of the peptone-tray liner samples were incubated at 37°C for 24 hours. Following selective enrichment in Bolton broth, each sample was streaked onto Campy-Cefex agar plates and incubated for 48 hours at 42°C in a microaerophilic environment (85% N_2, 10% CO_2, and 5% O_2)(Stern, 1992). Suspect colonies were confirmed as *Campylobacter* spp. by examination of colony morphology and motility on a wet mount under phase-contrast microscopy (Byrd, et al., 2007). One mL of peptone tray-liner sample was transferred in Rappaport-Vassiliadis (RV) (Becton Dickinson, Franklin Lakes, NJ) enrichment broth and incubated for 24 hours at 42°C. Following enrichment,

each sample was streaked onto plated onto Brilliant Green Agar (BGA) (Becton Dickinson, Franklin Lakes, NJ) with novobiocin (25 µg/mL) and incubated for 24 hours at 37°C. Colonies were analyzed for colony morphology.

Challenge

A strain of *Salmonella* Typhimurium (ST) used for the challenge was selected for resistance to novobiocin (NO) and nalidixic acid (NA). Media to culture the resistant strain contained 25 µg of NO and 20 µg of NA per mL. The challenge inoculum was prepared using an overnight culture, which had been transferred 3 times in trypticase soy broth. The culture was serially diluted in sterile phosphate-buffered saline to approximately 10^5 colony-forming units (cfu) per milliliter (mL) (Byrd, et al., 2008). The optical density of the cell dilution was measured with a spectrophotometer at 625 nm, and the number of cells for the inoculums was determined using a standard curve (Byrd, et al., 2001). Viable cell concentration of the challenge inocula was confirmed by colony units on Xylose-Lysine-Tergitol4 (XTL4) plates (Becton Dickinson, Franklin Lakes, NJ) in trial 1 and Brillant Green Agar (BGA) plates (Becton Dickinson, Franklin Lakes, NJ) in trial 2. Plates were incubated for 24 hours at 37°C and expressed as log10 ST to determine cfu/mL. A total of fifteen chicks were challenged with a 10^5 cfu dose of *Salmonella* Typhimurium (Corrier, et al., 1990) by crop gavage (0.5 mL) and were individually identified to indicate they were challenged with *Salmonella* (Table 1& Table 2). Fifteen chicks were then distributed with the groups (Trial 1: group 1 n=5, group 2 n=10; Trial 2: n=5 in all three groups). Fifteen chicks were challenged with a 10^4 cfu dose of *Campylobacter jejuni* (wild-type) by crop gavage (0.5 mL) and were individually

indentified to indicate they were challenged with *Campylobacter* (Table 1& Table 2). The fifteen chicks were then distributed with the groups (Trial 1: group 1 n=5, group 2 n=10; Trial 2: n=5 in all three groups).The remaining chicks which were not challenged or vaccinated acted as contacts in the trials.

Post-Challenge

Unchallenged and non-marked chicks were taken from their respective pens at various time points (Day 7, 14, 21, 28). Chicks were euthanized by cervical dislocation according to AVMA guidelines and subjected to necropsy. Cecal contents were aseptically collected, weighted and serially diluted at dilutions of 1:10, 1:100, 1:1,000, 1:10,000 in 9 mLs Butterfield's solution tubes and plated for quantitative bacterial re-isolation. For *Salmonella* reisolation, Xylose-Lysine-Tergitol4 (XLT4) plating (Becton Dickinson, Franklin Lakes, NJ) N/N media was used in Trial 1 and in Trial 2 Brilliant Green Agar (BGA) plating (Becton Dickinson, Franklin Lakes, NJ) N/N media was used. All XLT4 and BGA plates were incubated at 37°C for 24 hours and presumptive

Table 1: Experimental Challenge of *Salmonella* Typhimurium and *Campylobacter jejuni*, Vaccination and Booster of Infectious Bronchitis Virus of Day-of-Hatch Broiler Chicks in Trial 1

Groups	*Salmonella* Typhimurium Chicks Challenged (n)	*Campylobacter jejuni* Chicks Challenged (n)	Infectious Bronchitis Virus Vaccinated (x10) Chicks (n)	Revaccinated Infectious Bronchitis Virus (10x, Day 17) Chicks (n)	Contacts (Non-vaccinated And Unchallenged) Chicks (n)	Total Chicks Used (n)
CON	5	5	0	0	90	100
IBVV at Day 1 & 17	10	10	10	10	160	200

Table 2: Experimental Challenge of *Salmonella* Typhimurium and *Campylobacter jejuni*, Vaccination and Booster of Infectious Bronchitis Virus of Day-of-Hatch Broiler Chicks in Trial 2

Groups	*Salmonella* Typhimurium Chicks Challenged (n)	*Campylobacter jejuni* Chicks Challenged (n)	Infectious Bronchitis Virus Vaccinated (x10) Chicks (n)	Revaccinated Infectious Bronchitis Virus (10x, Day 17) Chicks (n)	Contacts (Non-vaccinated And Unchallenged) Chicks (n)	Total Chicks Used (n)
CON	5	5	0	0	90	100
IBVV at Day 1 & 17	5	5	5	5	80	100
IBVV at Day 1	5	5	5	0	85	100

colonies were analyzed for colony morphology and counted. *Salmonella* O Antiserum Poly-A was used for further verification of colonies (Becton Dickinson, Franklin Lakes, NJ) for needed. For *Campylobacter* reisolation, Campy-Cefex plates (Stern, 1992) was used. All Campy-Cefex plates were placed into a microaerophilic environment (85% N_2, 10% CO_2, and 5% O_2) and incubated at 42°C for 48 hours and were analyzed for colony morphology and counted. If further analyzes was needed, motility on a wet mount under phase-contrast microscopy was used for *Campylobacter* spp. confirmation.

Statistical Analysis

Salmonella and *Campylobacter* recovery levels (cfu/g of cecal contents) were compared by factorial ANOVA using the GLM procedure where Day X CFU were compared (SPSS, Armonk, NY). A significant interaction was observed so additional analyses involved comparing recovery levels on each day of sampling (d7, 14, 21, and 28) by one-way ANOVA using the GLM procedure. Significant differences ($P<0.05$) were further separated using Duncan's multiple range test (SPSS, Armonk NY).

Results and Discussion

Microorganisms such as *Salmonella* and *Campylobacter jejuni* occur naturally in the gastrointestinal intact of poultry and are usually non-pathogenic. Interruption in the homeostasis of the gastrointestinal environment can cause the microorganisms to become pathogenic and the shedding of the microorganisms via feces onto the litter can cause other birds to become infected. Infectious bronchitis virus is an acute, highly contagious upper respiratory disease of poultry. Weinack and coworkers (1984) examined the influence of *Mycoplasma galliseptium* and the infectious bronchitis virus (IBV) had on

the shedding of *Salmonella* Typhimurium or *Escherichia coli* in chickens that had established a native intestinal microflora. *Mycoplasma galliseptium* and infectious bronchitis both affect the respiratory system of birds and may cause decreases in body weight, feed efficiency and increases in the incidence of airsacculitis. In the study, they reported that the exposure to *Mycoplasma galliseptium* and infectious bronchitis significantly increased the shedding of *E.coli* and *S.* Typhimurium (Weinack, et al., 1984). The increase in *E.coli* and *S.* Typhimurium shedding may have been caused by a disruption in the intestinal microfloral due to respiratory stress.

At the hatchery, the IBV vaccine is given by mass administration to newly hatched chicks. However uniformity is a problem with this method of administration because vaccinated birds may horizontally shed the virus to "missed" vaccinated individuals within the flock. Transmission of the virus via this route may be variable causing a "rolling reaction". This reaction may cause birds to become immunocompromised and susceptible to *Salmonella* and/or *Campylobacter* colonization. A previous study reported that an increase dosage of IBVV applied via spray to 1-day-old birds was linked to a higher probability of detecting *Salmonella* in the flock during rearing and on the broiler carcasses at the pre-chilling (Volkova, et al., 2011a).

Table 3: *Salmonella* and *Campylobacter* Cecal Recovery from Young Broilers at Day 7 Post Challenge

Trial	Group	N	*Salmonella* (Log_{10} cfu/g of cecal contents)	*Campylobacter* (Log_{10} cfu/g of cecal contents)
1	1.CON-IBVV not administrated	10	3.29±1.34	5.72±1 30
	2. IBVV administration at Day 1 &17	20	4.57±1.48	5.92±1.28
2	1.CON- IBVV not administrated	10	3.78±1.53	4.47±1.38^{A}
	2.IBVV administrated at Day 1 &17	10	4.16±1.75	5.98±.89^{B}
	3.IBVV administrated at Day 1	10	4.41±1.38	4.80±.62^{A}

^{A,B,C}Means with no common superscript differ significantly ($P<0.05$)
Means ± Standard Deviation

Table 4: *Salmonella* and *Campylobacter* Cecal Recovery from Young Broilers at Day 14 Post Challenge

Trial	Group	n	*Salmonella* (Log_{10} cfu/g of cecal contents)	*Campylobacter* (Log_{10} cfu/g of cecal contents)
1	1.CON-IBVV not administrated	10	1.20±1.15	6.40±.32
	2. IBVV administration at Day 1 &17	20	1.57±1.08	6.57±.33
2	1.CON- IBVV not administrated	10	0.88±1.59	5.75±.82^{A}
	2.IBVV administrated at Day 1 &17	10	1.06±.32	6.57±.34^{B}
	3.IBVV administrated at Day 1	10	0.1±1.45	6.57±.24^{B}

^{A,B,C}Means with no common superscript differ significantly ($P<0.05$)
Means ± Standard Deviation

In our present study, we evaluated if birds vaccinated with a high dose of the IBVV would have a higher prevalence of *Salmonella* and/or *Campylobacter* in the ceca of day-of-hatch chicks. All tray liner samples tested negative for *Campylobacter* and only one tray liner sample in trial 1 tested positive for *Salmonella*. These test results may suggest that the chicks in the study were not colonized with either microorganism before the challenge. In trial 1 at Day 7, *Salmonella* cfu was at its highest for the first trial however there was no significant differences in *Salmonella* cfu in Group 1 (Control) and Group 2 (IBVV at Days 1&17) and no significant difference in *Campylobacter* cfu was seen in either groups (Table 3). On Days 14, 21 and 28, *Salmonella* cfu for both groups decreased with no significant differences between the two groups (Tables 4, 5, and 6). On Days 14, 21 and 28, *Campylobacter* cfu remained constant throughout with no significant differences between the two groups (Tables 4, 5, and 6). In trial 1, no significant differences were seen between Group 1 (Control) and Group 2 (IBV at Days 1&17) for either *Salmonella* or *Campylobacter* cfu on all the sample days.

Table 5: *Salmonella* and *Campylobacter* Cecal Recovery from Young Broilers at Day 21 Post Challenge

Trial	Group	n	*Salmonella* (Log_{10} cfu/g of cecal contents)	*Campylobacter* (Log_{10} cfu/g of cecal contents)
1	1.CON-IBVV not administrated	10	0.26±.83	4.99±.86
	2. IBVV administration at Day 1 &17	20	0.46±.56	5.72±.72
2	1.CON- IBVV not administrated	10	0±.00	6.11±.44
	2.IBVV administrated at Day 1 &17	10	0.1±.91	6.18±.33
	3.IBVV administrated at Day 1	10	0.41±.22	6.34±.31

^{A,B,C}Means with no common superscript differ significantly (P<0.05)
Means ± Standard Deviation

Table 6: *Salmonella* and *Campylobacter* Cecal Recovery from Young Broilers at Day 28 Post Challenge

Trial	Group	n	*Salmonella* (Log_{10} cfu/g of cecal contents)	*Campylobacter* (Log_{10} cfu/g of cecal contents)
1	1.CON-IBVV not administrated	10	0.44±.74	5.40±1.18
	2. IBVV administration at Day 1 &17	20	0.75±.57	5.15±.87
2	1.CON- IBVV not administrated	10	0.20±.63	6.28±.96
	2.IBVV administrated at Day 1 &17	10	0±.0000	6.35±.37
	3.IBVV administrated at Day 1	10	0±.0000	6.13±.68

[A,B,C]Means with no common superscript differ significantly (P<0.05)
Means ± Standard Deviation

Table 7: Overall (Main Effect) *Campylobacter* Cecal Recovery from Young Broilers – Trial 2

Trial	Group	n	*Campylobacter* (Log_{10} cfu/g of cecal contents)
2	1.CON- IBVV not administrated	40	$5.84 \pm .83^{A}$
	2.IBVV administration at Day 1 &17	40	$6.25 \pm .83^{B}$
	3.IBVV administration at Day 1	40	$6.06 \pm .83^{A}$

^{A,B,C}Means with no common superscript differ significantly ($P<0.05$)

Means ± Standard Deviation

In Trial 2, at Day 7 no significant difference in *Salmonella* cfu was seen between Group 1 (Control), Group 2 (IBV at Days 1&17) and Group 3 (IBV at Day 1). However at Day 7, a significant difference in *Campylobacter* cfu ($P<0.05$) was seen between IBV at Days 1&17 vs Control and IBV at Day 1 (Table 3). At Day 14, *Salmonella* cfu sharply decreased however no significant difference in *Salmonella* cfu between the groups was seen (Table 4). However on Day 14, a significant difference in *Campylobacter* cfu ($P<0.05$) was seen between IBV at Days 1&17 and IBV at Day 1 vs Control (Table 4). On Days 21 and 28, *Salmonella* cfu remain relatively low with no significant differences between the groups. On Days 21 and 28, although the *Campylobacter* cfu remained high, significant differences seen in the previous sample days were no longer observed. In Trial 2, no significant difference in *Salmonella* cfu was seen between Group 1 (Control), Group 2 (IBV at Days 1&17) and Group 3 (IBV at Day 1) however an increase in *Campylobacter* cfu was seen in Days 7 and 14 (Tables 3 &4).

In both trials, *Salmonella* cfu decreased over days 7, 14, 21 and 28. Although the vaccination program of the broiler breeders flocks used by the commercial hatchery from which the chicks were obtained is unknown, one could conclude breeders may have been vaccinated for *Salmonella*. In previous studies, *Salmonella* vaccination of broiler breeder flocks showed to be beneficial to the progeny. It has been reported that progeny from *Salmonella* vaccinated groups had lower ceca shedding counts when compared to progeny from unvaccinated groups, which would explain the decrease in *Salmonella* as the birds aged (Inoue, et al., 2008). Passive immunity could have been responsible for the low *Salmonella* recovery. It is also unknown if a "rolling reaction" occurred within

the groups. The trials were designed to optimize the chances of the occurrence of a "rolling reaction". In a "rolling reaction", vaccinated birds shed the virus to unvaccinated birds, which could cause the unvaccinated birds to develop a more severe form of the disease and become immunocompromised. However in trial 2, an increase in *Campylobacter* prevalence was seen on Days 7 and 14 in the IBVV groups when compared to the controls, which may indicate a "rolling reaction", may have occurred (Table 7). The increase in *Campylobacter* was in agreement with a study by Weinack and coworkers (1984), in which IBV was seen to increase the shedding of *Salmonella* in chickens. Shedding of the IBV by high dose vaccinated chicks could have the same implications as if the virus was naturally obtained and shedding to also be applied to *Campylobacter*. Although a higher prevalence of *Salmonella* was not seen in birds that received a high dose of the infectious bronchitis virus vaccine and increase in *Campylobacter* prevalence was seen in Trial 2 (Table 7). These results suggest that broilers vaccinated with IBV may lead to higher pathogenic bacterial contamination by using *Campylobacter* cfu post challenge versus non-vaccinated broilers as an example.

CHAPTER IV

EFFICACY OF COMBINING DIFFERENT *SALMONELLA* VACCINE FRACTIONS FOR PREVENTING *SALMONELLA* INTESTINAL COLONIZATION IN EXPERIENTIALLY CHALLENGED PULLETS

Description of Problem

Salmonella has been reported to cause 1.4 million cases of human foodborne illness each year in the United States (Mead, et al., 1999; Kimura, et al., 2004; Voetsch, et al., 2004; Braden, 2006). Although eggs have been frequently identified as a common source for the microorganism, poultry meat could also be a serve as an important source. In 2010 and 2011, two major *Salmonella* outbreak occurred which brought into question the safety of the food produced in the United States. Outcry from consumers put pressure on the government and the commercial poultry industry to take action. The use of antibiotics in animal feed, which are used to reduce pathogens, have also become under fire from consumers in fear of drug-resistant microorganisms (Zhang-Barber, et al., 1999). With the pressure to reduce the use antibiotics in animal feed, researchers have begun to look into possible alternatives that could help in controlling microorganisms such as *Salmonella*.

Vaccines are a powerful tool in the commercial poultry industry with their use being linked to controlling diseases such as Newcastle disease and Merck's disease that once plagued the industry (Fadly and Smith, 1991; Fussell, 1998). Studies found that the vaccination broiler breeder flocks against these diseases provided passive immunity to

the progeny. Maternal antibodies are transferred into the yolk, which are later ingested by the embryo during its development (Dohms, et al., 1978). Previous studies have looked into the use of *Salmonella* vaccines in both layers and broilers. *Salmonella* vaccination was shown to be effective in reducing the shedding of *Salmonella* and providing the progeny of the birds with some protection against early colonized of *Salmonella* (Hassan and Curtiss, 1997; Van Immerseel, et al., 2005; Barrow, 2007; Inoue, et al., 2008). The reduction in *Salmonella* shedding also decreases the possibility of horizontal transmission and environmental contamination.

Salmonella Enteritidis and *S*. Typhimurium are the two most common serotypes linked to foodborne illness. These serotypes are also two most problematic *Salmonella* serotypes plaguing the poultry industry. The development of a vaccine that could provide immunity against these serotypes as well as various other *Salmonella* serotypes would be ideal for the poultry industry. Previous studies have looked into the effectiveness of several individual *Salmonella* vaccines however research done looking into combination of *Salmonella* vaccines is limited. In this study, we evaluate the potential cross-protection of two previously-formulated vaccines when used in various dosage combinations.

Materials and Methods

Animals

Four-hundred and twenty Hy-line W-36 (Hy-line, West Des Moines, IA) female chicks were obtained from a local commercial hatchery. Upon arrival to the trial location, chicks were wing banded for identification and each chick was weighted and the

data was recorded. The chicks were then placed into two 6-stage electrically heat 14.72 ft^2 brooder battery units. On day 21, chicks were weighted and transferred into 4.69 ft^2wired floored grower battery units. All units were equipped with water and feed trays, which were monitored daily. Chicks were provided feed and water *ad libitum* from time of placement until termination of the trial. Layer feed was formulated using nutritional requirements found in the Hy-line W-36 management guide that made or exceeded the National Research guidelines. Animal husbandry was conducted in accordance to the Hy-line W-36 management guide and a protocol approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC).

Pre-Vaccination

Assignment of the chickens into treatment groups took place at time of vaccination (Table 8). Chickens (n=60) were randomly assigned to treatment groups and their wing band number were recorded. Whole blood (1-3 mL) was collected from the jugular veins of 25 birds from each treatment group into Serum Separator Tubes (SST) (Becton Dickinson, Franklin Lakes, NJ) for ELISA determination of anti-*Salmonella* antibody titer (data not shown). Weights for all birds were taken and recorded before birds are place into their assigned location.

Table 8: Group Assignment and Vaccine Administration of W-36 Pullets

Group	Pullets Used (n)	Fraction A[1] (mL)	Fraction B[2] (mL)
1	60	0.5*	0.5*
2	60	0.5	0.5
3	60	0.25	0.5
4	60	0.1	0.5
5	60	0.5	0
6	60	0.25	0
7	60	0	0.5

*Group 1 received 0.5 mL of two mock vaccines containing only the adjuvants used in Fractions A&B.
[1]Two killed *Salmonella* serotypes formulated in an aluminum hydroxide adjuvant.
[2]Single *Salmonella* serotype formulated in oil-in-water adjuvant.

Vaccination

Vaccines were administered by intramuscular injection (IM). Fraction A consisted of two killed *Salmonella* serotypes formulated in an aluminum hydroxide adjuvant and administered using 10 mL syringes with 5/8", 25 gauge needles (Becton Dickinson, Franklin Lakes, NJ). Fraction B consisted of purified proteins from a single *Salmonella* serotype formulated in oil-in-water adjuvant 10 mL syringes with 1", 23 gauge needles (Becton Dickinson, Franklin Lakes, NJ). Each vaccine fraction was administered IM into a separate breast muscle at 6 weeks-of-age (Table 8). The breast muscle (left or right) at which the fraction at the first vaccination was administered was maintained for the booster. The booster occurred 4 weeks after the first vaccination using the same vaccine.

Pre-Challenge

Before challenge at day 84, all chickens were screened for the presence of recoverable *Salmonella* by cloacal swabbing; swabs were placed into 2 mLs of Buffered Peptone Water (BPW), incubated at 37°C for 24 hours and plated onto Brilliant Green Agar (BGA) (Becton Dickinson, Franklin Lakes, NJ) with novobiocin (25 µg/mL). Wing band numbers were recorded for each bird to verify group assignment. Individual swab samples were pooled in groups of four chickens per pool for a total of 5 pools for each group of 20 animals and were cultured for the presence of *Salmonella* before challenge. If a swab pool sample was found to be positive for the presence of *Salmonella*, individuals from which samples were taken were removed from the study and euthanized by cervical dislocation according to the Texas A&M University IACUC guidelines. Whole blood was collected from the jugular vein of each bird into SST for

ELISA determination of anti-*Salmonella* antibody titer (data not shown) before challenge. Birds were then transported to USDA-ARS Southern Plains Agricultural Research Center BSL2 challenge facility for placement into challenge subgroups. 20 individuals from each of the vaccinated groups were divided into the three challenge subgroups. Each challenge subgroup was housed in three separate environment-controlled rooms to prevent cross-contamination between the evaluated challenged serotypes. At this location, the chickens were placed into 3.75 ft^2commercial layer battery units with the 20 individuals divided into three units for each group; two units contained seven pullets while one unit contained six pullets. The distribution of pullets within units was similar in all three rooms. All battery units were equipped with nipple drinkers and feed trays.

Challenge

The individual *Salmonella* serotypes included: *Salmonella* Enteritidis (SE) (Corrier, et al., 1991), *S*. Typhimurium (ST) (Corrier, et al., 1990) and *S*. Hadar (SH) (wild-type) that were selected for resistance to novobiocin (NO) and nalidixic acid (NA). Media to culture the resistant serotypes contained 25 µg of NO and 20 µg of NA per mL. The challenge inoculums were prepared from overnight cultures, which had been transferred 3 times in trypticase soy broth. The cultures were serially diluted in sterile phosphate-buffered saline to approximately 10^8 colony-forming units (cfu) per milliliter (mL). The optical density of the cell dilution was measured with a spectrophotometer at 625 nm, and the number of cells for the inoculums was determined using a standard curve (Byrd, et al., 2001). Viable cell concentration of the challenge inoculums were

confirmed by colony units on Brillant Green Agar (BGA) plates (Becton Dickinson, Franklin Lakes, NJ). Plates were incubated for 24 hours at 37°C and expressed as log10 SE, ST, or SH to determine cfu/mL. Challenge was administered by crop gavage at 2 mLs to each bird (Table 9). Individual *Salmonella* serotypes were challenged on different days to prevent cross-contamination. In a time period of three days, one serotype was administered to its assigned subgroup and same procedure was repeated for the remaining serotypes and subgroups.

Post-Challenge

Chickens were observed daily for 7 days after challenge. Before euthanasia, whole blood (1-3 mLs) was collected from the jugular veins into SST from each bird for ELISA determination of anti-*Salmonella* antibody titer (data not shown). According to the Texas A&M University IACUC guidelines, all chickens within each challenge subgroup were euthanized by CO_2 asphyxiation followed by cervical dislocation and subjected to necropsy. Tissue samples and wing band numbers were recorded for each bird to verify group assignment. One of the ceca from each bird was aseptically removed and cecal contents were aseptically collected, weighted and serially diluted at dilutions of 1:10, 1:100, 1:1,000, 1:10,000 in 9 mL Butterfield's solution tubes and plated onto BGA (Becton Dickinson, Franklin Lakes, NJ) N/N plates. All BGA (Becton Dickinson, Franklin Lakes, NJ) N/N plates were incubated at 37°C for 24 hours and presumptive colonies were analyzed for colony morphology and counted. The other ceca was placed into 20 mLs of Rappaport-Vassiliadis (RV) (Becton Dickinson,

Table 9: ***Salmonella*** **Challenge of W-36 Pullets for each of the Serotype Subgroups**

Group	Pullets Used (n)	Fraction A[1] (mL)	Fraction B[2] (mL)	Challenge dose (mLs)
1	20	0.5*	0.5*	2
2	20	0.5	0.5	2
3	20	0.25	0.5	2
4	20	0.1	0.5	2
5	20	0.5	0	2
6	20	0.25	0	2
7	20	0	0.5	2

*Group 1 received 0.5 mL of two mock vaccines containing only the adjuvants used in Fractions A&B.
[1]Two killed *Salmonella* serotypes formulated in an aluminum hydroxide adjuvant.
[2]Single *Salmonella* serotype formulated in oil-in-water adjuvant.

Franklin Lakes, NJ) enrichment broth, incubated at 42°C for 24 hours and streaked onto BGA (Becton Dickinson, Franklin Lakes, NJ) plates with novobiocin (25 g/L) and nalidixic acid (20 g/L) (N/N), incubated for an additional 24 hours at 37°C and examined for qualitative bacterial reisolation. *Salmonella* O Antiserum Poly-A was used for further verification of colonies (Becton Dickinson, Franklin Lakes, NJ).

Organ Colonization

Briefly, specimens of liver and spleen samples were collected asecptically and cultured as a single combined sample (Corrier, et al., 1991). The combined sample of liver and spleen was placed into 20 mLs of Rappaport-Vassiliadis (RV) (Becton Dickinson, Franklin Lakes, NJ) enrichment broth, incubated at 42°C for 24 hours and streaked onto BGA (Becton Dickinson, Franklin Lakes, NJ) plates with novobiocin (25 g/L) and nalidixic acid (20 g/L) (N/N), incubated for an additional 24 hours at 37°C and examined for qualitative bacterial reisolation.

Statistical Analysis

Body weights taken at pre-vaccination, pre-challenge and post-challenge were compared by factorial ANOVA using the GLM procedure where GROUP X WT were compared. *Salmonella* recovery levels (cfu/g of cecal contents) were compared by factorial ANOVA using the GLM procedure where GROUP X CFU were compared. Significant differences ($P<0.05$) were further separated using Duncan's multiple range test (SPSS, Armonk, NY). Chi-square analysis was done using the Excel software (Microsoft, Redmond, WA) to determine significant differences between groups in *Salmonella* colonization rate.

Table 10: Pre-vaccination Body Weights of W-36 Pullets in *Salmonella* Enteritidis (SE), *S*. Typhimurium (ST), and *S*. Hadar (SH) subgroups at 6 Weeks of Age

Group	Vaccine Received	SE Weight[1] (kg)	ST Weight[1] (kg)	SH Weight[1] (kg)
1	Mock vaccines	438.80±34.45	428.55±27.62^{B}	448.15±27.87^{C}
2	0.5 mL Frac A/B+	430.45±32.68	419.95±36.52^{AB}	417.88±38.75^{AB,3}
3	0.25 mL Frac A/B+	427.60±40.56	396.65±37.72^{A}	432.25±43.10^{ABC}
4	0.1 mL Frac A/B+	435.70±36.27	434.15±33.52^{B}	444.00±33.35^{BC}
5	0.5 mL Frac A/B-	436.75±41.76	418.62±29.12^{AB,2}	441.73±38.51^{BC}
6	0.25 mL FracA/B-	422.30±36.04	440.65±34.44^{B}	409.70±45.30^{A}
7	0 mL Frac A/B+	427.25±37.37	418.85±41.45^{AB}	439.40±28.59^{BC}

^{A,B,C}Means with no common superscript differ significantly ($P<0.05$)
Means ± Standard Deviation
[1]n=20
[2]n=16
[3]n=18

Table 11: Pre-challenge Body Weights of 12 Week-old W-36 Pullets in *Salmonella* Enteritidis (SE), *S*. Typhimurium (ST), and *S*. Hadar (SH) Subgroups 4 Weeks after First Vaccination

Group	Vaccine Received	SE Weight[1] (kg)	ST Weight[1] (kg)	SH Weight[1] (kg)
1	Mock vaccines	593.15±87.58^{A}	953.50±57.41^{AB}	978.73±50.98^{BC}
2	0.5 mL Frac A/B+	563.35±68.24^{A}	950.10±64.37^{AB}	936.33±68.89^{AB,3}
3	0.25 mL Frac A/B+	588.70±93.60^{A}	925.35±58.13^{A}	978.15±65.01^{BC}
4	0.1 mL Frac A/B+	669.50±164.22^{B}	961.90±61.08^{AB}	951.40±75.75^{ABC}
5	0.5 mL Frac A/B-	969.30±71.77^{C}	953.18±43.10^{AB,2}	989.73±51.81^{C}
6	0.25 mL FracA/B-	952.00±66.66^{C}	982.60±58.23^{B}	929.55±63.21^{A}
7	0 mL Frac A/B+	957.70±54.95^{C}	959.50±69.93^{AB}	978.60±53.32^{BC}

^{A,B,C}Means with no common superscript differ significantly ($P<0.05$)

Means ± Standard Deviation

[1]n=20

[2]n=16

[3]n=18

Results and Discussion

Salmonella is a microorganism that of great interest to the commercial poultry industry because of the millions of human foodborne illness cases is causes each year. Vaccines have been successful in controlling diseases that economically devastating to the commercial poultry industry (Fadly and Smith, 1991; Fussell, 1998). The success of these vaccines has gained the interest in developing vaccines to control *Salmonella* in poultry production flocks. Previous studies have shown the effectiveness of *Salmonella* vaccines in poultry. The used of killed *Salmonella* vaccines have shown to decrease *Salmonella* shedding versus non-vaccinated groups (Feberwee, et al., 2000; Berghaus, et al., 2011). The progeny of vaccinated breeders have also been reported to have protective immunity to *Salmonella* infection (Inoue, et al., 2008).

In the current study, we evaluated the effects of a combination vaccine used in various dosage combinations and its ability in proving protective immunity against three serotypes of *Salmonella*. Pre-vaccination body weights within the groups did have some significant differences however an explanation to the variation in body weight could not be determined since all birds were raised under the same conditions (Table 10). The differences in average body weights after vaccination could be associated with the energy used to react to the vaccines and the production of antibodies (Table 11).The maintenance of a competent immune or an immune response has been shown to be energy demanding (Lochmiller, et al., 1993). In the *S*. Enteritis subgroup, individuals in group 2 (0.5 mL Frac A/B+) and group 6 (0.25 mL Frac A/B-) had significantly lower average body weights, 990.95 kgs and 1002.75 kgs respectively, versus the control, 1057.05 kgs, at

post-challenge however it is hard to determine the cause for the difference in body weight (Table 12). In the *S*. Typhimurium subgroup, trends in average body weight remained constant throughout the trial. Individuals in group 6 (0.25 mL Frac A/B-) had the highest average body weights, 982.60 kgs at pre-challenge (Table 11) and 1058.60 kgs at post-challenge (Table 12). Individuals in group 3 (0.25 mL Frac A/B+) had the lowest average body weights, 925.35 kgs at pre-challenge (Table 11) and 1011.35 kgs at post-challenge (Table 12). There was no significant difference between the average body weights of the other groups.

In the S. Hadar group, trends in average body weight also remained constant throughout the trial. At pre-challenge (Table 11), individuals in group 5 (0.5 mL Frac A/B-) had the highest average body weights, 989.73 kgs and individuals in group 6 (0.25 mL Frac A/B-) had the lowest average body weights, 929.55 kgs. At post-challenge (Table 12), individuals in group 5 (0.5 mL Frac A/B-) had the highest average body weights, 1018.68 kgs and individuals in group 2 (0.5 mL Frac A/B+) had the lowest average body weights, 952.61 kgs. In both the S. Typhimurium and S. Hadar groups, the average body weights post-challenge were not significantly different when compared to the controls.

Table 12: Post-challenge Body Weights of 14 Week-old W-36 Pullets 14 in *Salmonella* Enteritidis (SE), *S*. Typhimurium (ST), and *S*. Hadar (SH) Subgroups 7 Days after *Salmonella* Challenge

Group	Vaccine Received	SE Weight[1] (kg)	ST Weight[1] (kg)	SH Weight[1] (kg)
1	Mock vaccines	1057.05±89.10^{C}	1022.55±55.40^{AB}	997.57±62.85^{ABC}
2	0.5 mL Frac A/B+	990.95±59.25^{A}	1023.00±68.91^{AB}	952.61±75.77^{A,3}
3	0.25 mL Frac A/B+	1038.55±64.25^{ABC}	1011.35±64.21^{A}	1002.65±62.88^{BC}
4	0.1 mL Frac A/B+	1047.80±77.50^{BC}	1027.85±62.92^{AB}	975.00±90.09^{ABC}
5	0.5 mL Frac A/B-	1016.55±76.54^{ABC}	1032.12±48.36^{AB,2}	1018.68±40.31^{C}
6	0.25 mL FracA/B-	1002.75±67.99^{AB}	1058.60±61.81^{B}	957.85±64.36^{AB}
7	0 mL Frac A/B+	1029.55±57.38^{ABC}	1032.80±70.79^{AB}	1001.55±59.95^{BC}

^{A,B,C}Means with no common superscript differ significantly ($P<0.05$)
Means ± Standard Deviation
[1]n=20
[2]n=16
[3]n=18

Table 13: *Salmonella* Enteritidis Cecal Reisolation, Organ Colonization, and Cecal Recovery in W-36 Pullets 7 Days after Challenge

Group	Vaccine Received	*Salmonella*-culture – positive/total (%) – Direct plating [a]	*Salmonella*-culture – positive/total (%) - Ceca [a]	*Salmonella*-culture – positive/total (%) – Liver and Spleen [a]	*Salmonella* Enteritidis (Log10 cfu/g of cecal contents)
1	Mock vaccine	13/20	18/20	4/20	2.65 ± 1.06^{B}
2	0.5 mL Frac A/B+	8/20	10/20*	5/20	1.64 ± 1.09^{A}
3	0.25 mL Frac A/B+	6/20***	7/20**	5/20	1.29 ± 1.62^{A}
4	0.1 mL Frac A/B+	6/20***	10/20*	6/20	1.45 ± 1.51^{A}
5	0.5 mL Frac A/B-	7/20	8/20**	2/20	1.29 ± 1.50^{A}
6	0.25 mL FracA/B-	5/20**	2/20**	2/20	0.70 ± 1.14^{A}
7	0 mL Frac A/B+	4/20***	7/20**	2/20	0.95 ± 1.20^{A}

[+/-]Fraction B was given at 0.5 mL

[A,B,C]Means with no common superscript differ significantly (P<0.05)

Means ± Standard Deviation

[a]Values followed by asterisks are significantly different from controls: *=P<0.01, **=P<0.001,***P<0.05

In the *Salmonella* Enteritidis subgroup, all the vaccinated groups had lower recovered cecal *Salmonella* cfu when compared to the control. These results are in agreement with previous studies in which *Salmonella* vaccination decreased the shedding of *Salmonella* (Zhang-Barber, et al., 1999; Van Immerseel, et al., 2005; Berghaus, et al., 2011). However there was not a significant difference between the variation in the fraction dosage and the fraction received. In relation to cecal re-isolation, the results were all significant different when compared to the control. However group 3 (0.25 mL Frac A/B+), group 5 (0.5 mL Frac A/B-), group 6 (0.25 mL Frac A/B-), and group 7 (0 mL Frac A/B+) had significant less positive samples ($p<.001$), which may indicated the vaccine dosages received by these pullets were more effective preventing cecal colonization (Table 13). Organ invasion throughout the groups were low and showed no significant difference between the groups. In the *Salmonella* Typhimurium group, there were no significant differences between the control and the vaccine groups (Table 14). There were also no significant differences seen in the cecal reisolation and organ invasion (Table 14). The lack in response seen in the *Salmonella* Typhimurium group could be related to an unsuccessful challenge of the individuals in this group. A previous study reported that older birds become more resistant to *Salmonella* infections due to having a well-developed microflora and a mature immune system (Gast and Holt, 1998).

Table 14: *Salmonella* Typhimurium Cecal Reisolation, Organ Colonization, and Cecal Recovery in W-36 Pullets 7 Days after Challenge

Group	Vaccine Received	*Salmonella*-culture – positive/total (%) – Direct plating [a]	*Salmonella*-culture – positive/total (%) - Ceca [a]	*Salmonella*-culture – positive/total (%) – Liver and Spleen [a]	*Salmonella* Typhimurium (Log10 cfu/g of cecal contents)
1	Mock vaccine	4/20	11/20	1/20	1.13±1.10
2	0.5 mL Frac A/B+	5/20	7/20	1/20	1.01±1.25
3	0.25 mL Frac A/B+	5/20	11/20	5/20	1.14±1.20
4	0.1 mL Frac A/B+	6/20	11/20	5/20	1.34±1.31
5	0.5 mL Frac A/B-	3/20	6/16	2/16	0.79±1.18
6	0.25 mL FracA/B-	5/20	9/20	5/20	1.30±1.38
7	0 mL Frac A/B+	3/20	7/20	3/20	0.76±1.04

[+/-]Fraction B was given at 0.5 mL

[A,B,C]Means with no common superscript differ significantly (P<0.05)

Means ± Standard Deviation

[a]Values followed by asterisks are significantly different from controls: *=P<0.01, **=P<0.001

Table 15: *Salmonella* Hadar Cecal Reisolation, Organ Colonization, and Cecal Recovery W-36 Hens 7 Days after Challenge

Group	Vaccine Received	*Salmonella*-culture – positive/total (%) – Direct plating [a]	*Salmonella*-culture – positive/total (%) - Ceca [a]	*Salmonella*-culture – positive/total (%) – Liver and Spleen [a]	*Salmonella* Hadar (Log10 cfu/g of cecal contents)
1	Mock vaccine	13/20	18/20	4/20	2.58±1.13
2	0.5 mL Frac A/B+	7/18	15/18	7/18	2.05±1.17
3	0.25 mL Frac A/B+	9/20	12/20*	8/20	2.09±1.74
4	0.1 mL Frac A/B+	12/20	15/20	8/20	2.53±1.62
5	0.5 mL Frac A/B-	9/20	10/20**	8/20	2.22±1.69
6	0.25 mL FracA/B-	6/20*	12/20*	12/20**	1.53±1.30
7	0 mL Frac A/B+	7/20	11/20*	4/20	1.59±1.46

[+/-] Fraction B was given at 0.5 mL
[A,B,C] Means with no common superscript differ significantly ($P<0.05$)
Means ± Standard Deviation
[a] Values followed by asterisks are significantly different from controls: *=$P<0.05$, **=$P<0.01$

In the *Salmonella* Hadar group, there was no significant difference between the control and the vaccine groups (Table 15). However a significant difference was seen in cecal reisolation. A significant difference ($P<0.05$) was seen in group 3 (0.25 mL Frac A/B+), group 6 (0.25 mL Frac A/B-), and group 7 (0 mL Frac A/B+) and a significant difference ($P<0.01$) was seen group 5 (0.5 mL Frac A/B-) (Table 15). This may indicate the vaccinated groups did have some resistance to the *Salmonella* challenge. In organ invasion, group 6 (0.25 mL Frac A/B-) was the only group that had a significant higher positive-samples ($P<0.01$) however an explanation cannot be made.

The age of the birds could have played a crucial role in the success of the *Salmonella* challenge. With older birds having a successful challenge can be more difficult as these birds have developed their immune systems (Gast and Holt, 1998). It could explain why weak/low responses were seen the *Salmonella* Typhimurium and *S*. Hadar groups. Access to the results of the ELISA determination of anti-*Salmonella* antibody titer could provide more insight on the effectiveness of the vaccines and if significant differences between dosages were seen. Based on the bacterial re-isolation and enumeration results, we can conclude that the combination vaccine was effective in reducing shedding of S. Enteritidis but cross protection against multiple serotypes was not observed.

CHAPTER V

CONCLUSION

This manuscript examined two different aspects of vaccination. In the first aspect, we evaluated the influence of infectious bronchitis virus (IBV) vaccination on the prevalence of *Salmonella* or *Campylobacter* colonization in young broilers. A previous study reported that a higher dose of infectious bronchitis virus increases detection of *Salmonella* in broiler at rearing and on carcasses pre-chiller. The increase in prevalence in *Salmonella* could correlate with the effects of a "rolling reaction". With vaccinated chicks shedding the virus to "missed" vaccinated chicks, virulence of the virus could worsen as the virus transfers from bird to bird. The respiratory stress caused by the virus may lead to disruptions in the intestinal mircoflora. However, the increase in Salmonella was not our current study but an increase in *Campylobacter* prevalence was seen at day 7 and day 14. This finding may indicated that a higher dose of the infectious bronchitis vaccine could increase the prevalence of *Campylobacter* in broilers.

In second aspect, we evaluated the cross-protection of a *Salmonella* combination vaccine when used in various dosage combinations. Reduction in the shedding of *Salmonella* Enteritidis was seen in all the vaccinated groups when compared to the control however no significant difference was seen between the different dosages. No signification differences in *Salmonella* shedding were seen in the *Salmonella* Typhimurium and *S.* Hadar vaccinated groups. This data might bring new incite to the poultry industry. Improvements in vaccine application of the infectious bronchitis virus

could reduce the incidence of *Campylobacter*. Further work is needed in the development of new *Salmonella* vaccines, which can provide cross protection of different serotypes.

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