

ABSTRACT

Title of Document: PREVALENCE OF *SALMONELLA* ON LAYING HEN FARMS AND CONTROL OF COLONIZATION IN POULTRY THROUGH EGG YOLK ANTIBODIES.

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In the United States, rates of foodborne illness caused by *Salmonella* have not changed significantly. One study in this thesis estimated *Salmonella* prevalence and antimicrobial resistance of various samples from conventional (n=181) and organic (n=252) farms. Rates of *Salmonella* contamination were significantly lower on conventional than organic farms. Antimicrobial resistance was significantly higher on isolates from conventional versus organic farms. These findings suggest that poultry production practices may have significant effects on prevalence and antibiotic resistance patterns of *Salmonella*.

The other study assessed the efficacy of a *Salmonella* control strategy using anti-*Salmonella* antibodies, two chicken cell lines, an HD-11 macrophage and a DF-1 fibroblast line, and *Salmonella* serovars Typhimurium and Enteritidis. In DF-1 cells, treatment showed decrease adherence of the pathogen. However, in HD-11 cells,

treatment showed an increase in pathogen adherence, indicating a more detailed understanding of chicken response to treatment with the antibodies is needed before full-scale implementation.

PREVALENCE OF *SALMONELLA* ON LAYING HEN FARMS AND CONTROL
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By

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Dedication

To my parents, brothers, friends and Veronica for your endless support, encouragement and inspiration throughout my study.

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Chapter 1: Introduction

Salmonella is a genus of Gram-negative bacteria that can persist in the gastrointestinal (GI) tract of humans and animals and causes the disease, called salmonellosis. The *Salmonella* genus is comprised of only two species, *S. enterica* and *S. bongori*. The *enterica* species is further branched into six subspecies *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*, and altogether it has more than 2,500 different serotypes. It is estimated by the Center for Disease Control and Prevention (CDC) that there are 48 million cases of domestically acquired foodborne illness annually, of which nontyphoidal *Salmonella* contributes to 1.03 million cases. Moreover, foodborne nontyphoidal *Salmonella* is estimated to cause 19,336 cases of hospitalization and 378 deaths annually (1). The majority of human outbreaks of *Salmonella* reported to the CDC are attributed to either chicken or egg products (2). Economic estimates of human nontyphoidal *Salmonella* infection place the annual burden between \$1.5 - \$10.9 billion, when accounting for medical costs and productivity loss (3). Adoption of *Salmonella* control measures may be able to decrease incidence of salmonellosis associated with poultry and poultry products. Cost-effective control measures that will maximize benefit to the consumers, producers and poultry industry/farms must be studied further.

While serovar specific rates of salmonellosis have changed since 1996-1998, the overall incidence of laboratory-confirmed *Salmonella* cases has remained consistent through 2012 (4). During 1998-2008, of the 1,491 reported *Salmonella* outbreaks, 403 outbreaks were caused by a single serotype and a single-food commodity. Eggs and chicken represented the majority of these outbreaks, with 28%

and 16%, respectively (2). Moreover, from these 403 outbreaks, 36% were caused by *Salmonella* Enteritidis and 14% were caused by *Salmonella* Typhimurium. As a hardy pathogen, *Salmonella* is able to survive in many different environments and *Salmonella* contamination on farms may come from a variety of sources, including wild animals and rodents, insects, feed and humans on the farms themselves (12, 67, 119, 120, 121). While decontamination of poultry meat or eggs may help decrease human foodborne illness, in order to successfully decrease the incidence of poultry associated *Salmonella* infections, multiple intervention strategies are needed.

It is also important to address the growing organic market in the United States. The Organic Trade Association reported that the organic food industry has grown from \$1.0 billion dollars in 1990 to \$26.7 billion in 2010. Further, organic poultry meat captured \$294 million of the total \$470 million spent on organic meat in 2010 and in 2008, total organic egg sales were valued at \$154.8 million (5). Compared to their conventional counterparts, organic operators certified by the United States Department of Agriculture are not allowed to use growth promotants, drugs or synthetic antibiotics. Studies focusing on the incidence and antimicrobial resistance of *Salmonella* associated with chicken have shown mixed results (17, 20, 21, 25). Many of these studies have focused on contamination in broiler hens and their carcasses, leaving us with gaps in our knowledge specifically for laying hens. Moreover, due to the lack of antibiotic use available on these organic farms, the efficacy of alternative control strategies such as passive immunization, pro or pre-biotics, bacteriophages, fermented feed or naturally occurring antimicrobial peptides must be studied. Combined with the growing organic egg sector and the lack of

information available for organic laying hens, these studies look to understand the epidemiology of *Salmonella* in organic chicken layer farms and efficacy of alternative *Salmonella*

1.1 Goal and Objectives

The goal of this study was to contribute to the growing body of knowledge associated with the epidemiology and control of *Salmonella* at the production level. The following objectives were set in this study:

- Prevalence, distribution, and antimicrobial resistance of *Salmonella* serovars isolated from organic and conventional laying hen farms
- Evaluate the efficacy of egg-yolk antibodies as a novel *Salmonella* in-feed additive control strategy using *in vitro* poultry cell models

1.2 Salmonella Overview

Salmonella represents a genus of Gram-negative, rod-shaped bacteria from the family *Enterobacteriaceae*. The pathogen is named after Daniel Salmon, an American veterinary pathologist, and can be found in a variety of different environmental sources. Human associated *Salmonella* infections, known as salmonellosis, are typically associated with the onset of gastroenteritis within 12 to 72 hours of infection. In mild disease, symptoms typically involve diarrhea and abdominal cramps, which may last 4-7 days. More severe cases of *Salmonella* infection may spread from the intestine to the blood stream to cause systemic illness; typically immunocompromised infants and elderly persons are at higher risk for severe illness.

To differentiate between *Salmonella enterica* at the subspecies level, a system was devised to characterize strains by serotyping based on the immunoreactivity against two surface structures, the O and H antigens. First, isolates are identified at the genus and species level by biochemical test. Next, a series of independent agglutination tests determine the O and H antigens on the surface; *Salmonella* are then placed into different serotypes based on a Kauffman-White scheme. In 2007, the CDC officially recognized over 2,500 different serovars of *Salmonella*, with approximately 60% belonging to subspecies *enterica* (122). However, only a relatively small subset of these serovars cause human illnesses, with the top 100 serotypes accounting for 98% of all isolated recovered by the CDC (122).

Salmonella is recognized as a causative agent of zoonotic and emerging infectious disease in the United States. *Salmonella* is often host-adapted, meaning certain serovars of the pathogens can cause disease only in particular species of animals. Humans can be infected from a variety of sources, however not all *Salmonella* serovars can cause illness in humans (2, 6). For example, *S. Pullorum* causes disease in chickens, while *S. Enteritidis* is able to colonize chicken without causing any symptomatic disease in adult chickens (9). Moreover, it has been demonstrated that *Salmonella* are able to survive in a variety of environments ranging in moisture content, pH, salinity and heat (124, 125). Since *Salmonella* contamination and persistence may occur in meats, vegetables, fruits and animals, human *Salmonella* infection remained relatively high and consistent for the past 15 years, compared to infection rate by other pathogens.

1.3 Human Associated Salmonella

In 2011, the CDC noted that the top five identified serovars of *Salmonella enterica* associated with humans were Enteritidis, Typhimurium, Newport, Javiana and I 4,[5],12:i:- (1). It has been reported that severity of salmonellosis is dependent on the serotype causing the illness. For example, a study focused on the outcomes of individuals infected with different serotypes of *Salmonella*, found that 24.2% of *S. Typhimurium* cases resulted in hospitalization, while *S. Enteritidis* cases resulted in hospitalization of 20.6%. In the same study, of the 2,830 individuals who were infected with *S. Heidelberg*, 13.5% of those led to invasive disease compared to 1.4% of *S. Newport* cases (7).

To better understand how the epidemiology of *Salmonella* infection has been changing, the Foodborne Disease Active Surveillance Network (FoodNet) compared the rates of the top, human associated laboratory-confirmed *Salmonella* serotypes in 2012 to previous years through 1996. When compared with the 1996-1998 rates, serovars Enteritidis and Newport were both higher, while the relative rate of laboratory confirmed *S. Typhimurium* infections was lower. Unfortunately, the changes over time for serotypes Javiana and I 4,[5],12:i:- were not tracked.

Between 1998 and 2008, a total of 1,491 outbreaks of *Salmonella* were reported to the Foodborne Disease Outbreak Surveillance System (FDOSS). Outbreaks are only defined as the occurrence of two or more cases of illness resulting from ingestion of the same food (2). Of the reported outbreaks, 403 were attributable to a single serotype and single food commodity and a total of 47 different serotypes were identified (2). The top four *Salmonella* serotypes, (Enteritidis, Typhimurium,

Newport and Heidelberg) caused more than 60% of the 403 outbreaks traceable to a single food commodity; *S. Enteritidis* caused 36% of the 403 outbreaks, while *S. Typhimurium*, Newport and Heidelberg caused 14%, 10% and 6%, respectively. Of the 144 *S. Enteritidis* outbreaks, eggs and chicken were implicated in 65% and 13% of the outbreaks, respectively. Out of the 58 *S. Typhimurium* outbreaks, eggs and chicken were implicated in 7% and 26% of the cases, respectively (2).

Salmonellosis may also occur due to contact with contaminated animals or environments. It has been estimated that contact with animals has led to 120,000 cases of *Salmonella* caused illness, leading to 2,400 hospitalizations and 47 deaths (8). Serovar specific rates of salmonellosis caused by contact have not been estimated, though several species have been implicated as important reservoirs for *Salmonella* illness through contact. Live poultry, dogs, cats, reptiles and rodents have all previously been implicated in multi-state outbreaks of *Salmonella*. It is important to better understand *Salmonella* persistence in these vectors and strategies for controlling infection from these sources.

1.4 Salmonella Associated with Poultry

Salmonella serovars associated with poultry have been dramatically changing since the early 1900's. Previously, serovars *S. Gallinarum* and *S. Pullorum* were endemic to poultry flocks in the United States. These two host-adapted serovars cause severe illness in poultry; symptoms include anorexia, diarrhea, dehydration, decreased egg production and increased mortality. In 1935, the United States Department of Agriculture (USDA) established the National Poultry Improvement Plan (NPIP) in order to eradicate the two pathogens and adoption of this program

allowed *S. Gallinarum* and *S. Pullorum* to be eradicated from commercial poultry flocks by the 1970's. It is believed that the disappearance of these pathogens left a niche for other pathogens to fill (9, 10). Another theory states that *S. Enteritidis* was associated primarily with rodents and the interaction between these two species allowed *S. Enteritidis* to transfer to poultry flocks (11). Others attribute the rise in *S. Enteritidis* in poultry to competitive exclusion by *S. Gallinarum*. It is thought that the presence of *S. Gallinarum* increased flock immunity against *S. Enteritidis*, ensuring colonization only by the host-adapted serovar (9).

Transmission of *Salmonella* can occur either horizontally and vertically. Horizontal transmission typically occurs through fecal-oral transmission; however other experiments acknowledge that *Salmonella* may be transmitted among poultry flocks through environmental contamination (12). Vertical transmission occurs when infected ovarian tissues pass on the bacteria to the developing egg before oviposition. In that case, the *Salmonella* will contaminate and persist in the egg yolk and will be found in the newly hatched chick. Should the new chick survive disease, they may act as a carrier to infect other hens (13).

While *S. Enteritidis* briefly surpassed *S. Typhimurium* as the number one human associated serovar in the 1990's, quality assurance programs put in place by NPIP and egg producers may account for the decrease in prevalence of *S. Enteritidis* in eggs and poultry (9, 14). Regulations to control *S. Enteritidis* have been put into place as recently as 2009, with the FDA's egg safety rule, which was meant to decrease infection in chickens and their associated eggs by the serovar (76). It is believed that these rules have led to the increase in other chicken associated

Salmonella serovars, *S. Kentucky* and *S. Heidelberg* (15). The present study was aimed to contribute to the current knowledge of work by testing the prevalence, distribution and antimicrobial resistance of *Salmonella* isolated from laying hen farms here in the United States. By understanding these characteristics of *Salmonella* in these farming systems, targeted control strategies can be put in place to better address the *Salmonella* serotypes most often associated with poultry products.

1.5 Antimicrobial Interventions in Poultry Practices

A number of studies have been performed to understand the effect of farming system on the prevalence and distribution of antimicrobial susceptible and resistant *Salmonella*. Many of these studies focused on the effect of organic or conventional farming practices on broiler chickens; while *Salmonella* contamination is quite variable between farming systems, antimicrobial resistance was typically found to be higher in *Salmonella* isolates recovered from conventional compared to organic farms (16-20).

A gap of knowledge exists in our understanding of *Salmonella* prevalence on laying hen farms in the United States, while many studies have been performed in the European Union (20-22). In the E.U., antimicrobials are not allowed to be used as a method to control *Salmonella* in poultry with the exception of a very small set of specific cases, such as poultry presenting clinical salmonellosis causing “undue suffering to the animals” or salvaging valuable genetic material from “elite” or research flocks. In either of these cases, administration of antimicrobial must be done under the supervision of a competent authority figure (23). However, in the United States, poultry may have no exposure to antimicrobials (organic hens) or may not be

exposed to different classes of antimicrobials, depending on poultry type (conventional laying hens, broilers) (24). Organic farms are certified by the USDA are not allowed to sell any products as organic when they come from animals treated with antimicrobials and the use of vaccines as a treatment strategy is encouraged. Meanwhile, conventional farming practices are allowed to use a variety of antimicrobials. Conventional laying hen farms are limited to using bacitracin, tylosin and chlortetracycline, while broilers can use a wider variety of antimicrobial classes (24).

1.6 Alternative Salmonella Poultry Control Strategies

Antimicrobial resistance has been documented in broilers and layers in both organic and conventional laying hen systems (20, 21, 25). Further, concerns about the use of antimicrobials and growth hormones in live-stock have been driving consumer demand to the organic sector, where the use of antimicrobials is banned. A variety of alternative antimicrobial intervention strategies exist to address the issue of *Salmonella* in poultry, however continued work is needed to better understand the efficacy of these strategies. Further, it is believed that reducing *Salmonella* at the poultry-production chain may minimize contamination as poultry products move from the farm to the table.

To help reduce *Salmonella* at the production level, a number of strategies are being developed. The majority of these strategies are meant to be added to the feed itself in order to reduce enteric colonization of *Salmonella*. The in-feed additives are devised to target the pathogen specifically or to modulate several factors of the chicken gastrointestinal tract including pH and the poultry microbiota (26-28).

One of these strategies is to acidify the environment of the gastrointestinal tract of the chicken, through the use of fatty-acids or fermented liquid feed. It is believed that the successful acidification of the gastrointestinal environment will affect the bacteria by either inhibiting their growth (bacteriostatic) or actively killing them (bactericidal) (29). Short-chain and medium-chain fatty acids have been demonstrated to have both bacteriostatic and bactericidal effects *in vitro*, though some of these tests suggest that exposure to fatty-acids may increase invasiveness and survival at low pH's (30, 31). Studies with fermented liquid feed have generally been more positive. Fermentation of the liquid feed may occur via back slopping (adding a previously fermented batch as inocula) or through addition of fresh lactic acid bacteria; these two processes lead to the increase concentration of lactic acids produced by lactic acid bacteria, leading to low pH's. Of the few trials testing the effects of fermented liquid feed, the general consensus showed the fermented liquid feed was able to significantly decrease *Salmonella* colonization when compared to their respective control groups (32, 33). More work is needed to better understand the best combination of treatments that will be able to significantly decrease *Salmonella* colonization in poultry.

The application of pro-, pre-, or synbiotics to modulate the microbiota of poultry represents other alternatives to antimicrobials. Probiotics are live bacteria that are meant to improve the intestinal microbial balance, while prebiotics are ingredients added to the feed in order to stimulate the growth and/or activity of beneficial bacteria; synbiotics represent the combination of both probiotic strains of bacteria and prebiotic ingredients meant to stimulate their populations. Probiotic bacteria used in

poultry include *Lactobacillus*, *Enterococcus*, *Pediococcus* and *Bifidobacteria* and the application of these probiotics have been shown to decrease in *Salmonella* contamination in broiler chickens (34-36). It is believed that these bacteria may overcome *Salmonella* through increased competition for receptor sites, nutrients and other metabolites. Designing of the most effective synbiotic will require a deeper understanding of the metabolism of these probiotic bacteria and their mechanism of action against enteric pathogens.

Vaccination may also be an alternative to antimicrobial therapy, though development of an effective vaccine requires a comprehensive understanding of host and pathogen interaction. Vaccines have been designed using a variety of different antigens; both killed and live-attenuated *Salmonella* strains have been used for vaccination and have shown a protective effect (37). After vaccination occurs, a phagocytic cell engulfs the foreign body; fragments of the foreign body is presented by a helper T cell to B-lymphocytes, leading to the production of antigen specific antibodies by plasmocytes. Antigen specific antibodies may help induce phagocytosis of foreign pathogens or help lead to the lysis of the bacteria. A class of the antigen specific antibodies, known as immunoglobulin-Y (IgY) are deposited specifically into the egg-yolk of the chicken, to deliver passive immunity to the developing chick. This antibody can be found exclusively in the egg-yolk and is analogous to mammalian Immunoglobulin-G, though it has an extra fragment in the Fc region (38, 39). Due to the complex nature of an immune response and the pathogenicity of *Salmonella*, development of a vaccine effective across the multiple serotypes found in poultry will need to be researched further.

Adhesins may prove to be an important target for vaccination. Adhesins represent a class of cell-surface components that are necessary for binding and colonization to a particular surface. *Salmonella* has a variety of adhesins, though it is not well understood which adhesins are required for adherence and colonization of poultry. Type 1 Fimbriae have been implicated in *S. Enteritidis* colonization of the oviduct tract in poultry, while flagellae were implicated in *S. Enteritidis* gut colonization (40, 41). For *S. Typhimurium* attachment to HeLa cells, a type 1 fimbriae leads to reversible binding (42). Furthermore, plasmid encoding fimbriae (*pef*), thin aggregative fimbriae (*tafi*) and long polar fimbriae (*lpf*) have been implicated in *S. Typhimurium* biofilm formation on chicken intestinal epithelium (43). In addition to these, *Salmonella* have other fimbral adhesins and atypical structures, such as flagellum and type three secretion systems, which contribute to adhesion to other tissues (44). Information specifically targeting *Salmonella* adhesion to poultry tissues must be studied in more detail for better understanding of serovar specific adhesion factors. Vaccination against these antigens could be useful for producing antibodies specifically to prevent initial colonization.

Like active immunization, passive immunization through oral-ingestion of anti-*Salmonella* antibodies may prove to be effective in decreasing *Salmonella* contamination in poultry (38, 45, 46). After immunization against adhesins, a hen will lay eggs with a high titer of specific antibodies that can be recovered from the yolk. After collection of these antibodies, they may be administered either in the feed or water provided to the hens, where they will preferentially bind to *Salmonella* adhesins, preventing the pathogen from adhering or colonizing the gastrointestinal

tract of the chicken. Several studies have been performed using non-poultry cells lines and anti-*Salmonella* antibodies have showed positive results; however, the follow up *in vivo* studies have showed poor efficacy and attributed this response to the antibodies being denatured in the gastrointestinal tract of the chicken (47, 48). The failure of passive immunity effectiveness may be attributable to an improper *in vitro* model of infection or too low of a small concentration range tested or an improper delivery system used. There is a need for more research to be done in the area of passive immunization using IgY: the development of a proper vaccine target that will work against multiple *Salmonella* serotypes and finding a delivery system that will allow antibodies to remain intact are both necessary before this strategy is adopted widely. One of the aims of the present study was to add knowledge to this body of work by testing whether or not antibodies produced against multiple *Salmonella* antigens can reduce their colonization using *in vitro* poultry cell models.

Chapter 2: Prevalence and Antibiotic Susceptibility of *Salmonella* serovars in organic and conventional laying hen farms

2.1 Abstract

Salmonella is a gram-negative pathogen, capable of causing foodborne-illness in humans. In the United States, of the 403 *Salmonella* outbreaks attributable to a single serovar and food commodity, 28% of these were traced back to chicken eggs (2). The majority of studies linking human *Salmonella* food-borne infection to chickens have focused on the environments and the carcasses of broiler laying hens. The growing demand for organic eggs market coupled with the inability of organic farmers to use antibiotics prophylactically, has made it important to study the prevalence of *Salmonella* contamination in conventional and organic laying hen farms. In this study, a total of 433 (181 conventional, 252 organic) samples were collected between April 2013 and January 2014, from a total of nine farms (three conventional, six organic) in order to estimate the contamination rates of *Salmonella* on these two farming systems. Samples were collected from the laying hen environments (bedding, dust baths), feed/water and the eggs themselves. We aimed to identify the top egg associated *Salmonella* serovars, *S. Enteritidis*, *S. Typhimurium* and *S. Heidelberg*, as well as determine antimicrobial resistant phenotypes of the *Salmonella* isolates. Results showed that the rates of *Salmonella* contamination were significantly ($p = 0.008$) higher on organic farms (20.2%, 51/252) than they were on conventional farms (10.5%, 19/181) and *S. Typhimurium* was the most prevalent serovar on both farming systems. Of *S. Typhimurium* isolates recovered, the serovar was identified in a higher proportion of conventional farm isolates (63.1% 12/19) than

organic farm isolates (43.1%, 22/51)). Moreover, isolates resistant to a single or multiple antimicrobial agents were found in significantly ($p = 0.026$) higher rates in conventional farm isolates (84.2%) than they were in organic farm isolates (52.9%). These findings indicate that implementation of proper biosecurity measures in both farming systems is important to control *Salmonella* contamination and a novel approach alternative to use of antimicrobials is essential to deal with the growing resistance of *Salmonella* which is a major public health concern.

2.2 Introduction

Salmonella enterica represent the most common bacterial foodborne pathogen, infecting a wide range of animal hosts and typically causes gastroenteritis in humans. In 2012, the Foodborne Diseases Active Surveillance Network (FoodNet) of the Center for Disease Control and Prevention (CDC) identified 7,800 laboratory confirmed cases of salmonellosis associated with foodborne disease, causing 2,284 hospitalizations and 33 deaths (51). Furthermore, the CDC estimates the rate of foodborne *Salmonella* infection to be approximately 16.42 cases per 100,000 people. While serovar specific rates of laboratory confirmed *Salmonella* infections have fluctuated, overall rates of laboratory confirmed infections with *Salmonella* have not changed significantly compared to average annual incidence since 1996-1998 (51).

From 1998 to 2008, a total of 1,941 *Salmonella* outbreaks were reported to the Foodborne Disease Outbreak Surveillance System (FDOSS) and 34% of these were assigned to a single food commodity. Eggs and chicken meat were the most commonly identified food commodities, accounting for 112 and 64 outbreaks, respectively. The major serovars contributing to the egg-associated outbreaks were

Salmonella enterica serovars Enteritidis, Heidelberg and Typhimurium, accounting for 83%, 9% and 4%, respectively (2). From 2009 to 2010, the CDC found that eggs and poultry were associated with 2,231 and 826 outbreaks, respectively (51).

The United States Department of Agriculture (USDA) revealed that USDA certified organic food growers in the US sold more than \$3.5 billion worth of organic commodities in 2011 and estimated that organic chicken eggs and broiler chicken sales accounted for \$276 million and \$115 million, respectively (52). Available information indicated that average annual growth of organic egg sales between 2005 and 2007 was 19% (53). Surveys indicate organic foods are perceived to be safer and healthier than their conventional counterparts and these attitudes have created higher demand for these products (54-56). To address the increasing consumer demand, acreage of certified organic farmland has been increasing and the Economic Research Service has estimated that organic acreage has increased from 1.3 million acres in 1997 to 4.8 million acres in 2008. Identifying the prevalence, diversity and resistance patterns of *Salmonella* in poultry can contribute to better our understanding of biosecurity measures in these farming systems.

USDA certified organically raised live stock must use 100% organic feed, provide animal access to outdoors and must withhold the use of antibiotics and growth hormones. These organic standards required by the USDA only address production and processing procedures and does not guarantee nutritional quality or safety of the products (60). On the other hand, in conventional laying hens, antimicrobial agents like bacitracin, chlorotetracycline and tylosin are approved as

therapeutic agents in feed or drinking water. In addition, tylosin is also approved as a growth promoting agent (24).

Currently, there are numerous studies estimating *Salmonella* prevalence and antimicrobial resistance in conventional and organic broiler hens and carcasses (20,21,61,62). Meanwhile, the number of studies comparing *Salmonella* in organic and conventional laying hen eggs and farming systems is relatively low and these studies have been reviewed predominantly performed in Europe (21). The objective of this study was to contribute to the body of knowledge surrounding *Salmonella* contamination in organic and conventional laying hen farms. Specifically, the study aimed to survey the environments (bedding, dust baths, past grass), fecal samples, feed/water and eggs for *Salmonella*, identify prevalence of commonly associated egg serovars and determine their antimicrobial resistance patterns of isolates present on these systems.

2.3 Materials and Methods

2.3.1 Sample Collection and Processing

A total of 433 samples from 9 farms (three conventional and six organic) were collected between April 2013 and January 2014. Farms sampled were selected based on convenience to the research team and with the agreement of farm managers. With the exception of one organic farm located in Pennsylvania, all farms sampled were located in Maryland. The numbers and types of sample take can also be seen in Table 1. A total of 181 and 252 samples were collected from conventional and organic laying hen farms, respectively. Organic farms tested in this study raised chickens free of antibiotics and allowed poultry access to an outside environment. Samples

analyzed in this study included feces, eggs, feed, water and environmental. Environmental samples obtained from conventional farms (n=38) were limited to litter and flies, as conventional poultry had no access to an outdoor area while organic farms (n=76) included soil from dust baths, pasture grass, bedding and flies. Feed samples consisted of trough feed and water from both conventional and organic farms, with 57 and 79 samples taken, respectively. Two conventional farms were high-rise cage hen facilities with a deep pit manure collection system; the other conventional farm was a non-cage facility with litter (shavings and manure) management. Fecal samples were collected around the entire conventional house (n=74) and throughout the pasture and hen houses from organic farms (n=69). Eggs were sampled from both conventional farms (n=12) and organic farms (n=28) prior to cleaning. All samples were aseptically collected and placed into Whirl-Park bags (Nasco, Fort Watkinson, WI) and were transported to the laboratory in an ice cold carrier.

Fecal and feed samples were subjected to enrichment in Luria-Bertani (LB) broth (AMRESCO, Solon, OH) supplemented with 5% Sheep blood (Ward's Science, West Henrietta, NY) and were incubated overnight at 37°C. A 10 µL aliquot was streaked on XLT-4 (Difco, Sparks, MD) Agar plate. An additional aliquot was selectively enriched in Tetrathionate broth with iodine for another 24 h at 37°C (HiMedia, Mumbai, India). The selectively enriched samples were also streaked out on XLT-4 Agar plates. Egg samples were processed following the method previously described (63). Briefly, eggs collected from each site were washed with 10 mL of Buffered Peptone Water (BPW) (HiMedia, Mumbai, India), and mixed vigorously for

1 minute. After the vigorous mixing, BPW was incubated for 18-24 hours at 37°C, and then samples were incubated in LB supplemented with 5% Sheep blood at a final ratio of 1:10 and enriched for another 24 h in Tetrathionate broth at 37°C. Grass samples were weighed and BPW was added to the samples at a 1:10 ratio for pre-enrichment, and mixed vigorously for 1 minute. Samples were then enriched and processed as described above. Presumptive positive colonies were black on XLT-4 agar; one to three presumptive colonies was picked and worked stocked in LB broth with 20% glycerol and stored at -20°C until further use. For biochemical tests, presumptive positive *Salmonella* colonies were inoculated into Simmons citrate, lysine iron (LIA) and triple sugar iron (TSI) agar (Difco) slant tubes and inoculated for 24 h at 37°C. Results were read and interpreted in accordance with the FDA's Bacteriological Analytical Manual (64).

2.3.2 *Salmonella* Identification by PCR

Biochemically identified presumptive positive *Salmonella* isolates were confirmed by a set of PCR assays modified from Hong et al., and O'Regan et al (65,66). *Salmonella* serovars Enteritidis, Typhimurium and Heidelberg were targeted specifically due to the high association of these serovars to egg and poultry products identified in foodborne illness outbreaks (51). Genomic DNA from isolates was extracted from overnight grown cultures using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Primers used for PCR assays were purchased from Eurofins Scientific and are listed in Table 2. An initial *aceK* (Isocitrate dehydrogenase kinase/phosphatase) gene-specific PCR was used to confirm the isolates as *Salmonella enterica* species. Next, four separate multiplex PCR reactions,

for O, H1-1, H1-2 and H2 were used to identify their serovars as *S. Heidelberg*, *S. Typhimurium* or *S. Enteritidis*. The scheme for serovar identification is mentioned in Table 3. PCR reaction were run with a final volume of 10 μ L using 1.5 mM $MgCl_2$, 0.04 U Choice-Taq DNA polymerase (Denville Scientific), 0.4 μ M primer and 0.1 mM dNTPs (Invitrogen, Carlsbad, CA). Thermocycler conditions for all PCR reactions were 94°C for 5 minutes, followed by 30 cycles of denaturation (94°C for 1 minute), annealing (55°C for 30 sec) and extension (72°C for 1 minute) and a final step at 72°C for 5 minutes before being held at 4°C. Positive control strains used in this study were *Salmonella enterica* serovar Typhimurium ATCC 19485, *Salmonella enterica* serovar Enteritidis ATCC 13076, and *Salmonella enterica* serovar Heidelberg ATCC 8326. *Escherichia coli* (EDL933) ATCC 700927 was used as a negative control. Resulting PCR product was separated on 1.5% agarose gel with ethidium bromide at 100V for 30 minutes and a 1 Kb plus DNA ladder (Invitrogen, Carlsbad, CA) was used as molecular mass standard.

2.3.3 *Salmonella* Antimicrobial Resistance Phenotyping

Resistance of *Salmonella* isolates to a selected panel of antimicrobials was tested using a standard agar dilution method by the Clinical Laboratory Standards Institute (CLSI) (66). Mueller-Hinton (MH) Agar (Himedia, Mumbai, India) plates supplemented with two-fold serially diluted antimicrobials were prepared by mixing molten agar to antimicrobial solutions according to manufacturer's instructions. Prior to testing, isolates were grown overnight on MH agar plates at 37°C. Using a sterile loop, isolated colonies were picked and bacterial cells were suspended and adjusted to McFarland Standard of 0.5 using 0.85% saline solution. An aliquot (0.1 mL) of this

suspension was added to 0.9 mL of saline solution and mixed well. Then, 2 μ L of each bacterial suspension ($\sim 10^5$ CFU) was added to MH agar plate containing antibiotic and incubated overnight at 37°C. The MIC was recorded as the lowest concentration of the antimicrobial agent that completely inhibited visible growth of a bacterial isolate. Results were interpreted in accordance with CLSI (2012) breakpoints and can be found in Table 4 (66). Isolates with intermediate resistance were classified as susceptible, since these isolates are able to be clinically treated with a higher than normal dosage of the drug.

2.3.4 Statistical Analysis

Comparisons of prevalence in farm type, sample type, antimicrobial resistance profiles and identification of *Salmonella* serovars, were carried out using SAS version 9.3 (SAS Institute, Inc., Cary, North Carolina). For analysis, the samples were either categorized as conventional and organic depending on the farming system they came from. The PROC FREQ statement was used to perform χ^2 tests to compare proportions of positive and antimicrobial resistant phenotypes. Fisher's exact test was also used to compare proportions of isolates coming from each sample type and their farm origin.

2.4 Results

2.4.1 Prevalence of *Salmonella* in Conventional and Organic Laying Hens Farms

In this study, a total of 433 samples were obtained from feces, feed, water and environments of three conventional (n=181 samples) and six organic (n=252 samples) poultry laying hen farms and were analyzed for *Salmonella enterica*. *Salmonella* was

identified in samples collected from both of the farming systems and overall prevalence was 16.1% (70/433). The prevalence of *Salmonella* in conventional and organic farms was significantly different ($p = 0.008$), with 10.5% (19/181) and 20.2% (51/252) contamination rates, respectively (Table 5).

Salmonella contamination rates by sample type were also compared in organic and conventional farms. The organic poultry feces showed a significantly greater prevalence relative to that of conventional farms. Fecal samples revealed *Salmonella* contamination in 27.5% (9/74) of organic farms and 12.1% (19/69) of conventional farms ($p = 0.03$), a 16.1% difference in point estimate (Table 5).

Overall, egg contamination on organic farms was not statistically significantly different from those samples on conventional farms, though *Salmonella* was found in 10.7% (3/28) of the organic egg samples taken, compared to 0% (0/12) in conventional eggs. Furthermore, no statistically significant difference was found in the rates of *Salmonella* contamination in feed or environmental samples collected from the two farming systems. Due to differences in available laying hen housing systems on conventional and organic farms, environmental samples collected from organic farms were more diverse. However, bedding and fly samples were available on both organic and conventional farms and thus could be compared; on bedding available from organic farms we identified *Salmonella* from 9.5% (2/19) of the samples 18.8% (3/16) from that of conventional farms, though this difference was not statistically significant (Data not shown). Fly samples were collected more often on conventional than organic farms. From the conventional farms, 13.6% (3/22) of the

fly samples were positive for *Salmonella* but 0% (0/3) of the fly samples from organic farms was positive.

Serovar prevalence was also studied against *S. Enteritidis*, *S. Typhimurium* and *S. Heidelberg* (Table 6). For each sample tested, one to three presumptive colonies were streaked and stored at -20°C. During testing for antimicrobial susceptibility, only one stock isolate was used per sample. Out of the recovered 70 *Salmonella* isolates, 34 (48.8%) and 1 (1.2%) were identified as *S. Typhimurium* and *S. Enteritidis*, respectively. However, none of the *Salmonella* isolates was identified as *S. Heidelberg*. The other 35 (50%) presumptive *Salmonella* isolates were collectively defined as *Salmonella enterica*. Among the 19 isolates recovered from conventional farms, 12 (63.2%) were *S. Typhimurium* while the remaining 7 (36.8%) were *S. enterica*. No *S. Enteritidis* was identified from the conventional isolates. Of the 51 isolates recovered from organic farms, 22 (43.1%) were identified as *S. Typhimurium*, and 1 (1.96%) was identified as *S. Enteritidis*. The other 28 (54.9%) were *S. enterica*. All three egg-shell samples isolates recovered from the organic farm were identified as *S. Typhimurium* (Table 6).

2.4.2 Antimicrobial resistance features of *Salmonella* isolates.

Antimicrobial susceptibility testing was performed on all 70 isolates against a wide range of antibiotic classes. Isolates were classified as either susceptible, antimicrobial resistant (AMR) or multidrug resistant (MDR). Pansusceptible isolates displayed no growth when challenged by all antimicrobials in the study, antimicrobial resistant isolates were resistant to only one antimicrobial and multidrug resistant isolates were resistant to two or more antimicrobial tested. The classification of these

isolates by sample and farm type is shown in Table 7. From conventional farms, 15.7% (3/19) of *Salmonella* isolates were pansusceptible, while 73.7% (14/19) displayed multidrug resistance and 10.5% (2/19) were single antimicrobial resistant. Isolates from the organic farms ($n=51$), 33.3% were multidrug resistant, 19.6% were single antimicrobial resistant and 47.1% were susceptible (Table 7). Statistical significance was not calculated for these values due to limitations of the χ^2 test.

Salmonella resistance to individual antimicrobials by farm type is shown in Table 8 and resistance patterns in Table 9. The percentage of isolates resistant to cefazolin was significantly higher ($p = 0.001$) on conventional farms (84.2%) than organic farms (39.2%) (Table 8). Ampicillin resistance was significantly higher ($p = 0.05$) in conventional isolates (73.7%, 14/19) when compared to organic isolates (51.0%, 26/51). From conventional farm isolates, 52.6% (10/19) showed an ampicillin-cefazolin resistant phenotype, followed by ampicillin-cefazolin-chloramphenicol resistant and susceptible phenotypes, which was found in equal proportions (15.8%) (Table 9). The leading phenotype for organic isolates was susceptible (45.0% 23/51) followed by ampicillin-cefazolin resistance (29.4%, 15/51). Regardless of the source of isolation and farm types, no isolate was resistant to gentamicin, ciprofloxacin, or trimethoprim-sulfamethoxazole.

A summary of *Salmonella* serovars resistance to antimicrobials can be found in table 10. The *S. Enteritidis* isolate was susceptible to all antibiotics tested and all *Salmonella* showed susceptibility to trimethoprim-sulfamethoxazole, gentamicin and ciprofloxacin. Ampicillin and cefazolin were the antibiotics with highest resistance rates. The only isolate of *S. Enteritidis* from organic farm recovered was susceptible

to all antibiotics tested. Of the 18 ampicillin resistant *S. Typhimurium* isolates, 9 (50%) were isolated from the conventional farm the other 9 (50%) were isolated from organic farms. In addition, 50% (17/34) of *S. Typhimurium* isolates were resistant to cefazolin and out of these cefazolin resistant *S. Typhimurium* isolates 9 (52.9%) of the isolates were from the conventional farm and 8 (47.1%) were from organic farms. Of the 7 other *S. enterica* recovered from conventional farms, 5 (71.4%) were resistant to ampicillin and all 7 (100%) were resistant to cefazolin as well. Of the other 28 *S. enterica* identified on organic farms, 14 (50%) were resistant to ampicillin and 11 (39.9%) were resistant to cefazolin.

2.5 Discussion

Here, we investigated the prevalence of *Salmonella* in conventional and organic laying hen farms and determined the antimicrobial resistance profiles of isolated strains. Our results showed that the overall rates of *Salmonella* contamination were significantly higher by about 10% on organic farms than they were on conventional farms and it was consistent to some degree for each sample type tested including fecal, egg, feed and environmental. Previous reports indicate *Salmonella* poultry infection may be related to the presence of other domesticated animals, infected farm workers, rodents, wild-birds and insects (18, 71, 72,78). Given the free-range and open grass land access to organic pasture layers, biosecurity level on these farms may be relatively decreased compared to conventional farms, thus increasing the possibilities of contamination with *Salmonella* and other zoonotic pathogens. With increased exposure to outdoors, chickens in these organic farming systems have more possible contact with other farms, rodents, insects, wild-life and

other domesticated animals, all who may introduce *Salmonella* into the farm (12, 15, 67,71). However, other reports on European farms indicated that contamination rates of *Salmonella* on conventional farms were slightly higher than organic farms which is opposite to our findings (20,21,67,68,69). This anomaly might be due in part to the difference in farming infrastructures and their biosecurity measures in US and Europe. Another study found that the effect of housing systems was dependent on the age of hens on the farm (72). Though age was not considered as a parameter in this study, in the future it will be important to see whether age has any significant effect on *Salmonella* contamination. As mentioned above, environmental samples on the organic farms were also found to have a higher contamination rate than conventional farms except samples from flies. The exposure to vectors for horizontal transfer has been noted to increase *Salmonella* serovar diversity in poultry (78). On organic farms sampled, none of the flies were contaminated with *Salmonella*, while we recovered *Salmonella* isolates from 13.6% of conventional flies tested, accounting for 50% of all positive environmental samples on the conventional farms. Due to the dense nature of housing on the conventional farms, flies represent an important vector for *Salmonella* contamination. However, our inability to detect *Salmonella* on fly samples from organic farms may be due to low sample availability (71,79).

In this study, *S. Typhimurium* was recognized as the most prevalent serovar in both farming systems but it was 20% higher on conventional farms than organic farms. Compared to *S. Typhimurium*, only one *S. Enteritidis* was identified and isolated from an organic farm. Previous studies have found *S. Enteritidis* as the predominant serovar and hence hypothesized that the serovar filled an ecological

niche when serovars Pullorum and Gallinarum were targeted for eradication (10,73). Other studies have reported a decrease in *S. Enteritidis* on conventional laying hen farms, while others reported a large prevalence of Enteritidis, followed by Typhimurium (18,22,74). This low *S. Enteritidis* isolation in our study may be a positive sign, as it follows the decreasing trend that has been started since the 1990's (9). More recently in 2009 the Food and Drug Administration had released final regulations with the intent to implement preventative measures, specifically with *S. Enteritidis* in mind. There are currently no concerted efforts targeting other individual *Salmonella* serovars. Results from studies focused on the changes of *Salmonella* serovar prevalence have indicated similar efforts to remove *S. Pullorum* of *S. Gallinarum* have resulted in an ecological niche modeled to be filled by *S. Enteritidis* (9). It will be important to continue surveillance of predominant *Salmonella* serovars related to chickens and their environments in order to detect the possible sources of new poultry associated *Salmonella* serovars.

Antimicrobial resistance was found in 84.2% and 52.9% of *Salmonella* isolates from conventional and organic farms, respectively. These results are in agreement with studies showing higher prevalence of antimicrobial resistant *Salmonella* on conventional poultry farms (17, 83, 84,85). Similar findings have been also identified in *Campylobacter* spp. isolated from the intestines of broilers and turkeys from conventional and organic farms (86). The administration of antimicrobials in feed may provide selective pressure on bacterial resistance on conventional farm environments (15,24). A study by Sapkota et al., (115) showed that after conventional broiler farms transitioned to organic farming practices, lower

prevalence of antimicrobial resistant *Salmonella* were found. Organic farms are not allowed to use antimicrobial agents to raise their poultry. However, our study showed significant number of organic isolates were also resistant to either single or multidrug resistance. Indeed, class I integrons have been found in antimicrobial resistant *Salmonella* isolates from pasture-flock hens and organic poultry carcasses, which may account for antimicrobial resistance seen on organic farms in this study (25).

Isolates from conventional farms showed high resistance to ampicillin, with 73.7% showing resistance. Organic farm resistance to ampicillin was significantly different, with only 51% resistant to ampicillin. Our results are similar to findings from Sapkota et al., (115), who found ampicillin resistance decreased after farms transitioned from conventional to organic farming practices. Alali et al., (17) reported similar high prevalence rate of ampicillin resistance on conventional and organic broiler farms. However, our results conflict with ampicillin resistance from chicken *Salmonella* isolates reported by NARMS, which showed 7.3% of isolates showed resistance. Further, the NARMS report shows an increasing resistance to ceftriaxone, from 0.5% in 1997 to 6.3% in 2011. In contrast, our rates of ceftriaxone resistance are lower than the 2011 rates, with 5.3% and 1.96% from conventional and organic farms, respectively. Rates of cefazolin resistance have been previously reported (117). The resistance to both cefazolin and ceftriaxone is troubling because these cephalosporins have been recommended to treat *Salmonella* human infection (118). In our study, no isolates were resistant to ciprofloxacin, gentamicin and trimethoprim-sulfamethoxazole and these may represent viable candidates for treating salmonellosis.

Without the use of antimicrobials on the organic farms, there seems to be a lack of direct selective pressure on the *Salmonella* isolates retrieved from these systems. However, naturally occurring compounds in these areas may be conferring the selective pressures. A number of antimicrobials are naturally produced by fungi and other bacteria, and these organisms may be present on the farms providing some pressure. Moreover, the wild-life, especially migratory birds, moving in and out of these environments may be coming from highly populated areas, bringing antimicrobial resistant *Salmonella* into these environments (128). The continued resistance seen in these *Salmonella* on organic farms may be indicative of certain increased fitness increases seen, possibly due to compensatory mutations, which has been shown *in vitro*. However, it is understood that generally the introduction of antimicrobial resistance comes at a decreased fitness cost and these compensatory mutations must be studied further (129). Moreover, environmental stressors, such as growth-compromising conditions, have been thought to help promote the development of antimicrobial resistance, though definitive links between environmental *Salmonella* and antimicrobial resistance have yet to be identified (130).

Chapter 3: The Efficacy of *Salmonella* Specific Egg-Yolk Antibodies (IgY) in Decreasing *Salmonella* Adherence to Chicken Cells *In Vitro*

3.1 Abstract

Salmonella remains an important causative agent of foodborne illness in the United States. Considering the majority of foodborne illness salmonellosis cases come from poultry and poultry products, controlling this pathogen has become an increasingly important objective for the poultry industry. In particular, the organic poultry industries, which are unable to use antibiotics, require new alternatives to control the pathogen at the production level. A possible alternative to antibiotics is the use of passive immunization through the oral ingestion of antigen-specific antibodies. The objective of this study was to determine the efficacy of antigen-specific antibodies to decrease the adherence of *Salmonella* to different chicken cells *in vitro*. *Salmonella* serovars *S. Enteritidis* and *S. Typhimurium* were tested for their ability to adhere to the DF-1 chicken fibroblast cell line and the HD-11 chicken macrophage cell line. Overall, when pre-treated with the different antibodies, the DF-1 fibroblast poultry model showed a significant decrease ($p < 0.05$) in the number of *S. Enteritidis* and *S. Typhimurium*, of approximately 1.5 log, depending on concentration and antibody treatment used. However, in the HD-11 cell line, antibody pre-treatment in some cases showed a significant increase in *Salmonella* adherence, of up to 0.5 log CFU ml⁻¹. While a modest decrease in *Salmonella* adherence may reduce the level of *Salmonella* contamination, further understanding of antigen-specificity and poultry response to the antibodies is needed before passive immunization may be adopted on organic farms.

3.2 Introduction

Salmonella is a Gram-negative enteric pathogen which is estimated to cause 1.4 million domestically acquired foodborne illnesses annually (49). Infection with the pathogen causes the disease salmonellosis, which occurs 12-72 hours after infection and manifests itself through diarrhea, fever and abdominal pain that lasts for 4-7 days. It is estimated that the economic burden of foodborne *Salmonella* infections ranges from \$1.5 billion to \$10.9 billion annually (3). In 1998-2008, out of 1,491 reported *Salmonella* foodborne outbreaks, 403 were traceable to a single serotype and single food source; out of the 403 outbreaks, *Salmonella enterica* serovar Enteritidis accounted for 144 outbreaks of which 65% were attributed to eggs and 13% were attributed to chicken products (2). On the other hand, *Salmonella enterica* serovar Typhimurium caused 58 outbreaks between 1998 and 2008, and eggs and poultry were implicated in 7% and 26% of those cases, respectively (2). It is believed that reduction of *Salmonella* in the intestines of birds will reduce contamination of future food-products from these livestock (46)

Adhesins play an important role in the pathogenesis of many enteric pathogens including *Salmonella* (44, 90). The presence of these surface structures allow *Salmonella* serovars to adhere to a variety of surfaces; in *S. Enteritidis* infection, type 1 fimbriae is implicated in poultry oviduct adherence and flagella is associated with poultry gut explant adherence (40, 91-93). *S. Typhimurium* utilizes type 1 fimbriae to attach to HeLa cells, and a variety of other fimbriae for biofilm formation on chicken intestinal epithelium (42, 43). Nevertheless, the role of fimbriae, flagella or other virulence factors in *Salmonella* adherence to chicken gut

tissues remains poorly understood, and hence requires further research. It is important to better understand adhesins necessary for *Salmonella* attachment, because this initial step is required for colonization and invasion (90, 94). One of possible strategy to block the initial adherence of *Salmonella* is proper vaccination. While successful vaccination resulting in protection against a pathogen depends on a number of factors, vaccination against antigens may prevent attachment of bacteria to the host cell and colonization of mucosal surfaces (95). Development of novel vaccine against these structures may be worthwhile to protect against *Salmonella* infection.

In contrast to active immunity provided by vaccination, passive protection through the oral ingestion of antibodies may be effective as a supplemental strategy in the protection of chicken from *Salmonella*. Hens are able to pass up to 100 mg of a single class of antibodies, known as immunoglobulin Y (IgY) from their serum specifically into the egg-yolk and after immunization against an antigen, approximately 2-10% of these IgY found in the egg-yolk will be antigen-specific (96, 97). Oral administration of specific antibodies found in egg-yolk from vaccinated laying hens may be able to prevent colonization from enteric pathogens, though previous studies have shown mixed results (39, 46, 48, 98, 99). While IgY produced against *Salmonella* outer membrane protein antigens have shown decreased adherence of *S. Enteritidis* and *S. Typhimurium* to Caco-2 cells *in vivo*, performance with these antibodies showed no protective effect against cecal contamination (103). Another study utilizing ducklings showed feed supplementation with probiotics and anti-*S. Enteritidis* IgY showed a significant decrease in *Salmonella* cecal, ileal, liver and splenic contamination when combined with probiotics (100). Though multiple *in*

in vivo studies have been performed on chickens, no studies have focused on the effects of anti-*Salmonella* IgY on *Salmonella* infection using *in vitro* chicken cell line models. The purpose of this study was to evaluate the efficacy of egg-yolk antibodies directed against different *Salmonella* antigens in preventing *Salmonella* adherence to different poultry cell lines: a DF-1 chicken fibroblast cell line and the HD-11 chicken macrophage cell line. Estimating the efficacy of the antibodies to decrease *Salmonella* adhesion with chicken cells *in vitro* may allow us to better design vaccines for effective passive immunization *in vivo*.

3.3 Materials and Methods

3.3.1 Bacterial Strains and Growth Conditions

Two strains, *Salmonella enterica* serovar Typhimurium LT2 ATCC 19485 (ST) and *Salmonella enterica* serovar Enteritidis ATCC 13076 (SE) were used in this study. These strains were kindly provided by Dr. Steven C. Ricke, University of Arkansas. The strains were cultivated and maintained regularly on Luria-Bertani agar (HiMedia, Mumbai, India) at 37°C overnight in aerobic conditions.

3.3.2 Cell Lines and Culture Conditions

The chicken macrophage-like cell line HD11 was kindly provided by Dr. Uma S. Babu, at the Immunobiology Branch, Food and Drug Administration (Laurel, MD). The chicken fibroblast cell line DF-1 used was kindly provided by Dr. John Song, University of Maryland. The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Corning Cellgro, Manassas, VA), with 2 mM L-glutamine, 4.5 g/L glucose and sodium pyruvate and supplemented with 10% heat-inactivated fetal

bovine serum (Corning Cellgro, Manassas VA) and were maintained at 37°C with 5% CO₂. Prior to infection, either 2×10⁵ or 2×10⁶ DF-1 or HD-11 cells, respectively, were seeded into 24- well culture plates (Greiner Bio-One, Monroe, NC) and allowed to proliferate to confluence for 24 h at 37°C. Before infection, the seeded culture-plates were washed with 1x phosphate buffered saline (PBS) and replaced with fresh, non-supplemented DMEM.

3.3.3 Immunization and Isolation of Immunoglobulin Y (IgY) from Egg Yolk Powders

We worked with the company ZymeFast Inc., in order to immunize and collect the eggs from hens vaccinated against four different antigens. Currently, the antigens used for vaccination are under patent review and must remain confidential until final approval. After immunization, egg-yolks were collected, lyophilized and sent to the University of Maryland; the antibody powders were stored at 4°C until used. Antibodies will be referred to as IgY1, IgY2, IgY3 and IgY4 for the remainder of the manuscript. The IgY was obtained from the egg-yolk powders using the method described by Lee *et al.* (48), with little modifications. In brief, approximately 10 g of egg yolk powder was added to 80 ml of cold acidified distilled water (pH 4.0, with 0.1 N HCl) and mixed gently. Then, 10 ml of cold acidified distilled water (pH 2.0) was added and mixed thoroughly before adjusting the final pH of solution to 5.0~5.2. This solution was incubated overnight at 4°C for at least 12 h. The solution was centrifuged at 3,500 x g at 4°C for 20 min in a Thermo Scientific Sorvall Legend XTR (Waltham, MA) and the supernatant was collected, neutralized with 0.1 N

NaOH and stored at -20°C until further use. IgY concentration was determined using Thermo Scientific Pierce BGG Protein Assay Kit (Rockford, IL).

3.3.4 Specific Activity of IgY

Specific activity of the IgY against *S. Typhimurium* and *S. Enteritidis* was carried out using an indirect enzyme-linked immunosorbent assay (ELISA) as described by Zhen *et al.* (127) and Biswas *et al.* (126). *Salmonella* cells were collected from overnight grown LB agar plates and suspended in PBS followed by centrifugation at 8,000 x g for 5 min. The supernatant was discarded and the bacterial cell pellet was then inactivated in 20 ml of 10% formalin and incubated for 3 hours at 37°C with agitation (100 rpm). The treated cells were washed with PBS to remove excess formalin. All of the following reagents were added at 100 µL/well. The bacterial suspension was spotted on LB agar and incubated overnight to confirm inactivation. The inactivated bacterial suspensions were diluted to an OD₆₀₀ of 0.4 using a PerkinElmer Lambda Bio + spectrophotometer (Waltham, MA), added to 96-well microtiter plates and incubated for 2 h at 37°C. The plates were washed 3-4 times with 200 uL of PBS plus 0.05% Tween-20 (PBST), and blocked with 5% non-fat dry milk in PBST for 2 h at 37°C. A washing step was performed again and IgY at a final concentration of 7.5 µg ml⁻¹ was added and incubated for another 2 h at 37°C. The plate was further washed with PBST and 100 uL of a peroxidase-conjugated goat anti-chicken IgG (Rockland, Boyertown, PA) was added to the wells and the plate was incubated for 1 h at 37°C. The microtiter plate was washed again and 3, 3', 5, 5'-Tetramethylbenzidine (AMRESCO, Solon, OH) was added to the plates and incubated for 15 minutes at 25°C. The reaction was stopped with 1 M H₂SO₄ and

absorbance at 450 nm (A_{450}) was recorded with a ThermoScientific MultiSkan (Waltham, MA) microplate reader.

3.3.5 Adherence Assays

The ability of the antibodies to block adherence of *Salmonella* was carried out using both DF-1 and HD-11 cells. Prior to infection, DF-1 and HD-11 cell monolayers were incubated with 1.5, 3 or 6 mg ml⁻¹ of IgY at 37°C for 1 hr in nonsupplemented DMEM. Controls were incubated with an equivalent volume of distilled water. Suspensions of *S. Enteritidis* and *S. Typhimurium* were adjusted to an OD₆₀₀ of 0.1 corresponding to 10⁸ CFU ml⁻¹; both bacteria were infected with a final MOI of 100 for DF-1 fibroblasts and a MOI of 10 for HD-11 cells and allowed to infect for 1 h at 37°C. After 1 h of infection, monolayers were washed twice carefully with 1 x PBS to remove nonadherent bacteria. The cells were then lysed by incubating monolayers with 0.1% Triton X-100 in PBS for 15 minutes at 37°C. The bacterial suspension was serially diluted and 50 uL of appropriate dilutions were plated on LB agar to enumerate the number of adhered *Salmonella* cells

To test the effect of antibodies after an initial infection of chicken cells with *Salmonella*, DF-1 monolayers in 24-well cell culture plates were infected *Salmonella* strains as previously described. After 1 h incubation at 37°C, 5% CO₂, the IgY was added to the cell/bacteria mixture at the same concentrations and allowed to incubate for an additional 1 h at 37°C. Enumeration of adhered bacteria was performed as described above. Each experiment was carried out in triplicate and replicated a total of 3 times.

3.3.6 Statistical Analysis

All experiments were conducted with three replicates in triplicate. Data were analyzed using Statistical Analysis Software version 9.3 (SAS Institute, Inc., Cary, North Carolina). The mixed model and Bonferroni's correction were used to evaluate the treatments as fixed effect and for multiple comparisons. Significant mean differences were considered at $P < 0.05$.

3.4 Results

3.4.1 IgY Antibody Reactivity

The ability of the four egg-yolk antibodies to bind was tested against both *S. Enteritidis* and *S. Typhimurium*. All antibodies were significantly more reactive for both *Salmonella* serovars when compared to the negative control. Moreover, antibodies were significantly more reactive ($p < 0.0001$) with *S. Enteritidis* than *S. Typhimurium*, with an overall mean average difference of 0.07. IgY 2 had the significantly highest reactivity with *S. Enteritidis* ($A_{450} 1.02 \pm 0.02$), followed by IgY 4, IgY1 then IgY 3 (Figure 1). The pattern of antibody reactivity for *S. Typhimurium* differed from the *S. Enteritidis* reactivity. For *S. Typhimurium*, IgY 2 (A_{450} of 0.91 ± 0.07) and IgY1 (A_{450} of 0.84 ± 0.03) showed statistically similar reactivity and both differed significantly from IgY 4 and IgY 3 (Figure 1). IgY 2 had similar reactivity against both *S. Enteritidis* and *S. Typhimurium*, while IgY 1 was the only other antibody treatment that had a higher reactivity with *S. Typhimurium* when compared to *S. Enteritidis*.

3.4.2 Adhesion Prevention Assay

The protective ability of the egg-yolk antibodies was tested in two different chicken cell lines with both pathogens. For *S. Enteritidis* infection, the preincubation of the antibodies with the DF-1 cell monolayer significantly changed the number of adhered bacteria ($p < 0.05$) and was dependent on the treatment used as well as the concentration applied (Fig 2A). When compared to the control treatment, of 0 mg ml^{-1} , there was a significant concentration dependent reduction of *S. Enteritidis* seen for each IgY treatment. Even the smallest reduction seen in IgY 2 at 1.5 mg ml^{-1} , was significant with an average reduction of $0.84 \text{ log CFU ml}^{-1}$ (Fig 2A). At the highest concentrations tested, IgY 4 and IgY 1 were the most effective against reducing *S. Enteritidis* adhesion, when compared with treatment with IgY 2 and IgY 3. At 6 mg ml^{-1} , the most significant decrease was found with antibody IgY4 showed a 1.78 log reduction in the pathogen adherence with the DF-1 fibroblast cell line, while the same concentration of IgY1 reduced *S. Enteritidis* adherence by $1.67 \text{ log CFU ml}^{-1}$ (Fig 2A). In contrast, the highest log reduction of antibody IgY2 and IgY3 was 1.43 log and 1.36 log , respectively.

For *S. Typhimurium*, preincubation of the antibodies also significantly decreased the adherence of the pathogen with the DF-1 cell monolayer when compared to the control. For *S. Typhimurium* infection, there was only one significant difference seen between the different antibodies at a concentration of 3 mg ml^{-1} ($p < 0.05$) and each treatment showed a significant effect of concentration ($p < 0.0001$). For example, when compared to the control, at 1.5 mg ml^{-1} IgY 2 showed an average $1.30 \text{ log CFU ml}^{-1}$ decrease in *S. Typhimurium* adhesion while the 6 mg ml^{-1}

treatment showed significantly different ($p < 0.0001$) 1.61 log CFU ml⁻¹ decrease in bacterial adhesion. A similar concentration dependent decrease in *S. Typhimurium* was seen in IgY 3, where 1.5 mg ml⁻¹ showed a 1.14 log CFU ml⁻¹ decrease compared 6 mg ml⁻¹, which showed a significantly different ($p < 0.0001$) decrease of 1.77 log CFU ml⁻¹ (Fig 2B).

Effect of preincubation of the antibodies with the macrophage HD-11 cells on *Salmonella* adherence was more nuanced. In *S. Enteritidis* infection overall, there was a significant effect of the different IgY concentrations, however this effect depended on the IgY treatment applied ($p < 0.0001$). As compared to the control, antibodies IgY 1 and IgY 4 caused an average decrease of *S. Enteritidis* adherence to the macrophages when compared to IgY 2 and IgY 3. A concentration of 1.5 mg ml⁻¹ of IgY 1 showed the highest average adherence decrease of 1.06 log CFU ml⁻¹ while 6 mg ml⁻¹ of IgY 4 caused an average 0.89 log CFU ml⁻¹ decrease (Fig 3A). Antibody IgY 2 showed an increase in the adherence of *S. Enteritidis* with HD-11 cells at the 3 and 6 mg ml⁻¹ of 0.40 and 0.52 log CFU ml⁻¹ respectively, when compared to the control. Treatment with antibody IgY 3 at 1.5 mg ml⁻¹ showed a significant decrease (0.84 log CFU ml⁻¹) while higher concentrations showed no significant effect.

Preincubation of the antibodies with the macrophages and subsequent infection with *S. Typhimurium* showed a similar pattern to *S. Enteritidis*. There was a significant effect of the concentration of IgY treatment, but this effect depended on the IgY treatment used ($p < 0.0001$). In *S. Typhimurium* infection, the 6 and 3 mg ml⁻¹ of IgY 2 showed a significant increase in the number of adhered bacteria to the monolayer of 0.66 log CFU ml⁻¹ and 0.57 log CFU ml⁻¹, respectively (Fig. 3B).

Antibody treatments IgY 1 and IgY 3 showed no significant impact on the number of adhered *S. Typhimurium*. Preincubation with antibody IgY 4 showed a significant, concentration dependent decrease in the number of adhered of *S. Typhimurium* with the HD-11 cells with the largest decrease of 0.8 log CFU ml⁻¹ at 6 mg ml⁻¹(Fig 3B).

The ability of the antibodies to decrease the adherence of *Salmonella* in a pre-existing infection was tested by incubating the monolayer after an initial infection period. For both *S. Enteritidis* and *S. Typhimurium* infection, there was no significant effect seen between each antibody treatment, however for there was a significant effect ($p < 0.0001$) of the concentration of antibody applied on the number of CFU ml⁻¹ adhered to the cells (Fig 4). After initial *S. Enteritidis* infection, antibody treatments at 6 and 3 mg ml⁻¹ were able to significantly decrease the adherence of the pathogen with the monolayer. The most marked decrease in adherence when compared to the control could be seen with IgY 4 at 6 mg ml⁻¹, with an average decrease 1 log CFU ml⁻¹. For *S. Typhimurium* infection, a significant concentration dependent decrease of adherence was seen in IgY 4, with an average decrease of 0.6 log CFU ml⁻¹.

3.5 Discussion

The ability of specific egg-yolk antibodies to prevent infection through enteric pathogens has been previously documented. Specifically, anti-*Salmonella* egg-yolk antibodies have been studied both in *vitro* and in *vivo* with varying success. As *S. Enteritidis* and *S. Typhimurium* possess diverse mechanisms to attach to different host tissues, a variety of reasons may account for the differences in success seen between *in vivo* and *in vitro* studied.

For adherence assays, both preincubation and postincubation of the antibodies showed a decrease in the adherence of *Salmonella* to the DF-1 fibroblast cell line. Preincubation of the antibodies with the cells resulted in a larger decrease of adhered *Salmonella* than treatment after an established infection. The ability of the antibodies to decrease the adherence of the *Salmonella* to the cells is in accordance with previous findings in both Caco-2 and HeLa cells (101-103). IgY 4 showed the second highest reactivity to *S. Enteritidis*, while showing the highest average decrease in adherence when DF-1 cells were pretreated. In contrast, IgY 2 showed the highest reactivity to *S. Typhimurium* and the greatest average decrease in adherence to pretreated DF-1 cells. Previous studies have looked at the effects of antigen specific IgY designed against outer membrane proteins (OMP, porins), lipopolysaccharides (LPS) or flagella on the adherence of *S. Typhimurium* or *S. Enteritidis* and have showed anti-OMP IgY exhibit the highest level of protection (39, 126). The current study indicates that while antigen specific IgY may show reactivity with multiple serovars, pathogen specificity may be needed for vaccination when designing antibodies; it will be important to further elucidate the most important proteins necessary to block *Salmonella* adhesion. It is thought that the binding of antigen specific IgY will bind to some specific antigen on the bacterial surface and which may lead to the impairment of adhesions. Alternatively, the binding of the IgY to the surface of the bacteria may modify the attachment and lead to changes in *Salmonella* morphology (48).

In comparison, treatment of the cells with any IgY after the infection had been established showed no significant differences between treatments, however a

concentration dependent effect was seen in *S. Enteritidis* infection. Views on *Salmonella* adhesion are contrasting and studies claim that either *Salmonella* the process is either slow or almost instantaneous (104 -106). Anti-*Salmonella* specific egg-yolk antibodies have been shown to have some inhibitory effects on the growth of *S. Enteritidis* and *S. Typhimurium* (48, 103). Low-density lipoproteins have been implicated as components present in the water-soluble fraction of the egg-yolk that may have this antibacterial activity, though activity has not been explored in *Salmonella* (103). These results may suggest that although specific anti-*Salmonella* egg-yolk antibodies may help prevent the association of the pathogen to the intestinal tract, other components of the egg-yolk may provide a more transient protection against *Salmonella* gastrointestinal infection in poultry.

Preincubation of the antibodies with the HD-11 macrophages showed a more varied response. Macrophages may be activated through the Fc receptor found on their surface; once the antibody binds to the surface of the pathogen, macrophage phagocytosis becomes more efficient (107). Further, the clearance of *Salmonella* depends on opsonization of the pathogen by specific antibodies (108). In both *S. Enteritidis* and *S. Typhimurium* infection, IgY 2 was able to increase the adherence of the pathogen with the macrophage, which may be indicative of this opsonization effect while antibodies 1, 3 and 4 generally decreased the adherence of *Salmonella*. Vaccination against only the O-antigen has been shown to be poorly immunogenic, however vaccination against an O-antigen covalently linked to carrier proteins have shown increased phagocytosis and bacterial clearance against *S. Typhimurium* and *S. Enteritidis* challenge in murine models (109, 110). Further, commercially available

Salmonella vaccines have been developed for both killed and live attenuated strains, though live attenuated strains are thought to better stimulate a cell-mediated and humoral immune response (27, 111).

Overall, anti-*Salmonella* specific egg-yolk antibodies caused a variety of responses in poultry cell culture models, suggesting the addition of the antibodies in poultry feed or water may cause a more complicated response in the poultry. The differences in the responses of the two cell-lines may be caused by differences in the *Salmonella* life cycle; while fibroblasts and macrophages may both play a role in persistent *Salmonella* infection, growth in these two cell lines is very different. Upon entrance to fibroblasts, *Salmonella* are thought to switch to a more nonproliferative and avirulent state while in macrophages, *Salmonella* promote replication and survival (112, 113). The results from the current study indicate that inclusion of the specific egg-yolk antibodies may be effective in providing protection against both *S. Enteritidis* and *S. Typhimurium* by decreasing adherence and increasing bacterial clearance. In order for adoption to occur at the farm level, the antibodies will need to block multiple serovars and strains from adhering to gastrointestinal tissue. Moreover, further work will need to be done to determine the efficacy of the antibodies following in *vivo* gastrointestinal digestion. These antibodies provide a promising therapy for the treatment of *Salmonella* in poultry as an alternative to antimicrobials.

Chapter 4: Conclusion

In summary, the results of the first baseline study sheds light on the prevalence and antimicrobial resistance patterns of three major *Salmonella* serovars associated with chickens and eggs products in conventional and organic laying hen environments in Maryland. These findings suggest that rates of prevalence and resistance of *Salmonella* on laying hen farms may be linked to poultry production practices. This study affirms the need for longitudinal studies to follow and confirm sources of *Salmonella* contamination on farms in order to identify where *Salmonella* are being introduced on the farm and how resistance is being developed. The results of the second study help uncover the efficacy of antigen-specific antibodies in blocking colonization of *Salmonella* in chicken. While showing promise, the study highlights the need for further studies understanding the host-pathogen interaction between *Salmonella* and the chicken. A more comprehensive understanding of *Salmonella* attachment will allow for a better antigen-specific IgY treatment and future studies should explore the possibility of combining other alternative therapies. Together, these studies contribute to the growing body of knowledge of *Salmonella* in laying hens and the feasibility of certain alternative therapies as organic production of food increases in the United States.

Chapter 5: List of Tables

5.1 Table 1. Total number of samples collected by farm and sample type

Subsection 1

| Sample Type | Conventional | Organic | Total |
|----------------------------------|---------------------|----------------|--------------|
| Fecal | 74 | 69 | 143 |
| Environmental^a | 38 | 76 | 114 |
| Feed^b | 57 | 79 | 136 |
| Egg | 12 | 28 | 40 |
| Total | 181 | 252 | 433 |

Organic environmental samples include free range pasture grass, soil, bedding & flies, while conventional environmental samples only included bedding & flies

^b Feed samples consisted of trough and water samples

5.2 Table 2. Primers used in the PCR identification of *Salmonella* species isolated in this study

| Target Gene ^a | Primer Sequence 5'-3' | Expected Size (bp) |
|--|---|-----------------------|
| <i>Salmonella enterica</i> . <i>aceK</i> ^b | F: CCGCGCTGGTGAGTGG R: GCGGGGCGAATTTGTCTTTA | 240 |
| O-antigen multiplex^c | | |
| <i>abe</i> ₁ (B) | F: GGCTTCCGGCTTTATTGG R: TCTCTTATCTGTTTCGCCTGTTG | 561 |
| <i>wbaD-manC</i> (C1) | F: ATTTGCCAGTTCGGTTTG R: CCATAACCGACTTCCATTTC | 341 |
| <i>abe</i> ₂ (C2) | F: CGTCCTATAACCGAGCCAAC R: CTGCTTTATCCCTCTCACCG | 397 |
| <i>prt</i> (A/D1) | F: ATGGGAGCGTTTGGGTTC R: CGCCTCTCCACTACCAACTTC | 624 |
| <i>wzx – wzy</i> (E1) | F: GATAGCAACGTTCCGAAATTC R: CCCAATAGCAATAAACCAAGC | 281 |
| H1-1 multiplex^c | | |
| <i>fliC</i> (i) | F: AACGAAATCAACAACAACCTGC R: TAGCCATCTTTACCAGTTCCC | 508 |
| <i>fliC</i> (g,m) | F: GCAGCAGCACCGGATAAAG R: CATTAACATCCGTTCGCGCTAG | 309 |
| H1-2 multiplex^c | | |
| <i>fliC</i> (r) | F: CCTGCTATTACTGGTGATC R: GTTGAAGGGAAGCCAGCAG | 169 |
| <i>fliC</i> (z ₁₀) | F: GCACTGGCGTTACTCAATCTC R: GCATCAGCAATACCACTCGC | 363 |
| H2 multiplex^c | | |
| <i>fliB</i> (I: 1,2; 1,5; 1,6; 1,7) | F: AGAAAGCGTATGATGTGAAA R: ATTGTTGGTTTTAGTTGCGCC | 294 |
| <i>fliB</i> (II: e,n,x; e,n,z15) | F: TAACTGGCGATACATTGACTG R: TAGCACCGAATGATACAGCC | 152 |

^a Primer set designed with genes or junctions between two genes used for designing primers

^b Primer set adapted from O'Regan et al., (66).

^c Primer set adapted from Hong et al., (65).

5.3 Table 3 Identification scheme for *S. enterica* serovars Enteritidis, Typhimurium and Heidelberg

| <i>Serovar</i> | <i>aceK</i> | <i>O</i> -multiplex | <i>H1</i> -multiplexes | <i>H2</i> -multiplex |
|----------------|-------------|---------------------|------------------------|----------------------|
| Enteritidis | aceK | A/D1 | g,m | - |
| Typhimurium | aceK | B | i | I |
| Heidelberg | aceK | B | r | I |

5.4 Table 4. Antimicrobials and resistance breakpoints according to CLSI Guidelines

| Antimicrobial | Class | MIC Interpretive Standard (µg/mL) | | |
|-----------------------------------|------------------------------|--------------------------------------|--------------|-----------|
| | | Susceptible | Intermediate | Resistant |
| Ampicillin | Penicillin | 8 | 16 | 32 |
| Cefazolin | Cephems | 2 | 4 | 8 |
| Trimethoprim- Sulfamethoxazole | Folate Pathway Inhibitors | 2/38 | | 4/76 |
| Chloramphenicol | Phenicol | 8 | 16 | 32 |
| Ceftriaxone | Cephems | 1 | 2 | 4 |
| Ciprofloxacin | Fluoroquinolone | .06 | .5-.12 | 1 |
| Gentamicin | Aminoglycoside | 4 | 8 | 16 |

5.5 Table 5. Prevalence (no.) of Salmonella by farm and sample type

| <i>Sample Type</i> | <i>Conventional % (n/N)^a</i> | <i>Organic % (n/N)</i> | <i>p-value^b</i> |
|--------------------|---|----------------------------|----------------------------|
| Fecal | 12.2 (9/74) | 27.5 (19/69) | 0.03 |
| Environmental | 15.8 (6/38) | 26.3 (20/76) | 0.24 |
| Feed | 7.0 (4/57) | 11.4 (9/79) | 0.56 |
| Egg | 0 (0/12) | 10.7 (3/28) | 0.54 |
| Total | 10.5 (19/181) | 20.2 (51/252) | 0.008 |

^a n = No. of positive isolates and N = Total no. of isolates

^b *p*-values were calculated for sample types using Fisher's exact test to compare prevalence of *Salmonella* in conventional and organic farms

5.6 Table 6 Proportion of *Salmonella* serovars by farm and sample type

| Sample Type | Conventional | | | Organic | | |
|---------------|---|----------------------------------|---------------------------------------|----------------------------------|----------------------------------|---------------------------------------|
| | <i>S. Typhimurium</i> % (n/N) ^a | <i>S. Enteritidis</i> % (n/N) | <i>Salmonella enterica</i> % (n/N) | <i>S. Typhimurium</i> % (n/N) | <i>S. Enteritidis</i> % (n/N) | <i>Salmonella enterica</i> % (n/N) |
| Fecal | 66.7 (6/9) | 0 (0/9) | 33.3 (3/9) | 52.6 (10/19) | 0 (0/19) | 47.4 (9/19) |
| Environmental | 50.0 (3/6) | 0 (0/6) | 50.0 (3/6) | 26.3 (5/20) | 5.0 (1/20) | 70.0 (14/20) |
| Feed | 75.0 (3/4) | 0 (0/4) | 25.0 (1/4) | 44.4 (4/9) | 0 (0/9) | 55.6 (5/9) |
| Egg | 0 - | 0 - | 0 - | 100 (3/3) | 0 (0/3) | 0 (0/3) |
| Total | 63.1 (12/19) | 0 (0/19) | 36.8 (7/19) | 43.1 (22/51) | 1.97 (1/51) | 54.9 (28/51) |

^an = number of isolates identified at serovar or species level and N = total number of strains of *Salmonella* from the sample type

5.7 Table 7. Prevalence of *Salmonella* antibiotic resistance by sample and farm type

| Sample Type | Conventional (n=19) | | | Organic (n=51) | | |
|---------------|----------------------------|-----------------|--|-------------------|-----------------|---------------------------|
| | AMR %(n/N) ^a | MDR % (n/N) | Pansusceptible % (n/N) ^a | AMR % (n/N) | MDR % (n/N) | Pansusceptible % (n/N) |
| Fecal | 11.1 (1/9) | 77.8 (7/9) | 11.1 (1/9) | 15.8 (3/19) | 26.3 (5/19) | 57.9 (11/19) |
| Environmental | 0 (0/6) | 66.7 (4/6) | 33.3 (2/6) | 20.0 (4/20) | 45.0 (9/20) | 35.0 (7/20) |
| Feed | 0 (0/4) | 100 4/4 | 0 (0/4) | 22.2 (2/9) | 11.1 (1/9) | 66.7 (6/9) |
| Eggs | - | - | - | 33.3 (1/3) | 66.7 (2/3) | 0 (0/3) |
| Total | 10.5 (2/19) | 73.7 (14/19) | 15.8 (3/19) | 19.6 (10/51) | 33.3 (17/51) | 47.1 (24/51) |

^a AMR isolates were resistant to only 1 antimicrobial, MDR isolates were resistant to 2 or more antimicrobial classes. Susceptible isolates tested were susceptible to all antimicrobials tested.

5.8 Table 8. Prevalence of *Salmonella* antimicrobial resistance by antimicrobial and farm system

| <i>Antimicrobial^a</i> | <i>Prevalence of Salmonella isolates across farm system</i> | | <i>p-value^b</i> |
|----------------------------------|---|---------------------------|----------------------------|
| | <i>Conventional %(n/N)</i> | <i>Organic %(n/N)</i> | |
| Cefazolin | 84.2 (16/19) | 39.2 (20/51) | 0.001 |
| Ampicillin | 73.7 (14/19) | 51 (26/51) | 0.05 |
| Chloramphenicol | 15.8 (3/19) | 1.96 (1/51) | 0.06 |
| Ceftriaxone | 5.3 (1/19) | 1.96 (1/51) | 0.47 |

^aAntimicrobials ciprofloxacin, gentamicin and trimethoprim/sulfamethoxazole are not listed since no isolates showed resistance to these antimicrobials

^b*p*-values are calculated using Fisher's exact test on the differences of resistant phenotype prevalence on farm type

5.9 Table 9. Antimicrobial resistance patterns of *Salmonella* from organic and conventional laying hens

| Antimicrobial resistance pattern ^a | Prevalence of <i>Salmonella</i> isolates across farm type | |
|---|---|-----------------------------|
| | Conventional isolates % (n/N) | Organic isolates % (n/N) |
| AmCzCl | 15.8 (3/19) | 2.0 (1/51) |
| AmCzCx | 5.3 (1/19) | 2.0 (1/51) |
| AmCz | 52.6 (10/19) | 29.4 (15/51) |
| Cz | 10.5 (2/19) | 5.9 (3/51) |
| Am | 0 (0/19) | 13.7 (7/51) |
| Susceptible | 15.8 (3/19) | 47.1 (23/51) |

^a Am, Ampicillin; Cz, Cefazolin; Cl, Chloramphenicol; Ce, Ceftriaxone

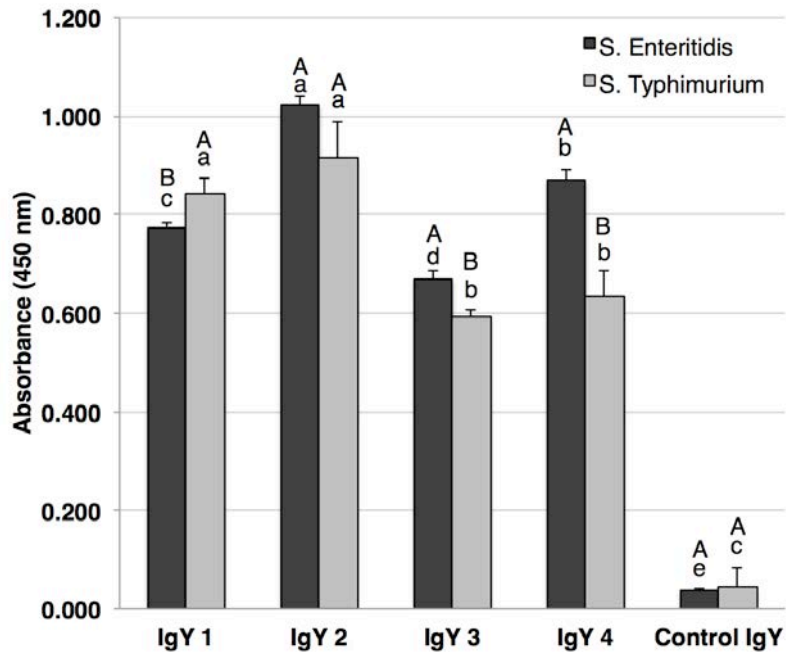
5.10 Table 10. Antimicrobial resistance of *Salmonella* serovars from organic and conventional laying hens

| Antimicrobial | Conventional | | Organic | |
|-----------------|-------------------------------------|---|-------------------------------------|---|
| | <i>S.</i> Typhimurium % (n/N) | <i>Salmonella</i> <i>enterica</i> % (n/N) | <i>S.</i> Typhimurium % (n/N) | <i>Salmonella</i> <i>enterica</i> % (n/N) |
| Am ^a | 75 (9/12) | 71.4 (5/7) | 41 (9/22) | 50 (14/28) |
| Cz | 75 (9/12) | 100 (7/7) | 36.3 (8/22) | 39.3 (11/28) |
| Cl | 8.3 (1/12) | 28.6 (2/7) | 0 (0/22) | 3.6 (1/28) |
| Ce | 8.3 (1/12) | 0 (0/7) | 0 (0/22) | 0 (0/28) |

^a Am, Ampicillin; Cz, Cefazolin; Cl, Chloramphenicol; Ce, Ceftriaxone

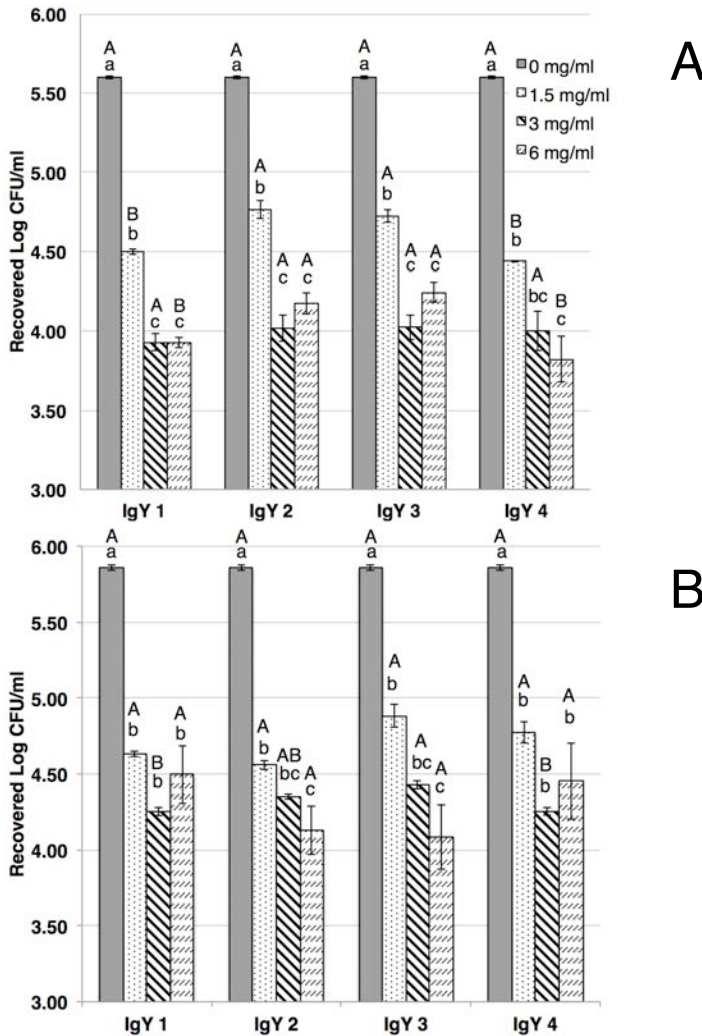
Chapter 6: List of Figures

6.1 Figure 1. Reactivity of anti-Salmonella Antibodies Against *S. Enteritidis* and *S. Typhimurium*



Indirect ELISA performed with four different IgY treatments against *S. Enteritidis* and *S. Typhimurium*. Data are represented as mean \pm S.D. Lower case letters represent significant differences ($p < 0.05$) in antibody reactivity for different treatments across a single *Salmonella* serotype. Upper case letters represent significant differences ($p < 0.05$) between reactivity of two *Salmonella* serovars for a single antibody.

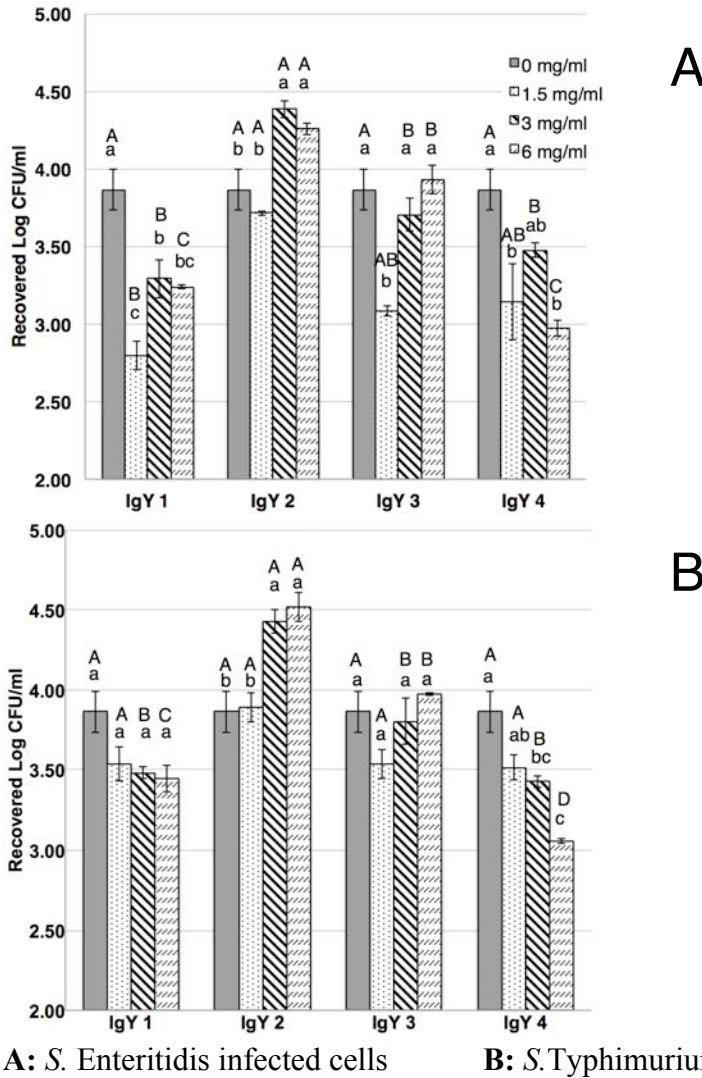
6.2 Figure 2. DF-1 Pretreatment Adherence Prevention



A: *S. Enteritidis* infected cells **B:** *S. Typhimurium* infected cells

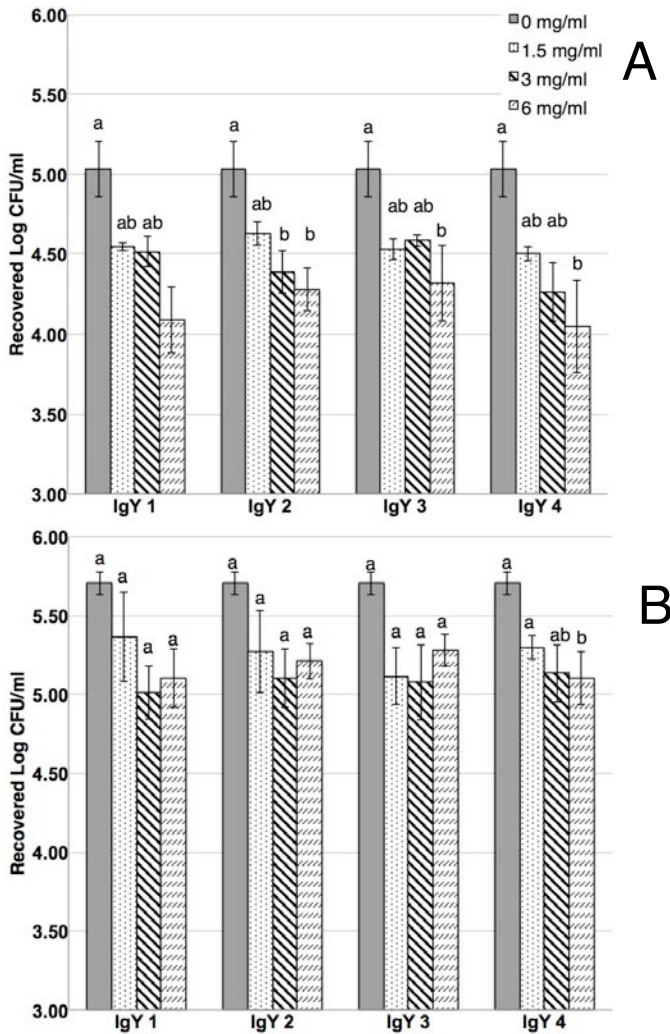
Bacterial adherence of *S. Enteritidis* or *S. Typhimurium* to DF-1 chicken fibroblasts before infection. Adhered bacteria are represented as mean log CFU ml⁻¹ ± S.D. Lower case letters represent significant differences ($p < 0.05$) in numbers of adhered bacteria between different concentrations in a single treatment. Upper case letters represent significant differences ($p < 0.05$) between number of adhered bacteria between different treatments at a single concentration, separated by serotype.

6.3 Figure 3. HD-11 Pretreatment Adhesion Prevention



Bacterial adherence of *S. Enteritidis* or *S. Typhimurium* to HD-11 chicken macrophages before infection. Adhered bacteria are represented as mean log CFU ml⁻¹ ± S.D. Lower case letters represent significant differences ($p < 0.05$) in antibody reactivity for different treatments across a single *Salmonella* serotype. Upper case letters represent significant differences ($p < 0.05$) between reactivity of two *Salmonella* serovars for a single antibody.

6.4 Figure 4. DF-1 Posttreatment Adherence Prevention



A: *S. Enteritidis* infected cells

B: *S. Typhimurium* infected cells

Bacterial adherence of *S. Enteritidis* or *S. Typhimurium* to DF-1 chicken fibroblasts after infection. Adhered bacteria are represented as mean log CFU ml⁻¹ ± S.D. Lower case letters represent significant differences ($p < 0.05$) in antibody reactivity for different treatments across a single *Salmonella* serotype.

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