

Colonization Sites of *Salmonella* Enteritidis and Heidelberg in Broilers when Exposed Continuously in Feed or Day of Hatch

by

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Abstract

Salmonella is the most common bacterial pathogen to cause foodborne illness in the United States with poultry acting as a main vector. In order to control this pathogen prior to the processing plant, consideration must be made in both entryways and colonization sites at the pre-harvest level. For these studies, the first aim was to determine colonization sites within broilers (meat birds) if they were given a constant exposure of *Salmonella* Enteritidis (SE) or Heidelberg (SH) in their feed at a constant dose of 10^2 CFU/gram. Supplementary studies included giving broilers either SE or SH on day 0 through one of five inoculation routes (oral, intratracheal, subcutaneous, ocular or cloacal) at 10^4 CFU. Birds were reared to market weight, then euthanized and samples collected. The samples included: breast, crop, a pooled sample of the bursa & thymus (B+T), spinal cord, trachea, skin from the neck area, thigh, kidney, a pooled sample of the liver & spleen (L+S), ceca, and crop. Swab samples were collected from the: abdominal cavity (ab cav), lung, bone marrow (bm), and cloaca.

A comparison of the recovery of *Salmonella* per serotype as well as inoculation route was analyzed using the GLM procedure and when appropriate, Tukey HSD ($P \leq 0.05$). A comparison of the means for the feed trial was determined by a T-Test. For all three presented studies, there was a greater recovery within the ceca in comparison to other collected samples. Other areas with high recovery included: B+T, crop, cloacal swab. The intratracheal inoculation route resulted in the greatest recovery of both SE and SH in comparison to the other investigated inoculation routes.

The recovery of SE and SH within the ceca indicates that this is the ideal area for colonization of *Salmonella* Enteritidis or Heidelberg at the pre-harvest level. The intratracheal inoculation route is in need of further analysis to determine this entryway's level of concern within the poultry industry. This holistic approach of analyzing both entryways and colonization sites will aid the poultry industry in determining pre-harvest control measures for *Salmonella*.

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List of Abbreviations

U.S.	United States of America
SPII	<i>Salmonella</i> Pathogenicity Island 1
SE	<i>Salmonella</i> Enteritidis
HACCP	Hazard Analysis Critical Control Points
CDC	Center for Disease Control and Prevention
SH	<i>Salmonella</i> Heidelberg
CCPs	Critical Control Points
FSIS	Food Safety Inspection Service
CFU	Colony Forming Units
mL	Milliliter
MDM	mechanically deboned meat
Mcg	Micrograms
Rpm	rotations per minute
Cm ²	centimeters squared
Cm ³	centimeters cubed
BPW	buffered peptone water
°C	degrees Celsius
GLM	General Linearized Model
<i>et al</i>	et alibi

HSD	honest significant difference
XLT4	xylose lysine agar supplemented with tergitol 4
μL	microliter
tt	Tetrathionate broth

Chapter 1. Introduction

Nontyphoidal *Salmonella* is considered one of the most influential bacteria within the human food systems. In the United States (U.S.), there is an estimated one million cases of salmonellosis each year, allowing for this bacterium to be the main cause of hospitalizations as well as deaths due to a foodborne illness (Alali and Hofacre, 2016). Chicken meat is often reported as the vector of *Salmonella* to humans and with a worldwide increase of poultry production and consumption, food safety initiatives have been directed towards pre-harvest and post-harvest control (Guran *et al.*, 2016). Because of the association between poultry and *Salmonella*, this pathogen has been well reviewed and monitored within the U.S. poultry and food systems (Ahmer and Gunn, 2011; Alali and Hofacre, 2016; Foley *et al.*, 2001; Holt *et al.*, 2011; Ricke *et al.*, 2013; Vandeplass *et al.*, 2009). The objective of these trials was to target both entryways and colonization sites that published literature has neglected. This was accomplished by utilizing two serotypes typically associated with causing contamination on poultry products as well as the documented human illnesses. These were *Salmonella* Enteritidis and Heidelberg (Alali and Hofacre, 2016). Analyzing pre-harvest interventions of these two serotypes is of utmost importance within the U.S. poultry industry and healthcare systems. Determining colonization sites of broilers following exposure to either serotype continuously through feed or day of hatch will aid the industry in a better understanding of *Salmonella* at the pre-harvest level.

Chapter 2. Literature Review

2.1 *Salmonella* General Characteristics

To recognize why *Salmonella* is an issue in poultry products, general characteristics must be understood. *Salmonella* are mesophilic, facultative, Gram-negative, rod-shaped bacterium with a petrichous flagella that is part of the family Enterobacteriaceae (Conner *et al.*, 2001). The shape of this pathogen allow is ideal for movement throughout the digestive tract of animals. The lipopolysacharride layer of the *Salmonella* cell allow for differentiating serotypes of *Salmonella*. General phenotypic characteristics of *Salmonella* include: growth on minimal nutrient agar, aero-anaerobes, ferment glucose, often produce a gas as a product of fermenting glucose, reduce nitrate to nitrite, and have a negative result for oxidase tests. *Salmonella* is an intracellular pathogen, meaning that it can colonize and establish within the gut of animals. It can tolerate acidic environments to allow for the movement through the digestive tract. It also induces inflammation within the area of colonization to decrease the competition with native and other pathogenic microflora (Schneitz and Mead, 2000).

The genus *Salmonella* contains two species, *S. enterica* and *S. bongori*, that are based on their phenotypic profile. As its name suggests, *S. enterica* will enter within a host and cause a reaction in the intestines and can be found within and cause harm between animal species (Brenner *et al.*, 2000; Hargis *et al.*, 2001; Conner *et al.*, 2001). When working with *S. enterica*, this species can be classified more specifically by one of the six subspecies or one of the 2,500 serotypes found within these subspecies (Brenner *et al.*, 2000). The 2,500 known serotypes within the genus of *Salmonella* vary on how each serotype affect humans (Humphrey, 2000; Beaumont *et al.*, 2010). The United States provides serotype-specific *Salmonella* surveillance to detect outbreaks, identify disease transmittance, and monitor control efforts already in place.

Salmonella enterica species act as residents of animal hosts and are genetically bestowed to thrive in this environment (Winfield and Groisman, 2003). An animal's gut will provide *Salmonella* serotypes with consistent temperature and high concentrations of free amino acids and sugars which are necessary for colonization and proliferation. *Salmonella* is considered a foodborne pathogen within food-producing animals, allowing for the asymptomatic transmission of this pathogen from food animals to human food systems. This transmission has been identified with *Salmonella* contamination within poultry (Crump *et al.*, 2002). The poultry industry is being forced to reanalyze management practices to reduce the incidence of *Salmonella* contamination on products meant for human consumption. Salmonellosis originating from processed meat and meat products has been an issue for decades due to food animals coming in constant contact with an array of microorganisms during their lifespan (Humphrey, 2000; McEntire *et al.*, 2014). It must be noted that the per capita consumption of poultry products has increased dramatically in the past one hundred years, which increases potential for exposure to pathogens, like *Salmonella*, when eating such products (Lynne *et al.*, 2009). It is known that microbial contamination of poultry carcasses is a natural result of producing meat from a live animal.

2.2 Poultry and Food Safety Concerns

Human salmonellosis is typically associated with the consumption of contaminated food products, specifically with poultry acting as a main carrier (Foley *et al.*, 2011). *Salmonella* bacteria are the primary cause of foodborne illness in many countries, including the U.S. (Kimura *et al.*, 2004; Fratamico, 2003; Parveen *et al.*, 2007; NARMS, 2014; Lee *et al.*, 2015). This pathogen causes a negative impact on the U.S. economy, healthcare systems, and food industry. *Salmonella* is assumed to cause 23,000 hospitalizations and 450 deaths in the United

States alone, according to the Center for Disease Control and Prevention. This is under the assumption that only 2% of the cases are reported, indicating that there could be a larger impact that is not documented (Lee *et al.*, 2015). It has been estimated that this pathogen has a direct medical cost of 3.6 billion dollars annually (NARMS, 2014). It is also estimated that 10-29% of salmonellosis cases are due to poultry products, the highest for any food group (Guran *et al.*, 2016; McEntire *et al.*, 2014). This is of main concern within this country due to the United States being one of the main poultry producing and exporting countries in the world (Alali and Hofacre, 2016). The need for reducing and controlling *Salmonella* within poultry demands control methods during both pre-and post-harvest to ensure that the microbial load is at a minimum (Corrier *et al.*, 1999).

Meat and poultry consumption has increased 6.5-fold since 1910, inferring that more meat is being consumed each year, increasing the risk of salmonellosis due to food animals containing this pathogen (Foley *et al.*, 2011; Alali and Hofacre, 2016). Between 2004-2008, poultry consumption increased by 14.4% worldwide and 2.9% in the United States, identifying an increase in the risk of consumers being exposed to pathogens on poultry products, like *Salmonella*. The United States Department of Agriculture- Food Safety Inspection Service (USDA- FSIS) has reported a 4.3% prevalence of *Salmonella* on whole broiler carcasses, indicating that of the nine billion broilers processed each year in the United States, 382 million would not pass the standards for *Salmonella* prevalence set by this agency (Alai and Hofacre, 2016). The poultry industry has a challenge of reducing bacterial loads on meat products, although most foodborne illnesses are a result of temperature abuse, mishandling, or improper preparation done by the consumer (Conner *et al.*, 2001). Educational efforts in the proper handling and cooking of poultry products are necessary in the desire to decrease or prevent

Salmonella illnesses. Implementation of safe food handling practices are readily available; however, few are translated into common practice (Kimura *et al.*, 2004). Due to the general consumer's lack of proper cooking and handling knowledge of raw poultry, it is of utmost importance that products contain the least amount of contamination when transitioning from farm to fork.

Non-typhoidal salmonellosis is known to cause illness within humans and ranges from mild to severe gastroenteritis within the lower intestinal tract. The infectious dose, or the minimum number of live *Salmonella* cells needed to cause illness, is noted to range from 10^4 to 10^6 cells and symptoms appear 12-36 hours after consumption. It is important to note that it is difficult to determine the minimum amount of exposure to this pathogen that will cause illness. The consumption of different amounts of this pathogen are associated with variable probabilities of illness and is dependent on host susceptibility and the serotype of interest, leading to inconsistency in the infectious dose and the reaction from the human intestine. It can be assumed that as the dose of *Salmonella* increases, the incidence of illness increases as well (McEntire *et al.*, 2014). Symptoms within humans include: nausea, vomiting, severe diarrhea, abdominal cramps, and discomfort (Kimura *et al.*, 2004; Hargis *et al.*, 2001; Conner *et al.*, 2001). However, long term affects can include arthritis, aortic aneurisms and ulcerative colitis (McEntire *et al.*, 2014).

Symptoms associated with *Salmonella* colonization in humans are due to the invasion of the intestinal cells by this bacterium. Pathogenesis of specific *Salmonella* species within the gut is expressed by type III secretions that are encoded by *Salmonella* pathogenicity island 1 (SPI-1). Type III secretions act as a virulence factor in the intestinal phase of the *Salmonella* infection. Type III secretions containing bacteria can invade non-phagocytic host cells. Such as those found

in the epithelial lining of the human intestine. SPI-1 is a large gene set that allows for communication, penetration and invasion of *Salmonella* into the epithelial layer of the intestines (Marcus *et al.*, 2000; Borsoi *et al.*, 2009). Another pathogenicity island associated with *Salmonella* (SPI-2), allows this bacterium to systemically spread and colonize other organs (Borsoi *et al.*, 2009). SPI-1 and SPI-2 require specific environmental cues in order for these genes to be transcribed.

Similar attachment and invasion can be identified within the poultry gut, even if the serotype appears asymptomatic. Epithelial invasion, synthesis of an enterotoxin, and induction of an inflammatory response are common steps within a *Salmonella enterica* infection; however, the exact mechanisms by which *Salmonella* causes mucosal damage are not well understood within poultry, specifically with serotypes that appear asymptomatic (Mehta *et al.*, 1998). Stress-induced perturbation of the normal intestinal microflora, specifically the epithelial lining, has been shown to allow for pathogen colonization and proliferation to occur within the gut of poultry (Burkholder *et al.*, 2008). There are multiple stress factors that occur during the rearing of a commercial flock. The response created in reaction to stress will create alterations within the mucus layer of the gut, altering the attachment potential for both beneficial and pathogenic bacteria (Burkholder *et al.*, 2008). For example, epithelial lesions are observed after *Salmonella* Enteritidis infection of broiler chickens but are very moderate compared with those in mammals (Awad *et al.*, 2012).

The animal gut is colonized by trillions of bacteria that exist in a balanced relationship within the host. Incoming pathogenic organisms that are not part of the normal gut microflora must pass barriers created by the intestinal microbiota to colonize. These barriers include:

physical, chemical, enzymatic, and/or immune. All areas must be considered when analyzing for *Salmonella* in both poultry and humans (Ahmer and Gunn, 2011).

Salmonella serotypes differ in reservoirs, specifically certain foods, and their ability to cause human infection (Jackson *et al.*, 2013). Of the 2,500+ serotypes, only about 10% are identified with the poultry and egg industry (Foley *et al.*, 2011). Salmonellosis incidence has not decreased over the past few decades but instead some serotypes have become more prevalent than others to appear within the U.S. food systems. *Salmonella enterica* serotypes Enteritidis and Heidelberg are predominately attributed to poultry food commodities as well as isolated from poultry farms and processing plants (Jackson *et al.*, 2013; Schlosser *et al.*, 2000). These serotypes are of high importance within the poultry industry and U.S. healthcare systems due to their ability to colonize asymptotically in poultry and symptomatically in humans.

Host- specific *Salmonella Gallinarum* and *Pullorum* have been identified to cause systemic disease in poultry once colonized in the ceca and bursa; however, asymptomatic serotypes, like Enteritidis or Heidelberg, can persist within the digestive tract of these animals for months without clinical signs, indicating variation of pathogenicity and virulence among serotypes (Sadeyen *et al.*, 2004). SE and SH resemble each other in that there are known virulence mechanisms in response to invasion, survival and growth within a host (Guard-Bouldin *et al.*, 2004). The animal intestine is in a constant state of (low) inflammation, creating an ideal environment for *Salmonella enterica* serotypes to outcompete both normal and invasive microflora (Ahmer and Gunn, 2011). In order to better understand both entryways and colonization sites of concern in commercial poultry, similarities and differences must be identified with SE and SH.

It has been identified that although similarities between serotypes exist, variation colonization mechanisms can depend on both host and serotype (Foley *et al.*, 2011; Porwollik *et al.*, 2004). There are subtle strain and serotype differences in acid tolerance and virulence gene expression which may explain some of the variation seen in frequency of SE and SH in poultry (Ricke *et al.*, 2013). Both SE and SH colonize within the reproductive tract of birds and contaminate eggs (Schoeni *et al.*, 1995; Gast *et al.*, 2007; Foley *et al.*, 2011). They have also been determined to be shed in feces and penetrate eggs during storage, indicating a pre-harvest concern with broilers (Schoeni *et al.*, 1995). Similarities have been identified with these two serotypes in that they share common surface antigens (Foley *et al.*, 2011). It has also been identified that both serotypes of interest contain SPI-1 and SPI-2 genes, indicating the use of these genes for colonization. Both can change their metabolism based on the environmental conditions to allow for a competitive edge over other microorganisms (Ricke *et al.*, 2013).

SE has been understood to be a concern in the poultry industry since the 1980's. It has been theorized that transition and proliferation of this serotype into poultry occurred when serotypes Gallinarum and Pullorum were eradicated from the U.S. poultry industry (Foley *et al.*, 2011). In the creation of a phylogenetic tree after microassays were conducted, there was great similarity of gene contents between these serotypes (Porwollik *et al.*, 2004). This is an indication as to why SE has become such a dominate serotype as Gallinarum and Pullorum have decreased, there is less competition for ideal nutrient sources.

Few studies have been conducted in examining colonization factors with SH due to its recent emergence within the poultry industry (Foley *et al.*, 2011). Borsoi *et al.* (2011) identified greater colonization within the ceca and it had greater fecal shedding in comparison to SE after the birds were orally dosed. Further analysis of colonization sites of this serotype is needed.

It has been hypothesized that varying recovery of either serotype could be due to virulence systems based from *Salmonella* pathogenicity islands (Borsoi *et al.*, 2011). Understanding colonization sites of both serotypes is of utmost importance due to the variation that can be found.

2.3 *Salmonella* Enteritidis

Salmonella enterica serotype Enteritidis (SE) has been identified as one of the most common serotypes documented to cause human infection since the 1980's, specifically in developed countries. This serotype was the cause of 841 outbreaks from 1985 to 1999 (Patrick *et al.*, 2004). After the implementation of Hazard Analysis Critical Control Point Program (HACCP), better control of this serotype was observed, but it still appears to cause outbreaks, illnesses, and recalls.

SE is most commonly known for being prevalent in layer eggs, however trends within illnesses found from consumption of poultry carcasses has identified a need for controlling this serotype within the broiler industry, potentially due to the transmission of SE within the eggs of breeders (Alteruse *et al.*, 2006; Jackson *et al.*, 2013). A challenge with SE is that it appears asymptomatic in poultry, allowing for SE to be able to infect birds and later our food systems without negative effects developing on or within the bird host (Beaumont *et al.*, 2010). In the 1990's, SE surpassed *Salmonella* Typhimurium as the predominant serotype that can be isolated from humans. While overall *Salmonella* incidence as decrease, SE prevalence at farms and within poultry products has increased (Altekruse *et al.*, 2006; Kimura *et al.*, 2004. A study by Altekruse *et al.* (2006) identified that there was less *Salmonella* recovered form whole carcass rinses, however there were more SE positive samples than previous years when serotyping was

performed. Carcass rinse samples are collected as the carcasses are leaving the chiller. At this point in production, the carcasses are sanitized, cooled to refrigeration temperatures, and are ready for packaging to go out on the market for consumer purchase and later consumption. With the increase in SE recovery from whole carcass rinses at this point in production, this identifies a common risk of *Salmonella* Enteritidis within poultry products. In a five-year study by Kimura *et al.*, there was an increase from fourteen to twenty-four states having products contaminated with SE. This serotype has been identified as an ongoing epidemic of both outbreaks and sporadic illnesses and will spread from layer flocks to breeders, affecting broilers. The egg shell has been identified as the main vehicle in transferring SE to broilers, allowing for interaction and colonization to occur when the chicks are young. It has been determined that the number of human SE infections increased as prevalence of SE within broiler chicken increased (Kimura *et al.*, 2004). This emphasized a positive correlation between chicken production and *Salmonella* Enteritidis illnesses in humans as well as demonstrated the need for *Salmonella* control in the poultry production plants.

SE outbreaks have been frequently traced to food served at restaurants, as opposed to food prepared at home. The Center for Disease Control and Prevention (CDC) reported that 59% of the SE outbreaks from 1985-1991 were associated with restaurants or other commercial food settings (Kimura *et al.*, 2004). This drew attention to SE contamination in further processed products. In 2015, the Minnesota Department of Health, Minnesota Department of Agriculture, and the USDA-FSIS identified SE within frozen, raw, stuffed and breaded chicken entrees produced by one company. This outbreak led to multiple companies that carried this product to recall their items, causing a large financial burden. From May to July of 2015, fifteen people from seven different states were infected with SE due to these products. Four people were

hospitalized but there were no recorded deaths. This caused over 2 million pounds of product to be recalled due to the potential of SE contamination. Indication that this product was the cause of the illnesses was due to consumers reporting to have eaten the frozen chicken product a week or so prior. Epidemiological tests identified similar characteristics between the SE found within the ill consumers and the SE found within these products, indicating infection due to this product. The SE isolated was determined to be resistant to common antibiotics used to treat *Salmonella* infections (U.S. Dept. of Health and Human Services, 2015).

Today, the number of outbreaks have been reduced, but there is still potential for contamination within poultry products. The exact mechanism for why SE has been reduced is still unknown, creating the need for a better understanding of this serotype within poultry products (Patrick *et al.*, 2004).

2.4 *Salmonella* Heidelberg

High percentages of *Salmonella* Heidelberg (SH) have been identified through case-control studies and previous research within eggs, chicken, and turkeys (Jackson *et al.*, 2013). SH is one of the top five most common serotypes associated with *Salmonella* infections in humans. In 2006, it was identified that salmonellosis had decreased by 9%, while SH infections had increased by 25%. In 2013, SH was the ninth most common serotype among human infections and the third most common serotype among isolated serotypes identified from retail chicken samples (Gieraltowski *et al.*, 2016). It is also a concern that SH isolates are continuously showing increased resistance to common antibiotics, demonstrating the need to control this serotype (Lynne *et al.*, 2009).

After HACCP was set into place in 1999, testing and surveys were performed on whole and ground poultry products to identify common serotypes found within these products after this ruling had taken place. Schlosser *et al.* (2000) identified that SH was the most commonly isolated *Salmonella* serotype on chicken carcasses and raw ground chicken. It was also commonly found with turkey carcasses and raw ground turkey. This brought attention to SH within poultry products, specifically if it had the potential to make humans ill after HACCP was set in place.

In 2013, 634 people from 29 states and Puerto Rico were infected with SH due to a single poultry company. Almost half of the cases reported were hospitalized. This was due to the SH strain being multi-drug resistant. Identification of this company as the source was due to sampling of ill-consumers' fecal matter, testing of leftover raw chicken that was collected from an ill-person's home, and a survey of what the ill ate a week prior to symptoms. The FSIS conducted in-facility testing for *Salmonella* at multiple production facilities specific to this company and six of the seven outbreak strains were isolated from raw chicken products. This outbreak is of major concern not only due to the number of people infected, but the variation in location of illnesses as well as the variation in products that were infected. Unopened chicken parts, rotisserie chicken, rotisserie chicken salad, boneless skinless tenders, boneless skinless chicken breasts, fryer chicken thighs, and drumsticks were all products that were positive for this SH contamination. This demonstrates the ability of SH to spread through multiple products that are produced in one processing plant. This has caused a financial burden on this company because of the cost of a recall, the cost of illness, and a lack of trust was created between consumer and producer (CDC, July 2014; Geiraltowski *et al.*, 2016).

In 2014, Tyson mechanically separated chicken products were also recalled due to contamination then outbreak of SH. Nine consumers from Tennessee were infected, and two were hospitalized. A recall of 33,840 pounds of poultry mechanically deboned meat (MDM) was required, causing a financial burden on the poultry company of concern (CDC, February 2014).

SH is among the most frequently isolated *Salmonella* serotypes from clinical cases, retail meats, and food animals in North America. It is also commonly derived and isolated from poultry products, specifically ground poultry. Outbreaks of SH present a significant public health concern and economic burden for both the producer and consumer in the United States (Zhao *et al.*, 2008).

2.5 Carcass Contamination at the Processing Plant

Detection of asymptomatic *Salmonella* serotypes in poultry can be difficult and can lead to a lack of knowledge in potential cross-contamination at processing plants (Edel and Kampelmacher, 1973). Chicken carcasses go through multiple steps within a processing plant to reach the acceptability of the consumer. These include, but are not limited to: bleeding, scalding, picking, washing, chilling, and secondary processing. All of these steps can be a source of *Salmonella* cross-contamination on poultry carcasses (Guran *et al.*, 2016). The Hazard Analysis Critical Control Point (HACCP) plan was created to analyze, reduce, and prevent physical, chemical, and biological contamination on meat and poultry products during processing to decrease the potential of foodborne illness. All poultry companies are required to following the HACCP ruling to maintain their processing establishments. The HACCP plan involves Critical Control Points (CCPs), which are critical areas in production the control of the physical, chemical, or biological hazards of utmost importance for the safety of products. CCPs monitor

temperature, humidity, pH, salt and chlorine concentration, and many other factors that could cause a change in the bacterial count on the poultry products. While conducting a hazard analysis at each CCP, the team in charge of that area should routinely check for biological, chemical, and physical issues with the poultry products. *Salmonella* was one of the main biological concerns that HACCP is focused on due to its prevalence in raw and ground meat products as well as the numerous methods that can be used to detect *Salmonella* (Bilgili, 2001; Conner *et al.*, 2001; McEntire *et al.*, 2014). Monitoring for this pathogen within retail meats includes: collection of cecal samples from the meat animals prior to slaughter as well as carcass rinses and sample collection at processing plants (NARMS, 2014). Because of this plan, *Salmonella* that had contaminated broiler chicken carcasses in the past was greatly reduced, however within the past decade, *Salmonella* contamination has steadily increased.

Once the broilers reach the processing plant, specific inspections carried out by plant employees and the Food Safety Inspection Service (FSIS) to ensure that products made are processed under sanitary conditions, are suitable for human consumption, and are free from adulteration. Epidemiological investigations are done to analyze for food-borne health hazards and disease outbreaks related to poultry products created in commercial facilities (Conner *et al.*, 2001). However, there are many areas in which cross-contamination of *Salmonella* can occur within the processing plant, demonstrating the need for better control at the pre-harvest level. The nature of modern poultry processing does not allow for an elimination of all bacteria, but a decrease in the bacterial load. Evisceration systems involve physical separation of the viscera from the carcass to decrease chances of cross-contamination, however if viscera tear, contamination of the carcass can occur with intestinal contents

It is routinely identified that *Salmonella* cross-contamination can occur when viscera is removed, but what is not understood is if abdominal fluid is also a potential contaminate for poultry product, specifically air sacs or the fluid surrounding the heart (Northcutt 2001; Bilgili, 2001). This area is in need of further analysis to determine potential colonization as well as contamination.

The crop is a storage site for ingested feed and other debris prior to digestion where food, water, and saliva create an optimal growth environment for bacterial species (McLelland, 1990; Ricke, 2003). Since the crop is the initial environment that *Salmonella* enters the body when orally ingested, it becomes an important determinant for colonization and contamination of *Salmonella* throughout the rest of the digestive tract (Ricke, 2003). The crop creates an ideal growth environment for *Salmonella*, where nutrients and moisture are readily available. It has been identified as a source of *Salmonella* contamination during processing due to broilers pecking at fecal material during feed withdrawal as well as the likelihood of this organ to tear during processing. Crops are eighty-five times more likely to break during processing in comparison to the ceca, an organ with high microbial load (Alali and Hofacre, 2016). *Salmonella* contamination has been shown to increase during feed withdrawal, indicating that feed patterns during pre-slaughter can alter *Salmonella* colonization within the crop (Corrier *et al.*, 1999; Alali and Hofacre, 2016). It has been suggested that normal microflora found within the crop is altered during feed withdrawal, causing an increase in pH, allowing for pathogens, like *Salmonella*, to bloom (Hargis *et al.*, 2001). Due to the functionality of the crop, feed and litter ingested that is contaminated with *Salmonella* will be held in this organ until the body is ready for digestion, allowing colonization to occur.

The ceca have been identified as one of the primary sites for microbial colonization within broilers. This is due to the fermentation processes that occur within the ceca, allowing for *Salmonella* contamination to reside within the normal gut microflora and for penetration of the mucosal epithelium (Berndt *et al.*, 2007). The ceca have been considered a primary source of *Salmonella* contamination during processing if the intestinal tract is ruptured (Corrier *et al.*, 1999; Alali and Hofacre, 2016). Contaminated floor litter that is ingested with cycle through the bird, allowing for increased fecal shedding of this pathogen. Studies have demonstrated that bacterial loads within the ceca remain relatively stable during feed withdrawal, allowing for this organ to be utilized for identifying *Salmonella* contamination (Corrier *et al.*, 1999). In 2013, the USDA FSIS integrated a cecal sampling program within the poultry industry. This sampling better reflects *Salmonella* contamination prior to birds arriving at processing plants in comparison to HACCP's sampling after processing (NARMS, 2014).

The liver and spleen work together and act as a filter for the circulatory system. The liver is also utilized as a part of giblets sold to consumers (McLelland, 1990). The spleen and liver have both been identified as common colonization sites of *Salmonella* in poultry (Alali *et al.*, 2016). This is due to the macrophages within the host disseminate *Salmonella* to the liver and the spleen (Henderson *et al.*, 1999). Infestation of *Salmonella* has the potential to be seen within these organs due to their functionality.

Previous studies have identified that *Salmonella* cells can attach firmly to chicken skin (Alali *et al.*, 2016, Guran *et al.*, 2016). This allows for cross-contamination of carcasses from pre-harvested poultry due to *Salmonella* presence on the skin. Chicken skin has been identified as a source of *Salmonella* contamination in processing due to this pathogen's ability to attach and be entrapped within skin layers, crevices, or feather follicles. Zhang *et al.* (2013) identified that

Salmonella are spread across skin surface and lodged into a specific depth of broiler skin prior to birds arriving at the processing plant. Another potential point of contamination is during processing, where the process of feather removal allows for deeper interaction and penetration of this pathogen into the skin, i.e. scalding and picking (Zhang *et al.*, 2013).

It is of utmost importance that the FSIS inspect, detect, and act on contamination of the carcass from spillage of digesta tract contents or fecal material due to the high instance of microbial contamination that can occur at these plants. However, even with a zero tolerance for fecal contamination, it cannot be completely prevented when working with meat products (Northcutt, 2001; Parveen *et al.*, 2007). The FSIS requires corrective action if *Salmonella* levels on the food products exceed the limit, but they must also implement new safety regulations if *Salmonella* cannot be controlled.

After inspection, carcasses are cooled in immersion chillers containing antimicrobials mixed with cold water to decrease bacterial loads. Chilling of poultry carcasses in the United States is usually done by immersion chilling, where large tanks of cold water containing antimicrobials acts as a bath for poultry carcasses after broilers have gone through evisceration. This process can be beneficial in that the rapid cooling decreases growth of mesophilic organisms, like *Salmonella*. The immersion chillers are countercurrent, meaning that the clean water enters where birds are leaving. This flow allows for more effective reduction in bacterial loads. However, if the antimicrobials are not monitored correctly, immersion chillers can also serve as an area of cross-contamination due to the large number of carcasses that pass through this system. *Salmonella* has the potential to wash off the skin of one carcass and move to many others, causing a negative effect on products created by these carcasses. It has been reported that chill water and the processing of cooling carcasses in an immersion chiller serve as sources of

pathogen contamination between carcasses. This can allow for a small number of contaminated carcasses to cross-contaminate many (Beery *et al.*, 1988; Parveen *et al.*, 2007). Parveen *et al.* (2007) were also able to identify no significant difference in *Salmonella* prevalence when comparing carcasses before and after chilling, indicating a lack of efficiency in decreasing bacteria within this chilling system.

Poultry processing facilities must reach the performance standards for *Salmonella* in each establishment (Bilgili, 2001). The 2016 Performance Standards include: whole carcasses at 9.8% acceptability, cut-up parts at 15.4% acceptability, and mechanically deboned meat at 25% acceptability (FSIS, 2016). However, it is important to note that the testing for *Salmonella* is a yes/no basis. Meaning that if a product contains one cell of *Salmonella* or one hundred cells of *Salmonella*, both will be treated in the same manner (McEntire *et al.*, 2014). It has been demonstrated that less than 5% of broilers going into the processing plants had *Salmonella* contamination while 35% of processed broilers were contaminated with *Salmonella*. The large number of potential (cross-) contamination of poultry at the pre- and post-harvest level limits the ability to be able to control *Salmonella* within poultry completely, but implementing integral control programs with a better understanding of contamination at the pre-slaughter level will allow for a decrease in *Salmonella* prevalence before birds reach the processing plant, later decreasing the potential of cross-contamination (Conner *et al.*, 2001).

Fifty-one carcasses are collected and whole carcass rinses are performed to monitor for *Salmonella* contamination. Five or less carcasses can be *Salmonella* positive. If greater than five carcasses have *Salmonella* recovery, the processing plant is required to collect follow-up samples and companies will monitor for *Salmonella* prevalence on products meant for human

consumption (Alali and Hofacre, 2016). Monitoring of antimicrobials and carcasses bacterial loads are essential in controlling cross-contamination at the plants (Conner *et al.*, 2001).

Carcasses can be shipped whole, cut- up, deboned, or further processed into ground poultry or mechanically deboned meat (MDM). Instruments utilized in created these products as well as increased contact with air allows for a plethora of *Salmonella* contamination as carcasses are further processed. Modern lifestyles have shifted the poultry industry into producing products that have partial preparation done at the processing plant. Cut-up parts, like the breast and thigh meat, are utilized at home for cooking convenience. These parts have been found to be more profitable for the poultry industry due to the high demand. However, with increased demand for these specific portions of meat, there is also an increased demand for monitoring and control of foodborne pathogens, like *Salmonella*, on commonly purchased poultry products. (Bilgili, 2001). In a study by Guran *et al.* (2016), *Salmonella* had significantly higher recovery on skin-on cut up parts in comparison to cut up parts without skin, identifying the attachment and contamination of *Salmonella* on the skin of poultry products. This must be taken into consideration while monitoring for *Salmonella* contamination in second processing.

With MDM, meat is removed from the skeletal bone tissues by grinding the frames, back, neck, drumsticks, skin, and other parts of the carcass that would otherwise go to waste and passing these parts through a sieve while under high pressure. Hand deboning allows for human error in the amount of meat removed from the carcass frame. To prevent this and other parts from going to waste, MDM utilizes human error and creates products from the leftover parts. (Savadkoohi *et al.*, 2013). Due to variability in what is available, the composition of MDM can vary slightly with each batch created. MDM cannot contain greater than 25% fat and no less than 14% protein if it is to be utilized as a product within the United States (Froning and McKee,

2001). In the industry, it is common practice for kidneys to not be used in mechanically separated poultry products; however due to the location of the kidneys, it is difficult to remove all kidneys from every carcass (Froning and McKee, 2001). Kidneys act as a filtering agent for the blood and if *Salmonella* is systemic, it would be recovered from this organ. Because of the process in creating MDM, some bone is incorporated into the product as well. The separation process to produce MDM from leftover carcass parts includes the neck, back, and thigh (Savadkoohi *et al.*, 2013). Skin is also utilized in MDM, increasing the potential of contamination with *Salmonella* on the further processed products (Alali *et al.*, 2016). Because of the high pressure used to create MDM, bone marrow and spinal cord have increased incidence of being incorporated in this product. It has been documented that broken bones used to create MDM allow for the incorporation of bone marrow (Froning and McKee, 2001). If *Salmonella* is prevalent within the spinal cord or bone marrow, it can proliferate within MDM, allowing for contamination of poultry products created from MDM. Deboning releases lipids and hemoglobin from the bone marrow of the poultry carcasses, allowing for poor oxidative stability in MDM which can lead to increased quantity of *Salmonella* contamination (McNeill *et al.*, 1988). In 2012, *Salmonella* positive samplings included 4.3% in carcasses and 28% in ground chicken (Department of Agriculture, 2016). The increase in acceptable *Salmonella* contamination as the carcass is further processed demonstrates the need for better control in pre-and post- harvested poultry. The greater prevalence of *Salmonella* in ground chicken in comparison to the whole bird could be due to one carcass containing high loads of *Salmonella* then this carcass is evenly distributed throughout multiple pounds of ground product. Ground products that contain increased bacterial loads may not be adequately cooked, leading to high contamination levels of this pathogen (McEntire *et al.*, 2014).

2.6 *Salmonella* Contamination at Poultry Farms

Pathogen prevalence on poultry products is of main concern to maintain biological control at the processing plants. Due to a lack of alteration in *Salmonella* prevalence, focus has shifted towards controlling contamination with pre-harvested poultry as well (Conner *et al.*, 2001). *Salmonella* food contamination has not decreased over the past decade, but rather different serotypes are becoming common in poultry and other food products (Jackson *et al.*, 2013).

There is a concern of both vertical and horizontal transfer of *Salmonella* within the poultry industry. *Salmonella* has been identified on the inside and outside of poultry eggs, indicating the potential of both vertical and horizontal transfer of this pathogen within a flock on or immediately after day of hatch (Hooft *et al.*, 2011).

Both SE and SH have shown to penetrate or be deposited into the egg after invading the reproductive tract of hens, indicating a vertical transfer of concern in the layer and broiler breeder industries (Gast *et al.*, 2007). *Salmonella* has demonstrated to survive on a fecal-contaminated eggshells as well as in the albumen and can multiply if it reaches the nutrient-rich yolk or yolk membranes. The latter rarely occurs; however, it is still possible (Gast *et al.*, 2007). The ability of this pathogen to colonize through the digestive tract as well as be shed through fecal material of the hen allows for contamination that can occur both within and on the outside of the egg, which can affect the progeny (Holt *et al.*, 2011). Although SE is most commonly noted on eggs, SH has also been recovered. Variation in SE and SH contamination in or on eggs could be due to varying colonization within the reproductive tract of breeders or variation in fecal shedding, either of which can contaminate the chick at the day of hatch (Hennessy *et al.*, 2004; Borsoi *et al.*, 2011).

Environmental stress has been identified as a main contributor to the colonization, shedding, horizontal transfer and carcass contamination of poultry (Burkholder *et al.*, 2008). Current commercial housing systems incorporate management and rearing practices that increase the potential of *Salmonella* contamination throughout grow-out. Direct contact of broilers on feces, ingestion of contaminated feed or feces, poor biosecurity practices, movement of equipment between farms and contaminated dusts or aerosols has all contributed to horizontal transfer (Conner *et al.*, 2001; Gast *et al.*, 2014). Due to the high population densities found within modern broiler houses, pathogens like *Salmonella* can bloom and spread throughout the house, causing (a) symptomatic contamination to the flock (Conner *et al.*, 2001).

As previously mentioned, the initial source of *Salmonella* in pre-harvested poultry can occur from several different vectors (Conner *et al.*, 2001; Gast *et al.*, 2014; Jarquin *et al.*, 2009). These studies were directed towards contamination at the farms, although hatcheries should also be considered as a source of this pathogen (Cox *et al.*, 1990).

Detection of *Salmonella* in the environment has shown to correlate with poultry prevalence found within further processed products, indicating that environmental contamination can happen. This will lead to cross contamination at the processing plants (Winfield and Groisman, 2003). Other factors that affect pre-slaughter poultry contamination include: a lack of uniformity in the bird sizes, feed outages during the withdrawal period, excessive personal activity in the poultry house during feed withdrawal which can cause stress on the birds, and a change in environmental conditions (Conner *et al.*, 2001). When focusing on *Salmonella* contamination at the farms that can later affect processing, it is important to analyze: air quality, litter, water, feed, and biosecurity practices.

A concern to mention is the immune responses developed within poultry for *Salmonella* colonization when chicks are exposed at a young age. Day-old chicks are vulnerable to *Salmonella* colonization due to an innate immune system (Cox *et al.*, 1996). The gastrointestinal tract of newly hatched chicks can be considered a “blank slate” and vulnerable to colonization with bacteria, specifically *Salmonella* (Holt, 2000). A hatching chick is vulnerable to both vertical and horizontal transfer of *Salmonella* (Alali and Hofacre, 2016). This allows for early contamination of poultry food products, where colonization occurs during the primary stages of life and has the potential to bloom and spread to other chicks (Barrow, 1991). Young chicks infected with *Salmonella* have both an immature immune system as well as the influx of bacteria, causing an obliteration of lymphoid tissue. As the chick hatches, their cloacal opening allows for fluid intake by the antiperistaltic reflex, bringing in microorganisms, like *Salmonella*, into the body near the bursa (Cox *et al.*, 1996).

A route of *Salmonella* contamination within a broiler house is infection by aerosols. *Salmonella* can be spread by dust, feed, down feathers, excrement, debris, and a plethora of microorganisms common in poultry houses (Aarnink *et al.*, 1999; Ritz *et al.*, 2005). Airborne microbial loads can contaminate a poultry house as well as the animals inside due to the movement and disturbance of poultry litter and the structural airflow created in modern poultry houses (Brooks *et al.*, 2010; Kallapura *et al.*, 2014b). Dust levels within each poultry house vary and can be dependent on the age of the house, animal activity, density, and the moisture of the litter (Ellen *et al.*, 2000; Ritz *et al.*, 2006) A study by Brooks *et al.* (2010) demonstrated that bacterial loads increased by 2-fold between outside and inside the poultry houses. This shows the increased concentration of bacteria that commercial poultry are exposed to during their lifespan while broilers are being reared in commercial housing systems. The study was also able to

identify *Salmonella* within air samples collected in the poultry houses, demonstrating that there is a potential for *Salmonella* to move throughout the house via aerosols. It has also been demonstrated that *Salmonella* can survive within an aerosol for hours, however this was in a lab setting (McDermid and Lever, 1996). Dust and debris can contain microorganisms, like *Salmonella*, which can contaminate flocks and later cross-contaminate poultry products meant for consumption.

Several studies have focused on dust levels in various poultry houses as well as its components, but there has been limited research on understanding *Salmonella* colonization within broilers through various orifices on the chicken that are affected by dust. Cox *et al.* (1996) identified that introducing low levels of *Salmonella* within the eye would allow for recovery within the ceca after only 7 days post-inoculation, indicating that the eye can act as a port of entry of *Salmonella* into broilers. Birds, as well as other animals, have nasolacrimal ducts. These ducts act as a drainage system to relieve debris from the eye into the nasal cavity where it can later be taken into the respiratory or digestive tracts (Williams, 2012). The nasolacrimal duct gives dust ridden *Salmonella* access to colonization sites within the broiler host.

Several studies have suggested airborne transmission of *Salmonella* into broiler carcasses and colonization through this route have been identified. Due to modern poultry houses being enclosed buildings with movement of high volumes of air throughout a house via negative pressure, lots of dust, debris, and microorganisms can travel and infect a flock, allowing for *Salmonella* colonization due to inhalation of this pathogen. The trachea has also been identified as a colonization site that can be utilized during processing to identify *Salmonella* contamination,

indicating that an intratracheal route can be utilized as a common way in which *Salmonella* is negatively affecting poultry products (Kallapura *et al.*, 2014a).

A cut or a scratch on the bird that could allow for *Salmonella* from the environment to enter the body. It has been identified that a subcutaneous injection of *Salmonella* will bring this pathogen rapidly to the liver and spleen of the birds (Barrow, 1991). Cuts and scratches can be common within a poultry house due to the high stocking density. The subcutaneous injection imitates if there is *Salmonella* present within the environment and the bird has an opened wound.

Differences between the susceptibility of the respiratory systems and the digestive tract maybe of little significance due to the anatomical connections found between these pathways. When an inoculation is exposed through an airway, there is potential for some to enter the digestive tract and vice versa (Cox *et al.*, 1996).

Litter is absorbent bedding material that is placed on the floor of a poultry house (Alali and Hofacre, 2016). Poultry are generally reared on litter consisting of wood shavings, rice and/or peanut hulls, shredded paper, and other finely torn wood materials. This can provide a source of *Salmonella* contamination due to the multiple sources in which litter can be obtained as well as the United States using “dirty litter”- litter that is recycled between flocks (Cressman *et al.*, 2010; Conner *et al.*, 2001; Alali and Hofacre, 2016). It is ideal to recycle litter to decrease the accumulated poultry litter; however, reused litter can affect the intestinal microbiota in the flocks. Used litter allows for bedding materials to consist of bird excreta, creating differences in bacterial loads in comparison to fresh litter. During broiler grow out, there is a constant influx of nutrients and environmental influences that alter litter microbiota and later poultry gut microbiota (Cressman *et al.*, 2010). Litter mates are known to ingest fecal droppings of each

other instead of only consuming food. This allows for the ingestion of *Salmonella* if present in the gut of the birds.

It has been demonstrated that under experimental conditions, young chicks that are exposed to *Salmonella* will have a fluctuation of *Salmonella* species between the litter and the gut of the chicken as it reaches market weight. With natural conditions, it has been identified that there is an influx of *Salmonella* until 3 weeks of age, then there is a rapid decline, especially in used litter where there is greater competition for resources (Renwick *et al.*, 1992). Pests also inhabit the litter, allowing for movement of pathogens, like *Salmonella*, to occur between the bird and other animals (Wales *et al.*, 2009). Chicks will sit on contaminated surfaces during their grow-out period. A moist or wet cloaca allows for a pathway in which *Salmonella* can enter broilers. The antiperistaltic reflex allows for the drawing in of fluid, debris, and microorganisms into the boiler's body to allow for exposure to the environment as well as proper development of the immune system. This route lacks the decreased acidity found when birds ingest *Salmonella*, giving an increased chance of *Salmonella* survival and colonization. As food is ingested and waste created, the fecal material will interact with the *Salmonella* found around the cloaca, allowing for environmental contamination that can lead to exterior *Salmonella* prevalence on birds. Intestinal proliferation of *Salmonella* allows for fecal shedding of this organism, which has the potential to contaminate the exterior of other chicks, like the skin (Cox *et al.*, 1996).

Colonization of *Salmonella* can occur over an entire flock in as little as a week. Fresh litter has increased *Salmonella* infection found within poultry in comparison to built-up litter. This can be due to competitive exclusion of the microflora within used litter. There is a positive relationship between litter moisture and *Salmonella* bacteria survival during grow-out. Higher amounts of litter moisture as well as a high pH allows for this pathogen to grow (Cressman *et al.*,

2010). Chemical treatments added to litter can alter the acidity of the litter, leading to a decrease in *Salmonella* prevalence (Conner *et al.*, 2001; Alali and Hofacre, 2016). Litter samplings have been utilized to monitor for *Salmonella* contamination within flocks due to the time efficiency in collecting samples, lack of stress on the birds, and minimal training and personnel are necessary to carry out the tests (Renwick *et al.*, 1992; Alali and Hofacre, 2016).

Salmonella is constantly released into the environment from infected animals and is regularly isolated from water sources contaminated with feces. Seepage from septic tanks, storm drains and well fields as well as using water that has been irrigated near farm animals facilitates the contamination of water with pathogens like *Salmonella*. In comparison to other bacteria, *Salmonella* has shown to have high survival rates in aquatic environments and prevalence is not altered by season of the year or water temperature (Winfield and Groisman, 2003). Secondary contamination of the drinkers is also possible, with fecal matter, litter, dust, and other residuals interacting with the water source and causing contamination. This pathogen's presence around or in the water source allows for opportunity for *Salmonella* to enter poultry through an oral route (Renwick *et al.*, 1992). Interventions of: acidifying the water, treating water with chlorate, and using essential oils within water have been studied to determine if there is a decrease in bacterial load (Alali and Hofacre, 2016). Specifically, the treatment of drinking water with organic acids has been identified to reduce *Salmonella* colonization, but these studies were mainly focused on feed withdrawal periods, indicating need to better understand how water can affect *Salmonella* within broilers and how it can be utilized to decrease this pathogen within poultry throughout grow out (Conner *et al.*, 2001).

Salmonella can frequently be isolated from feed (Northcutt 2001; Conner *et al.*, 2001; Alali and Hofacre, 2016). Poultry that consume contaminated feed will be colonized by

Salmonella as well as shed this pathogen in their feces onto the litter (Alali and Hofacre, 2016). Conditions of: source of ingredients, transportation to the feed mill, contamination at the feed mill, transportation to the farms, storage, and distribution of feed within the houses demonstrates the high potential of feed contamination with *Salmonella* and other pathogens (Andino *et al.*, 2014; Alali and Hofacre, 2016). Feed ingredients and dust have both demonstrated to be major sources of *Salmonella* contamination in feed mills and later to the animals eating the feed. Protein byproducts originating from animals have also been suggested as a source of zoonotic pathogen contamination (Andino *et al.*, 2014; Alali and Hofacre, 2016). Both mashed and pelleted feed have shown *Salmonella* contamination, however due to the process in creating each of these feeds, there is less contamination in the pelleted (Jones and Richardson, 2003; Alali and Hofacre, 2016). Both feed styles are utilized within the broiler industry, varying based on age of the bird. Bacterial contamination is due to the process in creating feed, where the combination of moisture and heat provide an ideal environment for *Salmonella* growth. If inadequate temperatures are used in the creation of mashed or pelleted feed, there is an increased likelihood of bacterial contamination (Alali and Hofacre, 2016). Broilers nibble at feed and eat regularly when the temperature remains constant and the lighting is continuous. When alterations of eating patterns occur, specifically prior to be collected for processing, there is a great variability in the content as well as the condition of their digestive tract. *Salmonella* serotypes brought into the birds by feed can harvest within multiple areas of the digestive tract and later lead to carcass contamination if feed withdrawal was done incorrectly and cause a loss in viscera integrity (Conner *et al.*, 2001).

The common route of microorganisms, like *Salmonella*, is direct ingestion (Kallapura *et al.*, 2014b). An oral inoculation allows for *Salmonella* to have contact with multiple areas of the

digestive tract and mimics the ingestion of this pathogen from the environment. This is the most common form of inoculation, due to the ease of administration for the researcher as well as the ease in the broilers receiving the inoculation. It has been demonstrated numerous times that an oral inoculation can give insight to areas in which commercial poultry will have *Salmonella* colonization (Barrow, 1991; Beaumont *et al.*, 2010).

Paratyphoid *Salmonella* serotypes that can asymptotically infect poultry and later cause gastroenteritis in humans are not host-adapted, a wide variety of animals can act as carriers of different serotypes into poultry (Conner *et al.*, 2001). Birds and bugs are important vectors for the widespread distribution of *Salmonella* in the environment, specifically poultry houses (Winfield and Groisman, 2003). Arthropods occur universally on livestock units, specifically within poultry houses and buried within the litter. Beetles are known for acting as carriers of a wide variety of pathogenic bacteria into poultry houses as well as into poultry if beetles are ingested. *Salmonella* has been shown to exist on the carcasses of beetles for at least 45 days after contamination. It has also been established that common flies found within poultry houses act as carriers of *Salmonella*. Not only can flies infect poultry with *Salmonella*, they can also infect other pests, like mice. Mice will shed *Salmonella* in their feces throughout poultry houses, later allowing for the pecking of poultry litter by the birds to create infection. Poultry mites have also been identified as a vector of *Salmonella*, specifically SE, throughout flocks. Mites are in issue with poultry because they create habitats near or on the bird, allowing for exchange of bacteria between the two animals. Insects and rodents can act as a vector in the direct transfer of *Salmonella* to animals on farms, including humans (Wales *et al.*, 2009).

Biosecurity is an important management tool to control pathogens from entering and leaving poultry farms. Biosecurity is the management strategies implemented to prevent and

control the introduction of pathogens to food animals raised on commercial farms (Alali and Hofacre, 2016). These practices aid in controlling diseases that can make both poultry and humans ill (Conner *et al.*, 2001). The presence of different niches throughout poultry houses determine the diversity of *Salmonella* prevalence found within broilers (Brooks *et al.*, 2010). The absence of effective control measures during live production can bring pathogens, like *Salmonella*, to the processing plant (Conner *et al.*, 2001).

The transmission of *Salmonella* into poultry is complex, requiring prevention and monitoring programs at both the pre-harvest and processing levels (Alali and Hofacre, 2016). A collection of measures has been shown to be (cost) effective in controlling *Salmonella*; however, research lacks a holistic viewpoint of a comparison of multiple ways in which this pathogen could be entering birds at the pre-harvest level. Knowledge of pre-harvest contamination will aid in decreasing *Salmonella* (cross-) contamination at processing plants, increasing food safety potential within poultry products.

Chapter 3. Continuous Exposure to *Salmonella* Enteritidis or Heidelberg in Broiler Feed

Introduction:

When analyzing ingredient formulation for commercial broilers, it is important to note the wide variety of sources that have the potential to contaminate the feed with *Salmonella*, allowing for colonization within broilers and later, contamination on chicken food products. To achieve the most cost-effective diet, ingredients come from all over the United States, allowing for multiple vectors of *Salmonella* contamination. Once the ingredients are shipped to a feed mill, they are mixed to create poultry feed, transported to farms, and held in storage until the broilers consume the feed. All areas serve as possibilities for *Salmonella* to integrate into the feed then into the broilers, later contaminating carcasses meant for human consumption (Andino *et al.*, 2014).

Salmonella enterica is estimated to cause over one million food-related illnesses each year in the United States, with the serotypes Enteritidis (SE) and Heidelberg (SH) being consistently associated with poultry products, specifically mechanically deboned meat (MDM) (Jackson *et al.*, 2013; Alali *et al.*, 2016). Both *S. Enteritidis* and *S. Heidelberg* have acted as causative agents linked to *Salmonella* outbreaks from poultry origins (Borsoi *et al.*, 2015). The presence of bacterial populations, like *Salmonella*, in poultry farm populations allows for the transfer of these pathogens into poultry products meant for human consumption (Borsoi *et al.*, 2015). The poultry industry is striving to control foodborne pathogens in pre- and post-harvested poultry while facing a demand for varying feed ingredients to maintain affordable birds meant for consumption (Tellez *et al.*, 2011).

Once contaminated feed has been ingested, it is stored in the crop with a mixture of water and saliva, providing optimal nutrients for bacterial growth (Ricke, 2003). Then, it travels through the digestive tract to the intestines, where interactions between *Salmonella* and intestinal epithelial cells occur. This interaction is known for causing a release of phagocytic cells to the site where *Salmonella* is present and cellular responses try to eradicate the invasive species. This reaction creates an inflammatory response within the intestine. *Salmonella* has demonstrated the ability to survive these attacks in multiple animal species and penetrate cells that carry this pathogen to lymphoid tissues (Henderson *et al.*, 1999). It has been identified that broad-host-range serotypes Enteritidis and Heidelberg can persist in the digestive tract of birds throughout grow-out without clinical signs of infection, indicating that there is no visual signs of illness with the birds when infected with either serotype (Sadeyen *et al.*, 2004). *Salmonella* colonizes within the intestine and can be carried to the liver and spleen, increasing the potential for contamination at processing plants (Henderson *et al.*, 1999). *Salmonella* has also been shown to shed through fecal matter of poultry which can lead to contact on the animal's skin as well as *Salmonella* becoming ingested by other members of the flock (Sadeyen *et al.*, 2004). All areas mentioned serve as potential colonization sites of *Salmonella* in poultry which can later cause contamination of poultry products at the processing plant, specifically mechanically deboned meat (MDM) which is made from the carcass frame after more profitable meat is removed from the carcass, poultry skin, and other parts of the carcass not utilized in alternative poultry products (Berghaus *et al.*, 2013; Froning and McKee, 2001; Savadkoobi *et al.*, 2013). MDM allows for the creation of a protein source that is widely used in the formulation of other meat products by utilizing parts of the carcass that contain no commercial value (Bigolin *et al.*, 2013; Froning and McKee,

2001). Although MDM is profitable, it must be closely monitored for *Salmonella* contamination to decrease the instance of an outbreak.

It has been numerously identified that *Salmonella* contamination can be found within poultry feed (Alali *et al.*, 2013; Andino *et al.*, 2014). It has also been identified that poultry carry *Salmonella* within their gut and other areas of their body, later contaminating or cross-contaminating carcasses at the processing plants, leading to increased exposure of this organism within the human food systems (Berndt *et al.*, 2007; Corrier *et al.*, 1999; Hargis *et al.*, 2001; Ricke, 2003; Sadeyen *et al.*, 2004; Schleifer *et al.*, 1983). Feed has been targeted as a source of contaminated for broilers at the pre-harvested level and specific organs and tissues have been identified as common colonization sites for *Salmonella*, however limited literature has focused on a continuous exposure of *Salmonella* through the feed and the identification of numerous colonization sites. The objective of this study was to compare the sites of establishment of *Salmonella enterica* serotypes Enteritidis and Heidelberg when broilers are given constant exposure through the feed until the broilers reach market weight.

Materials and Methods:

Bacterial cultures

Salmonella Enteritidis and Heidelberg isolates that are resistant to naladixic acid and novobiocin were utilized in these studies. These isolates were stored at -80°C until required. The frozen cultures were plated onto tryptic soy agar plates that contained 5% sheep's blood. Inoculated plates were incubated for 18-24 hours at 37°C. After that time, a single colony was selected and plated onto xylose lysine agar supplemented with tergitol 4 (XLT4) containing naladixic acid (100mcg/ml) and novobiocin (15 mcg/ml) and incubated for 48 hours at 37°C.

From the XLT4 plate, a single colony was removed and placed into 50 ml (milliliters) brain heart infusion broth (BHIB) to create the inoculations. The inoculated BHIB were put into a 37·C shaker incubator for 18 hours at 250 rpm. After which, the amount of incubated BHIB to add to feed gave a final count of 10^2 CFU/g (colony forming units per gram) of *Salmonella*. Dilutions were performed to ensure the desired CFU/g.

Experimental animals

Each study consisted of seventy-five straight run broiler chicks that were obtained on the day of hatch from a commercial hatchery and transported to the Auburn University Poultry Research Farm. Chicks were placed into floor pens with contaminated feed (25 birds/ pen) and allowed to grow for 32 to 36 days. Feed and water were provided to the birds *ad libitum*. Standard husbandry and biosecurity practices were followed when rearing the birds.

Method of inoculation

All broilers were fed an industry standard three phase diet consisting of a starter, grower, and finisher. The feed included an anticoccidial (Coban 90); however, no other feed additive was utilized. *Salmonella* Enteritidis and Heidelberg were mixed into the feeds prior to each feed being given to the birds using a Twin Shell Dry Blender (Patterson-Kelley Co.). Each inoculation was mixed into one pound of feed prior to placement into the blender to ensure thorough mixing. Then, 200 pounds of each feed were blended for 5 minutes. Low levels of SE and SH were utilized in this study in order to mimic contamination that can occur in the feed on commercial poultry farms.

Sample collection

On days 32 to 36, twenty to twenty-five birds per day were: euthanized with carbon dioxide, swabs were then taken of the cloaca for collection, then carcasses were sanitized by

immersion in a quaternary ammonia solution by submersing the body of the birds from below the head. The neck feathers of the birds were then removed and the birds were dipped a second time in the quaternary ammonia bath without wetting the head. Both times the birds were dipped attention was made to ensure that none of the detergent went into the birds' mouth. After the birds could drip dry, the head and neck were removed from the rest of the carcass. The carcass could exsanguinate as samples from the head and neck were removed. Samples were collected by placing the head onto a plastic cutting board that had been sanitized with 70% ethanol. Between each bird, the cutting boards were washed with water then sanitized with 70% ethanol. Approximately 5 cm² of skin from the neck area with feathers plucked was placed into a sterile bag (*Nasco*, Whirl-Pak). A thymus lobe, 5 cm of the trachea, and 3 cm of spinal cord were collected individually from the head sample and placed into individual sterile bags. Once exsanguinated, the carcass was placed onto a surface and sprayed with 70% ethanol. An incision was made distal to the keel bone towards the head as well as towards the abdomen of the carcass and skin was retracted to expose the fat pad and breast tissue. A 3-4 cm³ section of the breast muscle and the whole crop were removed and placed into individual sterile bags. Birds were not taken from feed due to the multiple day necropsy so feed from inside of the crop was removed before the crop was placed in the sterile bag. A section of the liver (one third of a lobe) and the whole spleen were removed and placed in a sterile bag together. One of the ceca was removed, placed into a sterile bag, and cut in half to expose the cecal contents. The bursa was removed and placed into the same sterile bag as the thymus. One kidney was excised and placed in a sterile bag. One leg was detached at the coxofemoral joint to expose the femoral head. Skin was pulled away from the thigh to expose thigh meat and approximately 3-4 cm³ of thigh meat was removed and placed into a sterile bag. All sterile bags containing samples were filled with 20 ml of

buffered peptone water (BPW) and homogenized using a stomacher for sixty seconds (Lab-Blender 80, Tekmar Company). The femoral head was truncated from the leg to expose bone marrow for collection. An abdominal swab of the interior of the body cavity was taken with a sterile swab. Then, a puncture of one lung was performed with another sterile swab. These samples were individually placed into sterile tubes containing 5 ml of BPW. These samples were then shaken. Strict aseptic techniques were enforced throughout the necropsies to ensure that minimal, if any, cross-contamination occurred.

Bacterial isolation

Due to the necropsies lasting over multiple days, BPW saturated samples were placed into a refrigerator at 4°C. On the last day of the necropsy, samples were moved into incubators set at 37°C and kept for 18-24 hours. Then, one ml of each BPW saturated samples was removed and placed into 5 ml of tetrathionate (tt) broth. Once all samples were transferred, the tt tubes were shaken then moved into an incubator set at 37°C for 48 hours. A loopful of the incubated tt tube samples were streaked onto XLT4 plates containing naladixic acid (100mcg/ml) and novobiocin (15 mcg/ml). The plates were incubated at 37°C for 48 hours. After this period, plates were analyzed on a yes or no basis centered on the growth of characteristic black colonies typical of *Salmonella enterica* serotypes on this medium.

Statistical Analysis

The SPSS version 22 software was used for analysis of the numerical and statistical differences between *Salmonella* positive organ and tissue samples collected from *S. Enteritidis* and *S. Heidelberg*. For each serotype, the GLM Procedure was utilized and when appropriate,

Tukey HSD. An independent T- test was utilized to identify differences, if any, between the serotypes for each sample collected. Differences were considered significant at $P \leq 0.05$.

Results

Figure 3.1 demonstrates the percentage of positive *Salmonella* Enteritidis samples found when given constant exposure of this serotype through the feed. The letters above each bar represent statistical similarities and differences determined by the Tukey HSD test. The crop (97.14%) was numerically the most significant, with the most prevalent *S. Enteritidis* colonization found within this sample. There is an intermediate statistical relationship identified with the crop as well as the bursa and thymus (B+T) (84.29%), ceca (88.57%), and cloacal swab (87.10%). Inferring that there are higher instances of finding *S. Enteritidis* colonization within the samples previously mentioned in comparison to other samples collected. The skin samples (68.57%) had a statistical intermediate relationship with the B+T, ceca, and cloaca as well as the L+S; however, the L+S (55.71%) did not have a statistical relationship with the previously mentioned samples except the skin. Other samples that had an intermediate with L+S include the kidney (42.03%), ab cav swab (28.57%), and the trachea (44.00%). The lung swab (20.00%) had similar recovery to the trachea. Samples with the lowest numerical value contained a similar statistical intermediate, which included the lung swab, bm swab (11.43%), breast (7.14%), thigh (5.71%), spinal cord (2.86%). *S. Enteritidis* was recovered from at least one sample from all different types of samples collected.

Figure 3.2 displays the percentage of positive *Salmonella* Heidelberg samples recovered after a constant exposure of this serotype within the feed. Like Figure 3.1, the letters above each bar demonstrate a statistical relationship between the prevalence of positive samples found. *S.*

Heidelberg was significantly more likely to be isolated within the crop (94.29%) and ceca (97.14%) compared to other samples collected. The B+T (68.57%), cloacal swab (55.70%), skin (52.86%), and the L+S (47.14%) all had similar recovery to each other. The L+S sample is statistically intermediate to the ab cav swab (27.14%) and the trachea (38.57%). The breast (12.86%), kidney (20.00%), lung swab (25.70%), and thigh (10.00%) have similar significance in the recovery of SH from these samples. These samples share an intermediate with the ab cav swab and trachea as well as the bm swab (1.43%) and spinal cord (2.86%). The samples with the lowest numerical recovery of SH had similar statistical recovery. All birds collected had at least one positive SH sample.

The relationship of colonization sites between the two serotypes found with an Independent T-test ($P \leq 0.05$) can be observed in Table 4.1. The B+T, bm swab, ceca, cloacal swab and kidney differed significantly in the recovery between *S. Enteritidis* and *S. Heidelberg*. The ab cav swab, breast, crop, L+S, lung swab, skin, spinal cord, thigh, and trachea had similar instances in recovery from birds given *S. Enteritidis* or *S. Heidelberg* continuously within the feed.

Discussion:

Day- old chicks have been shown to be susceptible to *Salmonella enterica* subspecies (Berndt *et al.*, 2007; Borsoi *et al.*, 2009). However, the serotypes of interest have shown variations in their ability to survive within poultry feed (Andino *et al.*, 2014). When broilers were given constant exposure to *Salmonella* Enteritidis or *Salmonella* Heidelberg, these serotypes were recovered from both local and systemic sites. With *Salmonella* Enteritidis, there was variation in what samples were statistically significant to each other but the crop sample had

the highest recovery numerically (Figure 3.1). However, with *Salmonella* Heidelberg (Figure 3.2), the ceca and crop samples are significantly more likely to be colonized than the other samples collected. When comparing the means of the samples collected between the two serotypes, the samples that showed significant variability ($P \leq 0.05$) between the serotypes were the B+T, BM swab, ceca, cloacal swab, kidney and lung. The variation in colonization demonstrates that each serotype can affect different parts of the carcass based on virulence factors.

As expected, continuous contamination of *Salmonella* within feed allowed for this pathogen to colonize throughout the digestive tract. The crop, ceca, and cloacal swab for both serotypes indicated high prevalence found within these birds with SE: 97.14%, 88.57%, 87.10% and SH: 94.29%, 97.14%, and 55.70%, respectively. The crops of the birds for both serotypes had high prevalence of *Salmonella* (SE: 97.14%, SH: 94.29%). Crop contents have been demonstrated to be a significant source of *Salmonella* contamination (Corrier *et al.*, 1999). Although high contamination is expected due to the constant presence within the feed and that the birds not having a period of feed withdrawal, it is important to note that the feed was removed from the crop prior to bag placement, and that standard error could have allowed for the few birds that were not positive for *Salmonella*. Colonization of *Salmonella* in the crop is common in broilers during the feed withdrawal period due to the pecking activity of the birds, however Corrier *et al.* (1999) found that there is also crop colonization in flocks before the withdrawal period. This is important for processing of the birds later, when cross-contamination can occur. It must also be considered that the withdrawal period could be a pre-harvest control point that can be utilized in reducing *Salmonella* contamination on broiler carcasses as they go

through the processing plant due to crop's vulnerability to *Salmonella* colonization and the crop's susceptibility to tear during processing (Corrier et al., 1999).

Little is known about how and to what extent *Salmonella* serotypes enter and infect the gut mucosa of poultry. To induce a systemic infection, these serotypes must reach the distal ileum and cecum and outcompete another microflora (Berndt *et al.*, 2007). The abdominal cavity swab demonstrated colonization of both serotypes (SE: 44.29%, SH: 27.14%) (Figures 3.1 and 3.2) inferring the movement of these serotypes through organs and tissues. This creates greater concern in the removal of viscera at the processing plant, where not only the tearing of viscera is of concern, but the viscera itself has potential to contaminate processing equipment. This can later cross-contaminate other birds going through the line. The ceca have been identified as the primary site of *Salmonella* colonization in poultry, allowing for samples from around the small intestine to have increased chances of *Salmonella* contamination (Corrier *et al.*, 1999). In this study, ceca samples were found significantly more positive for *Salmonella* than most other samples (SE: 88.57%, SH: 97.14%) (Figures 3.1 and 3.2), inferring the use of this organ as a target site for *Salmonella* contamination during grow out and processing.

The cloaca is the terminus of the digestive system where undigested food, urine, and other excrements not needed from the body are removed (USDA FSIS, 2014). Infection of chickens with *Salmonella* can lead to persistent shedding of this pathogen within fecal material, allowing for horizontal transmission of *Salmonella* serotypes through grow-out. Van Immereel and associates (2004) identified that shedding of SE after a 10^2 CFU oral inoculation allowed for high recovery within cloacal swabs until about 6 weeks of age. This indicates that SE has great potential to be shed through the feces. As shown in Figure 4.1, there was high prevalence of SE recovered within cloacal swabs taken from the infected birds (87.10%). However, there was

much lower incidence of *Salmonella* found within the cloacal swabs obtained from the SH infected birds (55.70%; Figure 3.2). There was a significant difference found between the two serotypes when comparing the means (Table 3.1). This indicates variation in colonization as well as shedding of the two serotypes of *Salmonella*. It also indicates that cloacal swabs are not a reliable source in identifying *Salmonella* contamination if the serotype of concern is unknown, like what Van Immerseel and associates identified (2004).

Lymphatic tissue, specifically the pooled samples of the bursa and thymus as well as the liver and spleen, harbor *Salmonella*. The present trials identified high colonization within the B+T, both numerically and statistically (SE: 84.29%, SH: 68.57%) for both serovars (Table 3.1). Also, observed in Table 3.1 is that there was a significant difference between the means of these two serotypes for the B+T. However, even though differences in recovery between serotypes were found, it is important to note that the presence of *Salmonella* Enteritidis or Heidelberg within lymphatic tissue could be utilized as an identification tool for contamination. The chicks were exposed to *Salmonella* Enteritidis or Heidelberg at day 0, at this age the immune system is immature, which easily allows for *Salmonella* to colonize. This has the potential to allow *Salmonella* to overcome immune defenses by the bursa and thymus that the bird develops later in life (Van Immerseel *et al.*, 2004). The exposure from day 0 on has demonstrated a reaction from within the immune organs.

Previously, studies have identified that *Salmonella* colonizes within the intestine and can migrate to the liver and spleen with prevalence like the samples collected from this study (Berndt *et al.*, 2007; Henderson *et al.*, 1999). *Salmonella* has demonstrated the ability to internalize within the spleen. Alali and associates (2016) utilized the spleen as a *Salmonella* identification method at processing plants. They identified that *Salmonella* prevalence within the spleen could

indicate a systemic infection. However, *Salmonella* positive spleen samples were only indicated to be within 15% of the samples they collected while these trials determined 55.71% of SE (Figure 3.1) and 47.14% of SH (Figure 3.2) spleen samples to be positive. The variation of positivity is due to that study collecting samples from processing plants as opposed to this study where broilers were given a known dosage of *Salmonella* for the duration of their lives. The birds examined by Alali and associates (2016) were not given a known and constant dosage of *Salmonella* in their feed. Another difference to note is that the samples collected from this experiment were pooled from the spleen and liver. However, this difference may be moot, since if *Salmonella* is systemic, both the liver and spleen would have colonization (Alali *et al.*, 2016).

Healthy poultry tissue from the breast and thigh are generally bought and eaten by the American consumer has little to no bacteria contamination prior to processing; however, this portion of the bird can become adulterated as it meets the outside of the animal as well as other organs and tissues that have bacteria (Barbut, 2015). The low levels of *Salmonella* prevalence found in either the breast or thigh samples that were collected during this study imply that these serotypes are not commonly found in the muscle. However, if birds are continuously exposed to either *Salmonella* Enteritidis or *Salmonella* Heidelberg contamination of these muscles is possible. This could be due to a systemic infection, where *Salmonella* entered the bloodstream and contaminated the muscles via the blood supply. These samples are important to note because the thighs and breasts are two of the most frequently consumed chicken parts in the United States. The USDA –FSIS verification program monitoring for *Salmonella* in poultry processing plants proposed a pathogen reduction of *Salmonella* contamination to 15.00% of samples collected from whole raw chicken carcasses (8/ 52 samples) (USDA 2014). When birds were constantly exposed to SE or SH within the feed, the breast and thigh meat samples that were

positive for either serotype fell below the pathogen reduction standard (Figures 1 and 2).

Meaning that, with constant exposure of 10^2 *Salmonella* in the feed, these portions of the bird will not be colonized to the point of rejection unless cross-contamination occurs.

When a *Salmonella*-positive broiler flock is processed and a mixture of parts are grinded together later in the plant, it can lead to carcass and product contamination (Alali *et al.*, 2016; Barbut, 2015). Mechanically deboned meat (MDM) is created from the chicken backs, necks, bones, skin, and other areas that are not of high profit to the general producer. The combination of undesirable parts increase profit by not allowing these portions of the carcass to go to waste (Bigolin *et al.*, 2013). However, a growing concern with MDM is *Salmonella* contamination, where the mixture of different portions of the carcass has demonstrated an increase in bacterial adulteration (USDA 2016).

Skin is commonly used as a *Salmonella* identification method at processing plants as well as a source of fat in MDM (Cox *et al.*, 1977; Bigolin *et al.*, 2013; USDA, 2016; Wu *et al.*, 2014). The focus of collecting skin would demonstrate if this sample could be utilized for detecting *Salmonella* prevalence as well as demonstrate if this is an area of cross-contamination occurring with producing MDM. This is due to the external surface of skin interacting with the poultry house environment, digesta, or fecal matter during grow-out (Kassem *et al.*, 2005). Skin that was collected from the neck area within these trials had a high prevalence of both serotypes of *Salmonella*, with *S. Enteritidis* having 68.57% and *S. Heidelberg* having 52.86% (Figures 3.1 and 3.2). The samples in this study differ from the neck samples collected at processing plants to monitor for *Salmonella* due to the lack of sanitation or disinfection prior to sampling. After carcasses leave the chiller, the collection of neck swabs occurs to determine bacterial contamination (USDA, 2016). *Salmonella* on flatter portions of the skin have been known to

wash off easily, but rigid skin or skin with feather follicles allow for protective pockets for *Salmonella* to be retained on the carcass. *Salmonella* on the skin allows for cross-contamination in the processing plant specifically during scalding. During this process, the chickens have been euthanized and the birds are dipped into a heated and agitated water bath to loosen both feathers and debris on the body. When the feathers are loosened, *Salmonella* can habitat within the skin, later leading to cross-contamination of whole or ground products (Diezhang *et al.*, 2014; Kassem *et al.*, 2005; USDA, 2016). Limited research has focused on the neck as a potential *Salmonella* carrier in pre-harvested poultry. Future studies should include analyzing *Salmonella* on the skin prior to processing to understand bacterial loads that are entering the processing plant.

Due to MDM containing: backs, necks, and bones it became pertinent to analyze if *Salmonella* colonization is occurring within the bone marrow and/or spinal cord. Colonization within these sites would also infer that there is a systemic infection. *Salmonella* within bone marrow or spinal cord of broiler carcasses has rarely been identified (Wu *et al.*, 2014). Previous studies have shown that around or less than one percent of bone marrow is contaminated with *Salmonella* (Wu *et al.*, 2014; Velaudapillai, 1964). In this study, broilers were constantly exposed to either serotype at a level of 10^2 cfu/gram of feed, findings of 11.43% positive bone marrow swab samples in *S. Enteritidis* and 1.43% positive bone marrow swab samples in *S. Heidelberg*. This demonstrates a variation of colonization ability of *Salmonella* (Figures 3.1 and 3.2; Table 3.1) and showed much higher colonization than previously reported. *Salmonella* Enteritidis is a highly invasive and a strong immune stimulator in comparison to other serotypes, specifically *S. Heidelberg*, allowing for increased colonization within the bone marrow (Brendt *et al.*, 2007). Both serotypes were recovered from the spinal cord samples at a level of 2.86% (Figures 3.1 and 3.2). This indicates that both serotypes have a similar likelihood to colonize

within the spinal cord (Table 3.1). Both the bone marrow and spinal cord should be further analyzed for *Salmonella* contamination if they will be continuously being part of the creation of MDM.

Conclusion

In conclusion, *Salmonella* serotypes Enteritidis and Heidelberg both systemically infected broilers when given with a constant exposure in the feed. This study demonstrates areas of the carcass that are likely to be infected with either or both serotypes of *Salmonella*. Monitoring the feed for *Salmonella* contamination could allow for a decrease of this pathogen's presence in the bird as well as within the environment of poultry houses, allowing for a decrease in exposure that can affect the *Salmonella* levels in the carcass. Monitoring at the pre-harvest level will allow for a decreased risk of exposure to zoonotic pathogens, like *Salmonella*, that can negatively affect human food systems. Although MDM utilizes parts of the carcass that contain minimal commercial value, precaution should be taken in creating this product with bones, skin, and other parts of the carcass due to the increased risk of *Salmonella* contamination. Future research should focus on the pathogenesis of *Salmonella* within poultry species. This area needs to be further examined to better understand colonization sites that can be identified in pre-harvested poultry.

Table 1. Comparison of <i>Salmonella</i> Recovery between Serotypes			
Sample	<i>S. Enteritidis</i>	<i>S. Heidelberg</i>	Significance (P≤0.05)
Ab. Cavity	44%	27%	0.171
B+T	84%	69%	0.029*
BM	11%	1%	0.016*
Breast	7%	13%	0.263
Ceca	89%	97%	0.049*
Cloacal Swab	87%	56%	0.000*
Crop	97%	94%	0.408
Kidney	42%	20%	0.005*
L+S	56%	47%	0.314
Lung	20%	17%	0.667
Skin (Neck)	69%	53%	0.058
Spinal Cord	3%	3%	1.000
Thigh	6%	1%	0.35
Trachea	37%	26%	0.114
* Statistical difference (P≤0.05) identified between serotypes			

Table 3.1 A comparison of *Salmonella* Enteritidis and Heidelberg using an Independent T-Test (P≤0.05). The numbers corresponding with each serotype and sample collected demonstrate the percent of positive samples for that sample within the specific serotype. The asterisk (*) symbolizes samples that had a significant difference in the prevalence of colonization between the two serotypes.

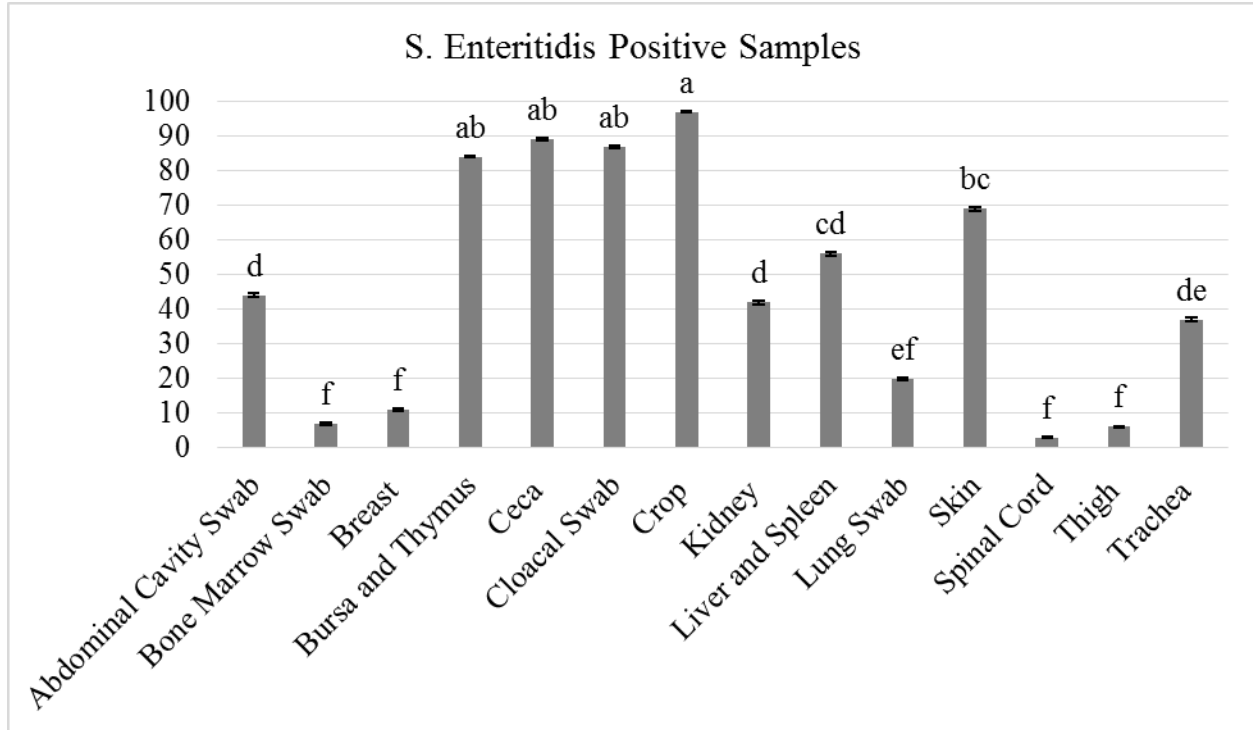


Figure 3.1 The percent of positive samples with *Salmonella* Enteritidis. The letters above each bar demonstrate the relationship of each sample's prevalence in comparison to the other samples collected by Tukey HSD ($P \leq 0.05$). Individual standard deviations were found and bars are utilized.

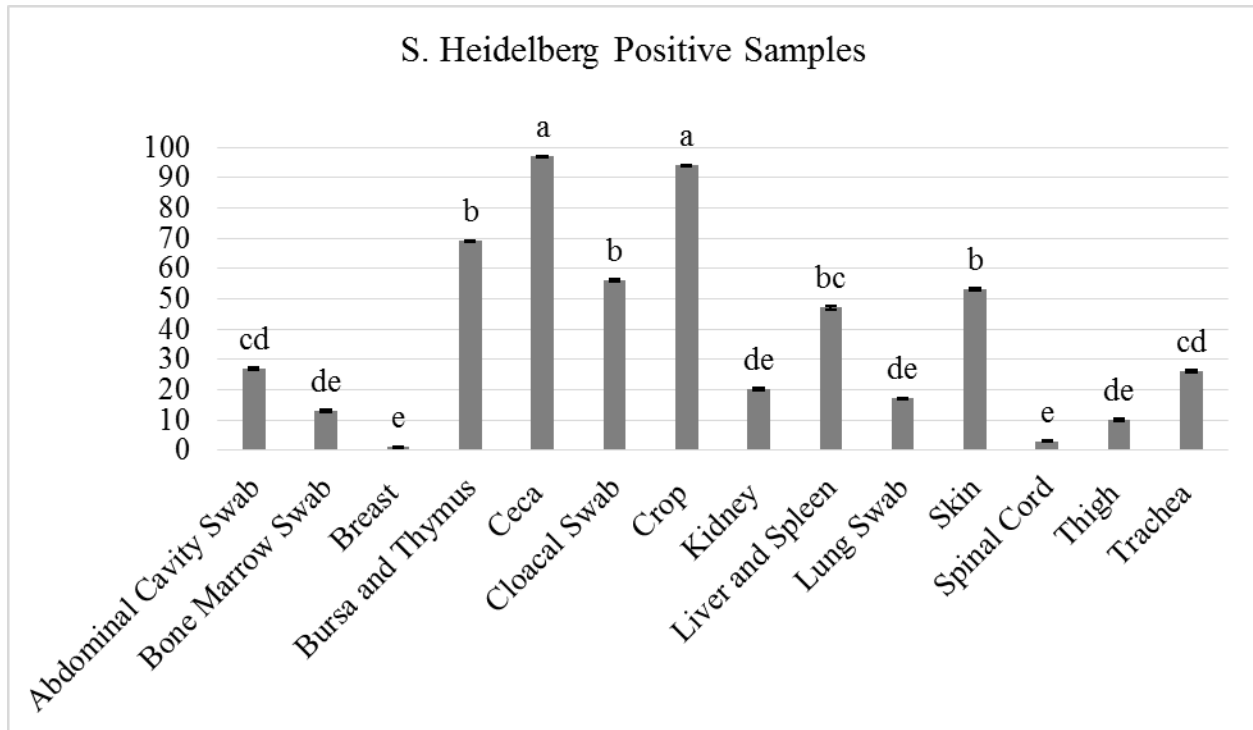


Figure 3.2 The percent of positive samples with *Salmonella* Heidelberg. The letters above each bar demonstrate the relationship of each sample's prevalence in comparison to the other samples collected by Tukey HSD ($P \leq 0.05$). Individual standard deviations were found and bars are utilized.

Chapter 4. A Comparison of Various Inoculation Routes and Colonization Sites of *Salmonella* Enteritidis when Administered to Broilers on Day of Hatch

Introduction:

Salmonella is known for causing over one million cases of foodborne illnesses each year. Specifically, *Salmonella enterica* serotype Enteritidis (SE) has been identified to cause human infections for numerous years with the number of infections increasing (Kimura *et al.*, 2004). It has been identified that chicken is one of the significant factors that caused SE infections in humans (Kimura *et al.*, 2004, Altekruise *et al.*, 2006). Although the numbers of cases of Salmonellosis have decreased over the past decade, the number of SE infections have increased, demonstrating the need for controlling this serotype within the poultry industry. Historically, SE has been an issue within the egg industry; however, SE has been recently identified to cause human infection by broilers (meat chickens) acting as carriers of this pathogen (Altekruise *et al.*, 2006).

Monitoring for *Salmonella* is currently done at poultry processing plants (Altekruise *et al.*, 2006, Berghaus *et al.*, 2013). Over a five-year span, SE recovery from poultry carcasses after exiting the chiller increased from 0.2% to 1.3%. This is of concern due to the timing of the sampling. At this point in the processing plant, chicken carcasses have been sanitized and are ready for further processing or packaging then distribution to the customer. It is also important to note that the distribution of SE within poultry processing plants were throughout the United States and not in one state (Altekruise *et al.*, 2006). Processing plant interventions are efficient in decreasing SE prevalence within the poultry products, but are not able to completely isolate these products from bacterial contamination (Berghaus *et al.*, 2013).

Control of SE infection in humans from commercially grown poultry must start at the farm level (Kimura *et al.*, 2004, Berghaus *et al.*, 2013; Corrier *et al.*, 1999). This can provide a

more thorough approach to controlling *Salmonella* in a vertically integrated industry. It is common to collect environmental samples from poultry houses then collect processing samples from poultry to examine for *Salmonella* prevalence (Berghaus *et al.*, 2013; Diezhang *et al.*, 2014). Although this gives a general idea of *Salmonella* prevalence within a flock, it does not target what organs and tissues are contaminated, later causing cross-contamination at the plants. Day-old chicks are more susceptible to SE colonization if exposed at a young age due to a lack of a developed immune system (Cox *et al.*, 1996; Kallapura *et al.*, 2014b) and environmental influences within poultry houses have been targeted as factors that contribute to SE colonization throughout growout. However, research lacks an understanding of inoculation routes that contribute to increased colonization within broilers (Corrier *et al.*, 1999). The objective of these studies was to target ways in which SE can colonize within broilers then identifying which organs and tissues are affected. Inoculations of day-old chicks with 10^4 colony forming units (CFU) SE included: oral, intratracheal, ocular, subcutaneous and cloacal.

Materials and Methods:

Bacterial cultures

Salmonella Enteritidis that is resistant to naladixic acid and novobiocin were utilized within these studies. These isolates were stored at -80°C until required. The frozen cultures were plated onto tryptic soy agar plates that contained 5% sheep's blood. These plates were incubated for 18-24 hours at 37°C. After that time, an isolated colony was selected and plated onto xylose lysine agar supplemented with tergitol 4 (XLT4) containing naladixic acid (100mcg/ml) and novobiocin (15 mcg/ml) and incubated for 48 hours at 37°C. A single unique black colony which is characteristic for *Salmonella* on this agar was removed and placed into 50 ml brain heart infusion broth (BHIB) to create the inoculations. The inoculated BHIB were put into a 37°C

shaker incubator for 18 hours at 250 rotations per minute. After which, the amount of incubated BHIB to add to the inoculum to give a final count of 10^4 CFU of *Salmonella*. Dilutions were performed to ensure the desired CFU.

Experimental animals

Each study consisted of seventy to one hundred straight run broiler chicks that were obtained on the day of hatch from a commercial hatchery and brought to the Auburn University Poultry Research Farm. Chicks were placed into floor pens with feed and water provided *ad libitum* (25 birds/ pen) and allowed to grow for 32 to 36 days. Standard husbandry and biosecurity practices were followed when rearing the birds. Environmental samples from the houses that would be rearing the birds as well as chick paper samples were collected and analyzed for SE. For all the studies, these samples were negative, indicating that there was no SE pre-exposure.

Method of inoculations

Oral: The chicks did not have access to food or water until after the inoculation was administered. For each chick, 500 μ L of inoculum was administered by inserting a 1 mL Tuberculin syringe directly into the crop. The chicks swallowed the inoculum prior to being placed onto litter.

Intratracheal: Sterile feed needles were utilized in administering SE to day old broiler chicks. Care was taken in administering SE through an intratracheal inoculation, where a worker held the chick and another administered 100 μ L of the inoculum into the trachea. The trachea was exposed by retracting the tongue and applying pressure under the lower beak to express the glottis. When the chicks took a breath and glottis was opened, the desired volume was administered.

Subcutaneous: 250 μ L of the inoculum was injected under the neck skin behind the head. Excess skin was pinched below the neck to administer the inoculum. Drawback was performed prior to injection to minimize an intravascular injection.

Ocular: A pipette was utilized to administer 100 μ L of the inoculum into the right eye of the chicks. The chicks could blink as the inoculum was given.

Cloacal: A pipette was used to administer the inoculum on the outside of the cloaca. The birds were held upside down and 100 μ L was placed onto the cloacal opening. The chick was held upside down until the cloacal drinking occurred and the inoculum was ingested through the cloacal lips.

Sample collection

On days 32 to 36, twenty to twenty-five birds per day were: euthanized with carbon dioxide, swabs were then taken of the cloaca for collection, then carcasses were sanitized by immersion in a quaternary ammonia solution by submersing the body of the birds from below the head. The neck feathers of the birds were then removed and the birds were dipped a second time in the quaternary ammonia bath without wetting the head. Both times the birds were dipped attention was made to ensure that none of the detergent went into the birds' mouth. After the birds dripped dry, the head and neck were removed from the rest of the carcass. The carcass was then exsanguinated as samples from the head and neck were removed. Samples were collected by placing the head onto a plastic cutting board that had been sanitized with 70% ethanol. Between each bird, the cutting boards were washed with water then sanitized with 70% ethanol. Approximately 5 cm² of skin from the neck area with feathers plucked was placed into a sterile bag (*Nasco*, Whirl-Pak). A thymus lobe, 5 cm of the trachea, and 3 cm of spinal cord were collected individually from the head sample and placed into individual sterile bags. Once

exsanguinated, the carcass was placed onto a surface and sprayed with 70% ethanol. An incision was made distal to the keel bone towards the head as well as towards the abdomen of the carcass and skin was retracted to expose the fat pad and breast tissue. A 3-4 cm³ section of the breast muscle and the whole crop were removed and placed into individual sterile bags. Birds were not taken off feed due to the multiple day necropsy so feed from inside of the crop was removed before the crop was placed in the sterile bag. A section of the liver (one third of a lobe) and the whole spleen were removed and placed in a sterile bag together. One of the ceca was removed, placed into a sterile bag, and cut in half to expose the cecal contents. The bursa was removed and placed into the same sterile bag as the thymus. One kidney was excised and placed in a sterile bag. One leg was detached at the coxofemoral joint to expose the femoral head. Skin was pulled away from the thigh to expose thigh meat and approximately 3-4 cm³ of thigh meat was removed and placed into a sterile bag. All sterile bags containing samples were filled with 20 ml of buffered peptone water (BPW) and homogenized using a stomacher for sixty seconds (Lab-Blender 80, Tekmar Company). The femoral head was truncated from the leg to expose bone marrow for collection. An abdominal swab of the interior of the body cavity was taken with a sterile swab. Then, a puncture of one lung was performed with another sterile swab. These samples were individually placed into sterile tubes containing 5 ml of BPW. These samples were then shaken. Strict aseptic techniques were enforced throughout the necropsies to ensure that minimal, if any, cross-contamination occurred.

Bacterial isolation

Due to the necropsies lasting over multiple days, BPW saturated samples were placed into a refrigerator at 4°C. On the last day of the necropsy, samples were moved into incubators set at 37°C and kept for 18-24 hours. Then, one ml of each BPW saturated samples was removed

and placed into 5 ml of tetrathionate (tt) broth. Once all samples were transferred, the tt tubes were shaken then moved into an incubator set at 37°C for 48 hours. A loopful of the incubated tt tube samples were streaked onto XLT4 plates containing naladixic acid (100mcg/ml) and novobiocin (15 mcg/ml). The plates were incubated at 37°C for 48 hours. After this period, plates were analyzed on a yes or no basis centered on the growth of characteristic black colonies typical of *Salmonella enterica* serotype on this medium.

Statistical Analysis

The SPSS version 22 software was used for analysis of the numerical and statistical differences between *Salmonella* positive organ and tissue samples collected from *S. Enteritidis*. The GLM Procedure was utilized and when appropriate, Tukey HSD. A comparative means test demonstrated differences, if any, between the serotypes. Differences were considered significant at $P \leq 0.05$.

Results

When day-old chicks were given a 10^4 inoculation of SE, the samples that had increased recovery for all inoculations included: ceca, skin from the neck area, crop, cloaca and the bursa and thymus (Table 4.1). All inoculation routes resulted in at least one positive sample for every different sample collected. There was no statistical difference found between inoculation routes for the samples collected from the: lung, bone marrow, breast, kidney, liver and spleen, skin from the neck area, spinal cord, and thigh (Table 4.1). The intratracheal challenge data presented in Table 4.2, shows that these samples have a SE prevalence of 17.6% followed by the oral challenge (12.3%), ocular challenge (11.2%), cloacal challenge (9.2%), and subcutaneous

challenge (5.2%). The analysis of each collected sample per inoculation route can be found in Figures 4.1-4.5. Within the oral inoculation (Figure 4.1), it was identified that the ceca had the greatest recovery numerically (34.3%) but the bursa and thymus (23.5%), crop (25.5%) and skin (26.5%) all had similar statistical recovery. With the intratracheal inoculation, the ceca had the greatest recovery numerically (49.0%) but similar recovery was identified with the bursa and thymus (36.3%) as well as the crop (32.4%; Figure 4.2). The subcutaneous inoculation (Figure 4.3) had the most recovery numerically from the skin (21.1%) followed by the crop (11.4%). All other samples were significantly less than what was identified with the skin. The ocular inoculation (Figure 4.4) had numerically greater recovery of SE within the crop (28.8%); however, statistical similarity can be found in the cloaca (22.5%), ceca (21.3%), bursa and thymus (17.3%), liver and spleen (12.3%) and skin (16.3%). Figure 4.5 shows the results from the cloacal inoculation, greatest recovery of SE numerically was collected from the bursa and thymus samples (18.8%). Similar statistical recovery was found with the abdominal cavity (12.5%), bone marrow (3.8%), ceca (3.8%), cloaca (13.8%), crop (16.3%), kidney (8.8%), liver and spleen (8.8%), lung (7.5%), skin (16.3%), thigh (5.0%) and trachea (8.8%). All other samples collected from this inoculation were identified to be significantly less than the bursa and thymus.

Discussion

Oral inoculation:

Previous experiments have suggested that the primary infection of SE in poultry is through the oral-fecal route (Cox *et al.*, 1996; Kallapura *et al.*, 2014b). When chicks were given an oral inoculation of SE, all organ and tissue samples collected contained at least one positive

sample for this serotype (Figure 4.1). The ceca had the highest statistical and numerical significance, followed by the skin from the neck area, the crop, and the bursa and thymus. Oral ingestion of SE allows for the movement of this serotype through the gastrointestinal tract to the ceca. The second most statistically and numerically significant sample that was positive for SE was skin collected from the neck area. This demonstrates that the fecal route allowed for excretion of this bacterium into the environment where the skin of the chicken became infected. It also shows that the fecal shedding infected the litter, where chickens peck at the ground and ingest the litter (Corrier *et al.*, 1999). This action allows for SE contact within the crop, the third most prevalent sample collected with this inoculation. SE throughout the digestive tract may have induced an immune response, which may explain the 24% recovery of this bacterium in the bursa and thymus. Resistance against *Salmonella* serotypes is done so by the bursa's production of B cells, which are part of the humoral immunity (Arnold and Holt, 1995). However, this serotype of *Salmonella* appears asymptomatic in poultry, meaning that the immune response to SE within birds is less severe than the immune response to this bacterium seen in humans (Beaumont *et al.*, 2010).

As observed in Figure 4.1, the trachea had a recovery of 11.8%, being statistically relative to all samples except the ceca. Kallapura and associates (2014a) identified that the recovery of *Salmonella* within the trachea of broilers on commercial farms was like the ceca. However, in this study with an oral inoculation given on day 0, these studies suggest that there is a significant difference between the recovery of SE in the trachea and ceca. This could be due to the intratracheal inoculation being given directly into the trachea while the oral inoculation was given in crop. This decreases the initial contact of *Salmonella* in the trachea through an oral inoculation in comparison to the intratracheal.

Recovery of SE from the kidney (10.8%) had significance relevant to the bursa and thymus (pooled), crop, trachea, abdominal cavity, bone marrow, breast, cloaca, liver and spleen (pooled) lung, spinal cord, thigh, and trachea. The kidney is not an ideal sampling organ due to its location in the broiler, making it difficult for the researcher to collect it. However, due to its location, it is of concern in mechanically deboned meat, where if the kidneys are not properly removed, they can be incorporated into further processed products (Froning and McKee, 2001).

The abdominal cavity (7.9%), bone marrow (3.0%), breast (2.0%), cloaca (8.0%), liver and spleen (4.9%), lung (3.0%), spinal cord (4.9%) and thigh (5.9%) had the least significant recovery in comparison to the other samples collected (Figure 4.1). However, it is important to note that of all samples collected, at least one of each sample was positive for SE, indicating the ability of this serotype to spread throughout a broiler if ingested orally.

Intratracheal inoculation:

Airborne transmission of SE has been neglected in poultry research. Modern poultry houses are densely stocked, enclosed buildings with movement of air by negative pressure to allow for ventilation. This dust laden air has the potential to carry pathogens, like SE, throughout a house and into broilers. Kallapura and associates (2014b) identified that the respiratory route is as viable as the oral route for allowing SE to colonize broilers; however, it was determined in these studies that SE had a greater colonization when administered through the intratracheal route (Table 4.2). When given an intratracheal inoculation, higher incidences of SE was observed within trachea samples as identified by Kallapura *et al.* (2014b) as well as in these studies (Intratracheal: 23.5%; Oral: 11.8%). Increased recovery of SE from the ceca as well as the liver and spleen was also identified in both studies in Table 4.1 (Intratracheal: 49.0%, 9.8%; Oral:

34.3%, 4.9%). It can be noted that when animals are exposed to SE through an intratracheal route, a portion of the inoculum will be swallowed as opposed to inhaled. This can be due to the anatomical connection between the two systems. Like what was identified by Cox *et al.* (1996), oral and intratracheal inoculations lead to more colonization within the ceca in comparison to the lungs (Table 4.1).

Table 4.2 demonstrates that there was an increased recovery of SE through the intratracheal inoculation route in comparison to all other inoculation routes considered in this study. There was statistically greater recovery within the ceca, like the oral inoculation, indicating that sampling this organ is ideal for determining SE prevalence within a flock, specifically if these inoculation routes are utilized. The bursa and thymus (pooled) and crop also had increased recovery in comparison to other samples collected, again like the oral inoculation (Table 4.1).

Subcutaneous inoculation:

The subcutaneous injection of SE had the lowest recovery and was statistically different in comparison to the other inoculation routes utilized within these studies, with only 5.2% of all samples collected having SE (Table 4.2). The subcutaneous injection was given to mimic cuts or scratches that commonly occur within poultry houses, specifically with older larger chickens in a commercial broiler house. The subcutaneous injection of SE did not administer the inoculum into the bloodstream due to subcutaneous tissue having few blood vessels. However, the subcutaneous injection did permit for a slow rate of absorption into the local area. Results from this route of inoculation are presented in Figure 4.3 and show that skin from the neck area (21.1%) had the highest recovery of SE followed closely by the crop (11.4%). All other collected

samples had similar recovery to the crop, but were recovered at less than 10% (Figure 4.3). All samples collected had at least one positive for every isolate (Table 4.2). This indicates that a cut or scratch can cause SE infection; however other routes of entry are of greater concern.

Ocular inoculation:

An ocular inoculation brings SE into the chicken's body via the nasolacrimal ducts. These ducts allow for the drainage of tears and other debris, like bacteria, from the eye into the respiratory or digestive tract (Williams, 2012). When analyzing the total number of positive samples per inoculation route, the ocular inoculation was statistically like the oral and cloacal inoculations (Table 4.2). To further demonstrate that the ocular inoculation allows for ingestion of the inoculation, the crop sample (28.4%) was the most statistically significant ($P \leq 0.05$) in comparison to other samples collected from this challenge (Figure 4.4). The cloacal swab (22.5%) and ceca (21.3%) were the next two most prevalent samples positive for SE after being challenged via ocular. These samples being the most common areas of isolation of SE demonstrate how an ocular inoculation has a similar movement of SE through the body as the fecal-oral route (Cox *et al.*, 1996; Kallapura *et al.*, 2014b).

The bursa and thymus (pooled) (17.3%), liver and spleen (pooled) (12.3%) and skin (16.3%) samples had recovery significant to all samples collected for this inoculation route. The skin recovery this demonstrates that ability of SE to colonize within the environment of poultry houses and continuously infect birds. Because of the increased incidence of recovery of SE from the ceca and cloacal swab, there is also the consideration of contaminated feces infecting the skin of the chicken during the rearing period. The trachea (11.3%) had similar recovery to all samples except the crop (Figure 4.4). When comparing all inoculations given, the ocular inoculation had

similar recovery to all others when collecting the trachea sample. This indicates a lack of diversity identified in the recovery of SE with an ocular inoculation.

As seen in Table 4.1 as well as Figure 4.4, the abdominal cavity (5.0%), lung (5.0%) and spinal cord (5.0%) had the same recovery for SE with the ocular inoculation. These samples were also like the bone marrow (2.5%), breast (2.5%), kidney (3.8%) and thigh (3.8%). All samples collected from the ocular inoculation contained at least one positive, indicating the ability of SE to spread through the bird if it enters in the eye.

Cloacal inoculation:

The cloacal inoculation mimics how young chicks can be susceptible to SE colonization due to the moist cloacum of the chick in combination with the placement onto contaminated litter (Cox *et al.*, 1996; Cressman *et al.*, 2010). This inoculation resulted in 9.2% of all samples collected having recovery of SE (Table 5.1). Numerically, recovery from all samples were similar. There was statistical variation identified between the bursa and thymus (pooled) and the breast and spinal cord samples. The results presented in Figure 4.5 show that the bursa is highly colonized after a cloacal inoculation. This is probably due to the anatomical position of the bursa in relation to the cloaca. Samples collected that had greater than 10% recovery include the: abdominal cavity (13%), cloacal swab (14%), crop (16%), neck skin (16%), and the bursa and thymus (19%). This indicates that if SE is absorbed through the cloaca, there is a likely chance of any organ or tissue collected from this study to have about a 10.0% recovery. This lack of variation indicates the potential of multiple sampling sites if this inoculation route is utilized.

SE given into the cloaca lacks the acidity found throughout the digestive tract; however, with fecal shedding as well as a chicken's natural habit of pecking at the litter in search for food,

there was some ingestion of this bacterium through the grow out period. The percentages of positive samples found from the cloacal challenge were statistically like the ocular and oral challenges (Table 4.2).

A comparison of inoculations:

Most the cellular and humoral defenses created by the broiler's immune system are done so within the bursa and thymus. These organs are in the neck and by the cloacal opening of the bird (Arnold and Holt, 1995). It was hypothesized prior to data analysis that the cloacal and subcutaneous inoculations would have increased SE recovery for the bursa and thymus in comparison to the other samples collected due to the location of the inoculations. However, as can be observed in table 5.1 the intratracheal inoculation resulted in the most statistically and numerically significant recovery of SE from these organ samples (36.3%) followed by the oral (23.0%), cloacal (19.0%), ocular (18.0%), and subcutaneous (7.0%). There was statistical difference ($P \leq 0.05$) found between the intratracheal, cloacal, ocular, and subcutaneous inoculation in SE recovery of this sample. This identifies that removal of these organs prior to processing would give varying levels of SE prevalence, making it un-ideal as the only sample to test for SE within a flock at the pre-harvest level.

The ceca have long been identified as the primary organ of *Salmonella* colonization within poultry (Corrier *et al.*, 1999; Hargis *et al.*, 1995; Ricke, 2003). Infection of young chicks with SE can lead to persistent shedding of this bacterium through fecal matter, which is fermented in the ceca then released through the cloaca. It was hypothesized that similar recovery of SE would be observed in the ceca and cloaca due to the ceca being known for such high colonization. The similarity between these two samples would provide a noninvasive method to

test for SE at the pre-harvest level by collecting cloacal swabs as opposed to euthanizing broilers to test for SE via ceca collection. Like what was identified by Van Immerseel and associates (2004), there is a lack of reliability of cloacal swabs to determine SE prevalence within broilers. From these studies (Table 4.1), the ocular inoculation had the greatest recovery of SE from the cloacal swab (22.5%) followed by the intratracheal (17.6%), cloacal (14.0%), oral (8.0%), and subcutaneous (3.0%). The ceca had statistically greater ($P \leq 0.05$) recovery with the oral and intratracheal inoculations and numerically greater recovery within the subcutaneous, ocular and cloacal inoculations in comparison to the cloacal swabs (Table 4.1). Variation of SE recovery from this sample indicates that cloacal swabs are not ideal as a detection method for SE in pre-harvested poultry. This brings direction of *Salmonella* sampling at the pre-harvest level towards the other organs that had greater recovery, like the crop, skin and the previously mentioned ceca.

The crop acts as a food storage site before movement to the gizzard and contains contents that allows for the growth of bacteria. The crop is the first host environment encountered by pathogens, like SE, after ingestion, which means that it can influence the survival of SE through the digestive tract. The crop composition can affect the gastrointestinal factors that are necessary to prevent or decrease SE from becoming systemic (Ricke, 2003). During processing, crops have been identified to be more likely to tear than other organs or tissues, therefore being a source of *Salmonella* cross-contamination (Corrier *et al.*, 1999; Hargis *et al.*, 1995). Hargis and associates (1995) identified that the crops cultured at a commercial processing plant had greater recovery of SE in comparison to the ceca that were collected. This was reiterated by Corrier *et al.* in 1999 and were like the ocular, subcutaneous, and cloacal inoculations with these studies. However, with the oral and intratracheal inoculation, the recovery of SE in the ceca was greater than the recovery of SE in the crop (Table 4.1). In comparing the routes of inoculation in these studies,

the only statistical difference ($P \leq 0.05$) of SE recovery in the crop was found between the intratracheal and the subcutaneous injection (Table 4.1). The general significance of the crop as a source of SE colonization at farms and contamination at the processing plants indicates that this organ could be utilized as a source of sampling for SE presence.

Table 4.1 also indicates that there were no statistical differences ($P \leq 0.05$) between the inoculations for SE recovery from the skin. The skin is the initial defense against environmental contaminants, including pathogens such as *Salmonella*. This allows for the nape to be consistently identified as contaminated with *Salmonella* (Hargis *et al.*, 1995). *Salmonella* species have been shown to attach to the skin of broilers prior to entering the processing plant and later cause contamination of poultry products. Fecal shedding during transport contaminates the feet, feathers, and skin of poultry with SE. Then, skin is in constant exposure to multiple machinery surfaces during poultry processing. Specifically, as the carcasses go through the chiller, the skin can act as a carrier of SE and lead to cross-contamination between carcasses. Multiple poultry products include the skin, such as the breast, thigh, drumstick, whole carcass, and mechanically deboned meat (Diezhang *et al.*, 2014; Froning and McKee, 2001). It has been suggested that neck skin is an ideal sampling spot to analyze *Salmonella* contamination after the chiller; it can also be inferred from these studies that skin collected from the neck area prior to processing will determine the *Salmonella* prevalence of poultry entering a processing facility (Cox *et al.*, 1977).

Variation of SE recovery lacked in the samples from: thigh, spinal cord, skin, liver and spleen (pooled), kidney, breast, lung and bone marrow when comparing all inoculation routes. This identifies these samples as not ideal in searching for SE when the inoculation route is (un)known due to the consistent lack of recovery through multiple inoculation routes. Because

SE was recovered from all inoculation routes and from every type of sample collected, SE can commonly be found within broilers if infected on day 0 at a 10^4 CFU.

Conclusion

Chicks at a younger age (less than a week) have been identified to be susceptible to SE infection and can lead to shedding and induction of carrier chickens (Kallapura *et al.*, 2014b; Van Immerseel *et al.*, 2004). The multiple inoculation routes that were administered allow for a comparison on how SE is entering broiler chickens at the pre-harvested level. This information will aid in targeting biosecurity and animal husbandry practices that can be improved to decrease SE exposure. The intratracheal route resulted in the most SE recovery, but it is important to note that all inoculations at 10^4 CFU resulted in recovery from multiple organ and tissue samples. Analyzing multiple organs and tissues for SE exposure through various inoculation routes will aid in determining areas of the bird that are likely to be contaminated when entering the processing plant. Organs and tissues of main concern include the: bursa and thymus, ceca, cloacal swab, crop and skin. These samples should be further analyzed as potential markers for SE contamination in pre-harvested poultry.

	Inoculations						
	Oral	Intra- Tracheal	Subcutaneous	Ocular	Cloacal	Overall (%)	
Samples	Abdominal Cavity Swab	8/101 (7.9%) ^{abD}	11/102 (10.8%) ^{abDE}	1/80 (1.3%) ^{bB}	4/80 (5.0%) ^{abCD}	10/80 (12.5%) ^{aAB}	7.5%
	Bursa and Thymus	24/102 (23.5%) ^{abABC}	37/102 (36.3%) ^{aAB}	5/79 (6.3%) ^{cB}	14/81 17.3% ^{bcABCD}	15/80 (18.8%) ^{bcA}	20.4%
	Bone Marrow Swab*	3/101 (3.0%) ^D	5/102 (4.9%) ^E	4/80 (5.0%) ^B	2/80 (2.5%) ^D	3/80 (3.8%) ^{AB}	3.8%
	Breast*	2/102 (2.0%) ^D	8/102 (7.8%) ^{DE}	2/80 (2.5%) ^B	2/80 (2.5%) ^D	2/80 (2.5%) ^B	3.5%
	Ceca	35/102 (34.3%) ^{abA}	50/102 (49.0%) ^{aA}	3/80 (3.8%) ^{cB}	17/80 (21.3%) ^{bABC}	3/80 (3.8%) ^{cAB}	22.4%
	Cloacal Swab	8/100 (8.0%) ^{bcD}	18/102 (17.6%) ^{abCDE}	2/80 (2.5%) ^{cB}	18/80 (22.5%) ^{aAB}	11/80 (13.8%) ^{abAB}	12.9%
	Crop	26/102 (25.5%) ^{abABC}	33/102 (32.4%) ^{aABC}	9/79 (11.4%) ^{bAB}	23/80 (28.8%) ^{abA}	13/80 (16.3%) ^{abAB}	22.9%
	Kidney*	11/102 (10.8%) ^{CD}	11/102 (10.8%) ^{DE}	1/80 (1.3%) ^B	3/80 (3.8%) ^D	7/80 (8.8%) ^{AB}	7.1%
	Liver and Spleen*	5/102 (4.9%) ^D	10/102 (9.8%) ^{DE}	4/79 (5.1%) ^B	10/81 (12.3%) ^{ABCD}	7/80 (8.8%) ^{AB}	8.2%
	Lung Swab*	3/100 (3.0%) ^D	4/102 (3.9%) ^E	2/80 (2.5%) ^B	4/80 (5.0%) ^{CD}	6/80 (7.5%) ^{AB}	4.4%
	Skin*	27/102 (26.5%) ^{AB}	32/102 (31.4%) ^{BC}	16/76 (21.1%) ^A	13/80 (16.3%) ^{ABCD}	13/80 (16.3%) ^{AB}	22.3%
	Spinal Cord*	5/102 (4.9%) ^D	1/102 (1.0%) ^E	4/80 (5.0%) ^B	4/80 (5.0%) ^{CD}	2/80 (2.5%) ^B	3.7%
	Thigh*	6/102 (5.9%) ^D	7/102 (6.9%) ^{DE}	1/80 (1.3%) ^B	3/80 (3.8%) ^D	4/80 (5.0%) ^{AB}	4.6%
	Trachea	12/102 (11.8%) ^{abBCD}	24/102 (23.5%) ^{aBCD}	4/80 (5.0%) ^{bB}	9/80 (11.3%) ^{abBCD}	7/80 (8.8%) ^{bAB}	12.0%
	Overall (%)	12.3% ^a	17.6%	5.2%	11.3% ^a	9.2% ^a	p≤0.05

Table 4.1 This table exhibits the comparison of all inoculation routes with every sample collected. Within each column and row, the number of positive samples for that inoculation, the total number of the specific sample collected for that inoculation and the percent of positive samples recovered for that inoculation are displayed. Lower-cased superscripts identify variation with a specific sample for inoculation routes. The asterisk next to samples indicate no variation identified for all inoculation routes. The upper-cased superscripts represent the statistical variation within an inoculation route for all samples collected. The percentages on the far and bottom portion of the table are the total for either each sample or each inoculation route. Analysis was conducted using the GLM Method and when appropriate, Tukey HSD ($P \leq 0.05$).

Treatment	N	Subset		
		1 ^c	2 ^b	3 ^a
Subcutaneous	1118	5.2% ^c		
Cloacal	1120		9.2% ^b	
Ocular	1120		11.2% ^b	
Oral	1422		12.3% ^b	
Intratracheal	1428			17.6% ^a

Table 4.2 Using the GLM Procedure and, when appropriate, Tukey HSD ($P \leq 0.05$), the comparison of all samples per inoculation route was observed. This table illustrates each treatment group, the total number of samples collected per treatment group (N) and the difference in statistical relevance indicated by each subset as well as differing letters next to the percentage of positive samples. Similar letters are considered statistically relevant to each other (i.e. oral, ocular and cloacal) and differing letters indicate statistical differences (i.e. intratracheal and subcutaneous).

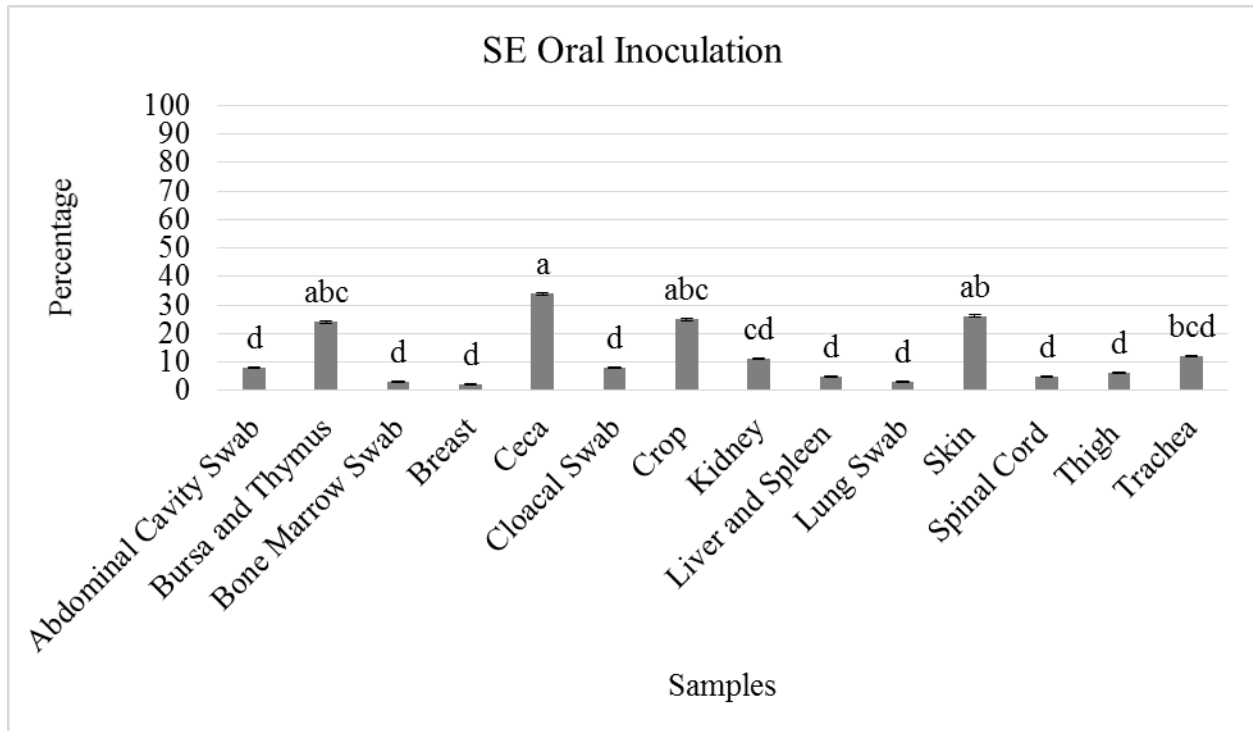


Figure 4.1 A comparison of the number of positive SE samples recovered through the oral inoculation route. The samples collected are along the x-axis. The y-axis represents the percentage. Letters above each bar graph indicate statistical differences identified using the GLM procedure and when appropriate, Tukey HSD ($P \leq 0.05$). Individual standard deviations were found and bars are used

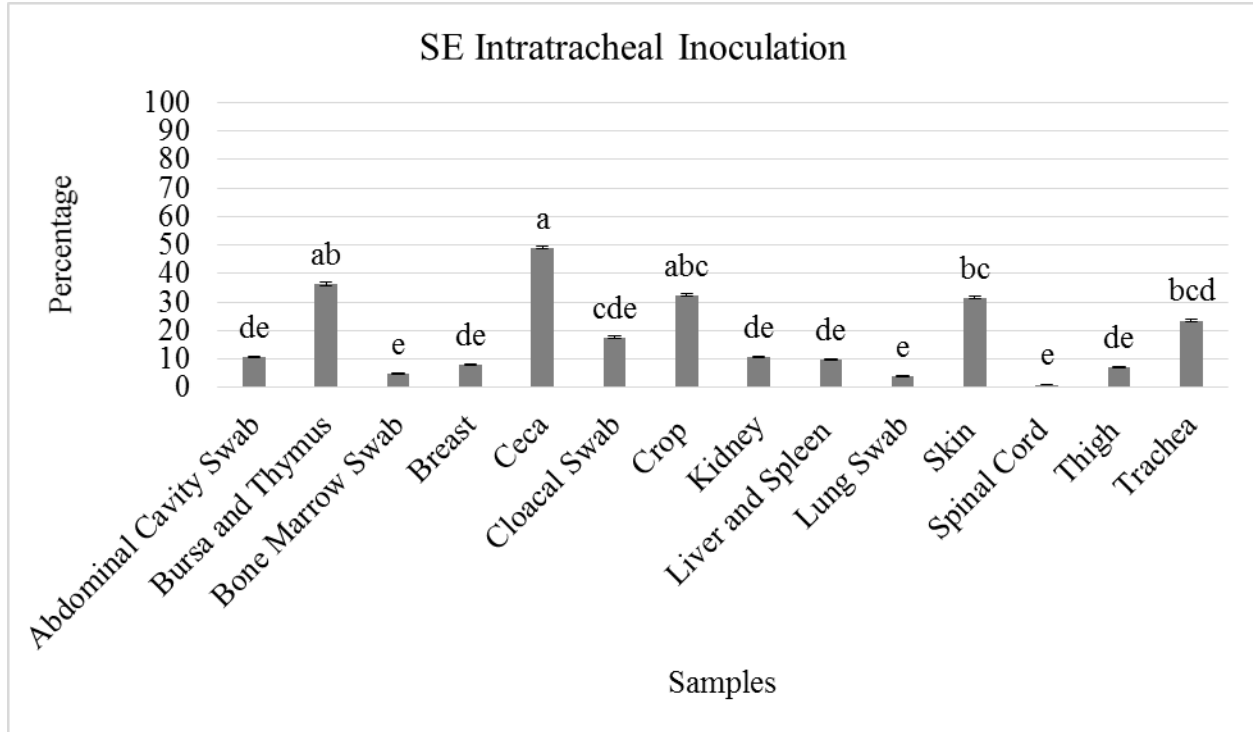


Figure 4.2 A comparison of the number of positive samples recovered through the intratracheal inoculation route. The samples collected are along the x-axis. The y-axis represents the percentage. Letters above each bar graph indicate statistical differences identified using the GLM procedure and when appropriate, Tukey HSD ($P \leq 0.05$). Individual standard deviations were found and bars are used.

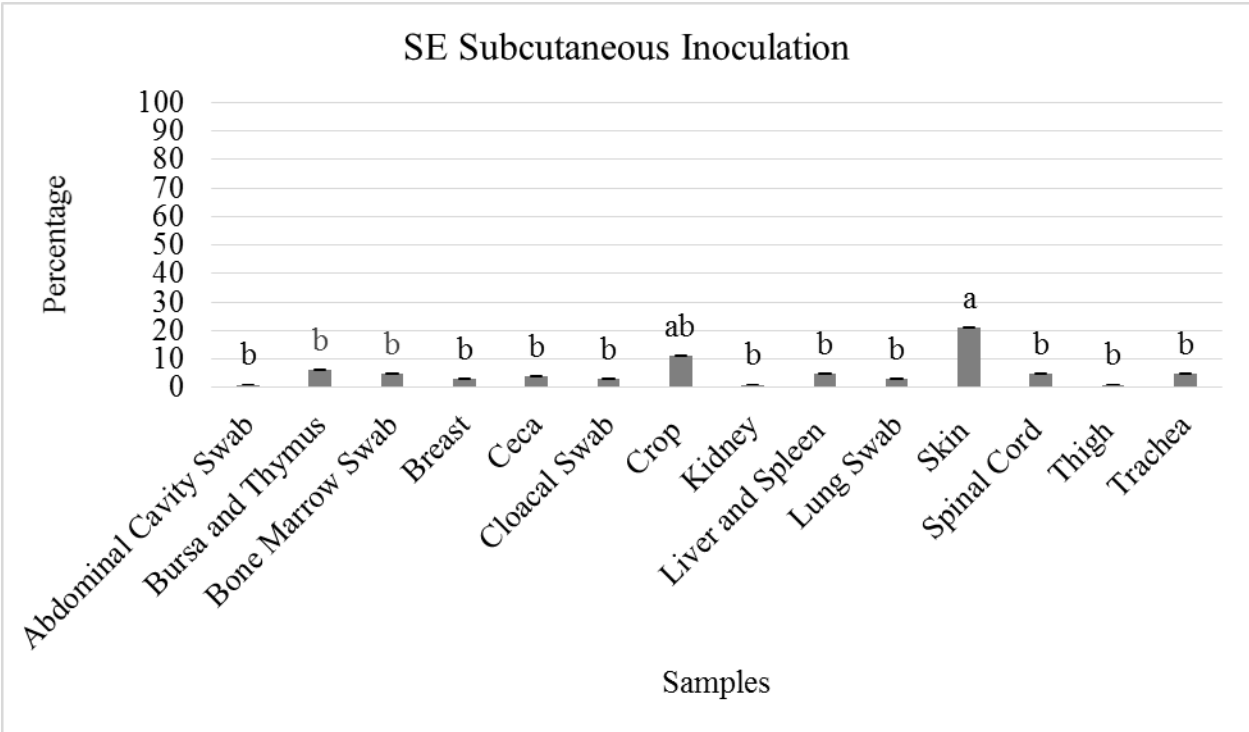


Figure 4.3 A comparison of the number of positive samples recovered through the subcutaneous inoculation route. The samples collected are along the x-axis. The y-axis represents the percentage. Letters above each bar graph indicate statistical differences identified using the GLM procedure and when appropriate, Tukey HSD ($P \leq 0.05$). Individual standard deviations were found and bars are used.

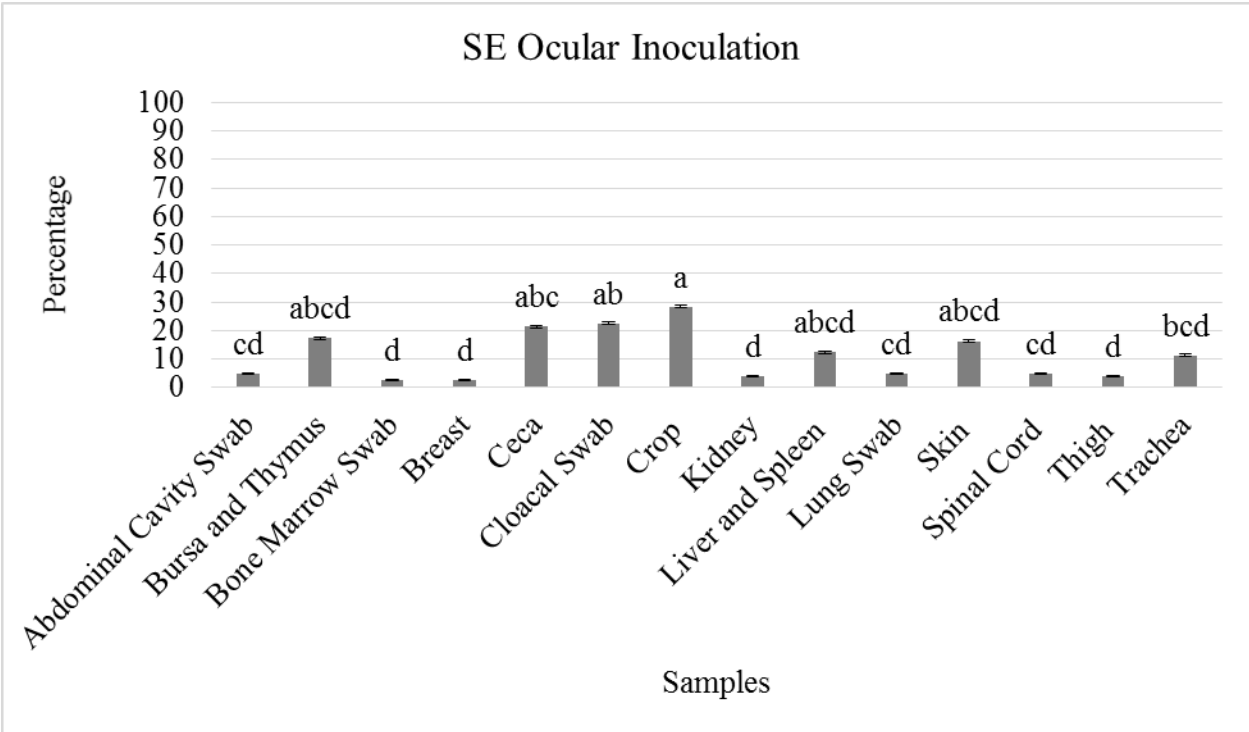


Figure 4.4 A comparison of the number of positive samples recovered through the ocular inoculation route. The samples collected are along the x-axis. The y-axis represents the percentage. Letters above each bar graph indicate statistical differences identified using the GLM procedure and when appropriate, Tukey HSD ($P \leq 0.05$). Individual standard deviations were found and bars are used.

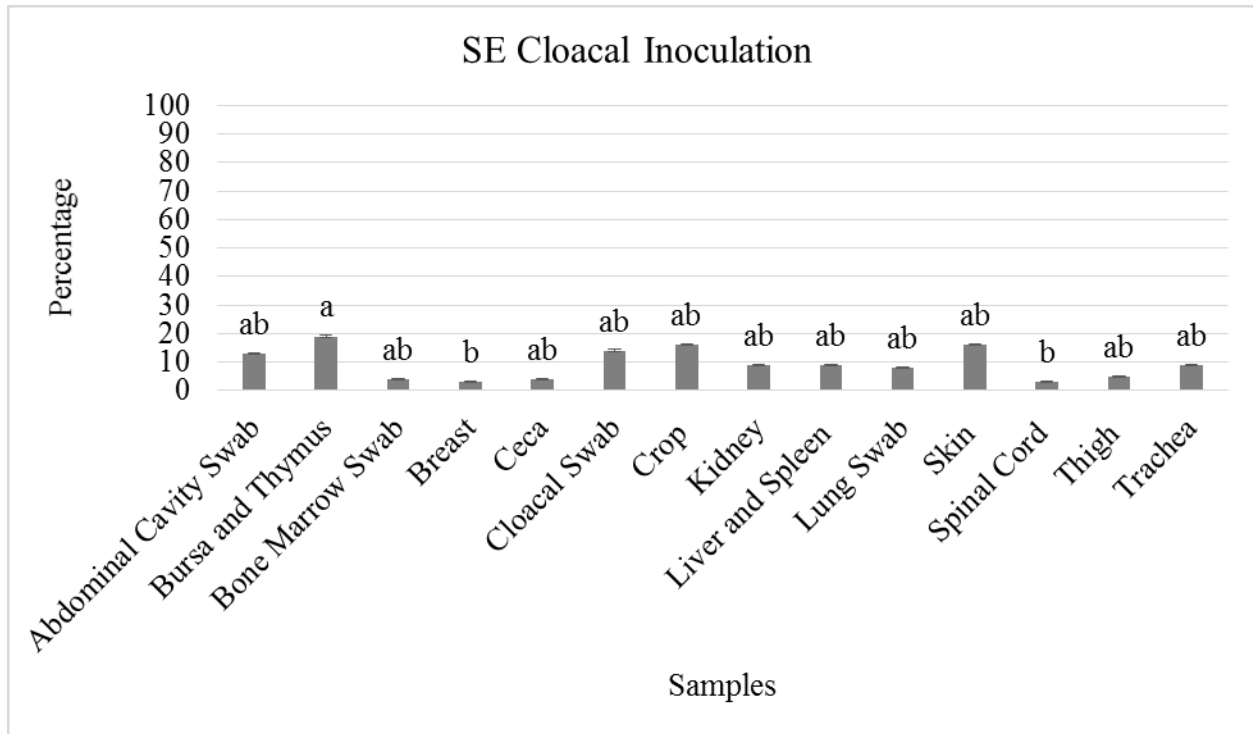


Figure 4.5 A comparison of the number of positive samples recovered through the intratracheal inoculation route. The samples collected are along the x-axis. The y-axis represents the percentage. Letters above each bar graph indicate statistical differences identified using the GLM procedure and when appropriate, Tukey HSD ($P \leq 0.05$). Individual standard deviations were found and bars are used.

Chapter 5. A Comparison of Various Inoculation Routes and Colonization Sites of *Salmonella* Heidelberg when Administered to Broilers on Day of Hatch

Introduction:

Over the past several decades, there has been variation in predominant *Salmonella* serotypes that have been associated with poultry. To date, *Salmonella enterica* serotype Heidelberg (SH) has become prominent in being detected with poultry as well as causing human illness. Fresh and processed poultry are of usual blame for *Salmonella* infections and SH is one of the most commonly identified serotypes detected in poultry (Alali and Hofacre, 2016; Chittick *et al.*, 2006). After the Hazard Analysis Critical Control Point (HACCP) Systems was put into place for the meat and poultry industry, SH was identified as a commonly isolated serotype within chicken and turkey carcasses as well as comminuted products (Schlosser *et al.*, 2000; Alali and Hofacre, 2016). There is concern with this serotype becoming multi-drug resistant, meaning that typical antibiotic treatment will not control this serotype from causing illnesses in humans (Rothrock *et al.*, 2015). This recognizes the need in controlling this serotype within poultry products.

SH has been identified to travel through the reproductive tract of birds and infect the eggs. Young birds are more susceptible to *Salmonella* colonization due to an immature immune response. This allows for both vertical and horizontal transfer of this pathogen; however, additionally this serotype appears asymptomatic in poultry (Chittick *et al.*, 2006; Foley *et al.*, 2011). This describes the need for controlling *Salmonella* in pre-harvested poultry, specifically in preventing this pathogen from colonizing young birds.

Salmonella is known to spread through the fecal-oral route, where it multiplies within the gastrointestinal tract of poultry and can be shed in the feces. It can also invade the cecal tonsils,

spread to the liver or spleen through the lymphatic system, and has the potential to become systemic. SH is also spread through fecal material, allowing for the cloacal opening to become contaminated when a bird defecates. Due to commercial chickens being reared on built-up litter in densely stocked houses, fecal contamination on the feathers and skin of neighboring chickens is inevitable (Foley *et al.*, 2011). Reducing *Salmonella* colonization in pre-harvested poultry is of high priority. Less contamination at the farms has the potential to lessen cross-contamination that occurs at poultry processing plants. The objective of these studies was to analyze SH colonization within poultry through various inoculation routes (oral, intratracheal, cloacal, subcutaneous, and ocular) if birds are infected on day 0.

Materials and Methods:

Bacterial cultures

Salmonella Heidelberg that is resistant to naladixic acid and novobiocin were utilized within these studies. These isolates were stored at -80°C until required. The frozen cultures were plated onto tryptic soy agar plates that contained 5% sheep's blood. These plates were incubated for 18-24 hours at 37°C. After that time, an isolated colony was selected and plated onto xylose lysine agar supplemented with tergitol 4 (XLT4) containing naladixic acid (100mcg/ml) and novobiocin (15 mcg/ml) and incubated for 48 hours at 37°C. A single unique black colony which is characteristic for *Salmonella* on this agar was removed and placed into 50 ml brain heart infusion broth (BHIB) to create the inoculations. The inoculated BHIB were put into a 37°C shaker incubator for 18 hours at 250 rotations per minute. After which, the amount of incubated BHIB was adjusted to give the final inoculum a count of 10^4 CFU of *Salmonella*. Dilutions were performed to ensure the target CFU was obtained.

Experimental animals

Each study consisted of ninety-nine to one hundred straight run broiler chicks that were obtained on the day of hatch from a commercial hatchery and brought to the Auburn University Poultry Research Farm. Chicks were randomly placed into floor pens with feed and water provided *ad libitum* (25 birds/ pen) and allowed to grow for 32 to 36 days. Standard husbandry and biosecurity practices were followed while rearing the birds. Environmental samples from the houses utilized as well as chick paper samples were collected and analyzed for SH. For all the studies, these samples were negative for SH. Prior to chick inoculations; two floor pens were dedicated as sentinel pens, where naive broilers chicks were placed. During feed changes, two broilers per sentinel pen (four total per feed change) were euthanized; ceca collected, and analyzed for SH colonization. One broiler for all studies and feed changes had a suspect *Salmonella* recovered; however the colony morphology was different than the characteristic colonies typically isolated with the SE and SH isolates utilized in these studies.

Method of inoculations

Oral: The chicks did not have access to food or water until after the inoculation was administered. For each chick, 500 μ L of inoculum was administered by inserting a 1 mL Tuberculin syringe directly into the crop. The chicks swallowed the inoculum prior to being placed onto litter.

Intratracheal: Sterile feed needles were utilized in administering SE to day old broiler chicks. Care was taken in administering SE through an intratracheal inoculation, where a worker held the chick and another administered 100 μ L of the inoculum into the trachea. Retracting the

tongue and applying pressure under the lower beak to express the glottis exposed the trachea.

When the chicks took a breath and glottis was opened, the desired volume was administered.

Subcutaneous: 250 μ L of the inoculum was injected under the neck skin behind the head. Excess skin was pinched below the neck to administer the inoculum. Drawback was performed prior to injection to minimize an intravascular injection.

Ocular: A pipette was utilized to administer 100 μ L of the inoculum into the right eye of the chicks. The chicks could blink as the inoculum was given.

Cloacal: A pipette was used to administer the inoculum on the outside of the cloaca. The birds were held upside down and 100 μ L was placed onto the cloacal opening. The chick was held upside down until the cloacal drinking occurred and the inoculum was ingested through the cloacal lips.

Sample collection

On days 32 to 36, twenty to twenty-five birds per day were: euthanized with carbon dioxide, swabs were then taken of the cloaca for collection, then carcasses were sanitized by immersion in a quaternary ammonia solution by submersing the body of the birds from below the head. The neck feathers of the birds were then removed and the birds were dipped a second time in the quaternary ammonia bath without wetting the head. Both times the birds were dipped attention was made to ensure that none of the detergent went into the birds' mouth. After the birds could drip dry, the head and neck were removed from the rest of the carcass. The carcass could exsanguinate as samples from the head and neck were removed. Samples were collected by placing the head onto a plastic cutting board that had been sanitized with 70% ethanol. Between each bird, the cutting boards were washed with water then sanitized with 70% ethanol. Approximately 5 cm² of skin from the neck area with feathers plucked was placed into a sterile

bag (*Nasco*, Whirl-Pak). A thymus lobe, 5 cm of the trachea, and 3 cm of spinal cord were collected individually from the head sample and placed into individual sterile bags. Once exsanguinated, the carcass was placed onto a surface and sprayed with 70% ethanol. An incision was made distal to the keel bone towards the head as well as towards the abdomen of the carcass and skin was retracted to expose the fat pad and breast tissue. A 3-4 cm³ section of the breast muscle and the whole crop were removed and placed into individual sterile bags. Birds were not taken off feed due to the multiple day necropsy so feed from inside of the crop was removed before the crop was placed in the sterile bag. A section of the liver (one third of a lobe) and the whole spleen were removed and placed in a sterile bag together. One of the ceca was removed, placed into a sterile bag, and cut in half to expose the cecal contents. The bursa was removed and placed into the same sterile bag as the thymus. One kidney was excised and placed in a sterile bag. One leg was detached at the coxofemoral joint to expose the femoral head. Skin was pulled away from the thigh to expose thigh meat and approximately 3-4 cm³ of thigh meat was removed and placed into a sterile bag. All sterile bags containing samples were filled with 20 ml of buffered peptone water (BPW) and homogenized using a stomacher for sixty seconds (Lab-Blender 80, Tekmar Company). The femoral head was truncated from the leg to expose bone marrow for collection. An abdominal swab of the interior of the body cavity was taken with a sterile swab. Then, a puncture of one lung was performed with another sterile swab. These samples were individually placed into sterile tubes containing 5 ml of BPW. These samples were then shaken. Strict aseptic techniques were enforced throughout the necropsies to ensure that minimal, if any, cross-contamination occurred.

Bacterial isolation

Due to the necropsies lasting over multiple days, BPW saturated samples were placed into a refrigerator at 4°C. On the last day of the necropsy, samples were moved into incubators set at 37°C and kept for 18-24 hours. Then, one ml of each BPW saturated samples was removed and placed into 5 ml of tetrathionate (tt) broth. Once all samples were transferred, the tt tubes were shaken then moved into an incubator set at 37°C for 48 hours. A loopful of the incubated tt tube samples were streaked onto XLT4 plates containing naladixic acid (100mcg/mL) and novobiocin (15 mcg/mL). The plates were incubated at 37°C for 48 hours. After this period, plates were analyzed on a yes or no basis centered on the growth of characteristic black colonies typical of *Salmonella enterica* serotype on this medium.

Statistical Analysis

The SPSS 22 software was used for analysis of the *Salmonella* positive organ and tissue samples collected. The GLM Procedure was utilized and when appropriate, Tukey HSD. A comparative means test demonstrated differences, if any, between the serotypes. Differences were considered significant at $P \leq 0.05$.

Results

When day-old chicks were given a 10^4 inoculation of SH, the sample that had significantly greater recovery compared to the other inoculations was the ceca (40.0%), (cloacal swab (27.0%), crop (27.0%) and skin (25.0%) when analyzing overall recovery for each sample.

There was similar recovery in all inoculations for the: bone marrow, breast, kidney, liver and spleen and lung (Table 5.1). The oral inoculation had significantly ($P \leq 0.05$) highest recovery within the ceca (Figure 5.1). The intratracheal inoculation (Figure 5.2) had significantly greatest recovery in the ceca (69.0%), skin (69.0%) and cloacal swab (68.0%). Figure 5.3 shows that the least amount of statistical variation was identified with a subcutaneous injection of SH, where the bursa and thymus (21.2%), skin (15.2%), ceca (11.1%) and crop (10.2%) had similar recovery. Recovery of SH through the ocular inoculation (Figure 5.4) was identified to be significantly greatest from the ceca sample (30.0%). The cloacal inoculation results can be observed in Figure 5.5, this Figure shows that the statistically highest recovery of SH was from the pooled bursa and thymus (16.0%). Similar recovery to the bursa and thymus was observed in the ceca (13.0%), crop (12.0%), trachea (11.0%) and cloacal swab (8.0%). When comparing inoculations (Tables 6.1 and 6.2), the intratracheal route (28.8%) had a significantly greater recovery of SH than oral (11.2%), subcutaneous (6.8%), ocular (8.1%) and cloacal (5.6%). There was no recovery of SH in the bone marrow of the subcutaneous inoculation or from the abdominal cavity, kidney and spinal cord of the cloacal inoculation.

Discussion

Oral inoculation:

It has been commonly identified that the movement of many *Salmonella* serotypes in poultry are through the fecal-oral route due to the broilers ingesting contaminated litter or fecal matter then shedding *Salmonella* into the environment (Kallapura *et al.*, 2014b). Great variation was identified within the recovery of SH through an oral inoculation (Figure 5.1; Table 5.1). The greatest recovery was identified within the ceca, where 50.0% of these samples were positive for

SH. Recovery of *Salmonella* within the ceca is common due to the functionality of this organ, where fermentation processes occur, increasing the general microbial load of this area of the body (Cox *et al.*, 1990; Corrier *et al.*, 1999).

Next, the crop (26.0%) had the second greatest recovery from the oral inoculation. This colonization site can be expected with an oral inoculation because the gavage with the inoculum is placed into the crop for ingestion. The crop also has a less acidic environment in comparison to the proventriculus and gizzard of the bird, both of which SH interacted with prior to colonizing within the ceca (Cox *et al.*, 1990). The crop also provides nutrient rich, moist environment due to the food storage that occurs within this organ. Similar recovery was identified with the cloacal swab (21.0%) and the bursa and thymus (16.0%). The abdominal cavity swab (2.0%), thigh meat (2.0%), spinal cord (2.0%), breast (3.0%), bone marrow swab (3.0%), liver and spleen (3.0%), kidney (4.0%), and lung swab (4.0%) samples all resulted with less than 5.0% SH recovery through the oral inoculation. The samples collected from the trachea (12.0%) and skin (8.0%) had similar recovery to the cloacal swab and bursa and thymus, but was also statistically related to the samples mentioned to have less than 5.0% recovery. All different areas of sampling from this inoculation resulted in recovery of SH. Recovery within the previously mentioned organs indicate the movement within the birds of SH through a fecal-oral route. The oral inoculation had similar overall SH recovery to the ocular inoculation, which will be discussed later (Table 5.2).

Intratracheal inoculation:

The intratracheal inoculation was to mimic poor air quality within a commercial poultry house, where dust and debris contaminated with *Salmonella* were prevalent. SH infected dust

and debris can be carried throughout a house and infect a flock due to the movement and disturbance of poultry litter during grow-out as well as the structural airflow common in commercial poultry houses (Brooks *et al.*, 2010; Kallapura *et al.*, 2014b). The administration for this inoculum directed SH directly into the trachea and into the lungs. This inoculation resulted in the greatest recovery of SH in comparison to all other inoculations in this study (Tables 5.1, 5.2). Table 5.1 shows that there was significantly greater recovery through the intratracheal inoculation in comparison to the other inoculations on the thigh (16.0 %), skin (69.0 %), crop (56.0 %), cloacal swab (68.0%) and trachea (44.4 %).

Table 6.1 and Figure 5.2 shows that the ceca (69.0%), skin from the neck area (69.0%) and cloacal swab (68.0%) had similar SH recovery and were also the most prevalent for this inoculation route. This indicates fecal shedding of this serotype into the environment, where it interacted with the skin. It has long been identified that shedding of *Salmonella* from the ceca could contaminate feathers and skin of other members of the flock. In the past few decades, crops have been identified as a source of contamination as well, indicating the ingestion of *Salmonella* (Hargis *et al.*, 2001).

The crop (56.0%) had similar recovery to the previously mentioned samples as well as the trachea (44.4%; Table 5.1, Figure 5.2). It has been suggested that tracheal sampling could be a viable method of analyzing *Salmonella* contamination pre-harvest, specifically if the inoculation is through aerosols (Kallapura *et al.*, 2014a). Although other samples within this inoculation demonstrated greater recovery, the trachea samples had greatest colonization within this inoculation in comparison to the four other routes of interest (Table 5.1), indicating the potential to utilize the trachea as a sampling method if intratracheal aerosol spreading of *Salmonella* is suspected. The similarity of recovery between the crop and trachea indicate the

connection between the respiratory and digestive tracts. The bursa and thymus (34.0%) had similar recovery to the trachea but was not like the crop (Figure 5.2). It is important to note that SH was administered into the trachea, increasing the potential of colonization and causing a reaction within immune organs, like the bursa and thymus.

Figure 5.2 and Table 5.1 also show that thigh meat (16.0%), pooled liver and spleen (12.0%), abdominal cavity swab (9.0%), spinal cord (8.0%), breast meat (8.0%), lung swab (5.0%), kidney (4.0%) and the bone marrow swab (1.0%) had similar recovery to SH, all statistically less than the other samples previously mentioned. Statistical variation between inoculation routes (Table 5.1) per sample had greatest recovery by the intratracheal route in the: thigh meat (16.0%), skin (69.0%), crop (56.0%), ceca (69.0%), cloacal swab (68.0%) and trachea (44.4%). This inoculation demonstrates the need for further analysis of dust and debris as carriers of *Salmonella* within a poultry house due to the recovery identified within this study.

Subcutaneous inoculation:

A subcutaneous inoculation was administered to mimic a cut or scratch on a chick prior to or during placement. The opening of a wound would allow for entryway of bacterial species, like SH, into the broiler and potentially colonize the bird. The injection was given in the back of the neck, close to the thymus glands.

Numerically, the greatest recovery from the subcutaneous inoculation (Figure 5.3) was identified within the bursa and thymus (21.2%) samples followed by the skin from the neck area (15.2%). This is expected due to the area of inoculation as well as the function of the bursa and thymus.

Similar recovery to the previously mentioned inoculation routes as well as all other samples collected includes the ceca (11.1%) and crop (10.2%). If colonization has occurred within the ceca, there is a strong probability that fecal shedding of SH is occurring. Although less recovery was identified, 8.1% of the cloacal swabs were positive for SH. This allowed for cloacal swabs to be statistically like both the ceca and crop. This also indicates fecal shedding of SH has occurred through this inoculation. Shedding of SH will allow for contamination on the litter used to rear the flock (Sadler *et al.*, 1969). This would introduce SH to the skin and feathers of the bird but also, due to the pecking habits of chickens, ingestion of the contaminated litter. Here, SH has the potential to colonize within the crop.

The liver and spleen (8.1%), trachea (4.0%), kidney (4.0%) and abdominal cavity (4.0%) were statistically similar in recovery of SH. Although recovery from these areas were numerically low, concern should be addressed due to their interactions with the broiler carcass during first processing. If contaminated and torn, these portions of the bird could result in contamination of the rest of the carcass as well as contamination of other carcasses (Sams, 2001).

The spinal cord (3.0%), thigh meat (3.0%), lung swab (2.0%) and breast meat (1.0%) had less than 3.0% recovery and no recovery of SH was identified in the bone marrow through this inoculation route. When comparing all inoculations, the subcutaneous injection had similar recovery to the cloacal and ocular inoculations (Table 5.2). Greater recovery from the oral and intratracheal inoculations in comparison to the subcutaneous inoculation indicates less concern for a subcutaneous infection during grow-out and more focus towards oral and intratracheal inoculations.

Ocular inoculation:

Like the oral inoculation (Figure 5.1), the greatest recovery for ocular inoculation (Figure 5.4) was identified within the ceca (30.0%) followed by the crop (17%). The eye contains a drainage system known as nasolacrimal ducts. These ducts allow for the removal of dust or debris from the eye into the digestive tract (Williams, 2012). This also allowed for the movement of SH from the eye into the rest of the body, where it passed through the small intestines and colonized the ceca. Similar recovery of SH can be expected between the ocular and oral inoculations due to the connection of the digestive tracts.

Samples with similar recovery to the crop included: bursa and thymus (13.0%), skin (9.0%), cloacal swab (9.0%), trachea (7.1%), liver and spleen (7.0%) and bone marrow (5.1%). The recovery from the bursa and thymus, liver and spleen and bone marrow imply an immune response was occurring within the broiler. The bursa and thymus are lymphatic organs located by the cloacal opening and the neck of the bird, respectively. They create B and T cells which, during an immune response like what is suggested with SH colonization, B and T cells will populate secondary lymphatic organs, specifically the spleen and bone marrow (Yegani and Korver, 2008).

The cloacal swab, skin and trachea SH recovery suggests the shedding of SH from infected birds into the environment. When sampling for *Salmonella* in the field, it is common to collect cloacal swabs due to the shedding of this bacteria from the ceca into the feces (Mueller-Doblies *et al.*, 2009). Broilers are raised on shared litter, meaning that the fecal material shed from one bird will encounter the others' feathers and skin. The negative pressure in commercial poultry houses that allow for the movement of air can also carry *Salmonella* ridden dust and debris throughout a house, infecting the air poultry breathe and later infecting the bird. The ridged structure of the trachea allows for movement of contaminated air into the bird's body

(Kallapura *et al.*, 2014b). The similarity of recovery for these samples indicates the fecal-oral movement of SH in this inoculation.

All other samples from the ocular inoculation resulted in less than 5.0% recovery. These included: abdominal cavity (4.0%), kidney (4.0%), breast meat (3.0%), thigh meat (3.0%), lung swab (1.0%) and spinal cord (1.0%). All samples collected had a least one SH recovery for this inoculation. The ocular inoculation had a similar recovery pattern to every inoculation except the intratracheal inoculation (Table 5.2).

Cloacal inoculation:

Young chicks are susceptible to *Salmonella* as well as other adhering bacterial species when they are placed onto litter after hatch. This is due to the damp pericloacum allowing for the entryway of bacteria into the chick's body. The cloaca is an ideal entryway for *Salmonella* because the pathogen does not have the natural defense of the acidic environment of the digestive system (Cox *et al.*, 1990). This allows for interaction with the bursa, an immune organ located near the cloacal opening.

With SH, the greatest statistical ($P \leq 0.05$) recovery for the cloacal inoculation (Table 5.1, Figure 5.5) was identified in the bursa and thymus samples (16.0%). Invasive SH was detected and interacted with the lymphatic tissue. It is apparent that the interaction occurred based off the recovery of this serotype from the bursa and thymus. Statistically similar recovery was identified in the ceca, cloaca, crop and trachea. This indicates that direct contact with the cloaca and bursa allowed for colonization, but a greater concern is the movement of SH through the environment and into the trachea and digestive tract.

Next, the ceca (13.0%) was identified as having the second highest recovery. For colonization, *Salmonella* must reach the cecum where it must outcompete both host and invasive microflora for nutrients (Berndt *et al.*, 2007). If *Salmonella* does not have to interact with the defense mechanisms of the digestive tract, fewer cells are injured in the process of reaching the ceca (Kallapura *et al.*, 2014b). However, when comparing inoculations, the recovery through the cloaca was statistically like the subcutaneous and ocular inoculations (Table 5.2). Overall, even without the acidic environment of the digestive tract, there was the least amount of recovery through the cloacal inoculation route. This indicates a decrease concern of contaminated litter being absorbed into the cloaca and allowing for SH colonization. However, it is important to note the previously mentioned movement of SH through the digestive tract, indicating that even if the cloaca does not allow absorption, movement into the digestive tract will still occur if the environment is contaminated.

Pecking is common with poultry, where birds will ingest food, litter, fecal material and feather debris (Aarnink *et al.*, 1999; Ritz *et al.*, 2005). If SH contamination has occurred on any of the previously mentioned sources, oral ingestion can occur. The crop, a food storage site in poultry, was the next common area identified to be contaminated with SH (12.0%). The crop contains ingested feed, water and other nutrients that are ideal for SH colonization (McLelland, 1990; Ricke, 2003). SH within the crop is a concern for processing due to the location of the crop by the breast meat. If improper feed withdrawal occurs and the crop is contaminated with *Salmonella*, there is concern that breast meat as well as other parts can become contaminated if the crop is ruptured during processing. This contamination of breast meat would be due to the infected crop spreading *Salmonella* when ruptured. After the crop, the trachea was identified as an area of colonization within the broilers (11.0%) challenged through the cloaca. Like what was

identified through the intratracheal inoculation, the statistically similar recovery between the crop and trachea indicate the previously mentioned connection between the respiratory and digestive tracts. It can be hypothesized that a cloacal inoculation allowed for contamination of the litter as well as the environment, bringing contaminated dust and debris into the trachea and crop of these birds.

Shedding of *Salmonella* through fecal material is common in poultry. This can allow movement of this pathogen into the litter, where flock mates have consistent contact with fecal droppings. The cloacal swabs resulted in 8.0% recovery, identifying that there is a strong chance that SH can be found within the excreta of the inoculated birds. Cloacal swabs are utilized in the poultry industry to identify *Salmonella* colonization. Mueller-Doblies *et al.* (2009) identified that sampling from cloacal swabs are insensitive to *Salmonella* recovery, potentially due to the small amount of fecal material that is collected. This study correlates with their identification, where only 8.0% recovery was found in birds through a cloacal swab, even though the inoculation was given at the same location.

All other samples resulted in 5.0% or less recovery (skin (5.0%), liver and spleen (4.0%), breast meat (3.0%), thigh meat (3.0%), bone marrow (2.0%), lung (1.0%), spinal cord (0.0%), kidney (0.0%) and abdominal cavity (0.0%)). The spinal cord, kidney and abdominal cavity swabs did not result in any recovery of SH through this inoculation. In comparing the cloacal inoculation with the others, similar recovery was identified with the subcutaneous and ocular inoculations. Overall, the cloacal inoculation had the lowest recovery of SH in comparison to the other inoculations given in this study (Table 5.2).

A comparison of inoculations

The intratracheal inoculation resulted in a significantly greater recovery overall, as well as with the thigh, skin, crop, cloacal swab and trachea samples (Tables 5.1 and 5.2). Air quality in relation to commercial poultry housing systems has been a concern for poultry health; however dust can contain a large number of microorganisms, like *Salmonella*, which can become a food safety issue at the pre-harvest level, if contaminated dust is inhaled. These studies indicate a need for controlling dust within a poultry house to improve animal health, worker health and pre-harvest food safety (Ritz *et al.*, 2006). One of the most important sources of dust within a poultry house is from the animals and their fecal material. Similar to the fecal-oral route of *Salmonella* previously mentioned, the fecal- intratracheal route through dust contaminates must also be considered (Ellen *et al.*, 2000). The similarity of recovery between the ceca, cloaca, crop, bursa and thymus, and trachea for most inoculation routes indicates the need for a better understanding of SH within the environment. The intratracheal inoculation must be further analyzed within a commercial setting to determine if this route of infection is of concern outside of an experimental setup as well as if it can be potentially controlled.

When comparing all inoculation routes, the ceca sample resulted in the greatest recovery in juxtaposition to the other thirteen samples collected, indicating the use of this organ when sampling for SH contamination at the farm. When the ceca become colonized with *Salmonella*, excretion through the feces will occur, continually allowing for interaction of this pathogen with the broilers during grow out (Borsoi *et al.*, 2010). Fanelli and associates (1970) inoculated birds with *Salmonella* then collected twelve samples from throughout the digestive tract. They were able to identify and this study reiterated that the ceca was the ideal sampling area when wanting to identify *Salmonella* colonization. Utilizing multiple inoculation routes and determining the ceca to have the highest recovery can aid the industry in the creation of a standardized sampling

method for *Salmonella*; however, other serotypes of concern must be examined in a similar manner to this study. Understanding of both entry ways and colonization sites allow for insight in controlling this as well as other unstudied serotypes at the pre-harvest level.

Conclusion:

It is a necessity within the United States poultry industry to analyze the integrated broiler production system to better understand the transmission of *Salmonella* as well as incorporate improved management and intervention strategies (Alali and Hofacre, 2016). The intratracheal, oral and ocular inoculations resulted in recovery of SH from each type of sample collected as opposed to the subcutaneous and cloacal inoculation where no recovery of SH was identified in certain samples.

	Inoculations					
	Oral	Intratracheal	Subcutaneous	Ocular	Cloacal	Overall (%)
Abdominal Cavity Swab	2/100(2.0%) abE	9/100(9.0%) ^{aD}	4/99 (4.0%) ^{abBC}	4/99 (4.0%) ^{abC}	0/100 (0.0%) ^{bE}	5.0%
Bursa and Thymus	16/100 (16.0%) ^{bBCD}	34/100 (34.0%) ^{aC}	21/99 (21.2%) ^{abA}	13/100 (13.0%) ^{bBC}	16/100 (16%) ^{bA}	21.0%
Bone Marrow Swab*	3/100 (3.0%) ^{DE}	1/100(1.0%) ^D	0/99(0.0%) ^C	5/99 (5.1%) ^{BC}	2/100 (2.0%) ^{CDE}	2.2%
Breast*	3/100 (3.0%) ^{DE}	8/100(8.0%) ^D	1/99(1.0%) ^C	3/100 (3.0%) ^C	3/100 (3.0%) ^{BCDE}	54.0%
Ceca	50/100 (50.0%) ^{bA}	69/100 (69.0%) ^{aA}	11/99 (11.1%) ^{dABC}	30/100 (30.0%) ^{cA}	13/100 (13.0%) ^{cdAB}	40.0%
Cloacal Swab	21/100 (21.0%) ^{bBC}	68/100 (68.0%) ^{aA}	8/99(8.1%) ^{bBC}	9/100 (9.0%) ^{bBC}	8/100 (8.0%) ^{bABCDE}	27.0%
Crop	26/100 (26.0%) ^{bB}	56/100 (56.0%) ^{aAB}	10/99 (10.2%) ^{cABC}	17/100 (17.0%) ^{bcB}	12/100 (12.0%) ^{bcABC}	27.0%
Kidney*	4/100 (4.0%) ^{DE}	4/100(4.0%) ^D	4/99(4.0%) ^{BC}	4/100 (4.0%) ^C	0/100 (0.0%) ^E	4.0%
Liver and Spleen*	3/99 (3.0%) ^{DE}	12/100 (12.0%) ^D	8/99(8.1%) ^{BC}	7/100 (7.0%) ^{BC}	4/100 (4.0%) ^{BCDE}	8.0%
Lung Swab*	4/100 (4.0%) ^{DE}	5/100(5.0%) ^D	2/99(2.0%) ^C	1/99 (1.0%) ^C	1/100 (1.0%) ^{DE}	3.0%
Skin (Neck)	8/100 (8.0%) ^{bCDE}	69/100 (69.0%) ^{aA}	15/99 (15.2%) ^{bAB}	9/100 (9.0%) ^{BC}	5/100 (5.0%) ^{BCDE}	25.0%
Spinal Cord	2/100 (2.0%) ^{bE}	8/99(8.0%) ^{aD}	3/99(3.0%) ^{abC}	1/99 (1.0%) ^{bC}	0/100 (0.0%) ^{bE}	4.0%
Thigh	2/100 (2.0%) ^{bE}	16/100 (16.0%) ^{aD}	3/99(3.0%) ^{bC}	3/100 (3.0%) ^{bC}	3/100 (3.0%) ^{bBCDE}	6.0%
Trachea	12/100 (12.0%) ^{bCDE}	44/99 (44.4%) ^{aBC}	4/99(4.0%) ^{bBC}	7/99 (7.1%) ^{bBC}	11/100 (11.0%) ^{bABCD}	17.0%
Overall (%)	11.2%	28.8% ^u	6.8%	8.1%	5.6%	p≤0.05

Samples

Table 5.1 This table exhibits the comparison of all inoculation routes with every sample collected. Within each column and row, the number of positive samples for that inoculation, the total number of the specific sample collected for that inoculation and the percent of positive samples recovered for that inoculation are displayed. Lower-cased superscripts identify variation with a specific sample for inoculation routes. The asterisk next to samples indicate no variation identified for all inoculation routes. The upper-cased superscripts represent the statistical variation within an inoculation route for all samples collected. The alpha (α) indicates the inoculation route with a significantly greater recovery when comparing all inoculations. The percentages on the far and bottom portion of the table are the total for either each sample or each inoculation route. Analysis was conducted using the GLM procedure ($P \leq 0.05$).

Treatment	N	Subset		
		1 ^c	2 ^b	3 ^a
Cloacal	1400	5.6% ^c		
Subcutaneous	1385	6.8% ^c		
Ocular	1396	8.1% ^{bc}	8.1% ^{bc}	
Oral	1399		11.2% ^b	
Intratracheal	1398			28.8% ^a

Table 5.2 Using the GLM procedure and when appropriate, Tukey HSD ($P \leq 0.05$), the comparison of all samples per inoculation route was observed. This table illustrates each treatment group, the total number of samples collected per treatment group (N) and the difference in statistical relevance indicated by each subset as well as differing letters next to the percentage of positive samples. Similar letters are considered statistically relevant to each other (i.e. cloacal, subcutaneous and ocular) and differing letters indicate statistical differences (i.e. intratracheal and cloacal).

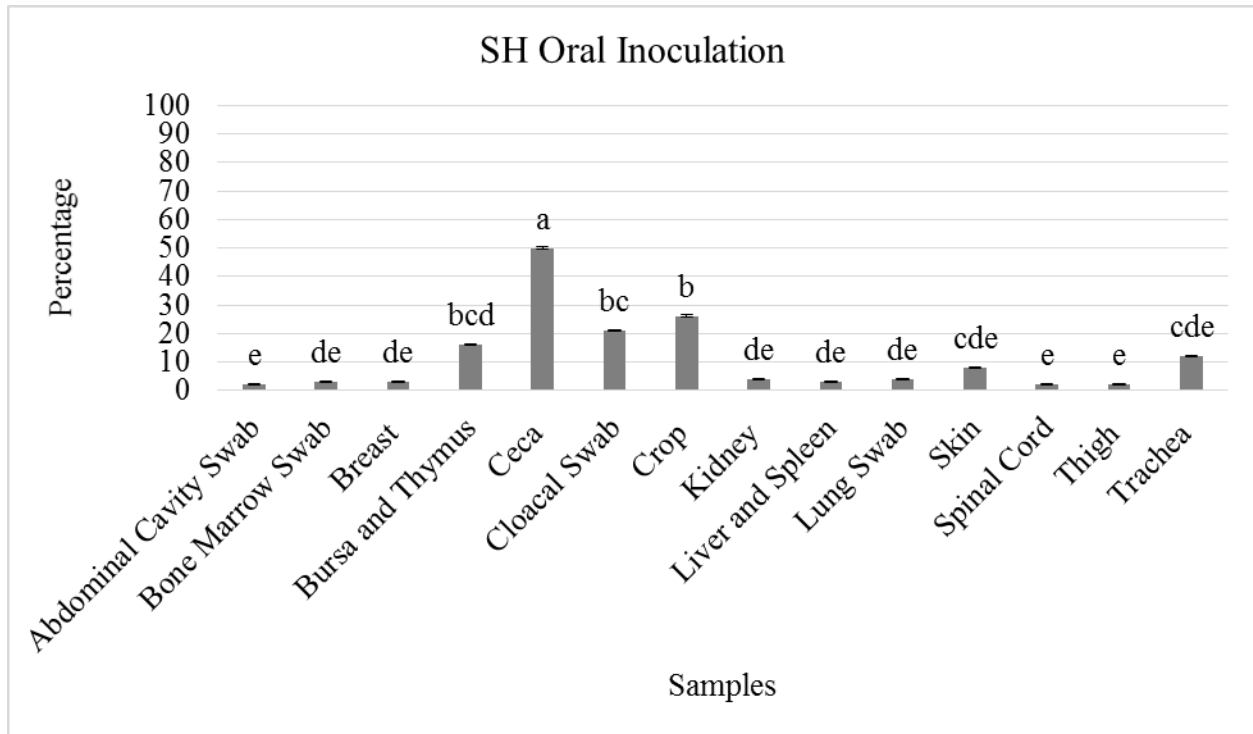


Figure 5.1 A comparison of the number of positive SH samples recovered through the oral inoculation route. The samples collected are along the x-axis. The y-axis represents the percentage. Letters above each bar graph indicate statistical differences identified using the GLM procedure and when appropriate, Tukey HSD ($P \leq 0.05$). Individual standard deviations were found and corresponding bars are used.

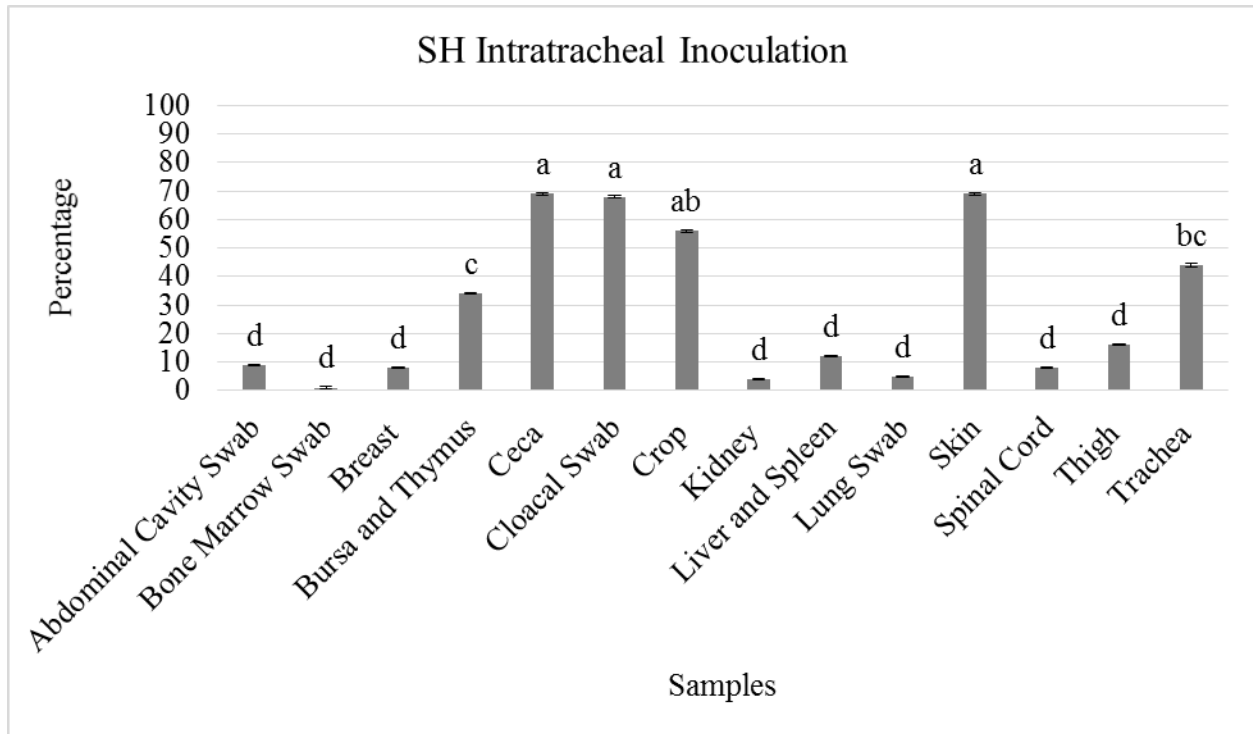


Figure 5.2 A comparison of the number of positive SH samples recovered through the intratracheal inoculation route. The samples collected are along the x-axis. The y-axis represents the percentage. Letters above each bar graph indicate statistical differences identified using the GLM procedure and when appropriate, Tukey HSD ($P \leq 0.05$). Individual standard deviations were found and corresponding bars are used.

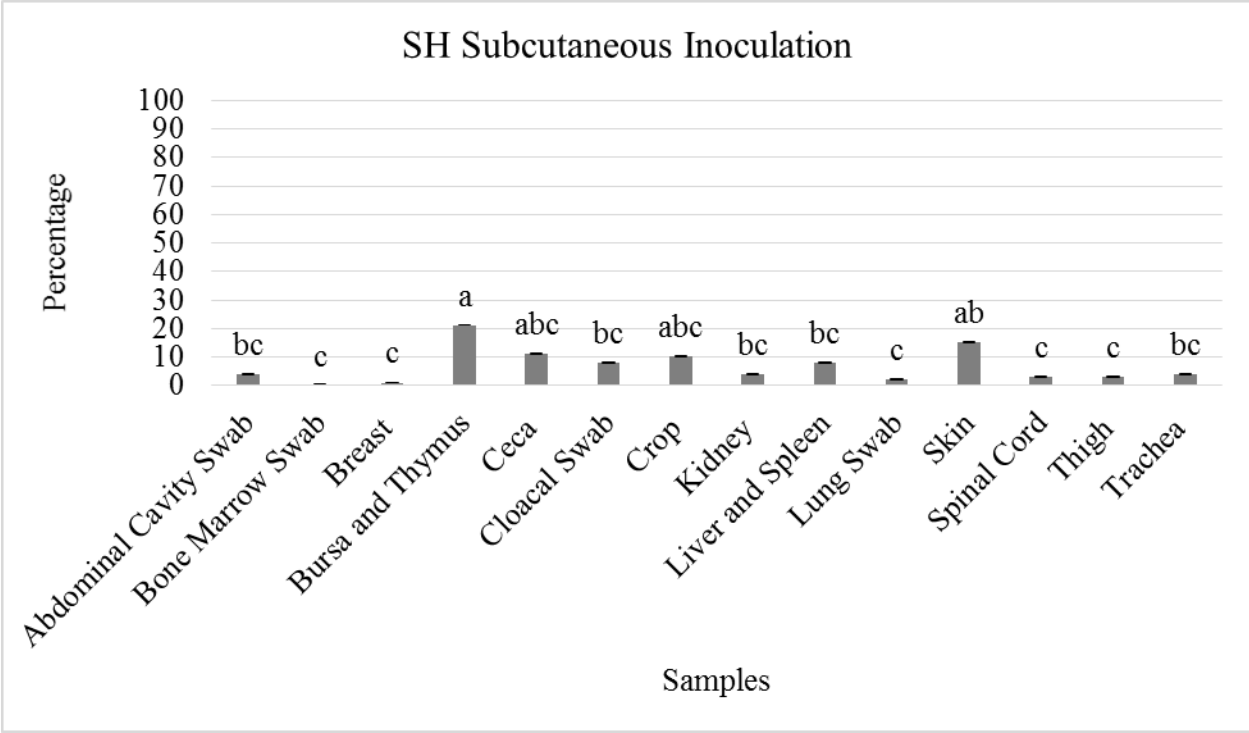


Figure 5.3 A comparison of the number of positive SH samples recovered through the subcutaneous inoculation route. The samples collected are along the x-axis. The y-axis represents the percentage. Letters above each bar graph indicate statistical differences identified using the GLM procedure and when appropriate, Tukey HSD ($P \leq 0.05$). Individual standard deviations were found and corresponding bars are used.

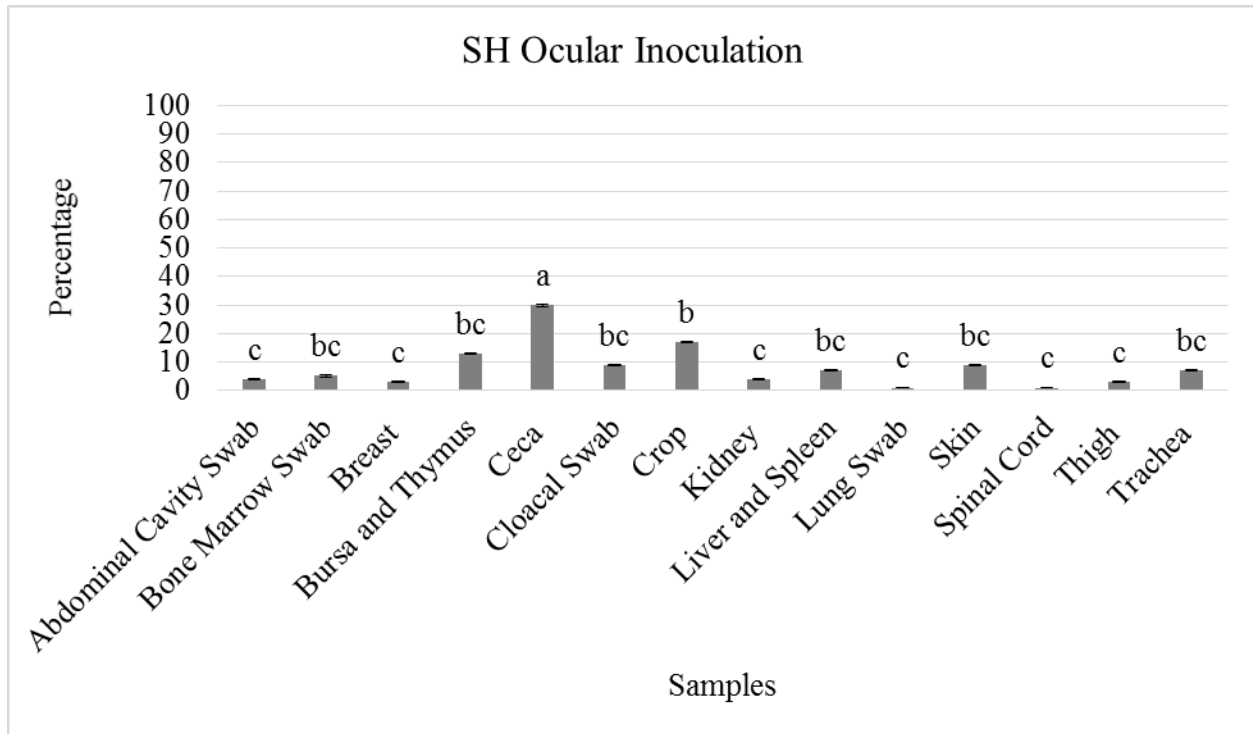


Figure 5.4 A comparison of the number of positive SH samples recovered through the ocular inoculation route. The samples collected are along the x-axis. The y-axis represents the percentage. Letters above each bar graph indicate statistical differences identified using the GLM procedure and when appropriate, Tukey HSD ($P \leq 0.05$). Individual standard deviations were found and corresponding bars are used.

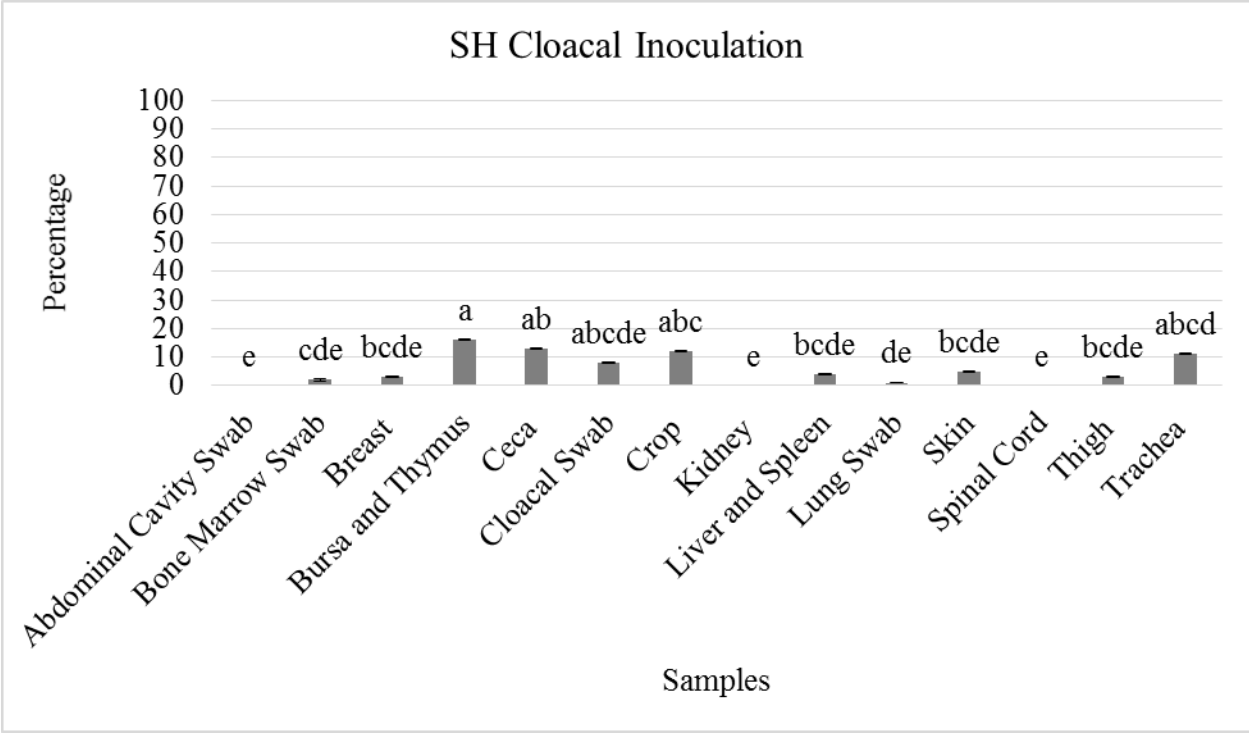


Figure 5.5 A comparison of the number of positive SH samples recovered through the ocular inoculation route. The samples collected are along the x-axis. The y-axis represents the percentage. Letters above each bar graph indicate statistical differences identified using the GLM procedure and when appropriate, Tukey HSD ($P \leq 0.05$). Individual standard deviations were found and corresponding bars are used.

Chapter 6. Summary and Conclusion

Poultry is the fastest growing meat sector and the United States is ranked as one of the top poultry producers in the world (Alali and Hofacre, 2016). With this volume and demand, the safety of poultry products is of high priority to consumer, producers, and governmental officials. This brings the necessity to control *Salmonella* within the poultry industry, due to poultrys' ability to act as a main vector of *Salmonella* into human food systems (Alali *et al.*, 2016; Alali and Hofacre, 2016). Poultry carcass contamination at the processing plant cannot be avoided if chickens arrive prevalent with *Salmonella* (Vandeplass *et al.*, 2010). During rearing and processing of broilers, there are a multiple of sources (feed, biological vectors and aerosols) that can allow for *Salmonella* contamination (Ricke *et al.*, 2013). Both preventative and curative strategies have been considered; however, an understanding of entryways and colonization sites needed further analysis.

Greater colonization of other bacteria through an intratracheal inoculation in comparison to other inoculation routes have also been identified (Toth *et al.*, 1988; Kallapura *et al.*, 2014a). The intratracheal inoculation was given to mimic poor air quality. The reduction of dust within commercial houses has been identified to correspond with a decrease in airborne bacteria (Ritz *et al.*, 2006). Sources of dust within a house can include, but are not limited to: feed, down feathers, excrement, bacteria, and crystalline urine. Controlling dust content has the potential to improve animal health, human (worker) health, and pre-harvest food safety, indicating a need for the poultry industry to consider this vector as the area of concern (Ritz *et al.*, 2006; Ellen *et al.*, 2000).

The ceca have been long identified as a main colonization and contamination site for *Salmonella*. This organ is essential for the establishment as well as the maintenance of *Salmonella* infection and has the potential to serve as a mechanical localization site (Fanelli *et al.*, 1970). The ceca and intestinal contents are established in literature as the primary source of *Salmonella* contamination both pre- and post-harvest (Corrier *et al.*, 1999). Utilizing both a continuous and one day inoculation of two serotypes reiterated the importance of this organ as a colonization site for *Salmonella*.

Feed has been determined to be a vehicle for *Salmonella* contamination within poultry. Feed can be positive for *Salmonella* and not be detected due to: the organisms not being evenly distributed, the organisms being damaged or injured making them difficult to identify and the ability to detect this organism is difficult due to the large volume of feed being made daily (Alali *et al.*, 2016). If contamination of the feed cannot be properly controlled, the industry must set a standardized sampling method for *Salmonella* at the pre-harvest level. From these studies, it was identified that the ceca and crop had a numerically greater recovery of both SE and SH when birds were given a continuous dose at 10^2 CFU/gram. There was a significantly greater recovery through the ceca in the SH inoculation, indicating that this would be the ideal sample for collection. The ceca had a similar statistical recovery to the crop when the birds were inoculated with SE. It is important to note that in these studies feed wasn't removed before the birds were necropsied, so contamination within the crop was expected.

If only a limited number of samples can be collected for *Salmonella* detection, the ceca would be the ideal sampling site based on the results from these studies as well as previous experiments (Fanelli *et al.*, 1970; Hargis *et al.*, 2001; Yegani and Korver, 2008; Ahmer and Gunn, 2011; Borsoi *et al.*, 2011; Kallapura *et al.*, 2014b). If gut physiology is an important

reason why salmonellae persistently colonize farm animals, then the cecal physiology of the bird may play a specific role in supporting the emergence of *S. enterica* serovars (Guard-Bouldin *et al.*, 2004). Further analysis should be focused towards how various entryways alter the ability of *Salmonella* to colonize within this organ.

To minimize the opportunity of introduction, persistence and transmission of *Salmonella* at the pre-harvest level, multiple inoculation routes must be considered to determine entryways of concern (Alali *et al.*, 2016; Sadeyen *et al.*, 2004). Patterns of *Salmonella* throughout poultry production (both pre- and post-harvest) are still considered unpredictable due to limited knowledge in the pathogenesis and ideal environmental conditions with this bacterium and the broiler host (Ricke *et al.*, 2013). This indicates a need for further analysis within the colonization sites identified in these studies. However, colonization was identified under very specific conditions. Further research with varying inoculation days and levels must also be considered.

Chapter 7. References

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