A Comparison of Sites Colonized in Broilers Challenged Through Various Routes and Feed Administration with *Salmonella* Enteritidis and *Salmonella* Heidelberg at Day 14

by

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

Auburn, Alabama
August 3, 2019

Keywords: *Salmonella*, poultry, food safety, preharvest contamination

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Abstract

Salmonella Enteritidis (SE) and *Salmonella* Heidelberg (SH) are among the top *Salmonella* serovars associated with poultry. Broiler grow-out facilities may contain these organisms and can be introduced to the broiler through contact with the environment, utilizing various body cavities for entry. For successful reduction or elimination of these organisms during the harvest and post- harvest period, greater attention must be placed on evaluation of the portals of entry utilized by the organism and the tissues colonized during the preharvest period. The objectives of the three experimental groups (Group 1: SE experiments; Group 2: SH experiments; Group 3: SE vs SH experiment) were to determine: (1) the effect of different inoculation routes (oral, cloacal, intratracheal, ocular, and subcutaneous) on tissue colonization of *Salmonella* (Groups 1 & 2), (2) the effect of the two serovars used on feed administration (Group 3), and (3) to determine *Salmonella* incidence within sampled organs (Groups 1-3) when challenged on d 14. For Groups 1 & 2, the birds were challenged with 1 x 10^4 colony forming units (CFU)/ bird at d 14 through the above-mentioned inoculation routes. For Group 3, pens were each given access to 15.87 kg of 1 x 10^4 CFU/g of SE or SH inoculated feed. This feed was fully consumed in approximately six to seven days before re-exposure to non-inoculated feed. The following ten tissue samples were collected from 100 birds approximately 21 days post inoculation: breast, bursa and thymus (pooled), ceca, crop, kidney, liver and spleen (pooled), skin, spinal cord, thigh, and trachea. Additionally, four swab samples were taken from the abdominal cavity, bone marrow, cloaca, and lung. For Groups 1 & 2, data were analyzed using

General Linear Model procedure and for Group 3 data was analyzed using an independent t- test. Differences were reported at $P \leq 0.05$, and if applicable, means were separated using Tukey's HSD. The ocular route produced the greatest percentage of *Salmonella* positive birds and its incidence within the samples (Groups 1 & 2). The birds challenged with SE contaminated feed produced the greatest number of positive birds and positive samples within the body (Group 3). Results from each of the studies varied considerably; however, samples most often affected include the ceca, crop, cloaca swab, bursa and thymus, skin, and trachea (Groups 1-3). Differences in incidence between serovars may be attributed to the isolate used and pathogenicity of the organism influencing survival, establishment, colonization, and invasion in the bird. With the greatest effect occurring in birds challenged through the ocular route, greater attention should be placed on evaluation of this route as a mode of transmission.

Acknowledgments

I would first and foremost like to thank my family for every single thing you have done for me. To my mom, Esterline, I will never forget the sacrifices you have made to make a better life for me. You are such a great person and a simple thank you will never be enough to show my appreciation. To my dad, Rahim, I value your words of wisdom and your encouragement have helped me so much over the years. To my stepdad, David, thank you for all the love and support you have given me over the years. Since childhood, you all have pushed me to be the best person I can be and taught me that hard work will always pay off in the end. You have always encouraged and supported my endeavors and I am forever grateful to you all for that. To my sister, Leanndra, you are literally my heart in human form. Since we were little you have been my biggest cheerleader, my closest confidant, and the best sister and best friend I could have ever asked. To my sister, Lisa, thank you for all the love and support. To my niece and nephews, I love you all and I hope that I have set a good example for your future.

To Moustapha, thank you for being so supportive of me. I am so happy to have you in my life. You have been incredibly understanding and I love you for that. A special thank you goes to my best friend Alasia and best squirrel friends: Porshia, Danielle, Japaira, and Mia. I would have never been able to get through this experience without you guys. Each of you has brought something special into my life and I am so happy to call you my best friends. To my friends Corenia, Lindsey, Luipe, Danny and fellow graduate students that I have met in Auburn, you guys are awesome. I have learned so much from you and I am very thankful for the friendship

and support you guys have given over the last two years. I know you each of you will be successful in your future endeavors. To my mentors, Dr. Brigid McCrea and Dr. Jon Moyle, you were able to see my potential even when I could not see it. Thank you for all your support over the years.

To Steve Martin, thank you for your friendship. I will genuinely miss the conversations we've had during my time here. You have been someone I could speak to for a pick me- up and I appreciate you for that. A big thank you also goes to the farm crew for all their help for my research. Aside from the research, I have genuinely enjoyed our conversations and I will miss each of you.

The last and most important thank you goes my advisor, Dr. Ken Macklin, and the Macklin Lab. To Dr. Macklin, I cannot thank you enough for giving me this opportunity. I thank you for your mentorship and undeniable support. I hope that we are able to keep in contact for years to come. To James, I have learned so much from you in this short time. I am thankful for your patience as a teacher and I have really enjoyed your friendship during this time. A special thank you goes to my fellow lab mates Kaicie, Luis, Brittany, Ammunay, undergraduates, and interns. You guys have been so helpful for my project. I know it was tough, but I have learned so much from you guys during our time together. I wish the best of luck to you all as you finish your own projects and in your careers.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ºC	degrees Celsius
μL	microliter
BapA	biofilm- associated protein
BHIB	brain heart infusion broth
BPW	buffered peptone water
CDC	Center for Disease Control and Prevention
CE	competitive exclusion
CFU	colony forming units
cm^2	centimeter squared
d	day
DC	dendritic cells
DPI	days post inoculation
ERS	Economic Research Service
et al.	et alia (and others)
FSIS	Food Safety Inspection Service
g	gram
GALT	gut- associated lymphoid tissues
GCC	gastrointestinal colonization control
GIT	gastrointestinal tract

GLM	General Linear Model
h	hours
HCl	hydrochloric acid
HSD	honest significant difference
kg	kilogram
PT	phage types
MCFA	medium chain fatty acid
mL	milliliter
NN	nalidixic acid and novobiocin
NTS	Non- Typhoidal *Salmonella*
RPM	revolutions per minute
RTE	Ready-to- Eat
SCFA	short chain fatty acid
SCV	*Salmonella*-containing vacuole
SE	*Salmonella enterica* subspecies *enterica* serovar Enteritidis
SH	*Salmonella enterica* subspecies *enterica* serovar Heidelberg
SPI	*Salmonella* Pathogenicity Island
ST	*Salmonella enterica* subspecies *enterica* serovar Typhimurium
T1SS	type I secretion systems
T3SS	type III secretion systems
TTB	tetrathionate brilliant green broth

US	United States
USDA	United States Department of Agriculture
VFA	volatile fatty acid
XLT4	xylose lysine tergitol- 4

Chapter 1.0 Introduction

With steady increases or insufficient decline in incidence of those *Salmonella* serovars which cause the most issues for food safety in the US (i.e. Enteritidis, Typhimurium, Heidelberg, Newport, and etc.; Hendriksen et al., 2011), greater attention has been placed on minimizing microbial exposure of pathogenic organisms at the preharvest level (Gast, 2007). During preharvest, natural infection of *Salmonella* in poultry flocks can occur and is influenced by the factors outlined by Bailey (1987) including: the age of the bird at the time of infection, ability of *Salmonella* to survive the gastric barrier passage, competition from other organisms, ability of the organism to locate a hospitable attachment site, diet, health and disease status, environmental stresses (e.g. temperature, stocking density, etc.), physiological status, medication, and host genetics. In addition to these, the route of inoculation and infectious dose may also play a role (Chadwick, 2017; Cox et al., 1996). Therefore, determining the most suitable mitigation strategy to reduce the amount of *Salmonella* entry into flocks requires baseline knowledge through an evaluation of these factors. A majority of the studies conducted have evaluated colonization within broilers when challenged with an oral gavage followed by sampling restricted primarily to the ceca. However, previous research conducted in our lab on day-of-hatch chicks highlighted the importance of evaluating other tissues colonized through various routes of challenge (Chadwick, 2017). Additionally, in a separate set of experiments Chadwick (2017) also revealed

differences in incidence attributed to the serovar utilized during feed administration. Therefore, this same impact must be evaluated in older broilers. Thus, the objectives of these experiments were to determine the effect of the route of inoculation and the serovar utilized during feed administration on tissue colonization in broilers challenged at d 14.

Chapter 2.0 Review of Literature

2.1 General *Salmonella* Characteristics

2.1.1 Structure, Classification, History, and Antigenic Types

Salmonella are widely ubiquitous bacteria belonging to the family Enterobacteriaceae, which are a group of important pathogens known to infect both humans and animals. *Salmonella* are known to be mesophilic, facultatively anaerobic, Gram-negative rods motile via peritrichous flagella, and are generally 2-5 microns long by 0.5- 1.5 microns wide (Giannella,1996). Depending on the serotype, the size of the genome can range from 4460 to 4857 kilobases (Andino and Hanning, 2015). These bacteria have the ability to ferment glucose, usually with the production of gas (Giannella,1996). The majority of *Salmonella* are hydrogen sulfide producers, oxidase negative, and catalase positive (Giannella,1996). They are considered prototrophic, which allows growth in minimal media with glucose as a carbon and energy source and ammonium ions as a nitrogen source (Giannella,1996). Characteristics typically displayed by most serovars that can be used for identification include the hydrolysis of urea, decarboxylation of lysine and ornithine, and growth on Simmons citrate agar (Giannella,1996). The ability of this organism and other genera of Enterobacteriaceae to resist novobiocin, selenite, tergitol, and bile salts, more so than other bacteria, have allowed for ease in selective isolation and colony differentiation (Grimont et al., 2000).

The genus *Salmonella* was first identified by Karl Eberth in Germany. Years later, in 1886, Theobald Smith isolated *Salmonella* Choleraesuis from pigs and considered it to be the causative agent of swine fever (hog cholera). Thus, the bacterium was named in honor of his

superior Daniel Salmon (Grimont et al, 2000). Since then, the species has grown to be more inclusive of organisms demonstrating similar behavioral patterns. The unique classification of *Salmonella* species contributes to 2,579 identified serotypes known as serovars. The complex group of *Salmonella* is comprised of two major species of *Salmonella*: *Salmonella enterica* (n=2,557) and *Salmonella bongori* (n=22). *S. enterica* is the largest of the two species of *Salmonella* and can be further subdivided into six subspecies: *enterica* (I) (n=1,531), *salamae* (II) (n=505), *arizonae* (IIIa) (n=99), *diarizonae* (IIIb) (n=336), *houtenae* (IV) (n=73), and *indica* (VI) (n=13) (Grimont and Wiell, 2007).

Salmonella possess three major antigens: H (flagellar) antigen, O (somatic) antigen, and Vi (superficial) antigen (Giannella,1996). The Vi antigen is found only in a few *Salmonella* serovars, primarily those which are not directly related to foodborne illness (i.e. *Salmonella* Typhi). Analysis of these antigens can offer clinical and epidemiological advantages through categorization into specific serogroups that may allow for a determination of the source of infection and the spread of the organism (Giannella,1996). The most common O- antigen serogroups of *Salmonella* are A, B, C1, C2, D, and E, with groupings based on antigenic similarities between serovars (Brenner et al., 2000). The cellular envelope of *Salmonella* contains a lipopolysaccharide (LPS) structure, similar to other Gram-negative rods, is an important structure for determining the virulence of this organism (Giannella,1996). The LPS layer may function as an endotoxin consisting of an outer O-polysaccharide coat, the R core, and the inner lipid A coat (Giannella,1996). The endotoxin could be responsible for the pathogenesis of many of the clinical signs typically associated with *Salmonella* infections (Giannella,1996).

2.1.2 Groupings of Serovars

Of the more than 2,500 *Salmonella* serovars characterized, three major groups can also be identified through the host range of infection (Singh, 2013). The first group known as "unrestricted serovars" have the capacity to nearly infect all animal species (Singh, 2013). It includes serovars such as *Salmonella* Typhimurium and *Salmonella* Enteritidis that cause mild enteric diseases and unsurprisingly hold the greatest zoonotic potential and food safety impact, as they have developed mechanisms to invade a variety of different hosts without resistance (Singh, 2013; Clarke and Gyles, 1993). "Host- adapted" serovars include those that have the capacity to cause severe systemic infection in the preferred or adapted hosts (Singh, 2013). Infection in non-preferred host results in a carrier state, in which the host sheds the bacteria asymptomatically (Singh, 2013; Clarke and Gyles, 1993). It includes serovars such as *Salmonella* Dublin and *Salmonella* Choleraesuis. The third and final group known as "host- restricted" comprise those which are strictly restricted to one very specific host (Singh, 2013). They are characterized by systemic infections resulting from the bacteria's ability to modulate the natural host environment, which often leads to fatality within the host (Singh, 2013; Uzzau, 2001). Examples of this group include *Salmonella* Typhi and *Salmonella* Gallinarum.

2.1.3 Evolution of *Salmonella*

Evolution along with the assistance of horizontal gene transfer has played a vital role in the emergence, divergence, and longevity of this organism (Singh, 2013). The evolution of its virulence has been characterized by three distinct evolutionary phases (Baumler et al., 1998). Phase one involves the acquisition of *Salmonella* Pathogenicity Island -1 (SPI-1) pathogenic determinant by plasmid- or phage-mediated horizontal gene transfer. This encodes for genes and virulence factors that are responsible for the invasion of *Salmonella* within the host (Singh, 2013;

Baumler et al., 1998). Molecular methods used for epidemiology suggests that *Salmonella* spp. diverged from *Escherichia coli*, and unsurprisingly all *Salmonella* serovars contain SPI-1, whilst *E. coli* and other Gram-negative bacteria do not (Singh, 2013). Pathogenicity islands are clusters of virulence genes present in pathogenic organisms and not in non- pathogenic organisms (Marcus et al., 2000). *Salmonella* have five pathogenicity islands, along with many other smaller pathogenicity clusters called islets, and at least one virulence plasmid (Groisman and Ochman, 1997; Salama and Falkow, 1999).

The second evolutionary phase is characterized by the emergence of the two *Salmonella* species: *Salmonella bongori* and *Salmonella enterica* (Singh, 2013). As a result of point mutations and acquisition of *Salmonella* Pathogenicity Island -2 (SPI-2) pathogenic determinant by horizontal gene transfer, *S. enterica* can be differentiated from *S. bongori* (Baumler et al., 1998). SPI-2 pathogenic determinant is responsible for the establishment within a host and also establishment intracellularly following invasion (Singh, 2013). The third and final phase triggered the branching of *S. enterica* species into several different subspecies and adaptation to specific hosts (Singh, 2013; Baumler et al., 1998). *S. enterica* subspecies I became mainly associated with warm-blooded vertebrates (birds and mammals), while all others (*S. bongori* and subspecies II, IIIa, IIIb, IV, and VI) became primarily associated with cold-blooded vertebrates (Baumler et al., 1998).

2.1.4 Invasion Within a Human Host

For illness to occur, *Salmonella* must enter, evade host defenses, colonize, and invade host tissue through a number of mechanisms. Once contaminated food has entered the GI tract, it is met with gastric juice in the stomach containing hydrochloric acid (HCl), necessary for proper absorption (Andreoli et al., 1997). Gastric HCl is a major defense mechanism against pathogens

that may be ingested with food or water (Andreoli et al., 1997). However, food can act as a buffer, especially those with high protein content, to protect bacteria against detrimental effects of gastric acid (Watermann and Small, 1998; Smith, 2003). *Salmonella* mechanisms such as the log-phase acid tolerance response allow for survival at a 3.0 pH environment for several hours (Smith, 2003).

The human digestive tract, similar to that of the respiratory and urogenital tracts, contains mucosa that are approximately 300-400 square meters in size (Ribet and Cossart, 2015). The three layers that make up the mucosa including the epithelia, loose connective tissue called the lamina propria, and a thin layer of smooth muscle are primary barriers to invasion by pathogenic and commensal bacteria (Ribet and Cossart, 2015). The intestinal mucus layer found directly above the epithelia contains glycoproteins called mucins produced by the goblet cells, digestive enzymes, antimicrobial peptides, and immunoglobins that are also important for protection against pathogenic and commensal bacteria from invading deeper tissues (Johansson et al., 2008). Normal gut commensals known as the microbiota found in the gut lumen are key actors in the control of pathogenic organisms such as *Salmonella* (Ribet and Cossart, 2015). Through competitive exclusion (CE), these organisms can reduce the likelihood of foodborne infection by utilizing nutrients found in the intestinal lumen, making it unavailable for pathogens (Stecher and Hardt, 2011). In addition, many of these organisms may have the capacity to produce inhibitory metabolites such as acetate or butyrate, which can create unfavorable conditions for pathogens (Stecher and Hardt, 2011).

Despite these natural host defenses, pathogens have evolved mechanisms to penetrate the mucus layer and establish themselves on the epithelium (Ribet and Cossart, 2015). One such method is triggering inflammation of the gut, which allows for alteration of the microbiota and

can cause pathogens to outcompete gut commensals (Pédron and Sansonetti, 2008). For *S.* Typhimurium specifically, mucosal inflammation can lead to the production of compounds such as tetrathionate needed as a terminal electron acceptor in anaerobic respiration, giving the bacterium a growth advantage over commensals (Winter et al., 2010).

The intestinal surface (epithelia) is a mixture of cells including goblet cells (mucus), Paneth cells (antimicrobial peptides), columnar absorptive cells (formation of tight impermeable junctions), and membranous cells (M cells) (Brumell and Finlay, 2000). M cells are specialized epithelial cells that have the capacity to internalize small and large particles in the lumen (Neutra, 1999). They act as a conduit for the presentation of antigens to the immunological barriers of the intestinal mucosa provided by gut-associated lymphoid tissues (GALT), inclusive of Peyer's patches, isolated lymphoid follicles, the appendix, and mesenteric lymph nodes (Neutra, 1999; Hein, 1999).

Following contact with the host epithelial lining, *Salmonella* must counterbalance intestinal peristalsis and adhere to the intestinal lining for successful establishment through fimbrial and non-fimbrial adhesions (Wagner and Hensel, 2011). Fimbriae are proteinaceous surface appendages, which allow fimbrial adhesins to bind to a specific cell receptor on the host cell (Wagner and Hensel, 2011). Interestingly, each serovar can display a distinct combination of fimbriae that can determine their affinity for the human epithelial lining (van de Velden et al., 1998). For instance, the long polar fimbriae gene cluster found in serovars such as Enteritidis, Typhimurium, and others that play a role in adhesion to Peyer's patches, which are believed to be the site of entry for a *Salmonella* infection (Baumler et al., 1996).

Non- fimbrial adhesins are a heterogeneous group of adhesins secreted by the type I secretion system (T1SS) auto-transported across the bacterial membrane (Wagner and Hensel,

2011). For *S. enterica*, cellulose and thin aggregative fimbriae are the principle compounds responsible for the biofilm matrices (Wagner and Hensel, 2011). Lastasa et al. (2005) found overproduction of BapA (biofilm-associated protein) promoted pellicle and biofilm formation for *S.* Enteritidis. Biofilms are matrix-enclosed microbial assemblies or extracellular polymeric substances, that can lead to increased virulence of an organism, as they are more resistant to host defenses and antibiotics, once established (Wagner and Hensel, 2011). A number of adhesins used to attach to host surfaces, which will not be discussed in detail, can also facilitate entry into host cells (Wagner and Hensel, 2011).

Intracellular survival within host cells provides pathogens with diverse advantages including inaccessibility from humoral and complement-mediated attack, shear stress- induced clearance by the host, accessibility to host cell nutrients, and it facilitates the dissemination of bacteria into host tissue as they travel throughout the host (Ribet and Cosart, 2015). Thus, the internalization of *Salmonella* within host cells is vital to the success of this pathogen and can occur through at least two distinct processes (Ibarra and Steele- Mortimer, 2009). One process occurs when professional phagocytes such as enterocytes (M cells of intestinal Peyer's patches) and macrophages, which are on the frontline defense against pathogens, utilize phagocytosis to recognize and internalize pathogens (Ribet and Cosart, 2015; Ibarra and Steele- Mortimer, 2009).

Another process may involve invasion of phagocytotic and non- phagocytotic cells through a type III secretion system (T3SS) via a trigger mechanism, T3SS1, which manipulates host cell signals (Ibarra and Steele- Mortimer, 2009; Schlumberger and Hardt, 2006; Brumell and Finlay, 2000). Though phagocytosis is an innate immune function designed to sample a vast majority of pathogens, T3SS1- mediated invasion by *Salmonella* is a highly specific process (Takaya et al. 2005). The T3SS1 are needle-like structures that deliver effector proteins to

interact with the host signaling system (Kubori et al., 1998). Along with phagocytosis and T3SS1-mediated invasion, fimbriae and/or non-fimbrial adhesins on the surface of *Salmonella* can mediate attachment and internalization (Guo et al., 2007).

Following entry into the host cells, *Salmonella* is localized in a membrane compartment known as the *Salmonella*- containing vacuole (SCV), or a modified phagosome (Ibarra and Steele- Mortimer, 2009; Brumell and Finlay, 2000). Within the SCV, *Salmonella* have the ability to replicate, kill the host cell, and release replicated *Salmonella* into the extracellular medium, facilitating the infection of other cells (i.e. macrophages) (Brumell and Finlay, 2000). Factors affecting survival intracellularly also allow *Salmonella* to bind to host epithelia: T1SS (BapA and SiiE), fimbriae, and flagella, T3SS1, T3SS2, virulence plasmids, superoxide dismutase (confers protection from extracellular reactive oxygen), and ion acquisition (Ibarra and Steele-Mortimer, 2009).

Sentinel cells such as M cells and dendritic cells (DCs) are targets for pathogenic entry because they are constantly exposed to pathogenic organisms to coordinate the innate and adaptive immunity (Ribet and Cosart, 2015). M cells can be exploited as a route of entry by allowing *Salmonella* to cross the barrier via these cells and infect deeper tissues (Jones et al., 1994). Moreover, infection of M cells leads to the destruction of M cells, thereby causing breaches in the intestinal barrier (Jones et al., 1994). DCs are antigen-presenting cells located in the mucosa that play a role in adaptive immunity (Ribet and Cosart, 2015). The phagocytic nature of these cells and their ability to migrate to mesenteric lymph nodes and interact with the lymphocytes are exploited by *Salmonella* (Niedergang et al., 2004). *Salmonella* is taken up by DCs in the Peyer's patches and inter-epithelial facilitate the rapid crossing of *Salmonella* across the epithelia (Rescigno et al., 2001). Thereby, contributing to dissemination in the host through

the bloodstream (Rescigno et al., 2001). Once *Salmonella* has invaded the bloodstream, they can ultimately reach filtration organs through the hepatic portal veins.

2.2 Poultry Food Safety Concerns and *Salmonella*

Consumption of poultry meat has increased dramatically since the 1930s and is currently the second most commonly consumed animal protein (Daniel et al., 2011). Poultry has become increasingly more important as an animal protein through changes in consumer lifestyles such as heightened health awareness and smaller family dynamics. In the United States (US), per capita consumption of poultry has doubled within 37 years between 1970- 2007 and from 2004 to 2008 consumption slightly increased by 2.9%, whereas worldwide consumption increased 14.4% (Alali and Horface, 2016; Daniel et al., 2011). Such dramatic increases in consumption can also increase the likelihood of exposure with organisms such as Non- Typhoidal *Salmonella* (NTS) and *Campylobacter* that are most often implicated in disease outbreaks relating to poultry (Alali and Horface, 2016). Therefore, knowledge of potential food safety implications resulting from the constant consumption of this protein is very important.

Human salmonellosis can occur through the consumption of contaminated meat or eggs. Other food matrices such as red meat, dairy, fruits, nuts, sprouts, and vegetables may have associations with NTS (CDC, 2019). However, its presence in poultry is of particular concern because of the commensal relationship this organism shares with poultry (Andino and Hanning, 2015). Clinical manifestations of NTS in humans, collectively known as salmonellosis, are brought about by a symptomatic infection with the bacteria. Salmonellosis occurs when the bacteria have colonized and invaded the epithelial lining of the host gastrointestinal tract (GIT) (Giannella,1996). Symptoms, known as gastroenteritis, including nausea, vomiting, diarrhea, fever, chills, and abdominal cramping usually begin 6 to 48 h after ingestion of the contaminated

food product (Giannella,1996) and are typically self- limiting after seven days (Andino and Hanning, 2015). Aseptic reactive arthritis and Reiter's syndrome are chronic conditions resulting from *Salmonella* infection (Andino and Hanning, 2015). The incubation period of the bacteria, defined as the period between infection and the appearance of clinical signs, is highly dependent upon the host and the amount required to cause infection or infectious dose (Acheson and Hohmann, 2001). Hosts most susceptible to salmonellosis infection include individuals with age extremes (young children and elderly), pregnancy, alteration of microbiota (antimicrobial therapy), and immunocompromisation (e.g. diabetes, HIV infections, and immunosuppressive drugs) (Acheson and Hohmann, 2001).

As a result of underreporting from individuals who do not seek medical care and misreporting from health professionals, the health burden from NTS and other pathogens cannot be clearly identified or may not be truly reflective of its actual burden (Mead et al, 1999). However, organizations such as the Center for Disease Control and Prevention (CDC) and the United States Department of Agriculture- Economic Research Service (USDA- ERS) extrapolate estimates from confirmed data. Collectively, the CDC estimates foodborne illness accounts for 48 million cases, 128,000 hospitalizations, and 3,000 deaths annually (Scallan et al., 2011). Currently, NTS are one of the leading foodborne bacterial pathogens causing illness, death, and financial burden in the US with over one million cases, 23,000 hospitalizations, and 450 deaths attributed to NTS each year (CDC, 2016; Scallan et al., 2011). Additionally, economic losses from medical expenses, productivity, and premature deaths are estimated at $3.6 billion for NTS (USDA- ERS, 2014). With over 29% of NTS attributed to fresh and processed poultry products, poultry continues to be a primary reservoir for many of the major serovars responsible for human illnesses (Foley et al., 2011; Gast, 2007).

It is widely known that *Salmonella* infection in poultry, in most cases, results in the asymptomatic carriage of this organism (Andino and Hanning, 2015). Exceptions include infection with the avian-adapted serovars, and interestingly, these serovars have little to no effect on human health (Andino and Hanning, 2015). Many of the mechanisms mentioned in the previous section (see invasion within a human host) involved in attachment, colonization, and invasion of *Salmonella* into the body for humans and other mammals reflect the same processes within the chicken, though chickens are virtually unaffected (Kogut and Arsenault, 2017). Infection and invasion do occur, as evidenced by the bacteria's presence in the GIT and systemically; however, the mechanisms facilitating persistence in the body without clear clinical signs are still not clearly understood (Kogut and Arsenault, 2017). Recently, it has been proposed that *Salmonella* infection in poultry induces disease tolerance (Kogut and Arsenault, 2017), defined as the ability of the host to limit damage incurred by the organism or the immune system (Ayres and Schneider, 2012). It is characterized by the bacteria triggering inflammation for a short period of time, followed by an alteration in host responsiveness in which the host does not recognize *Salmonella* as a pathogen ultimately leading to persistence within the ceca (Kogut and Arsenault, 2017). In the last stage, occurring approximately four days post infection, reprogramming of the local microenvironment leads to a homeostatic status. Thus, a commensal relationship is established.

One major industry concern of *Salmonella* is its persistence within the broiler house and processing facilities despite control strategies utilized during preharvest, harvest, and post-harvest stages (Alali and Horface, 2016). A study conducted by the United States Department of Agriculture- Food Safety Inspection Service (USDA-FSIS) revealed *Salmonella* prevalence on 3.9% of broiler carcass in processing plants in 2012 (USDA- FSIS, 2014a). Theoretically, of the

8.9 billion broilers produced each year, 347 million contaminated carcasses could possibly be sold to consumers (Alali and Horface, 2016). Therefore, consumers must adhere to proper food handling and preparation techniques, including but not limited to, cooking poultry to 165ºF, minimizing cross-contamination between cooked and uncooked foods, and proper storage of cooked foods safeguards against illness (Chai et al., 2017).

2.3 Serovars of Interest

Based on information provided in the previous section (see general *Salmonella* characteristics), the number of *Salmonella* serovars described total over 2500. However, approximately only about 10% of these serovars have ever been isolated from poultry and an even smaller portion are relevant to poultry and human illness (Gast, 2007). The distribution of *Salmonella* can vary based on the geographic region and shifts in predominating serovars can change over time, which may ultimately have an impact on effective control (Gast, 2007). For example, a global monitoring study by Hendriksen et al. (2011) from 2001 to 2007 placed Typhimurium in the top position among the most frequently identified serovars in North America and Oceania, whereas Enteritidis dominated in all other world regions, namely Africa, Asia, Europe, and Latin America. As of 2013, the top five serotypes associated with broilers were Kentucky, Enteritidis, Typhimurium, Infantis, and Heidelberg; however, in 1998, Kentucky, Heidelberg, Typhimurium var. Copenhagen, Typhimurium, and Hadar were the predominating serovars (USDA- FSIS, 2014b). Current cultured- confirmed data lists common poultry- associated serovars Enteritidis, Typhimurium, and Infantis in the top ten of frequently reported serovars, but Heidelberg was listed as number 12 (CDC, 2016). Another study utilizing surveillance data provided from FoodNet from 1996 to 2006 lists Typhimurium, Enteritidis, Newport, Heidelberg, and Javiana in the top five most commonly serotyped, which accounted for

61% of the total isolates (Jones et al., 2008). This highlights the variability of available data as a result of the different reporting agencies.

Major shifts in serovar dominance have the capacity to open favorable ecological niches for other serovars to take their place, in other words, the decline of one important serovar may facilitate the rise of another. One such example, as it has been proposed, is the relationship between the avian-adapted serovars and Enteritidis. Prior to the establishment of the National Poultry Improvement Plan in the 1930s, Pullorum and Gallinarum dominated; however, by the 1960s these serovars were eradicated from commercial poultry (Foley et al., 2011; Baumler et al., 2000). The decline in these diseases correspond to the emergence of Enteritidis, which prior to that point had associations with rodents. Baumler et al (2000), along with other authors, theorized Enteritidis filled an ecological niche since these organisms have antigenic similarities in serogroup D. Changes such as these demonstrate shifts in dominance possibly resulting from human intervention. In addition to human intervention, *Salmonella* distinction between serovars occurs as a result of the individuality of each of the organisms. This distinction can influence the organism's host range and pathogenicity attributed to factors such as acid tolerance mechanisms, virulence gene expression, cell structure, flagellin, and pathogenicity islands, ultimately influencing survival through the gastric barrier, affinity and adherence to host colonization sites, nutrient acquisition, invasiveness of the organism, and others (Andino and Hanning, 2015; Ricke et al., 2013).

2.3.1 *Salmonella* Enteritidis

Salmonella enterica subspecies *enterica* serovar Enteritidis (SE) is one of the most common serovars associated with chicken and is a common contaminate of shell eggs (Guard-

Petter, 2001). The rise of SE corresponds to the fall of the avian-adapted serovars that occurred since the mid- 1970s and by the 1990s it replaced *Salmonella* Typhimurium (ST) as a primary serovar associated with human salmonellosis (Guard-Petter, 2001; Baumler et al., 2000). Shell eggs provide a biological niche for SE as it has evolved mechanisms to colonize the reproductive organs for transovarian transmission into the shell egg or mechanisms evolved for penetration, survival, and growth of the shell egg, despite the physical and chemical barriers designed to reduce bacterial contamination (Gantois et al., 2008). As a result of this, quality assurance programs and other preharvest mitigation implemented in the 1990s have led to decreased infection rates (Patrick et al., 2004). However, it is still one of the most frequently reported and isolated serovars (CDC, 2016; USDA- FSIS, 2014b; Hendriksen et al., 2011).

Along with eggs, SE also has large associations with broiler carcasses. Kimura et al. (2004) identified eating chicken either prepared in the home or outside the home as the top factor causing SE illness. It was among the top five serovars isolated from broilers and ground chicken from 2003 to 2014 and 2004 to 2013, respectively (USDA- FSIS, 2014b). A study on broiler carcasses following chilling revealed two of the most common phage types (PT), SE PT4 and SE PT13 accounted for the greatest percentage of positive samples (Altekruse et al., 2006). According to data collected from 2000-2005 (Altekruse et al., 2006), prevalence of SE in broilers appeared to be increasing; however, more current data displays that other serovars have become more dominant (i.e. *Salmonella* Kentucky). In spite of this, more intervention and control strategies could be used to further decrease its prevalence.

Outbreak information provided by organizations such as the CDC reveals the most recent outbreaks of SE in chicken occurred as a result of raw, frozen, stuffed, and breaded chicken products in two outbreaks. In the first, the outbreak onset illness dates ranged from May 2015 to

July 2015 causing a recall of over two and a half million pounds of product affecting five people and leading to two hospitalizations (CDC, 2019). The second outbreak from April 2015 to July 2015 involved fifteen individuals with four hospitalizations. Similar incidences occurred in Minnesota from August 2005 to February 2006 with a frozen, stuffed, and prebrowned chicken product (Smith et al., 2008). In the end, thirteen cases of illness occurred with six hospitalizations including elderly individuals subjected to long- term hospitalizations. A large health concern surrounding these products are their prebrowned nature that lead consumers to believe these products are fully cooked and only require reheating in the microwave (Dominguez and Scaffner, 2009). Also, other contributing factors of *Salmonella* presence in these products is the long- term storage nature of Non-Ready-To-Eat (NRTE) products coupled with the ability of *Salmonella* to survive freezing (-21ºC) for at least sixteen weeks (Dominguez and Scaffner, 2009).

2.3.2 *Salmonella* Heidelberg

Salmonella enterica subspecies *enterica* serovar Heidelberg (SH) is also commonly associated with poultry and eggs. Along with SE, SH is a common shell egg contaminant due to its ability to colonize the reproductive tract or transovarian transmission (Gast et al., 2004). According to USDA- FSIS (2014b), from 1998 to 2013 SH has been among the top five serovars associated with chicken, turkey, ground chicken, and ground turkey. Between 1997 to 2006, the decline in SE prevalence corresponding to the rise in SH indicates the organism was possibly filling an ecological niche (Foley and Lynne, 2008). Compared to other *Salmonella* serovars, SH is among the serovars that tend to cause more invasive infections, as opposed to the mild to moderate self- limiting illness from others (Foley et al., 2011). This difference could be due in part to a distinct phylogenetic lineage from that of SE and the other avian-adapted serovars, but it

is similar and shares a serogroup (group B) with ST (Foley et al., 2013). Moreover, these organisms contain plasmids encoding resistance to disinfectants and heavy metal, resistance to antimicrobials, and genes for iron acquisition, though the serotype-specific virulence plasmid for SH has not been identified (Foley et al., 2013).

One of the largest outbreaks in recent history occurred as a result of chicken linked to a single poultry company with 634 individuals affected spanning twenty-nine states and Puerto Rico, though most occurred in California (Gieraltowski et al., 2016). The outbreak period was March 2013 to July 2014. A total with nine outbreak strains were implicated with resistance to common human antibiotics. The products related to the outbreak include Ready- To- Eat (RTE) rotisserie chicken, tenders, boneless skinless breast, thighs, and drumsticks. In previous years from 2012 to 2013, 134 individuals in thirteen states were affected by SH contaminated chicken from the same previously mentioned producer, leading to thirty-three hospitalizations (CDC, 2019).

SH outbreaks have also occurred as a result of further processed poultry products. One such example were infections linked to kosher broiled chicken liver sold as a NRTE product (CDC, 2019). A food safety concern associated with the consumption of liver is often the result of contamination or inadequate cook time (Lanier et al., 2018). The cases were reported to PulseNet from April 2011 to November 2011 with a total of 190 illnesses occurred from six states. Two separate outbreaks linked to frozen and prebrowned chicken products occurred in Minnesota and Canada. In the most recent case, frozen, breaded, and stuffed chicken purchased in Minnesota resulted in an outbreak from January 2005 to March 2005 affecting four people (Smith et al., 2008). The outbreak resulted in a recall of the product and modification of future packaging to include the term “uncooked” with verified cooking instructions. The second

outbreak occurred in British Columbia, Canada with frozen chicken nuggets and strips implicated as the cause (MacDougall et al., 2004).

2.4 On- Farm Contamination

Understanding the transmission of *Salmonella* within poultry companies is multi- faceted due to the integration of the food system. In the US, commercial poultry production is run primarily through vertical integration. Initial contamination of poultry can occur at any point along the continuum (i.e. breeder farm, hatchery, grow- out facility, transportation, and processing and further processing) and become easily transported throughout the various stages. However, recent efforts to combat microbial contamination have been focused at the pre- harvest level to reduce initial introduction, persistence, and transmission of *Salmonella* (Gast, 2007). At the breeder facility, vertical transmission (transovarian or fecal contamination of the egg) of *Salmonella* into the egg can produce a contaminated chick. Horizontal transmission (bird to bird) can occur during hatching, grow- out, and transport through contact with the environment and other birds. Therefore, successful management of critical control points during each of the stages can only occur with in depth knowledge of the organism and its spread.

2.4.1 Vertical Transmission

Transovarian transmission, in which the reproductive system is colonized with *Salmonella*, leading to transmission into the egg has been a characteristic identified within SE and SH (Gast et al., 2004). One study revealed inoculation of laying hens led to infection within the hatching chick and once sexually mature could produce a contaminated egg. For shell penetration, the temperature gradient between the warm egg and the cool environment leads to contraction of the shell membrane and can draw contaminated material such as feces found on the outside of the egg in through the egg pores (Gantois et al., 2009). Once internalized, the

chemical barriers present from bacteriostatic and bactericidal substances such as lysozyme (hydrolyzes peptidoglycan) and ovotransferrin (chelates iron) within the albumen are relatively ineffective (Gantois et al., 2009; Board and Fuller, 1974). Factors influencing egg shell penetration using an agar model revealed the motile and non- clustering properties of *Salmonella* favor penetration into the shell egg (De Reu et al., 2006). However, vertical transmission is generally difficult to study in naturally infected flocks. In one study, Wilding and Baxter- Jones (1985) isolated one *Salmonella* positive egg from over 10,000 sampled over several years.

2.4.2 Hatchery Contamination

The hatchery has long been identified as a major source of contamination. Contamination of eggs through vertical transmission can create seeder chicks infected with *Salmonella* (Cox et al., 1996). Moreover, *Salmonella* detected on egg fragments (71.4%), belting material (80%), and paper pads (74.2%; Cox et al., 1990a) can be easily aerosolized leading to rapid transmission throughout the hatching cabinets (Cox et al., 2000). At such a young age, chicks are very susceptible to *Salmonella* due to the lack of an established immune system and microflora that offer protection against pathogen colonization (Cox et al., 1996). As little as two cells administered intra- cloacally have been shown to colonize day-old chicks (Cox et al., 1990b). This same study revealed 100-fold fewer cells were necessary for colonization of day-old chicks than three-day old chicks with either oral or cloacal inoculation. Environmental samples of two different hatcheries revealed 0.32% (Heyndrickx et al., 2002) and 7.1% (Jones et al., 1991) in *Salmonella* prevalence. Authors have also estimated that of the chicks infected through vertical transmission or hatchery- acquired *Salmonella*, 4.8% (Bailey et al., 1994), 5% (Lahellec and Colin, 1985), 9% (Jones et al., 1991), and 37.5% (Dougherty, 1976) would be contaminated leaving the hatchery for grow- out.

2.4.3 Horizontal Contamination

Transmission through contact with the environment creates the widest variety of potential vectors during the grow-out period. Such vectors include feed, water, litter, feces, feathers, dust, insects, rodents, shared equipment, personnel, and other contaminated fomites (Poppe, 2000). Many of these vectors have been evaluated and monitored by authors over the years to provide insight into critical control points found within these houses (Rodriguez et al., 2006; Liljebjelke et al., 2005; Heyndrickx et al., 2002; Bailey et al., 2001; Jones et al., 1990; Doughterty, 1976), As changes occur in how birds are reared, these environments must be continuously monitored as shown in studies such as one conducted by Alali et al., 2010. These vectors may utilize different entryways of the bird to effectively cause infection. Therefore, preharvest food safety efforts geared towards reduction or elimination can only occur through evaluation of these vectors and the significant role they play in facilitating spread of the organism.

As an enteric pathogen, *Salmonella* is known to cause infection primarily through the GIT. As a result, this has been the most commonly investigated route of infection (Kallapura et al., 2014a). Though the studies challenging birds through the oral route are important and provide necessary information, it relies on only one aspect of epidemiology. Entryway through the oral cavity facilitates movement into the gastrointestinal tract with possible systemic infection through the blood system. Materials that enter in through the mouth make contact with the crop, an organ unique to birds allowing for rapid consumption of feed and swift departure to escape predation (Kieronczyk et al., 2016). The materials can then move from the crop to the proventriculus, acid stomach, followed by the gizzard, mechanical stomach, for grinding to increase the surface area of the material for absorption. Successful movement through the GIT can lead to colonization of these tissues and result in attachment to favorable colonization sites,

which have been identified in intestinal mucin found within the small intestine and the ceca (Craven and Williams, 1997). Once present within these tissues, stress from the environment and transport may induce fecal shedding of *Salmonella*. Birds infected with *Salmonella* but do not shed the bacteria known as carriers. Carriers of the organisms make detection very difficult and in some cases detection methods such as cloacal swabs may be ineffective (van Hoorebeke et al., 2009; van Immerseel et al., 2004).

In addition to other birds contributing to environmental contamination, rodents and insects may also play a significant role. Broiler houses are attractive environments because of the warm and moist conditions, access to feed, protection from the outside environment, and space available for reproduction (van Immerseel et al., 2009). As a result, pests such as insects and rodent often find refuge in these environments. The presence of these animals can be problematic as they can act as mechanical and biological vectors of *Salmonella* (Henzler and Optiz, 1992; Jones et al., 1990). One survey conducted by Henzler and Optiz (1992) revealed the greatest percentage of *Salmonella* present within the environment was also present in mice captured around the farm. Moreover, bacterial counts of mice fecal pellets yielded 100,000 *Salmonella* cells per pellet. With greater *Salmonella* on the outside rather than inside of insects, Jones et al. (1990) concluded insects act primarily as mechanical vectors. Insects of the concern include litter beetles, mites, and flies (Wales et al., 2010).

Of the materials entering through mouth, feed has been identified as an important vector for introduction into the poultry house. It can serve as a direct, through the contamination of the feed ingredients, or indirect, through contamination during mixing or prior to the consumption of the feed, source of contamination (Maciorowski et al., 2004). Feed ingredients included for poultry feeds can be animal or plant- based. Li et al. (2012) revealed animal- derived ingredients

had higher *Salmonella* prevalence when compared to plant- derived ingredients. Advantages of pelleting feed include feed uniformity, increased feed consumption and thus increases in body weight gain occur, but the pelleting process also destroys pathogenic organisms in the feed (Enberg et al., 2004; Veldman et al., 1995). However, concerns associated with pelleted feed include post- processing cross- contamination or recontamination. This has been demonstrated with *Salmonella* prevalence of 35% in mash feed versus 6.3% pelleted feed (Jones et al., 1990) and also 21% in mash feed versus 1.4% in pelleted feed (Veldman et al., 1995). Cross-contamination and recontamination could occur from dust, rodents, and other contaminated materials found within the feed mill (Alali and Horface, 2016).

One of the challenges associated with detection of *Salmonella* in feed is the uneven distribution of the organisms within feed, damaged or injured cells making detection and isolation difficult, and the appropriate number of samples necessary to account for the viable but non-culturable cells (Alali and Horface, 2016). On average, *Salmonella* present in feed and feed ingredients have been about 20 colony forming units per gram of feed (CFU/g), but levels greater than 1,000 CFU/g have also been found within the environment (Jones, 2011). With no active bacterial replication occurring in the feed as a result of the low water activity, feed acts as a vehicle to transport *Salmonella* into the body of the bird. The protein and lipids found within feed can buffer the microorganisms and offer protection from acidic conditions in the upper GIT (Ha et al., 1998). Differences in the survivability of *Salmonella* serotypes have also been identified (Andino et al., 2014). *Salmonella* prevalence in feed evaluated by several authors have resulted in varying results with results ranging from 2.3% (Jones et al., 1990) to 27.5% (Alali et al., 2010). Though highly variable, the presence of *Salmonella* in feed can create food safety

concerns as shown by Shirota et al. (2001) demonstrating the link between SE in feed and SE isolated from egg contents identified through pulsed- field gel electrophoresis.

Aside from ingestion of feed, water can also play a role in the spread of *Salmonella.* Nakamura et al. (1994) demonstrated the spread of infection though the drinking water with hens inoculated with 1 x 10^5 CFU/ mL. Studies evaluating *Salmonella* presence within the water have revealed 1.4% (Bailey et al., 2001), 1.8% (Liljebelke et al., 2005), and 7.8% (Heyndrickx et al., 2002) prevalence within the water lines or feed cups. Differences between drinker types can also influence *Salmonella* prevalence found within the water, with the greatest chances of contamination from plastic bell drinkers and metal troughs than nipple waterers (Renwick et al., 1992). *Salmonella* can take residence in poultry water lines through an establishment of a biofilm, at which point they will be a continuous source of water recontamination (van Immerseel et al., 2009).

Litter in the poultry house is used to cover the chicken house and the type of litter used is dependent on the price and availability of the bedding material (Alali and Horface, 2016). In the US, litter is typically reused for several flock cycles with benefits occurring as a result of economic feasibility and microbiota establishment (Cressman et al., 2010). However, reused litter has also been identified as a source of *Salmonella* as a result of the feces, feathers, spilled feed, and high moisture content from the previous flock (Alali and Horface, 2016). During grow-out, *Salmonella* presence found within the litter has been as high as 21.8% in a commercial house (Liljebjelke et al., 2005). An earlier study by Goren et al. (1988) correlated the *Salmonella* present within the litter at five weeks of age with the *Salmonella* present in the ceca during processing. This indicates ingestion of litter does occur, especially during times of feed withdrawal when birds peck at the litter in search of dropped feed.

The cloacal opening of the birds provides a unique and direct entry point of *Salmonella* into the gastrointestinal tract as the bird's vent is in constant contact with the litter. Agitation of the cloacal region by contaminated materials leads to contraction of these tissues (Schaffner et al., 1974). At which point, contaminated materials may be drawn into the cloaca through anti-peristaltic reflex action known commonly as "cloacal drinking" (Cox et al., 1996). Cells entering in through the cloaca maybe more viable as they are not subjected to the acidity found within the upper regions of the GIT (Cox et al., 1990). In addition, contact with the bursal tissues is inevitable because the plica of the bursa are open to the lumen of the cloaca (Schaffner et al., 1974). Bailey et al. (2005) demonstrated that birds infected via the cloaca had the fastest spread of *Salmonella* throughout the body, which may be attributed to the bursal lymphocytes found within the bursa.

In modern poultry production, regulation of air quality involves the movement of massive volumes of air through tunnel ventilation by negative pressure to remove ammonia and dust particles (Chinivasagam et al., 2009; Ritz et al., 2006). Movement of this air throughout the house can facilitate aerosol transmission from the contamination dust or bioaerosols present (Kallapura et al., 2014a). Aerosolization of dust can also occur during load out periods prior to transport or scratching of the litter in search of dropped feed (Harbaugh et al., 2006). The presence of the organisms within the air can be influenced by the temperature and humidity found within the houses (Kallapura et al., 2014b) These contaminated particles make contact with the respiratory system, namely the mouth, nares, and the conjunctiva and can enter through any of these orifices. The size of the particle may influence the system affected. Particles less than one micrometer in size are likely to pass through the nasal cavity and stay confined within the alveoli and the lung (Chinivasagam et al., 2009). On the contrary, the fraction of aerosols

greater than seven micrometers reach the pharynx and can enter the GIT (Chinivasagam et al., 2009).

The effect of aerosol transmission on broilers has not widely been reviewed. However, recent papers from Kallapura et al. (2014a, b) demonstrated that birds affected through the intratracheal route could receive a combined effect from *Salmonella* incidence found within the respiratory and GIT, simultaneously. Similarly, birds infected through the conjunctival or ocular route would receive a similar combined effect. In this case, cells that make contact with the conjunctiva drain into the nasolacrimal or tear duct, reach the pharynx, and are then swallowed (Humphrey et al., 1992). In day-old chicks, an inoculation dose of 100 cells led to a greater percentage of birds positive and greater recovery of *Salmonella* when challenged through the ocular route compared to those challenged through the trachea (Cox et al., 1996). Despite this, Chart et al. (1992) found the ocular route to be an insignificant mode of transmission.

Inoculation routes most commonly investigated include oral, cloacal, and most recently, respiratory inoculation. However, the navel has also been investigated as an inoculation route in young chicks (Cox et al., 1996). Subcutaneous inoculation could occur if birds were scratched with contaminated materials found within the house. But, an infection in day-old birds revealed birds challenged through the skin would receive a localized infection (Chadwick, 2017).

2.5 On-Farm Methods of Control

Due to the ubiquitous nature of *Salmonella*, initial contact with the bacterium from other birds and the environment is almost inevitable. As a result of this, control measures are put in place as a contingency plan to protect birds, and thus consumers, from colonization. Traditionally, control of these organisms in food occurred through safeguarding the microbial integrity of food or decontamination before consumption, if or when it occurs (Gast, 2007).

Antimicrobials and other bacterial reducing agents utilized during processing to decontaminate cannot overcompensate for lax management during the grow-out period, thereby highlighting the need for preharvest control measures. On-farm measures can range from least to most specific: management and sanitation, gastrointestinal colonization control, and vaccination.

2.5.1 Management and Sanitation

Proper biosecurity and general hygiene are some of the most important and useful management tools necessary to effectively reduce and eliminate *Salmonella* presence during grow- out. Without these tools, other methods such as gastrointestinal colonization control and vaccination implemented to reduce and eliminate *Salmonella* presence are practically useless (van Immerseel et al., 2009). Effective management and sanitation is a large umbrella encompassing all aspects of the poultry production continuum including *Salmonella*- free breeding flocks, hatchery management, proper cleaning and disinfection, effective insect and rodent management programs, strictly enforced biosecurity programs, and decontamination programs for litter, feed, and water. (Gast, 2007).

Salmonella are known to colonize reproductive organs of hens leading to the deposition of the bacteria into the eggs (transovarian transmission), possibly producing a *Salmonella*-positive chick (Gast et al., 2004; Berchieri et al., 2000). Therefore, sourcing of hatching eggs from *Salmonella*-free breeders is optimal (Gast et al., 2007). The egg may be exposed to pathogenic organisms from fecal material on the surface of the egg or the air may be contaminated. Sanitization methods that remove the egg cuticle are not preferred because removal can expose egg pores allowing an entry point through eggshell penetration and also affect hatchability (Wang and Slavik, 1998). In its place, methods such as ultraviolet irradiation of hatching eggs can be utilized without affecting hatchability (Coufal et al., 2003).

Since *Salmonella* can be introduced to the grow- out facility by shared equipment and personnel, enforcement of biosecurity measures including, but not limited to, monitoring of movement onto the farm, use of protective clothing and designated footwear between house, and proper cleaning and disinfection of shared equipment prior to movement around the farm is necessary (van Immerseel et al., 2009). Cleaning and disinfection programs are geared toward reducing the microbial load in the grow- out house. Four basic principles should be followed to ensure the best possible outcome: 1) dry cleaning followed by wet cleaning to remove dirt and organic matter that could impair disinfectant use, 2) appropriate use of disinfectants to kill microorganisms, 3) rinsing to clear residue and 4) fumigation (Morgan- Jones, 1987). Common disinfectants used include aldehydes, peroxides, quaternary ammonium compounds, and phenolic substances (van Immerseel et al., 2009). The efficacy of these programs can vary due to the procedure and the proper use of products (Davies and Breslin, 2003).

Rodents and insects may act as mechanical and biological vectors involved in the introduction of *Salmonella* within the house, but also spreading this organism around the farm. Methods to control these pests can involve physical control methods such as preventing access to the building, traps and bait stations, and clearing of vegetation around the house or rotational chemical control with insecticides and rodenticides (van Immerseel et al., 2009).

Decontamination of water, feed, and litter are important as these could be potential sources of *Salmonella* introduction. Chlorine can be used to sanitize the water lines, with varying efficacy, but does not necessarily result in reduced cecal colonization (Poppe et al., 1986). Chemical poultry litter amendments such organic acid, formalin, sodium bisulfate, sodium sulfate, and sulfuric acid are used primarily to reduce ammonia emission by pH modification of the litter; however, control of *Salmonella* and other pathogens may be a secondary effect of this

pH modification (Vicente et al., 2007). Feed decontamination can occur primarily through physical methods, but chemicals may also be added during feed processing. Physical methods such as pelleting (thermal processing) utilize heat and steam during conditioning, or formation of pellets, to effectively kill pathogens such as *Salmonella* present (Jones, 2011). A number of factors including temperature, time at a given temperature, and moisture influence the number of cells killed during processing (Jones, 2011). For successful elimination of *Salmonella* in feed, conditioning temperatures of 80 to 85ºC have been proposed (Jones and Richardson, 2004; Veldman et al., 1995). Chemical additives, namely organic acids formic and propionic acids, can be used to offer protection against recontamination when used correctly (Jones, 2011).

2.5.2 Gastrointestinal Colonization Control

Gastrointestinal colonization control (GCC) programs broadly refer to methods involved in reducing pathogen colonization or the numbers of the organism found within the GIT (Gast, 2007). GCC programs can include a wide variety of methods such as dietary modification, antibiotics, organic acids, prebiotics, probiotics, synbiotics (prebiotics and probiotics), CE, and others (i.e. antimicrobial peptides, essential oils, and bacteriophages) (Vandeplas et al., 2010; van Immerseel et al., 2009).

Dietary modification may occur through the alteration of feed structure (particle size and feed form), formulation of optimal amino acid balance, incorporation of chelators, and early or in ovo feeding concepts (Vandeplas et al., 2010). Alteration of the diet through changes in feed structure and balance of amino acids can increase volatile fatty acid (VFA) production, thereby lowering the pH of the gut, and discouraging the growth of pathogenic organisms (Hume et al., 1996). Additionally, a proper balance of amino acids in the diet creates growth- limiting nutrient conditions, in which the indigenous microflora use the nutrients making them unavailable for

pathogens (Ha et al., 1994). Chelators in the diet such as phytic acid can bind micronutrients such as iron that increase bacterial virulence of *Salmonella* and other pathogens (Miyamoto et al., 2000; Jones et al., 1977). Early and in ovo feeding programs are designed to accelerate small intestinal development (Vandeplas et al., 2010) leading to an enhanced epithelial barrier and more mature enterocytes (Cheled-Shoval et al., 2011) or increased goblet cells with acidic mucin production (Smirnov et al., 2006), thus possibly reduced colonization ability from *Salmonella*.

Consistent use of antibiotics in poultry have been for growth- promoting effects; however, an older study by Smith and Tucker (1975) also demonstrated the effectiveness of various antibiotics on ST. When the antibiotics were used fecal shedding was reduced, but once removed, shedding levels mirrored untreated birds. Widespread attempts of the poultry industry to reduce or eliminate antibiotic use from resistance concerns in human health has brought about more "natural" methods (Vandeplas et al., 2010). One such example is organic acids in the form of short- chain (SCFA) and medium chain fatty acids (MCFA) (van Immerseel et al., 2009). As little as 1.25 minimolar (mM) of SCFA monocaprin in an emulsion resulted in a bactericidal effect (6- 7 log decrease) against SE (Thormar et al., 2005).

Prebiotics (inclusion of non- digestible feed ingredients to stimulate the growth of beneficial bacterial species), probiotics (addition of live organisms to alter natural microbiota), and synbiotics (prebiotic substrates to favor a probiotic strain) also offer potential in reducing colonization of *Salmonella* in live birds (Vandeplas et al., 2010; Schrezenmeir and Vrese, 2001). For classification as a prebiotic, criteria outlined by Gibson and Roberfroid (1995) must be met such as these substances cannot be absorbed or hydrolyzed in the stomach or small intestine so that it may interact with the indigenous microflora in the lower region of the GIT. The inclusion of mannooligosaccharides at 4,000 parts per million led to a 34% decrease in cecal colonization

with *S.* Dublin in 10 day- old birds (Spring et al., 2000). Bacterial species typically associated with probiotics are *Lactobacillus*, *Enterococcus*, *Pediococcus*, and *Bacillus* spp. (Vandeplas et al., 2010). In vitro, 12 *Lactobacillus* strains used were effective against *Salmonella* attachment to ileal epithelial cells by blocking attachment sites making them unavailable to *Salmonella*, but also through the production of lactic acid as an inhibitory substance against *Salmonella* (Jin et al., 1996). Studies evaluating the use of synbiotics against *Salmonella* in poultry have been conducted through competitive exclusion cultures. One such example is a significant decrease in the recovery of SE in birds administered 0.1% fructooligosaccharide and 0.1% fructooligosaccharide plus CE culture compared with the control and birds administered CE 1- and 7-days post inoculation (Fukata et al., 1999).

CE treatments utilize a defined or undefined bacteria culture, typically sourced from mature birds, to minimize chick susceptibility to *Salmonella* colonization before the establishment of their own resident microflora (Gast, 2007). Similar to their inclusion as a probiotic, bacterial species most often included are: *Lactobacillus*, *Bifidobacterium*, and *Bacillus* spp. and can be administered in many ways through installation into the crop, mist, drinking water, or used as a feed additive (Gast, 2007). Protection of the chick by *Salmonella* colonization through CE results from interference with *Salmonella* attachment and inhibition of growth through VFA production (Schneitz, 2005).

2.5.3 Vaccination

Use of live or inactivated *Salmonella* vaccines only reduces the susceptibility to *Salmonella* and cannot create an impermeable barrier against infection (Gast, 2007). For broilers, the use of vaccines in production is not at all feasible, but vaccination of broiler breeders is worthy of investigation. In this case, vaccination can reduce the prevalence of *Salmonella* in

breeding hens, thus their progeny, but also increases passive immunity in broilers (Dorea et al., 2010). This has been demonstrated with decreased prevalence of *Salmonella* prevalence on breeder carcasses compared to unvaccinated breeders, but also in the broiler progeny with differences in *Salmonella* prevalence in chick box liners, litter swabs, dust, and on the carcasses compared to the progeny of the unvaccinated breeders.

Chapter 3.0 A Comparison of Sites Colonized in Broilers Through Various Inoculation Routes when Challenged with *Salmonella* Enteritidis at Day 14

Introduction:

Salmonella remains one of the leading foodborne bacterial pathogens causing illness and death in humans. In the United States (US) alone, the Center for Disease Control and Prevention (CDC) estimates that over one million salmonellosis cases occur each year with 23,000 hospitalizations and 450 deaths (CDC, 2016; Scallan et al., 2011). Infection with these organisms also carry large financial burden with an estimated $3.6 billion in economic losses from medical expenses, productivity, and premature deaths (USDA-ERS, 2014).

Among the >2500 serovars identified, *Salmonella* Enteritidis (SE) continues to be one of the most frequently isolated serovars in all regions of the world, with the exception of North American and Oceanic regions (Hendriksen et al., 2011). The Laboratory- based Enteric Disease Surveillance (LEDS) places SE as the top most frequently reported *Salmonella* serovar in foodborne salmonellosis cases (CDC, 2016). SE illnesses are commonly associated with the consumption of raw or undercooked poultry and eggs; however, pork, leafy greens, and other vegetables can also be a source of SE-related illness (Andino and Hanning, 2015; Alali et al., 2010). More specifically, SE was among the top five serovars isolated from broilers and ground chicken from 2003 to 2014 and 2004 to 2013, respectively (USDA- FSIS, 2014b). Its strong association with poultry and eggs gives insightful evidence that poultry is an important and primary reservoir for SE and other *Salmonella* serovars (White et al., 1997). Historically, control of pathogens such as SE have depended on safeguarding microbial integrity of food or decontamination before consumption (Gast, 2007). However, steady increases in incidence of SE

and other serovars have led researchers to focus food safety efforts to control microbial introduction, persistence, and transmission at the preharvest level (Gast, 2007).

Salmonella infects live broilers and the susceptibility of the broilers to the infection is influenced by a number of factors including: the age of the bird at the time of infection, ability of *Salmonella* to survive the gastric barrier passage, competition from other organisms, ability of the organism to locate a hospitable attachment site, broiler diet, health, physiological and disease status, environmental stresses (e.g. temperature, stocking density, etc.), medication administered, and host genetics (Bailey, 1987). Additionally, the route of inoculation and inoculation dose may also play an important role (Cox et al., 1996, Chadwick, 2017). During rearing, infection can occur via horizontal (bird-to-bird) or vertical (hen-to-egg) transmission. Horizontal transmission of *Salmonella* through contact with the environment (water, feed, litter, feces, fluff/ feathers, dust, insects, rodents, shared equipment, personnel, and other contaminated fomites) create the widest variety of potential vectors during the grow-out period (Poppe, 2000).

There have been many studies conducted to evaluate the prevalence of *Salmonella* on broiler farms (Alali et al., 2010; Rodriguez et al., 2006; Liljebjelke et al., 2005; Heyndrickx et al., 2002; Bailey et al., 2001; Jones et al., 1990; Dougherty, 1976). However, a better understanding of on- farm prevalence in relation to the impact of routes of inoculation and how it affects *Salmonella* colonization on farms is still necessary for successful preharvest control. A majority of the studies conducted to determine *Salmonella* colonization have focused on birds challenged via oral gavage with samples restricted to the liver, spleen, ceca, and reproductive organs. Traditionally, the intestines, namely the ceca, have been the prime area of *Salmonella* recovery within the bird (Kallapura et al., 2014a). However, isolation of *Salmonella* from organs such as the crop (Hargis et al., 1995) and trachea (Kallapura et al., 2014a; Kallapura et al.,

2014b) will give insightful information about on-farm contamination. Previous research conducted on birds challenged on day- of- hatch demonstrated *Salmonella* recovery in the ceca was significantly higher than the other 13 investigated samples (Chadwick et al., 2017). However, the intratracheal route imposed the greatest effect on the tissues and swabs sampled after five weeks compared to the oral, ocular, cloacal, and subcutaneous routes investigated. Further investigation was warranted to understand the effect of age on *Salmonella* tissue colonization patterns via different routes in broilers. Therefore, the objective of these experiments was to determine the incidence of SE in various tissues in 14-day old broilers challenged through five inoculation routes: oral, cloacal, intratracheal, ocular, and subcutaneous.

Materials and Methods

Isolation of Bacteria and Use of Cultures

Naladixic acid and novobiocin resistant *Salmonella enterica* subspecies *enterica* serovar Enteritidis (SE) isolates were used for this experiment. Beads of the SE isolate held in a -80ºC freezer were placed onto a plate of Tryptic Soy Agar II containing 5 % Sheep Blood (BBL™, Beckon, Dickinson, and Company, Sparks, MD). They were then incubated for 18-24 h at 37ºC. The isolates were confirmed as *Salmonella* using slide agglutination with *Difco*™ *Salmonella* O Antiserum Poly A- I & Vi (Beckon, Dickinson, and Company, Sparks, MD). The isolates were then plated onto Xylose Lysine Tergitol- 4 Agar (XLT4; Criterion™, Hardy Diagnostics, Santa Maria, CA) containing naladixic acid (100 μg/mL; Alfa Aesar, Wand Hill, MA) and novobiocin (15 μg/mL; Alfa Aesar, Wand Hill, MA (NN), for confirmation, and incubated for 18-24 h at 37ºC. A colony from the XLT4 + NN plate was selected and used to inoculate 50 mL of Brain Heart Infusion Broth (BHIB; Hardy Diagnostics, Santa Maria, CA). The BHIB was placed into a shaking incubator for 18-24 h with 200 revolutions per minute (RPM) at 37ºC. Following

incubation, serial dilutions were made from the stock solution to determine the colony forming units per milliliter (CFU/ mL), which was approximately 10^9 CFU/ mL.

Broiler and Farm Management

Broilers used for the trial were housed at the Auburn University Poultry Research Farm in Auburn, AL. A total of 150 chicks for each inoculation route/experiment (150 chicks x 5 inoculation routes = 750 total) were sourced from a commercial broiler hatchery. For all experiments, chicks used were randomly allocated into five floor pens (25 birds/ pen/experiment), with excess birds placed into an unchallenged pen. For convenience, groups of two experiments were conducted simultaneously i.e. oral and cloacal; intratracheal and ocular, while the subcutaneous inoculation experiment was conducted as an individual stand-alone experiment (October 2017- June 2018). The birds were reared using standard conditions (stocking density, photoperiod, light intensity, temperature, and ventilation) appropriate for the age of the bird for the duration of the experiments. The experiments ran for a total of 34 to 36 d or 39 to 41 d, depending on when the birds were set and the time necessary to perform the necropsies.

Route of Inoculation

The inoculum was prepared as mentioned in the section above. The inoculum was adjusted to obtain a final inoculation dose of 1 $x10^4$ CFU per broiler and the inoculum levels were confirmed. The birds were inoculated at d 14 post-hatch throughout the experiment.

Oral: Using a one mL tuberculin syringe (BD™, Beckon, Dickinson, and Company, Franklin Lakes, NJ), each broiler was dosed with 500 µL of inoculum directly into the esophagus.

Cloacal: To inoculate via the cloaca, each broiler was inverted to access the cloaca. Using a one mL tuberculin syringe (BD™, Beckon, Dickinson, and Company, Franklin Lakes, NJ), each broiler was given 100 µL of the inoculum.

Intratracheal: Special care was taken to avoid damage to the trachea. A 20-gauge animal feeding needle attached to a one mL tuberculin syringe (BD™, Beckon, Dickinson, and Company, Franklin Lakes, NJ) was used to apply pressure to the tongue to open the passageway into the trachea. Once opened, the feeding needle was inserted and 100 µL of the inoculum was delivered directly into the trachea.

Ocular: Using a one mL tuberculin syringe (BD™, Beckon, Dickinson, and Company, Franklin Lakes, NJ), the birds were given 50 µL of the inoculum into each eye, totaling 100 µL.

Subcutaneous: To inoculate subcutaneously, a 22- gauge needle was attached to a one mL tuberculin syringe (BD™, Beckon, Dickinson, and Company, Franklin Lakes, NJ). With two pinched fingers, the skin at the nape (neck skin) was lifted, the needle inserted under the skin, and 250 µL was injected. The presence and feel of a "fluid bubble" indicated the inoculation was performed correctly.

Sampling

To maintain aseptic conditions during necropsy, all instruments (stainless steel round-end forceps, stainless-steel scissors, and stainless-steel poultry shears) were initially dipped into water, then 10% bleach, and finally 70% ethanol, respectively, before and after each tissue collection. In addition, the instruments were cleaned and sanitized as mentioned above when contact was made with surfaces other than the appropriate tissue. The sanitizing agents and water were replaced intermittently throughout the necropsy. Cutting boards used for necropsy were washed with water, scrubbed, and sprayed with 70% ethanol following sampling of each bird. All tissues samples were collected into a sterile Whirl-Pak™ bag (*Nasco®)*; 20 mL of Buffered

Peptone Water (BPW; HiMedia®, HiMedia Laboratories Pvt. Ltd, Mumbai, India) was then added to each bag and stomached onsite for one minute. The swab samples were collected using a sterile cotton swab **(**Puritan®, Puritan Medical Products Company LLC, Guilford, ME) and then placed into five mL of BPW tubes.

Samplings were performed between 20-22 (34- 36 d of the experiment) or 25-27 (39-41 d experiment) days post inoculation (DPI). Randomly selected broilers (n=100, 20 broilers/pen x 5 pens/experiment) were used for necropsy. Each bird was given a unique identifier to differentiate during necropsy. The birds were euthanized using a carbon dioxide asphyxiation. Once confirmed dead, cloaca swabs were collected using a swab and this was placed into five mL of BPW. The birds were then dipped into a quaternary ammonia immersion solution from the base of the neck near the shoulders down to the feet sanitize and remove excess dirt, feathers, and fecal material from the body.

Necropsies were carried out utilizing a two-person sampling system for the following 14 tissue and swab samples collected: abdominal cavity swab (ab cavity), bone marrow swab (bone mar), cloaca swab (cloaca), lung swab (lung), breast, bursa and thymus (bur and thy), ceca, crop, kidney, liver and spleen (liv and sple), skin, spinal cord, thigh, and trachea. Person A was responsible for all the samples pertaining to the head and leg: skin, trachea, thymus (pooled with bursa), and spinal cord and leg: thigh meat and bone marrow swab. Person B was responsible for all the samples pertaining to the body: crop, breast meat, abdominal cavity swab, lung swab, liver and spleen (pooled), ceca, bursa (pooled with thymus), and kidney.

Person A:

Following immersion into the quaternary ammonia solution, neck feathers were plucked to the base of the head to expose the neck skin. Using the shears, the neck of the bird was cut off,

near the base of the neck being careful to avoid cutting the crop. The bird was allowed to bleed out into kill cones. At which point, one of the legs was also removed for further sample collection. Approximately 5 cm^2 of neck skin (representative skin sample) was removed from the pre-plucked neck section and placed into a bag. To access the thymus, the fold of skin previously cut from the neck skin was then cut upwards, parallel to the side of the neck to expose the thymus. A portion of the thymus, approximately 3-4 cm^2, was removed and placed into the bag with the bursa (pooled samples). Following removal of the thymus, all remaining skin attached to the neck was removed. The bird was laid flat against the side of the cutting board to expose the trachea. Once exposed, the trachea was then pulled away from the neck and approximately 3-4 cm^2 was removed and placed into a bag. With the head held flat against the cutting board, the neck was cut upwards using the shears, perpendicular to the beak to expose the spinal cord. Approximately 2-3 cm^2 of the spinal cord was removed and placed into a bag.

To sample the thigh meat, skin overlaying the muscle was peeled back with connective tissue also cut to free skin and feathers, as they could act as a potential source of contamination. Approximately 3-4 cm^2 of the thigh meat was removed and placed into the bag. To access the bone marrow, the femoral head was cut at an angle using the shears. A swab was then used to dig into the bone marrow and then placed into a BPW tube.

Person B:

Once bled, the skin at the cranial portion of the body was cut open to expose the crop. Approximately 3-4 cm^2 of the crop was taken from the bird and placed into a bag, with excess feed and/or water removed using the back of the scissors. With the forceps, the skin overlaying the breast tissue was peeled back with connective tissue also cut along the way to free the skin and feathers away from the body, as they could act as a potential source of contamination to the

rest of the body. Approximately 3-4 cm^2 of the breast tissue was removed and placed into a bag. The ribs of the bird were cut open using the shears and an abdominal swab was taken and placed into a BPW tube. Using a swab, a section of the lung was stabbed and placed into a BPW tube.

To remove the liver and spleen, a lobe of the liver was drawn away from the body and approximately 3-4 cm^2 was removed and placed into a bag. To access the spleen, the intestines were pulled away from the body cavity and inverted to expose the spleen, with caution not to pull the intestines completely out of the body cavity, as this could be a potential source of contamination. The base of the spleen was grabbed and the entire organ was removed and placed into a bag. The ceca tonsils, proximal to the spleen, were exposed following unraveling the intestine. One of the cecal tonsils were cut at the cecal neck to avoid leakage of its contents into the body cavity and approximately 5-6 cm^2 was cut, removed, and placed into the bag. Once removed, the intestines were then completely pulled away from the body cavity to expose the bursa. Approximately all of the bursa was removed and placed into a bag. The kidney presented a greater challenge to remove, as they are so delicate, difficult to remove, and incorrect removal could result in shredding of the organ. Therefore, removal of the kidney occurred using the scissors to dig underneath the organ to remove a substantial section; it was then placed into the bag. Following sampling of challenged birds, five birds were randomly selected from unchallenged pens and cloaca swabs were taken to determine horizontal transmission to unchallenged birds.

Microbiological analysis

All the stomached tissue samples were stored at 4ºC until further analysis. The samples were incubated for 18-24 hours at 37ºC in 20 mL BPW. Following pre-enrichment, one mL of each sample was placed into five mL of Tetrathionate Brilliant Green Broth tubes (TTB;

HiMedia®, HiMedia Laboratories Pvt. Ltd, Mumbai, India) and incubated for 48- 72 h at 37ºC. Enriched samples were then streaked onto XLT4+ NN plates using 1 µL disposable loops (VWR International, LLC, Radnor, PA) and incubated for 48 h at 37ºC. From those plates, isolated colonies demonstrating typical *Salmonella* colonies were slide agglutinated with *Difco*™ *Salmonella* O Antiserum Poly A- I & Vi (Beckon, Dickinson, and Company, Sparks, MD). The presence of SE was indicative of a score of one (positive), whereas a score of zero (negative) was indicative of SE absence.

Data Analysis

All statistical analyses were conducted using IBM® SPSS® software version 22. Data pertaining to a percentage of birds positive overall for the challenge, a percentage of samples positive overall for the challenge (calculated by dividing the total number of positive samples by the total number of samples collected for each inoculation), and a comparison of differences in incidence among each sample types for SE for each inoculation were analyzed using a General Linear Model (GLM). Significant differences were reported at $P \leq 0.05$, and if applicable, means were separated using Tukey's HSD.

Results:

Table 3.1 summarizes the percent incidence for all fourteen tissue and swab samples collected following inoculation of birds at d 14 across all inoculation routes. With the exception of the abdominal cavity and bone marrow samples, SE incidence was not significantly different between all the routes ($P > 0.05$). However, for the remaining twelve tissue and swab samples, incidence was significantly different between the routes .

Samples taken from the cloaca, ceca, and trachea formed more distinctive relationships between inoculation routes, with significant differences occurring between the groups. With the cloaca swab samples, the routes with the greatest incidence were the ocular (60.61%) and

intratracheal (48.00%) routes, the routes with the intermediate incidence were the oral (18.37%) and cloacal (30.00%) routes, and the subcutaneous route (0.00%) had the least incidence. The ceca samples were also arranged very similarly with the ocular (61.00%) and intratracheal (51.00%) routes incurring the greatest incidence, the oral (28.00%) and cloacal (31.00%) routes with the intermediate incidence, and the subcutaneous route (0.00%) with the least incidence. Two groups were formed between routes for the trachea samples: SE incidence occurred the greatest in the oral (20.20%), ocular (21.21%), and (33.00%) routes and the least in the cloacal (2.02%) and the subcutaneous (0.00%) routes.

The remaining samples were less distinctive, indicating incidence between the routes had greater variation. For the lung samples, the ocular route (8.33%) produced the greatest incidence when compared to the other routes. The oral, cloacal, and subcutaneous routes were the least affected with 1.01, 1.01, and 0.00% incidence, respectively, though incidence from the intratracheal route (3.00%) was not significantly different from the ocular and the oral, cloacal, and subcutaneous routes ($P > 0.05$). The inoculation routes for the breast samples followed a similar pattern. Incidence within the bursa and thymus samples was greatest in the cloacal route (75.76%; $P < 0.05$). Routes in the intermediate group included the ocular (39.00%) and intratracheal (28.00%) routes, though incidence for intratracheal route was not significantly different from the oral route (21.00%; $P > 0.05$). Incidence within the crop samples was greatest in birds challenged through the ocular route (59.60%), followed by the intratracheal and oral routes with 32.00 and 20.00%, respectively. The greatest incidence in the kidney samples occurred in birds challenged through the cloacal (19.00%) and the ocular (12.00%) routes, though incidence from the ocular route was not significantly different from the oral (7.00%) and intratracheal (5.00%) routes. The liver and spleen samples within cloacal inoculation produced

the greatest incidence (30.30%). The intermediate group consisted of the oral (15.15%), ocular (16.16%), and the intratracheal (7.00%) routes, though the subcutaneous (0.00%) route was not significantly different from incidence in the intratracheal route ($P > 0.05$). The skin samples for the subcutaneous route (25.51%) had the highest recovery of SE followed by ocular (21.00%) and intratracheal (20.00%); these were all similar ($P > 0.05$). In addition, the incidence from intratracheal route was not significantly different from the cloacal (9.00%) and oral (6.12%) routes ($P < 0.05$). Incidence within the thigh samples was greatest in the intratracheal route (6.00%), though this was not significantly different from incidence within the oral, cloacal, and ocular routes with 1.00, 1.01, and 3.06, respectively ($P > 0.05$).

In Figure 3.1, highly significant differences were noticed through a comparison of the routes using a GLM ($P < 0.05$) and three groups were formed from means separation ($P < 0.05$). Birds challenged via the cloacal, ocular, and intratracheal routes had the highest percentage of SE-positive birds with 89, 88, and 85%, respectively ($P > 0.05$). Compared to other routes, oral gavage and subcutaneous routes yielded 48% and 28% of SE positive birds, respectively ($P < 0.05$). Figure 3.2 demonstrates the overall percent incidence of positive samples by inoculation route. Overall incidence in positive samples from the routes were 2.3, 10.7, 15.5, 17.8 to 23.1% for the subcutaneous, oral, cloacal, intratracheal, and ocular route, respectively. Although broilers challenged through oral gavage had different levels of SE recovered from tissues, the differences were statistically insignificant ($P > 0.05$). Oral gavage resulted in the highest recovery of SE in the ceca (28.00%) followed by the bursa and thymus (21.00%), trachea (20.20%), crop (20.00%), cloaca (18.40%), and liver and spleen (15.20%; Figure 3.3). The lowest recovery (1-4%) of SE by oral gavage was observed in abdominal cavity swab, bone marrow swab, lung swab, breast, and thigh ($P > 0.05$).

Figure 3.4 displays SE recovery from broilers challenged through the cloacal route. When challenged through cloacal route, the bursa and thymus samples exhibited the highest recovery ($P < 0.05$) of SE compared to other tissues. The greatest recovery of SE occurred in the cloaca swabs samples (75.80%) and incidence found here was significantly different from all other samples ($P < 0.05$). The intermediate group included the cloaca (30.00%), liver and spleen (30.30%), ceca (31.00%), and kidney (19.00%) with incidence that was not significantly different between samples ($P > 0.05$). The lowest (1-3%) recovery of SE was observed in the abdominal cavity swab, lung swab, breast, spinal cord, thigh, and trachea but were not significantly different from bone marrow swab (6.00%), crop (7.00%) and skin (9.00%) ($P > 0.05$).

The greatest recovery of SE in birds challenged through the intratracheal route occurred in the ceca (51.00%) followed closely by the cloaca swab (48.00%; $P > 0.05$; Figure 3.5). The intermediate group included the bursa and thymus (28.00%), crop (32.00%), trachea (33.00%), and skin (20.00%), with incidence in the crop and trachea similar to cloaca swab recovery. The group with lowest recovery included abdominal cavity swab, bone marrow swab, and lung swab with incidence ranging from 2.00- 3.00% ($P > 0.05$); however, recovery in these tissues were not different from breast, kidney, liver and spleen, spinal cord, and thigh, with incidence from 5.00-7.00% ($P > 0.05$).

Birds challenged through the ocular route, displayed in Figure 3.6, had the greatest recovery of SE in the cloaca swab (61.00%), ceca (61.00%), and crop (60.00%), these were significantly different from all others ($P < 0.05$). These were followed by bursa and thymus (39.00%), which also significantly different from all others ($P < 0.05$). The samples with the least recovery of SE were bone marrow and thigh with 2.00 and 3.00% incidence, respectively.

These were comparable to abdominal cavity swab, lung swab, breast, kidney, liver and spleen, and spinal cord with ranges in recovery from 5.00 to 16.00% ($P > 0.05$), though significantly different from trachea and skin, both with 21.00% incidence ($P < 0.05$).

Results from the subcutaneous inoculation can be observed in Figure 3.7. The sample with the greatest recovery of SE was the skin sample (25.50%), which was significantly higher than the bursa and thymus (7.00%) ($P < 0.05$). Incidence within both samples were significantly higher than the other twelve samples in which incidence did not occur ($P < 0.05$).

Discussion:

The fecal- oral route has been cited as the primary route of *Salmonella* infection in chickens and is the most commonly investigated route of inoculation (Kallapura et al., 2014a). *Salmonella* infection through the fecal-oral route can occur when birds ingest *Salmonella*-contaminated material, including feed, litter, water, feathers, feces, and others (Poppe, 2000). The contaminated material along with the pathogen travels to the GIT where *Salmonella* colonizes and is then shed in the feces. Although this route offers important and invaluable information, it relies primarily only on one aspect of epidemiology, which is infection through the gastrointestinal tract.

Only 48% of the birds in the study were SE positive (Figure 3.1), whereas 95.7% of broiler chicks were affected when challenged on day-of- hatch in a different study (Cox et al., 1996). The difference in birds affected could be the result of the lack of a mature immune system leading to increased susceptibility to colonization of the gut (Cox et al., 1996). However, similar work conducted by Chadwick (2017) for birds challenged on day- of- hatch observed 12.3% recovery of SE.

Organs affected (Figure 3.3) greatest by the oral challenge are ceca, bursa and thymus, crop, trachea, and cloaca with a recovery of 28.00, 21.00, 20.20, 20.00, and 18.37%,

respectively. These results can be explained by the pathway of the organism from ingestion to excretion. Compared to the findings from Kallapura et al. (2014a), the SE recovery in the ceca was similar and approximately twice in the trachea as reported by the researchers. Differences could possibly be attributed to the age of the bird when challenged, the length of time from challenge to sample collection, or aspiration of the inoculum into the trachea during oral gavage.

The cloaca, a common cavity for excretory and digestive waste products, offers unique entry of *Salmonella* and other pathogens into the GIT. *Salmonella* infection via the cloaca can occur when birds sit on contaminated material or a contaminated surface found within the grow-out facility (Cox et al., 1996). Agitation of the cloacal region causes the sphincters in the cloacal opening to contract (Schaffner et al., 1974). Solids and fluids possibly containing *Salmonella* or other pathogens are then drawn into the cloaca through anti- peristaltic reflex action known as "cloacal drinking" (Cox et al., 1996). Entry of *Salmonella* cells into the body in this manner could lead to better survivability of these cells since they are not subjected to the high degree of acidity found within the upper regions of GIT including the crop, proventriculus, and the gizzard (Cox et al., 1990). As such, a smaller number of cells have the ability to colonize favorable attachment sites within the ceca (Cox et al., 1990). In day- old chicks only two cells were necessary to colonize intra- cloacally, as compared to 100- fold greater in chicks that were orally inoculated (Cox et al., 1990). In the same fashion, our experiments demonstrated a greater overall incidence in positive samples from birds inoculated via the cloaca (15.5%) as compared to birds inoculated orally (10.7%; Figure 3.2).

Cloacal inoculation resulted in the overall higher ($P < 0.05$) incidence of SE (89%) compared to oral and subcutaneous routes (Figure 3.1) and can be attributed to the prevalence of

SE found in the bursa and thymus samples of a majority of the birds sampled. This incidence level was similar to the ones reported by Cox et al. (1996) and Chadwick (2017) indicating that inoculation levels and bird age may not affect SE incidence in broilers. As previously mentioned, inoculation through the cloacal cavity resulted in the highest colonization of SE in the bursa and thymus (pooled) samples (Figure 3.4). Anatomically, the plica of the bursa are exposed to the cloacal lumen through the bursal duct (Schaffner et al., 1974). As a result, microbial contact with the bursa is inevitable. However, findings from Schaffner et al. (1974) demonstrated the lack of viable organisms (*Escherichia coli* and *Staphylococcus albus*) found within these tissues following inoculation but macrophages containing these organisms were present within the interfollicular connective tissue. Thus, SE cells present within these tissues may not guarantee infection of the bird through systemic infection but may only indicate the SE presence due to its location. This has been observed in the present experiment with the bursa and thymus as the only positive sample in many of the birds sampled.

Traditionally, infection through the fecal- oral route of *Salmonella* and other pathogens has been most commonly observed, both naturally and experimentally. However, other routes such as the intratracheal route (used to simulate infection via the respiratory route) would provide an explanation for the colonization of both the digestive and respiratory systems simultaneously (Kallapura et al., 2014a). As it has been observed in other aerosol challenges (Baskerville et al., 1992), even if the exposure dose is low, this route appears to provide a combined effect of simultaneous infection of the respiratory and the GI tracts , which can usually be compared to that of an oral challenge at a higher dose. Though birds for each inoculation were given a 1×10^4 CFU dose, the ability of an intratracheal challenge to cause a greater

impact has been demonstrated by the high incidence of SE in the ceca, cloaca swabs, trachea, and, crop samples with 51.00, 48.00, 33.00, and 32.00% (Table 3.1), respectively.

Salmonella infection in broilers can occur with movement of bioaerosols, contaminated dust particles, or both moved through the air by negative pressure through tunnel ventilation for bird comfort (Kallapura et al., 2014a; Chinivasagam et al., 2009). Following exposure to these aerosols, a fraction of the organisms are inhaled, reach the pharynx, and are swallowed (Cox et al., 1996; Baskerville et al., 1992) thus infection of the respiratory and the GI tract can occur. A number of factors within the grow- out facility including temperature and humidity can play a role in the survivability of these organisms in the environment (Kallapura et al., 2014b). Additionally, airborne particles greater than seven micrometers in size are more likely to stay trapped within the upper respiratory tract, nose, and throat, and can therefore enter the GIT, whereas particles less than one micrometer pass through the nasal cavity and stay confined within the alveoli and the lung (Chinivasagam et al., 2009).

Typically, it is known that day- old chicks are more vulnerable to *Salmonella* colonization compared to older birds due to the lack of a well- established immune system and competitive exclusion from an established microflora (Cox et al., 1990). Studies have been able to successfully duplicate *Salmonella* infection and colonization of day- old chicks challenged through the intratracheal route or by aerosol (Lever et al., 1996; Cox et al., 1990). The intratracheal route in birds challenged at d 14 produced results in overall incidence in positive samples comparable to a similar study by Chadwick (2017) with birds challenged at day- of-hatch with the same infectious dose. Similarities in the results of these experiments offer opposition to this idea of increased resistance to infection in older birds (Leach et al., 1999; Baskerville et al., 1992).

Previously, *Salmonella* colonization of the crop has been shown to parallel with incidence in the ceca coupled with a greater likelihood to rupture during processing (Hargis et al., 1995). The similarity in incidence (Figure 3.5) between the cloaca swabs (48.00%) and the crop (32.00%), which are usually used as indicators of *Salmonella* presence, with the trachea (33.00%) is in agreement with previous research findings using the trachea as an indicator organism for *Salmonella* infection (Kallapura et al., 2014b). Colonization of *Salmonella* within the trachea can be attributed to the rate at which clearance occurs within the trachea (Mensah and Brain, 1982) as well as a reduced inflammatory response from the cells resulting in a commensal and longer lasting infection (Okamura et al., 2005).

Investigation of SE infection via the ocular inoculation route (also referred to as conjunctival in the literature) similar to the intratracheal inoculation route in broilers has been reduced due to the lack of emphasis placed on this route as a viable mode of transmission. However, in the present experiment, birds challenged through the ocular route produced the highest incidence of SE colonization in six of the fourteen samples collected including the abdominal cavity swab, breast, ceca, cloaca swab, crop, and lung swab. It has also produced the highest overall incidence of positive samples with 23.1% incidence, despite the lack of available or supporting research as an important inoculation route from authors such as Chart et al. (1992). Very similar to that of infection via the intratracheal or respiratory routes, movement of air throughout the house by negative pressure may carry pathogens in the form of bioaerosols or contaminated dust (Kallapura et al., 2014a). Particulates could land in the eye of the bird, with drainage of organisms into the nasolacrimal duct (Humphrey et al., 1992). These organisms reach the pharynx and are swallowed (Humphrey et al., 1992). Swallowing of these organisms is

followed by movement through the GIT and subsequent shedding in the feces (Humphrey et al., 1992).

A study conducted by Cox et al., (1996) in which day- old chicks were challenged through the ocular route with *S.* Typhimurium (ST) at 1 x 10^2 CFU/ mL found a similar percentage of overall birds positive with 83% compared to 88% of birds in our study. Samples with the greatest SE incidence including the crop, cloaca swab, and ceca with approximately 60% incidence demonstrate that once contact has been made with the conjunctiva, has been the organisms can move from the nasolacrimal duct to the GIT. In this case, similar to that of the intratracheal inoculation route, birds receive a combined effect in which the respiratory and GIT are affected simultaneously (Kallapura et al., 2014a). In laying hens, even after 20 to 28 days post infection (DPI), SE could be recovered from the reproductive organs, kidney, liver and spleen, jejunum, ileum, the ceca indicating systemic infection (Humphrey et al., 1992). Similar conclusions can be drawn from the current study. The bursa and thymus at 39.00% incidence were affected by the ocular inoculation route. However, Chart et al. (1992) found that laying hens challenged by SE via the conjunctiva at 1 x 10^3 CFU/ mL produced a poor immune response and when challenged at 1 x 10^8 CFU/ mL produced mainly IgM antibodies at low titers. Differences in the responses can be attributed to age of the bird at infection and the methodology used for detection of the organism.

Subcutaneous inoculation can occur when chickens are scratched with contaminated materials found within the chicken house. Birds kept in higher density conditions are 2.9 times more likely to be scratched than those kept at normal conditions (Elfadil et al., 1995). Subcutaneous inoculation is most often used for determining vaccine efficacy (Desin et al., 2014); however, Chadwick (2017) proposed that birds infected in this manner with *Salmonella*

receive a localized infection. In the present experiments subcutaneous inoculation had the lowest incidence (Figure 3.2) of all other inoculation routes (2.3%) and also the least number of positive birds (23%; Figure 3.1).

With the exception of the skin and bursa and thymus samples, there was no recovery of SE in any other samples in this inoculation. The administration of the bacteria for this route was performed in the neck area, which could explain the recovery of SE found there. For systemic infections of SE to occur, the bacteria must invade the bloodstream for dissemination throughout the body. However, the small number of blood vessels found within the skin restricted SE to the area of inoculation and little other areas throughout the body, leading to a localized infection. Though localized, this may present a problem for ground chicken since skin is normally added in the preparation of non- mechanically separated (non-MSC) ground chicken to meet the target fat content. Subsequent grinding can distribute the bacteria throughout the product leading to proliferation throughout the product (Alali et al., 2016).

The previously discussed inoculations (i.e. oral, cloacal, intratracheal, ocular, and subcutaneous) represent the ways *Salmonella* enter the body. Though the oral route has been the most commonly investigated route of inoculation, the data presented above indicate that other inoculation routes should be included in future studies. Moreover, infection by other routes, particularly those in which two passageways are affected simultaneously (i.e. ocular and intra-tracheal) may have a greater impact on the individual samples. Therefore, it is important to determine the effect on tissues based on what has been shown from the routes and the implications of *Salmonella* recovery within those tissues.

For successful infection of the host, *Salmonella* must evade host defenses, colonize, and invade host tissue. Though exact mechanisms by which these processes are carried out in the

chicken are not fully understood, attempts must be made from the bacteria to adhere to the intestinal lining through fimbrial and non- fimbrial adhesions that bind to specific cell receptors (Wagner and Hensel, 2011; Craven and Williams, 1997). Some of the cell receptors for enteropathogens have been identified within the intestinal mucin (Craven and Williams, 1997). Once adhesion has been made, colonization of the GIT can occur, which may eventually lead to entry into the blood stream. The bacteria's affinity for the chicken digestive system, namely the ceca, allows for persistence within the body without producing clinical signs of infection (Andino and Hanning, 2015). For this reason, the ceca have been a standard for *Salmonella* recovery within birds (Kallapura et al. 2014a; Corrier et al., 1999; Hargis et al. 1995; Snoeyenbos et al., 1982; Fanelli et al., 1971). In broilers, intermittent shedding of the bacteria can be exacerbated from stress resulting from transport to the processing facility (Rigby and Petit, 1980). Hence, the similarities in SE incidence among the cloaca and ceca incidence among all routes ($P > 0.05$; Table 3.1).

In addition to the ceca and cloaca, the crop has also been identified as a source of contamination within the processing facility and a major site of contamination with the GIT (Hargis et al., 1995). Upon evaluation of the crops and ceca of three broiler flocks at processing, the overall percentage of SE- positive crops (52.00%) were greater than that of the ceca (14.60%) (Hargis et al., 1995). Conversely, this finding was not observed for any of our inoculations. They also observed that the crops were 86 times more like to rupture than that of the ceca, which would result in spillage of possibly contaminated ingesta onto broiler carcass. Contamination of the ingesta can result from contaminated litter consumed through the feed withdrawal period and has been shown to increase SE incidence (Corrier et al., 1999) or colonization of the crop (Ramirez et al., 1997). Colonization of the crop may occur when the pH

is increased through insufficient activity which creates a favorable environment for pathogen invasion into the tissues (Kieronczyk et al., 2016). In our experiments, birds were not subjected to a feed withdrawal period in any of the inoculations, therefore, this could explain the differing results. In the present experiment, it was observed that colonization within the crop was greatest with birds challenged through the ocular route (59.20%; Table 3.1). Higher crop incidence of birds challenged in this manner could be attributed to the combined effect of an aerosol challenge.

As previously mentioned, recent data using the trachea as an indicator of *Salmonella* presence has produced promising results (Kallapura et al., 2014a; Kallapura et al., 2014b). Kallapura et al. (2014b) found that *Salmonella* was more readily recovered from the trachea than from the ceca in all trials with *Salmonella* samplings in four different countries. However, Kallapura et al. (2014a) found that the ceca were more readily colonized than the trachea with SE challenged birds, but studies using ST as the challenge isolate observed the trachea was more readily colonized than the ceca at all infectious doses. These data are consistent with findings from our trial since the incidence for tracheal samples in SE positive birds (Kallapura et al., 2014a) was not greater than the incidence in cecal samples. Additionally, with low incidence found in the cloacal (2.02%) and subcutaneous (0.00%) routes demonstrate that infection via these routes may not be anatomically relevant to the trachea. Together, these results suggest that evaluation of the trachea for *Salmonella* presence could be used in conjunction with collection of samples such as the ceca and crop and was also proposed by Kallapura et al. (2014b).

Previously it was mentioned that contaminated aerosols or dust may be carried throughout the house with tunnel ventilation. If particles sizes are small enough, they can pass down into the alveoli and the lung (Chinivasagam et al., 2009). In the present study, colonization

of the lung was relatively low (Table 3.1) in comparison to work by Cox et al. (1996). Colonization of the lung tissue in broiler chicks with inoculation doses ranging from 10^2 to 10^4 CFU/dose resulted in 18, 34, and 16% of birds positive when challenged by oral, respiratory system (fog), and intratracheal inoculations, respectively (Cox et al. 1996). Differences in number of birds positive between the two studies could be attributed to age differences but also differences in culturing techniques. In the present study, lung swabs were used for detection; however, Cox et al. (1996) harvested the entire lung of the bird.

Along with the lung tissue, the abdominal cavity, namely the air sacs, could also be another area within the respiratory system affected by *Salmonella* infection. (Gorham et al. 1994). Airsacculitis found in birds during processing can be an indication that pathogenic *E. coli* and *Salmonella* are present (Russell, 2003). Additionally, bacteria affecting the air sacs could be an indication of systemic infection, as these organs are found throughout the body cavity. Despite this, no significant ($P > 0.05$) recovery of SE within the body was observed in any of the inoculations (Table 3.1).

Among other factors, transport has been identified as a major external source of *Salmonella* entry into the processing facility because it can cause increased excretion rates of contaminated fecal material and possibly increased shedding of the bacteria through stress (Rigby and Petit, 1980). *Salmonella* found within the fecal material can land on the skin. Once firmly attached to the skin through fimbrial adhesions, the bacteria may lodge themselves in the crevices of the skin and accessibility may become more difficult (Lillard, 1989b). In the European Union excised neck skin used for sampling of broiler carcasses to determine *Salmonella* presence has been shown to produce similar result as whole carcass rinsing, commonly performed in the US (Cox et al., 2010). Though different, each of the methods are

designed to evaluate external contamination found on the skin. Incidence of SE on skin in these experiments showed the ocular (21.00%), intratracheal (20.00%), and subcutaneous (25.51%) routes are comparable to commercial broilers originating from *Salmonella*- confirmed breeders (21.4%; Wu et al., 2014). The incidence of SE recovery on skin is similar in the cloacal, ocular, and intratracheal routes ($P > 0.05$; Table 3.1) to recovery of SE within the ceca and cloaca. This indicates that skin can also be used in conjunction with these samples to determine *Salmonella* presence.

Following penetration of the mucosal epithelium, interaction between *Salmonella* and the epithelium triggers inflammation, which can then cause phagocytic cells to uptake *Salmonella* (Bohez et al., 2007). As an intracellular pathogen, phagosomes (modified macrophages) offer diverse advantages including inaccessibility from humoral and complement- mediated attack, sheer stress- induced clearance by the host, and accessibility to host cell nutrients (Ribet and Cosart, 2015). Furthermore, it facilitates dissemination into other internal organs such as the liver, spleen, and kidney which indicates that a systemic infection has occurred (Bohez et al., 2007). Studies evaluating *Salmonella* colonization include the liver, spleen, or both as indicator organs (Kaseem et al., 2012; Cox et al., 2007).

Along with indicating systemic infection within the birds, the presence of *Salmonella* within these tissues also has food safety implications. Chicken liver can be prepared and consumed in a variety of ways, but inadequate cooking and pathogen contamination offer the greatest risk for consumption (Lanier et al., 2018). Anatomically, the kidneys are located firmly attached to back and, unless removed, could be consumed as part of a whole chicken legs or ground into MSC. With respect to the present experiments, as shown in Table 3.1, recovery of SE in the kidney was greatest in birds challenged through the cloacal routes (30.30%), which

was twice the recovery of SE from birds challenged through the oral (15.15%) and ocular routes (16.16%) for the liver and spleen. Similarly, recovery of SE was greatest in the cloacal route (19.00%) but was also comparable to the ocular route (12.00%) ($P > 0.05$). The cloacal route may offer better survivability of cells into the gastrointestinal tract, and thus more viable and non- injured cells available to colonize and invade the blood stream (Cox et al., 1990). This was also observed in the present experiments with the greatest effect on the filtration organs in birds challenged in this manner.

Following entry into the processing facility a series of steps including killing, bleed- out, scalding, defeathering, evisceration and crop removal, washing and chilling, leading to processing of carcasses (Alali et al., 2016; Buncic and Sofos, 2012; Carrasco et al., 2012). These carcasses can be marketed for retail as a whole bird, cut up parts, or further processed products. Products such as the thigh and the breast are typically sold as cut up parts for consumer convenience. A great majority of the contamination found is the result of external contamination from rupturing of the GIT or crop (Buncic and Sofos, 2012). The relatively small amount of incidence found within our experiments confirm that a majority of incidence would be attributed to external contamination during processing. However, Leach et al., (1999) reported that muscle contamination following oral inoculation was rare, but inoculation through aerosol led to a significantly higher ($P < 0.05$) increased incidence. They proposed the increase was the result of increased number of SE positive blood samples, or septicemia. Within our experiments, a trend was observed with most of the muscle contamination (thigh and breast) greater than 5% occurring in those challenged by routes that could be affected through aerosol, i.e. intratracheal and ocular routes.

Together the bursa and thymus are the central lymphoid organs of birds (Cooper et al., 1965). The "bursa of Fabricius" is a lymphoid organ located near the cloaca in which cells destined for use in humoral immunity mature, B lymphocytes (Ribatti, 2017). An earlier study also demonstrated the important role the bursa plays in antibody production (Glick et al., 1956). Similarly, the thymus is the lymphoid organs clustered in the neck of the bird in which cells destined for use in cell- mediated immunity mature and differentiate, T lymphocytes (Ribatti, 2017). In the present study, the response of either of these organs to *Salmonella* infection was not evaluated. However, the presence of *Salmonella* within these tissues indicate that it prompted either cell- mediated or humoral immunity, or both. Similar observations were proposed by Berndt and Methner (2004) who revealed a participation of both the humoral and cellular immunity against ST five days following oral inoculation of immunized day- old chicks The greatest recovery of SE was found in birds challenged through the cloacal route (75.00%) followed by the ocular route (39.00%). This demonstrates that the route of inoculation and also the ease of the organism to cause a systemic infection will result in a greater response of the immune system.

The bone marrow of birds is a site of new cell production or hematopoiesis and also a key element of the lymphatic system through the generation of lymphocytes (Campbell, 1967). Few papers address *Salmonella* contamination of the bone marrow (Wu et al., 2014; Kaseem et al., 2012, Velaudapillai, 1964); however, since bone- in chicken parts are utilized in the preparation of MSC, it is quite conceivable that *Salmonella* contamination in ground chicken resulting from bone marrow may occur. In commercial flocks, Wu et al. (2014) found that 0.7% of the bone marrow samples were *Salmonella*- positive and is comparable to Velaudapillai (1964) with 0.8% *Salmonella*- positive bone marrow samples. *Salmonella* bone marrow incidence in control birds

orally inoculated with 2 x 10^5 was (2/10) 20% (Kaseem et al., 2012). For the present work, we found no significant difference in incidence of bone marrow samples between inoculations ($P > 0.05$). The greatest SE incidence occur in birds challenged through the cloacal route and coincides with high incidence of the liver and spleen (30.30%), which can be indicative of a systemic infection.

Bacterial contamination of the spinal cord resulting from *Salmonella* has not been widely reported in the literature. The spinal cord (central nervous system) forms the connection between the brain and the peripheral nervous system. *Salmonella* is not traditionally known to take residence within the spinal cord of birds; however, bacterial cells found within these tissues are subject to entry into the food supply since the backs and necks are ground for MSC (Alali et al., 2016), but recovery was low in all routes (< 6.00% incidence for all inoculations) indicating that is may not be a major area of *Salmonella* recovery.

Conclusion:

Introduction of SE can occur at any point along the poultry production continuum. However, when birds were exposed at d 14, results imply that the ocular and intratracheal routes have the greatest incidence and overall effect on the birds when evaluated approximately 21 DPI. Persistence of this organism for extended periods of time in grow- out facilities could possibly lead to contamination going into the processing facility. Thus, mitigation strategies geared towards reducing contaminated dust and aerosols should be evaluated further.

	Oral (%)	Cloacal (%)	Ocular (%)	Intratracheal (%)	Subcuta-neous (%)	P-value (P ≤ 0.05)
ABDOMINAL CAVITY*	3.03	3.03	5.10	3.00	0.00	0.323
BONE MARROW*	4.04	6.06	2.00	2.00	0.00	0.109
CLOACA*	18.37^{b}	30.00^{b}	60.61^{a}	48.00^{a}	0.00^{c}	**<0.001**
LUNG*	1.01^{b}	1.01^{b}	8.33^{a}	3.00^{ab}	0.00^{b}	**0.002**
BREAST	4.04^{ab}	1.01^{b}	9.09^{a}	5.00^{ab}	0.00^{b}	**0.007**
BURSA & THYMUS	21.00^{cd}	75.76^{a}	39.00^{b}	28.00^{bc}	7.00^{d}	**<0.001**
CECA	28.00^{b}	31.00^{b}	61.00^{a}	51.00^{a}	0.00^{c}	**<0.001**
CROP	20.00^{bc}	7.00^{cd}	59.60^{a}	32.00^{b}	0.00^{d}	**<0.001**
KIDNEY	7.00^{bc}	19.00^{a}	12.00^{ab}	5.00^{bc}	0.00^{c}	**<0.001**
LIVER & SPLEEN	15.15^{b}	30.30^{a}	16.16^{b}	7.00^{bc}	0.00^{c}	**<0.001**
SKIN	6.12^{c}	9.00^{bc}	21.00^{ab}	20.00^{abc}	25.51^{a}	**<0.001**
SPINAL CORD	1.01	1.00	5.00	6.00	0.00	**0.022**
THIGH	1.00^{ab}	1.01^{ab}	3.06^{ab}	6.00^{a}	0.00^{b}	**0.033**
TRACHEA	20.20^{a}	2.02^{b}	21.21^{a}	33.00^{a}	0.00^{b}	**<0.001**

Table 3.1 Comparison of *S.* Enteritidis recovery between the five inoculation routes

* indicates swab samples instead of tissue samples

Data were analyzed using a GLM and **bold** values indicate significant differences at $P \leq 0.05$.

$^{(a\text{-}d)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation

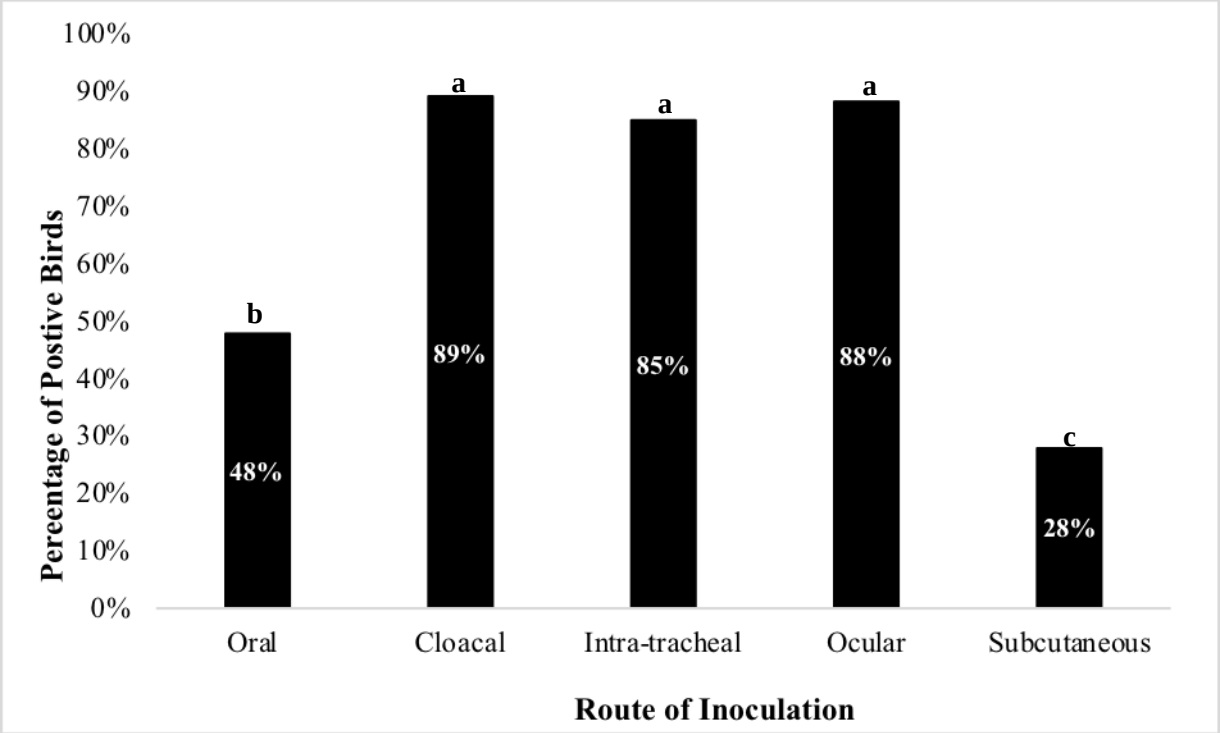

Figure 3.1 Comparison of the percentage of SE positive birds for each route of inoculation Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$. $^{(a\text{-}c)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation

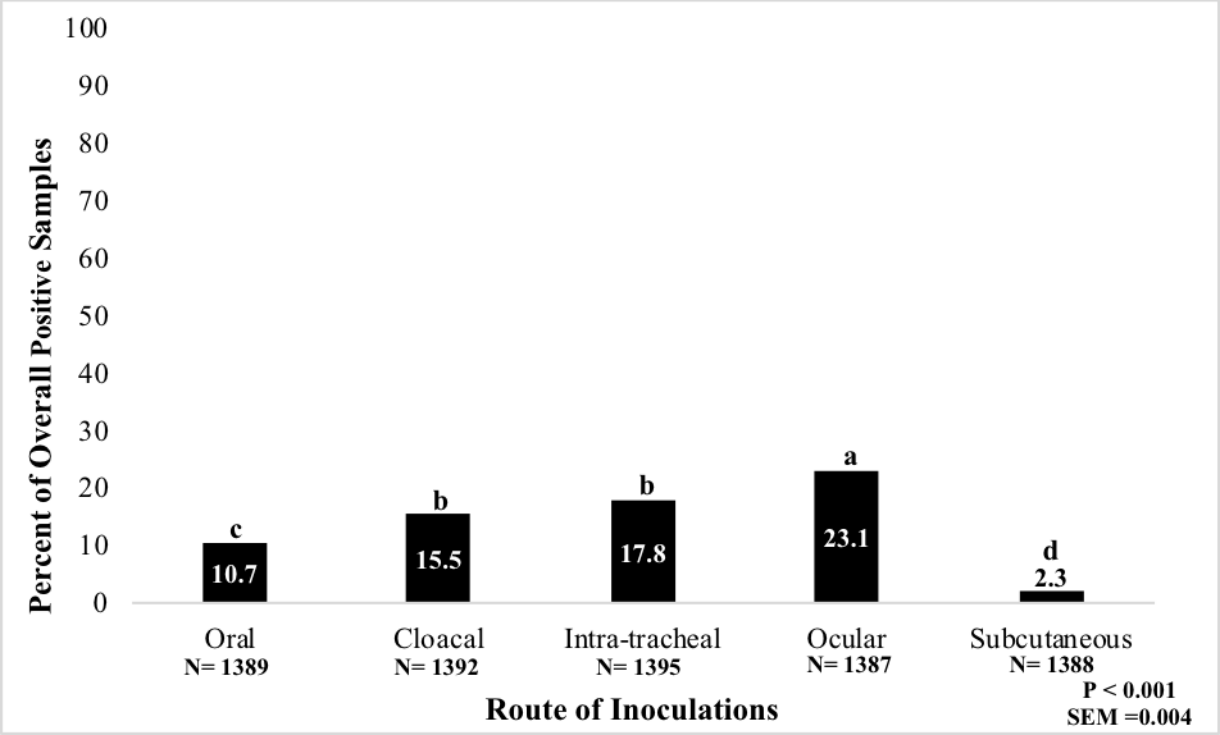

Figure 3.2: Overall percent incidence of positive samples by inoculation route

N= number of samples collected for each of the routes. Variation between routes occurred as a result of missed samples during necropsy

Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$.

$^{(a\text{-}d)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation

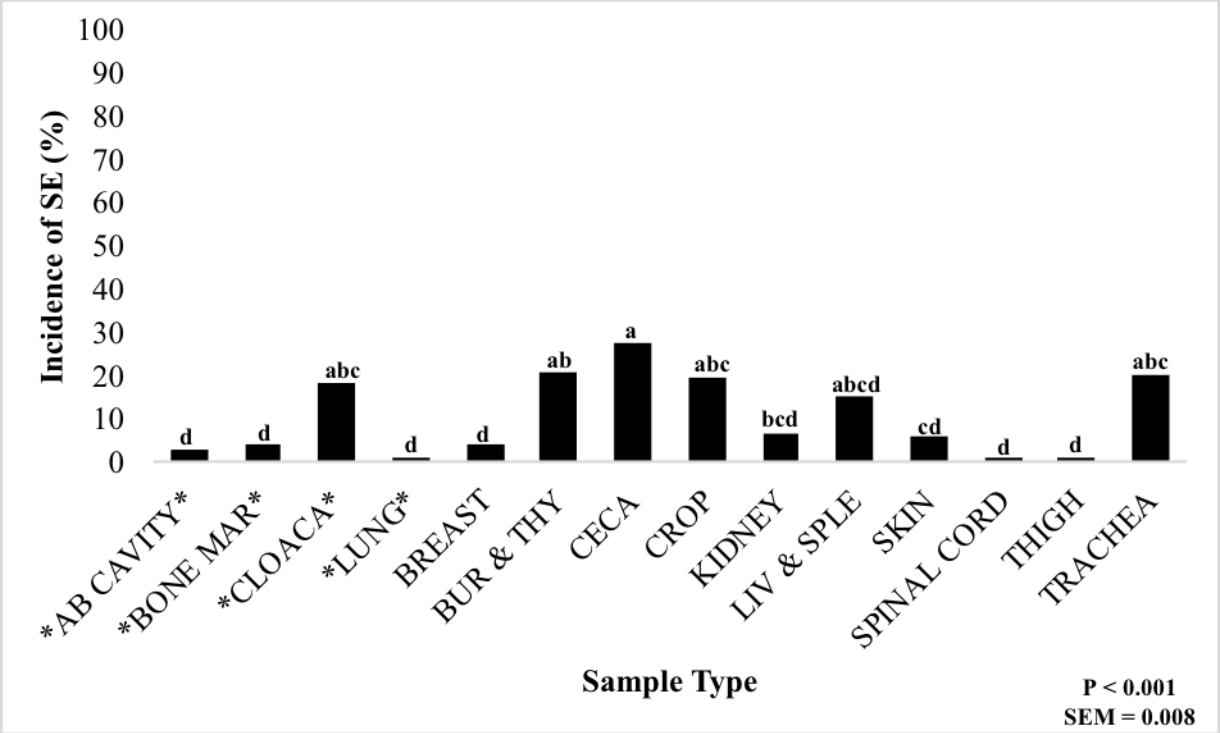

Figure 3.3: Comparison of incidence between the 14 sample types for oral inoculation

Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$.

$^{(a\text{-}d)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation

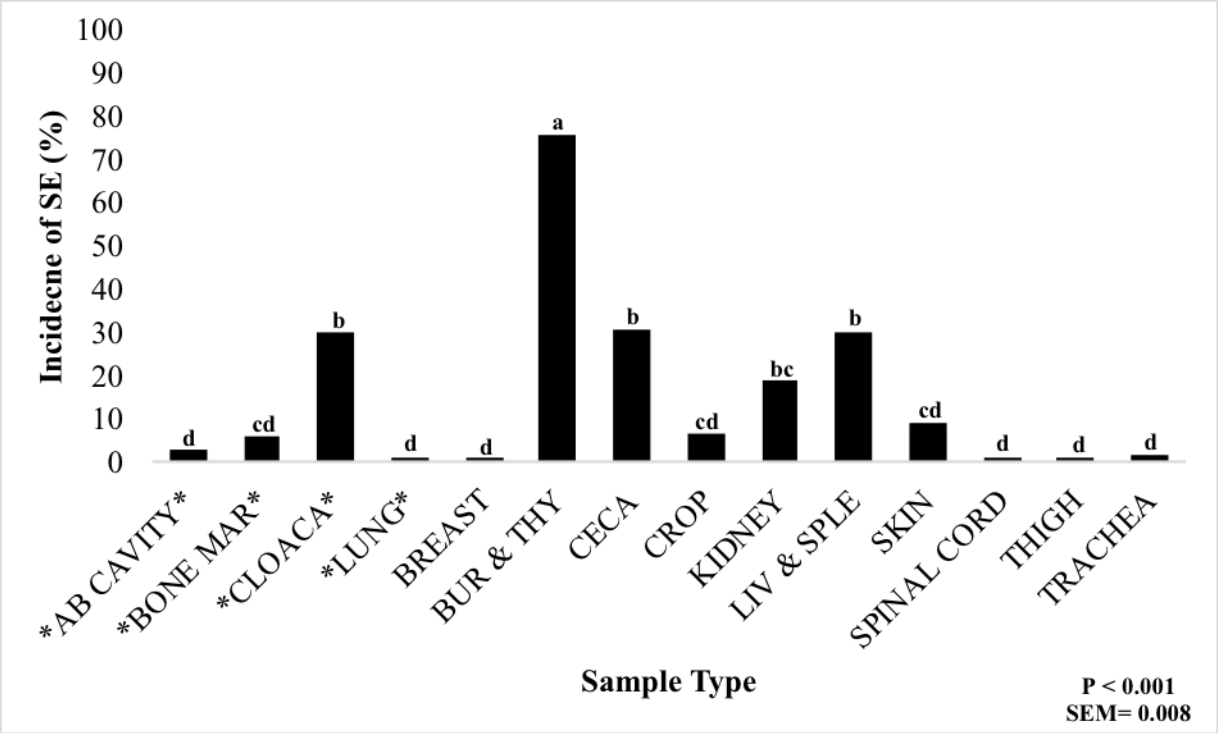

Figure 3.4: Comparison of incidence between the 14 sample types for cloacal inoculation

Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$.

$^{(a\text{-}d)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation

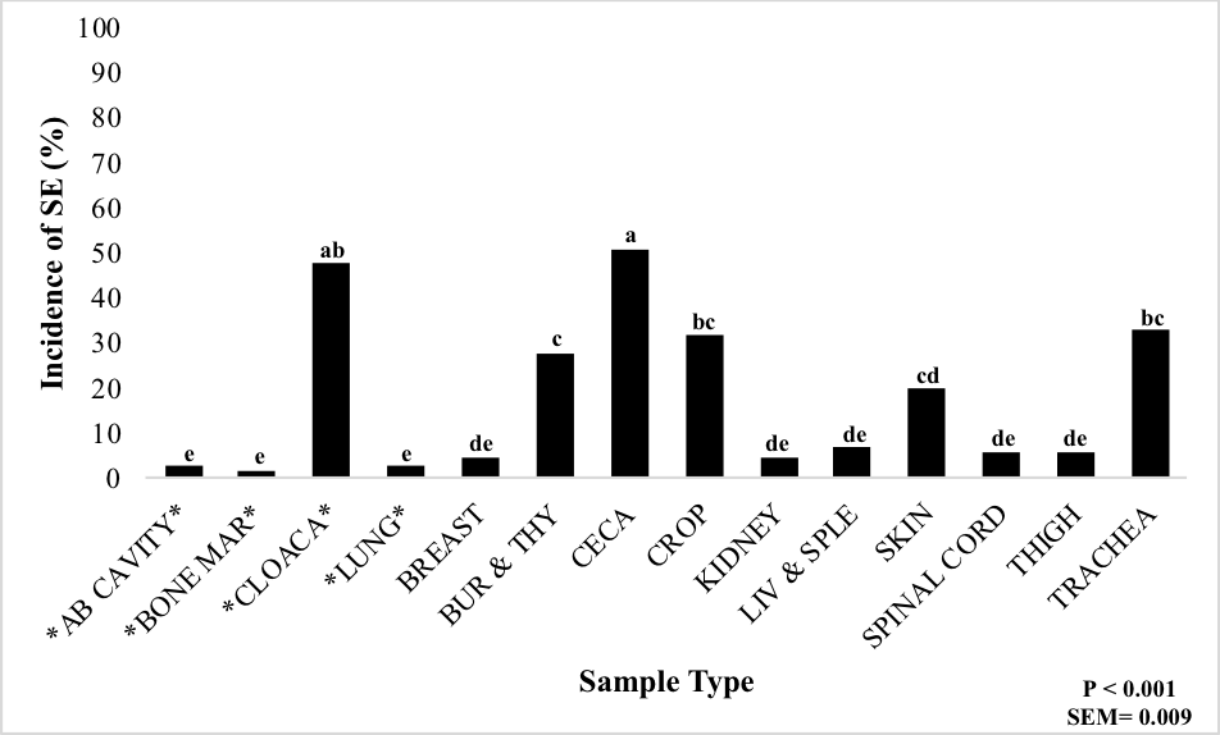

Figure 3.5: Comparison of incidence between the 14 sample types for intratracheal inoculation Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$ $^{(a\text{-}e)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation.

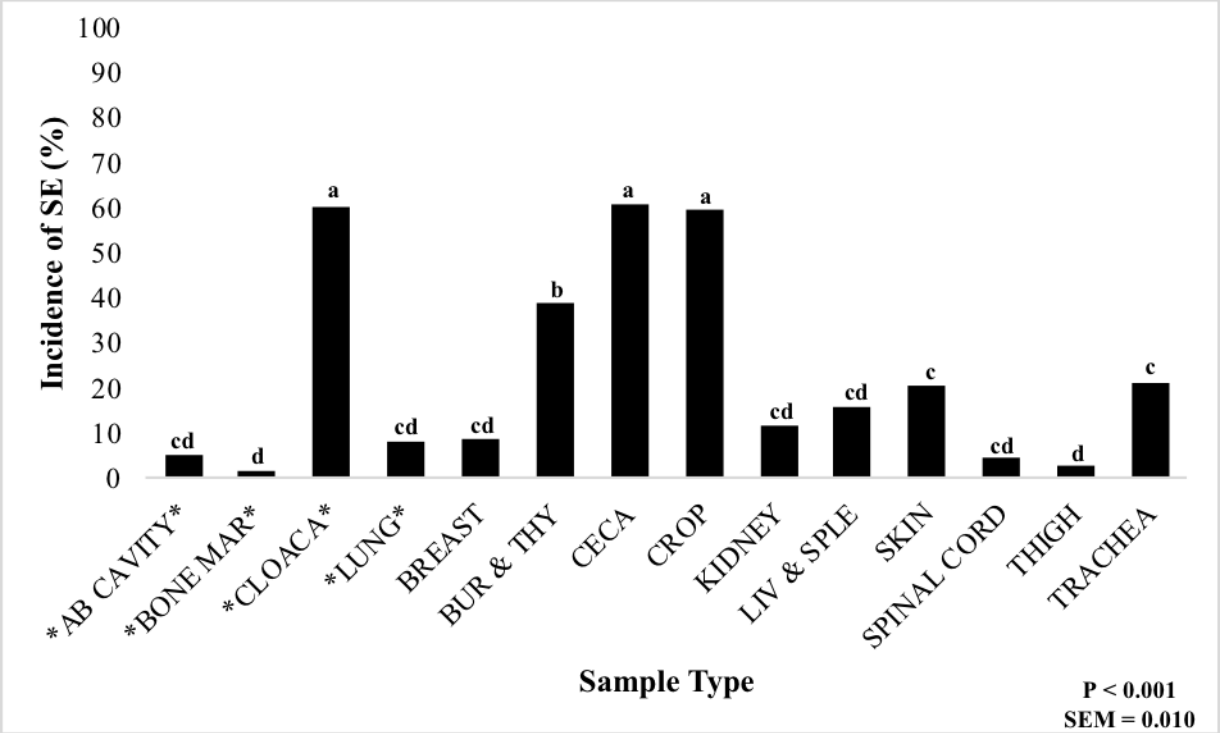

Figure 3.6: Comparison of incidence between the 14 sample types for ocular inoculation Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$ $^{(a\text{-}d)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation.

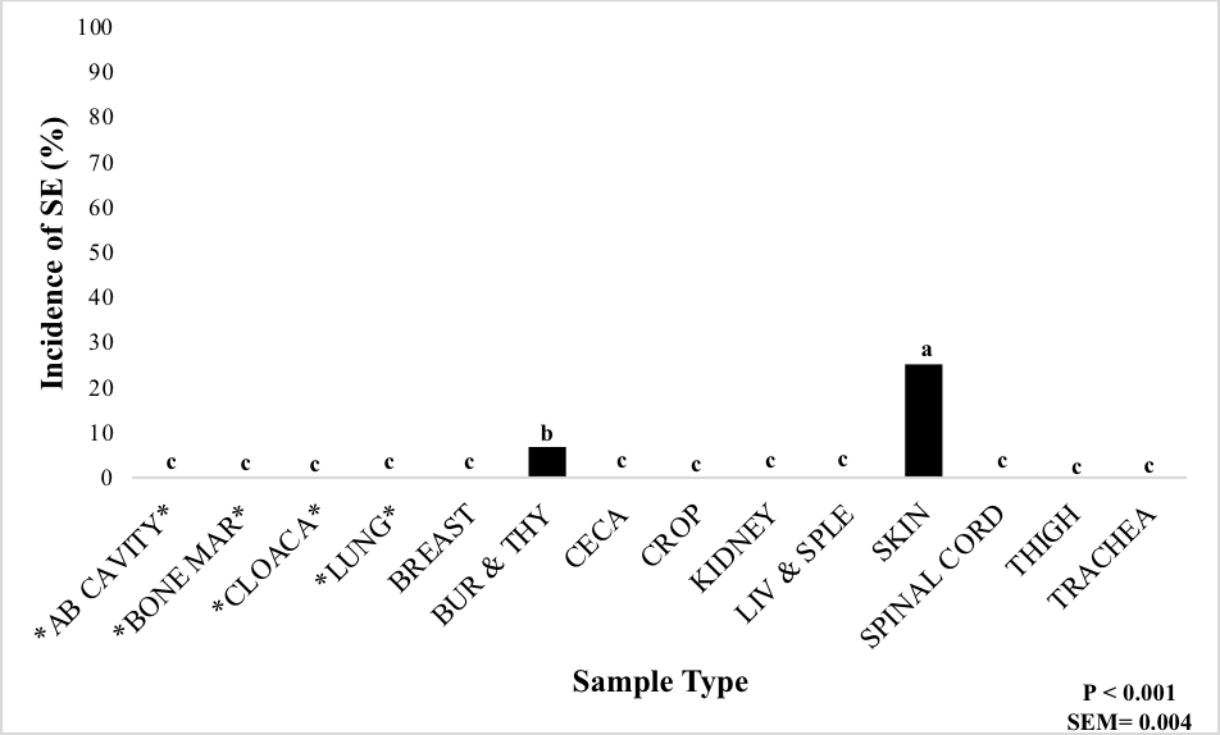

Figure 3.7: Comparison of incidence between the 14 sample types for subcutaneous inoculation Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$ $^{(a\text{-}c)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation

Chapter 4.0 A Comparison of Sites Colonized in Broilers Through Various Inoculation Routes when Challenged with *Salmonella* Heidelberg at Day 14

Introduction:

In the United States (US) alone, the Center for Disease Control and Prevention (CDC), estimates that approximately 48 million cases illnesses, 128,000 hospitalizations, and 3,000 deaths occur as a result of foodborne illness (Scallan et al., 2011). Of these, *Salmonella* remains one of the leading foodborne bacterial pathogens causing illness and death in humans. The CDC also estimates that over one million salmonellosis cases, 23,000 hospitalizations, and 450 deaths each year (CDC, 2016; Scallan et al., 2011). Additionally, the estimated financial burden due to economic losses from medical expenses, productivity, and premature deaths total $3.6 billion (USDA- ERS, 2014).

There has been more than 2500 different *Salmonella* serovars identified. Among these, *Salmonella* Heidelberg (SH) was ranked in the 12th position in the top most *Salmonella* serotypes reported to the Laboratory- based Enteric Disease Surveillance (LEDS) system (CDC, 2016). It is also among the top serovars isolated from poultry. More specifically, SH placed in the top five serovars isolated from chicken, turkey, ground chicken, and ground turkey from 1998 to 2013 (USDA-FSIS, 2014b; Foley et al., 2011). A recent outbreak of SH affecting 634 individuals was linked back to one poultry company in California from the consumption of contaminated meat (Gieraltowski et al., 2016). Additionally, egg contamination resulting from SH through egg- shell penetration or colonization of the reproductive organs has been shown (Chittick et al., 2006). Therefore, the relationship that SH contamination has with poultry and egg products support

strong evidence that poultry is a primary reservoir for this serovar (White et al., 1997). Steady increases in *Salmonella* serovars present during processing, such as SH, have led researchers to shift food safety efforts of controlling microbial introduction, persistence, and transmission towards preventing preharvest contamination (Gast, 2007).

Bailey (1987) outlined factors that may influence the susceptibility of broilers to *Salmonella* infections including: the age of the bird at the time of infection, ability of *Salmonella* to survive the gastric barrier passage, competition from other organisms, ability of the organism to locate a hospitable attachment site, broiler diet, bird health, physiological and disease status, environmental stresses (e.g. temperature, stocking density, etc.), medications administered, and host genetics. Additionally, the route of inoculation and inoculation dose have been shown to influence cecal colonization (Cox et al., 1996; Chadwick, 2017). Infection with SH can occur at any point along the poultry production continuum either vertically or horizontally. Though vertical transmission (hen to egg) may occur with low incidence, it can lead to seeder chicks that can infect chicks nearby (Cox et al., 1996). Horizontal transmission (bird-to- bird) of *Salmonella* through contact with the environment including: water, feed, litter, feces, fluff/ feathers, dust, insects, rodents, shared equipment, personnel, and other contaminated fomites create the widest variety of potential vectors during the grow-out period (Poppe, 2000).

There have been many studies available to evaluate *Salmonella* on broiler farms (Alali et al., 2010; Rodriguez et al., 2006; Liljebjelke et al., 2005; Heyndrickx et al., 2002; Bailey et al., 2001; Jones et al., 1990; Dougherty, 1976). However, greater understanding of this prevalence as it relates to the impact of the inoculation routes and tissues affected by *Salmonella* colonization is necessary for preharvest control. The impact of the inoculation route on colonization within birds have been evaluated using *Salmonella* serovars such as Enteritidis and Typhimurium with

broiler chicks (Kallapura et al., 2014a; Cox et al., 1996, Cox et al., 1990). Moreover, a great deal of these studies has also evaluated birds challenged though oral gavage with samples collected restricted to the liver, spleen, ceca, and reproductive organs. Recovery of *Salmonella* has been mostly focused on the ceca, since the bacteria is known to take residence in these areas of the gastrointestinal tract (GIT; Corrier et al., 1999). However, areas such as the crop (Hargis et al., 1995) and trachea (Kallapura et al., 2014a; Kallapura et al., 2014b) have also been shown as sites of colonization within the chicken. An evaluation of organs such as these in conjunction with other tissues that have direct food safety implications will provide necessary information for on-farm contamination and effective control.

Previous research conducted on birds challenged at day- of- hatch demonstrated *Salmonella* recovery in the ceca was significantly higher than the other thirteen investigated samples (Chadwick et al., 2017). However, the intratracheal route imposed the greatest effect on the tissues and swabs sampled after five weeks compared to the oral, ocular, cloacal, and subcutaneous routes investigated. Thus, further investigation was warranted to determine the effect on the bird age at infection on *Salmonella* colonization in older birds. Therefore, the objective of these experiments was to determine the effect of SH recovery in various tissues with birds challenged on d 14 through oral, cloacal, ocular, intratracheal, and subcutaneous routes.

Materials and Methods:

Isolation of Bacteria and Use of Cultures

Naladixic acid and novobiocin resistant *Salmonella enterica* subspecies *enterica* serovar Heidelberg (SH) utilized for the experiments. The SH isolates used were stored in a -80ºC freezer were placed onto a plate of Tryptic Soy Agar II containing 5 % Sheep Blood (BBL™, Beckon, Dickinson, and Company, Sparks, MD). They were then incubated for 18-24 h at 37ºC. The isolates were confirmed as *Salmonella* using slide agglutination with *Difco*™ *Salmonella* O

Antiserum Poly A- I & Vi (Beckon, Dickinson, and Company, Sparks, MD). The isolates were then plated onto Xylose Lysine Tergitol- 4 Agar (XLT4; Criterion™, Hardy Diagnostics, Santa Maria, CA) containing naladixic acid (100 μg/mL; Alfa Aesar, Wand Hill, MA) and novobiocin (15 μg/mL; Alfa Aesar, Wand Hill, MA (NN), for confirmation, and incubated for 18-24 h at 37ºC. A colony from the XLT4 + NN plate was selected and used to inoculate 50 mL of Brain Heart Infusion Broth (BHIB; Hardy Diagnostics, Santa Maria, CA). The BHIB was placed into a shaking incubator for 18-24 h with 200 revolutions per minute (RPM) at 37ºC. Following incubation, serial dilutions were made from the stock solution to determine the colony forming units per milliliter (CFU/ mL), which was approximately 10^9 CFU/ mL.

Broiler and Farm Management

Broilers used for the trial were housed at the Auburn University Poultry Research Farm in Auburn, AL. A total of 150 chicks for each inoculation route/experiment (150 chicks x 5 inoculation routes = 750 total) were sourced from a commercial broiler hatchery. For all experiments, chicks used were randomly allocated into five floor pens (25 birds/ pen/experiment), with excess birds placed into an unchallenged pen. For convenience, groups of two experiments were conducted simultaneously i.e. oral and cloacal; intratracheal and ocular, while the subcutaneous inoculation experiment was conducted as an individual stand-alone experiment (October 2017- June 2018). The birds were reared using standard conditions (stocking density, photoperiod, light intensity, temperature, and ventilation) appropriate for the age of the bird for the duration of the experiments. The experiments ran for a total of 34 to 36 d or 39 to 41 d, depending on when the birds were set and the time necessary to perform the necropsies.

Route of Inoculation

The inoculum was prepared as mentioned in the section above. The inoculum was adjusted to obtain a final inoculation dose of 1 x10^4 CFU per broiler and the inoculum levels were confirmed. The birds were inoculated at d 14 post-hatch throughout the experiment.

Oral: Using a one mL tuberculin syringe (BD™, Beckon, Dickinson, and Company, Franklin Lakes, NJ), each broiler was dosed with 500 µL of inoculum directly into the esophagus.

Cloacal: To inoculate via the cloaca, each broiler was inverted to access the cloaca. Using a one mL tuberculin syringe (BD™, Beckon, Dickinson, and Company, Franklin Lakes, NJ), each broiler was given 100 µL of the inoculum.

Intratracheal: Special care was taken to avoid damage to the trachea. A 20-gauge animal feeding needle attached to a one mL tuberculin syringe (BD™, Beckon, Dickinson, and Company, Franklin Lakes, NJ) was used to apply pressure to the tongue to open the passageway into the trachea. Once opened, the feeding needle was inserted and 100 µL of the inoculum was delivered directly into the trachea.

Ocular: Using a one mL tuberculin syringe (BD™, Beckon, Dickinson, and Company, Franklin Lakes, NJ), the birds were given 50 µL of the inoculum into each eye, totaling 100 µL.

Subcutaneous: To inoculate subcutaneously, a 22- gauge needle was attached to a one mL tuberculin syringe (BD™, Beckon, Dickinson, and Company, Franklin Lakes, NJ). With two pinched fingers, the skin at the nape (neck skin) was lifted, the needle inserted under the skin, and 250 µL was injected. The presence and feel of a "fluid bubble" indicated the inoculation was performed correctly.

Sampling

To maintain aseptic conditions during necropsy, all instruments (stainless steel round-end forceps, stainless-steel scissors, and stainless-steel poultry shears) were initially dipped into water, then 10% bleach, and finally 70% ethanol, respectively, before and after each tissue

collection. In addition, the instruments were cleaned and sanitized as mentioned above when contact was made with surfaces other than the appropriate tissue. The sanitizing agents and water were replaced intermittently throughout the necropsy. Cutting boards used for necropsy were washed with water, scrubbed, and sprayed with 70% ethanol following sampling of each bird. All tissues samples were collected into a sterile Whirl-Pak™ bag (*Nasco®)*; 20 mL of Buffered Peptone Water (BPW; HiMedia®, HiMedia Laboratories Pvt. Ltd, Mumbai, India) was then added to each bag and stomached onsite for one minute. The swab samples were collected using a sterile cotton swab **(**Puritan®, Puritan Medical Products Company LLC, Guilford, ME) and then placed into five mL of BPW tubes.

Samplings were performed between 20-22 (34- 36 d of the experiment) or 25-27 (39-41 d experiment) days post inoculation (DPI). Randomly selected broilers (n=100, 20 broilers/pen x 5 pens/experiment) were used for necropsy. Each bird was given a unique identifier to differentiate during necropsy. The birds were euthanized using a carbon dioxide asphyxiation. Once confirmed dead, cloaca swabs were collected using a swab and this was placed into five mL of BPW. The birds were then dipped into a quaternary ammonia immersion solution from the base of the neck near the shoulders down to the feet sanitize and remove excess dirt, feathers, and fecal material from the body.

Necropsies were carried out utilizing a two-person sampling system for the following 14 tissue and swab samples collected: abdominal cavity swab (ab cavity), bone marrow swab (bone mar), cloaca swab (cloaca), lung swab (lung), breast, bursa and thymus (bur and thy), ceca, crop, kidney, liver and spleen (liv and sple), skin, spinal cord, thigh, and trachea. Person A was responsible for all the samples pertaining to the head and leg: skin, trachea, thymus (pooled with bursa), and spinal cord and leg: thigh meat and bone marrow swab. Person B was responsible for

all the samples pertaining to the body: crop, breast meat, abdominal cavity swab, lung swab, liver and spleen (pooled), ceca, bursa (pooled with thymus), and kidney.

Person A:

Following immersion into the quaternary ammonia solution, neck feathers were plucked to the base of the head to expose the neck skin. Using the shears, the neck of the bird was cut off, near the base of the neck being careful to avoid cutting the crop. The bird was allowed to bleed out into kill cones. At which point, one of the legs was also removed for further sample collection. Approximately 5 cm^2 of neck skin (representative skin sample) was removed from the pre-plucked neck section and placed into a bag. To access the thymus, the fold of skin previously cut from the neck skin was then cut upwards, parallel to the side of the neck to expose the thymus. A portion of the thymus, approximately 3-4 cm^2, was removed and placed into the bag with the bursa (pooled samples). Following removal of the thymus, all remaining skin attached to the neck was removed. The bird was laid flat against the side of the cutting board to expose the trachea. Once exposed, the trachea was then pulled away from the neck and approximately 3-4 cm^2 was removed and placed into a bag. With the head held flat against the cutting board, the neck was cut upwards using the shears, perpendicular to the beak to expose the spinal cord. Approximately 2-3 cm^2 of the spinal cord was removed and placed into a bag.

To sample the thigh meat, skin overlaying the muscle was peeled back with connective tissue also cut to free skin and feathers, as they could act as a potential source of contamination. Approximately 3-4 cm^2 of the thigh meat was removed and placed into the bag. To access the bone marrow, the femoral head was cut at an angle using the shears. A swab was then used to dig into the bone marrow and then placed into a BPW tube.

Person B:

Once bled, the skin at the cranial portion of the body was cut open to expose the crop. Approximately 3-4 cm^2 of the crop was taken from the bird and placed into a bag, with excess feed and/or water removed using the back of the scissors. With the forceps, the skin overlaying the breast tissue was peeled back with connective tissue also cut along the way to free the skin and feathers away from the body, as they could act as a potential source of contamination to the rest of the body. Approximately 3-4 cm^2 of the breast tissue was removed and placed into a bag. The ribs of the bird were cut open using the shears and an abdominal swab was taken and placed into a BPW tube. Using a swab, a section of the lung was stabbed and placed into a BPW tube.

To remove the liver and spleen, a lobe of the liver was drawn away from the body and approximately 3-4 cm^2 was removed and placed into a bag. To access the spleen, the intestines were pulled away from the body cavity and inverted to expose the spleen, with caution not to pull the intestines completely out of the body cavity, as this could be a potential source of contamination. The base of the spleen was grabbed and the entire organ was removed and placed into a bag. The ceca tonsils, proximal to the spleen, were exposed following unraveling the intestine. One of the cecal tonsils were cut at the cecal neck to avoid leakage of its contents into the body cavity and approximately 5-6 cm^2 was cut, removed, and placed into the bag. Once removed, the intestines were then completely pulled away from the body cavity to expose the bursa. Approximately all of the bursa was removed and placed into a bag. The kidney presented a greater challenge to remove, as they are so delicate, difficult to remove, and incorrect removal could result in shredding of the organ. Therefore, removal of the kidney occurred using the scissors to dig underneath the organ to remove a substantial section; it was then placed into the bag. Following sampling of challenged birds, five birds were randomly selected from

unchallenged pens and cloaca swabs were taken to determine horizontal transmission to unchallenged birds.

Microbiological analysis

All stomached tissue samples were stored at 4ºC until further analysis. The samples were incubated for 18-24 hours at 37ºC in 20 mL BPW. Following pre-enrichment, one mL of each sample was placed into five mL of Tetrathionate Brilliant Green Broth tubes (TTB; HiMedia®, HiMedia Laboratories Pvt. Ltd, Mumbai, India) and incubated for 48- 72 h at 37ºC. Enriched samples were then streaked onto XLT4+ NN plates using 1 µL disposable loops (VWR International, LLC, Radnor, PA) and incubated for 48 h at 37ºC. From those plates, isolated colonies demonstrating typical *Salmonella* colonies were slide agglutinated with *Difco*™ *Salmonella* O Antiserum Poly A- I & Vi (Beckon, Dickinson, and Company, Sparks, MD). The presence of SH was indicative of a score of one (positive), whereas a score of zero (negative) was indicative of SH absence.

Data Analysis

All statistical analyses were conducted using IBM® SPSS® software version 22. Data pertaining to a percentage of birds positive overall for the challenge, a percentage of samples positive overall for the challenge (calculated by dividing the total number of positive samples by the total number of samples collected for each inoculation), and a comparison of differences in incidence among each sample types for SH for each inoculation were analyzed using a General Linear Model (GLM). Significant differences were reported at $P \leq 0.05$, and if applicable, means were separated using Tukey's HSD.

Results:

Table 4.1 summarizes recovery of SH for all fourteen tissue and swab samples collected after the inoculation of birds on d 14. SH recovery from the abdominal cavity swab, kidney, liver

and spleen, spinal cord, and thigh were not significantly different across inoculation routes ($P > 0.05$). For the nine remaining samples, significant differences were noticed between inoculation routes ($P < 0.05$).

Bone marrow swab and cloaca samples formed more distinctive groups between routes, with significant differences occurring between the groups ($P < 0.05$). For bone marrow swab samples, the greatest incidence occurred in bird challenged through the intratracheal route with 8.16% incidence. The remaining four routes were the least affected with incidence varying from 0.00 to 2.04% ($P > 0.05$). The ocular (23.00%) and intratracheal (14.14%) routes were affected the greatest of the cloaca swab samples ($P > 0.05$). In contrast, the oral, cloacal, and subcutaneous routes had significantly lower incidence with 0.00, 1.00, and 0.00%, respectively.

The remaining samples displayed less distinctive groups, indicating interactions between routes occurred. The greatest incidence in lung swab samples occurred in birds challenged through the intratracheal route (4.00%). The next highest incidence occurred in birds challenged through the ocular route (1.00%), though no significant differences were shown between the intratracheal route and the other routes in the remaining routes i.e. oral, cloacal, and subcutaneous with 0.00% incidence. For the breast samples, the greatest incidence occurred in the ocular route (5.00%) and the least incidence occurred in the intratracheal and subcutaneous routes with 0.00%. However, incidence in birds challenged through oral (1.01%) and cloacal (2.00%) routes were not significantly different from the three previously mentioned routes ($P > 0.05$). Incidence in the bursa and thymus samples was greatest in the cloacal (20.20%), ocular (15.00%), intratracheal (12.12%), and subcutaneous (9.09%) routes. Significant differences in incidence were only observed between the cloacal (20.20%) and oral (5.00%) routes ($P < 0.05$). Similar arrangements between the routes were observed between the ceca and crop samples. The

greatest incidence was shown in birds challenged through the ocular route (ceca: 50.51% and crop: 37.00%). The intermediate group includes birds challenged through the intratracheal (ceca: 18.00% and crop: 14.00%), oral (ceca: 6.00% and crop: 5.00%), and cloacal (ceca: 8.00% and crop: 3.00%). However, incidence in birds challenged subcutaneously (ceca: 1.00% and crop: 0.00%) were not significantly different from incidence in the oral and cloacal routes ($P > 0.05$). For the skin samples, incidence was highest in the ocular (16.00%), subcutaneous (14.29%), and intratracheal (10.10%) inoculations and lowest in the cloacal (4.00%) and oral (0.00%). The intratracheal route (42.42%) significantly higher incidence among the trachea samples.

Figure 4.1 illustrates the percentage of birds positive for SH and highly significant differences were observed ($P < 0.05$). The ocular and intratracheal routes produced the highest percentage of birds positive with 73 and 67%, respectively. The percentage of positive birds in the ocular and intratracheal routes were significantly higher than the other routes ($P > 0.05$). The subcutaneous, cloacal, and oral routes formed a group with 30, 28, 16% of positive birds, respectively ($P > 0.05$).

As illustrated in Figure 4.2, the overall percent incidence in descending order was 12.8, 10.4, 4.2, 2.2, and 1.9% for the ocular, intratracheal, cloacal, subcutaneous, and oral routes, respectively. Highly significant differences in the percent incidence were shown ($P < 0.05$). Highly significant differences among the fourteen tissue and swab samples were observed for each of the routes ($P < 0.05$) and are presented in Figures 4.3-4.7.

Birds challenged through the oral route (Figure 4.3) had the greatest recovery of SH in the ceca (6.00%) With such a low incidence among samples, no significant differences in incidence were shown among them ($P < 0.05$). Birds infected through the cloacal route had the had the greatest recovery of SH in the bursa and thymus (20.20%; Figure 4.4). Incidence within

these samples were significantly higher than all other samples ($P < 0.05$). The next group of samples with no significant differences in incidence include the ceca, kidney, trachea, liver and spleen, and the skin with 8.00, 6.00, 6.00, 4.00, and 4.00% incidence, respectively ($P > 0.05$). Figure 4.5 displays sample incidence of birds challenged through the intratracheal route. The greatest incidence occurred in the trachea samples (48.00%) and incidence was significantly different from all other samples ($P < 0.05$). Significant differences in recovery of SH were not shown in incidence between the ceca (18.00%), cloaca (14.14%), crop (14.00%) and bursa and thymus (12.12%) samples ($P > 0.05$). SH was not recovered (0.00%) in the breast and spinal cord samples. In birds challenged through the ocular route, incidence occurred in all fourteen tissue and swab samples (Figure 4.6). The greatest recovery of SH occurred in the ceca (50.51%) followed by the crop (37.00%; $P > 0.05$). Incidence within these tissues were significantly different from all other samples. Recovery of SH within the skin (16.00%) and bursa and thymus (15.00%) samples were similar in incidence to the cloaca swab samples (23.00%; $P > 0.05$). In Figure 4.7, birds challenged through the subcutaneous route formed two significantly different groups. The first group ($P > 0.05$) consisted of the skin (14.29%), followed by bursa and thymus (9.09%). Incidence in the second group, which consisted of the twelve remaining samples, ranged from 0.00 to 3.03%.

Discussion:

Historically, investigation of *Salmonella* colonization in poultry has been most often performed by oral administration (Kallapura et al., 2014a). Within the grow- out facility, *Salmonella* can be ingested through many introductory vehicles, not limited to feed alone, but also including water, litter, feces, feathers, and others (Poppe, 2000). Once entry has been made into the body, acidic conditions and digestive enzyme production found within the upper GIT can be buffered by proteins and lipids found within ingested products such as meat and bone

meal and soybean meal (Ha et al., 1998). If these products contain *Salmonella*, there is a chance that the bacteria may be protected. Successful movement of *Salmonella* through the proventriculus and the gizzard can result in colonization of favorable attachment sites found within the small intestine and ceca. As a result, intermittent shedding of these organisms may occur, perpetuating the cycle of excretion and ingestion by other birds within the same facility. Though important, other portals of entry into the bird may offer greater incidence of the *Salmonella* within investigated tissues.

Although frequently investigated, birds in the present study challenged with SH at d 14 by oral inoculation (1.9% incidence) had the lowest incidence when compared to the four routes investigated (Figure 4.2). Additionally, only 16% of birds challenged were positive for the organism (Figure 4.1). In comparison to other studies, this value is low. In day- old chicks challenged orally with 1 x 10^4 CFU of *Salmonella* Typhimurium (ST), cecal colonization incidence was 93% and on day- three cecal colonization incidence was 39% (Cox et al., 1990). Cox et al. (1996) found that 95.7% of day- old broiler chicks inoculated with SE were positive when evaluated one- week post inoculation. In comparison to other research, these results demonstrate that the birds were not largely affected by oral inoculation with SH. Birds challenged in older stages of life, compared to younger birds, are expected to be more resistant to colonization resulting from acquired immunity and competitive exclusion from an established microflora (Cox et al., 1990). However, such dramatic differences in incidence when compared to other studies could result from a number of factors attributed to differences in pathogenicity between SH and other serovars such as the of survivability of the organism through the gastric barrier and ability to effectively colonize internal organs (Bailey, 1987).

Salmonella infection through the cloaca, or cloacal inoculation, is one of the more unique orifices *Salmonella* and other pathogens can use to enter the body. In comparison to the oral route, the cloaca, a common cavity for excretory and digestive waste products, offers direct access to areas such as the ceca. The tissues are known to be favorable colonization sites of most enteric pathogens because of nutrient availability (Dunkley et al., 2009). Infection through the cloaca may occur once the cloacal region encounters material possibly containing *Salmonella* (Cox et al., 1996). This material agitates the sphincters found within this region leading to contraction of these tissues. Once contracted, material can be rapidly drawn into the body cavity through anti- peristaltic reflex action (Cox et al., 1996; Schaffner et al., 1974). Bacterial cells that make entry through this orifice can bypass the acidic conditions found within the upper regions of the GIT (Cox et al., 1990). Bypassing acidic conditions and direct entry to a favorable colonization site may allow for a greater number of non- injured cells available for colonization (Cox et al., 1990). In broiler chicks, inoculation through the cloaca with two ST cells led to colonization of cecal tissues and incidence was comparable to birds challenged orally with 100 cells (Cox et al., 1990). In another study, broiler chicks inoculated via the cloaca with ST led to colonization of 84% of the chicks, which is 3- fold higher than colonization of birds in the present study (28%) (Cox et al., 1996; Figure 4.1). Differences can be attributed to age of bird at the time of infection and the differences in pathogenicity of the the isolates utilized for the experiments i.e. ST vs. SH.

Contact with bursal tissue during cloacal inoculation is inevitable due to location of these tissues to the cloacal lumen (Schaffner et al., 1974). Thus, significant differences in incidence were observed between the bursa and thymus (pooled) samples and the other thirteen tissue and swab samples ($P < 0.05$; Figure 4,4). However, there were no significant differences in overall

sample incidence with birds challenged through the cloaca or the oral cavity ($P > 0.05$; Figure 4.2). This indicates survivability of the bacteria was largely unaffected by entry through the cloaca or the mouth.

Intratracheal inoculation of birds is performed to simulate infection of the respiratory tract. In modern poultry production, regulating air quality geared towards removing ammonia, carbon dioxide, and dust is essential for bird comfort (Ritz et al., 2006). In attempts to remove particulate matter from the air, tunnel ventilation systems carry massive volumes of air throughout the house by negative pressure (Kallapura et al., 2014a; Chinivasagam et al., 2009). Movement of air can facilitate aerosol transmission from contaminated dust or bioaerosols present, which may then become inhaled and/ or swallowed (Kallapura et al., 2014a). The presence of these organisms within the air can be affected by the temperature and humidity found within the houses (Kallapura et al., 2014b). Furthermore, particle size of the aerosols can influence the systems within the body affected. Smaller particles (<1 μm in size) are likely to pass through the nasal cavity and infect the alveoli and the lung (Chinivasagam et al., 2009). On the contrary, the fraction of aerosols greater than seven micrometers are more likely to stay confined to the upper respiratory tract, reach the pharynx, and enter the gastrointestinal tract (Chinivasagam et al., 2009). In this case, the bird would receive a combined effect of simultaneous infection of the respiratory and GI tracts.

As an intratracheal challenge high incidence in the trachea (42.42%) is expected and was significantly higher than all other investigated samples ($P < 0.05$). In a study by Kallapura et al. (2014a), tracheal samples collected from seven-day old birds challenged with ST and a similar inoculation dose as the birds in the present study resulted in 66.66% incidence. Differences could be attributed to the pathogenicity of the isolates used. Additionally, differences could also be

attributed to the time in which the necropsies occurred. In the present experiment, necropsies were performed approximately 21 DPI and during that time period bacterial clearance is expected. In the previously mentioned study, necropsies were performed 24 h post inoculation and any bacteria present during this time period inoculation may have been transient. In spite of the differences in the results between the experiments, the incidence found within the trachea may result from the inability of macrophages to effectively clear the bacteria from these tissues (Mensah and Brain, 1982). This response is coupled with an apparent lack of inflammation in the this region and may ultimately allow for persistence in these tissues for a greater period of time (Okamura et al., 2005).

Tracheal incidence in the present study was significantly greater ($P > 0.05$) than incidence within the ceca and the crop, which are considered most susceptible to *Salmonella* colonization (Corrier et al., 1999; Hargis et al., 1995). These results offer validation to findings from Kallapura et al. (2014b) using the trachea as an indicator organ in *Salmonella* infection. In different study, day- old chicks challenged with 100 cells of ST led to colonization in 53% of the birds (Cox et al., 1996), in comparison to the present study with 67% using a 1×10^4 dose of SH. Differences may be attributed to susceptibility of the bird due to age at infection and the pathogenicity from the use of different isolates.

Ocular or conjunctival inoculation can occur when contaminated dust or bioaerosols are moved through the house by negative pressure (Kallapura et al., 2014a). If particles containing *Salmonella* or other pathogens land in the eye, drainage into the nasolacrimal or tear ducts may occur (Humphrey et al., 1992). Similar to the intratracheal inoculation, this could result in introduction of the organism into the respiratory and digestive tracts, simultaneously (Kallapura et al., 2014a). Experimental investigation of the ocular route has been limited or not widely

supported (Chart et al., 1992). Despite this, birds in the present study challenged through ocular inoculation had the greatest incidence of positive samples (12.8%; Figure 4.2) and the greatest number of positive birds (73%; Figure 4.1). Day- old chicks challenged with 100 cells of ST through the ocular route resulted in 88% percent of the birds positive, which is slightly greater than percentages found in our study (73%). Differences can be attributed the age at which the birds were inoculated and serovar utilized for the experiments (Cox et al., 1996).

Infection through the ocular route also produced the greatest incidence compared to the other inoculations in five of the fourteen tissue and swab samples collected (shown in Figure 4.6): ceca (50.51%), cloaca (23.00%), crop (37.00%), breast (5.00%), and skin (16.00%). Incidence of the crop, ceca, and cloaca demonstrate that infection through this route can result in simultaneous infection of the respiratory and GI tracts. Humphrey et al., (1992) found that ocular inoculation of laying hens with SE resulted in a systemic infection with the reproductive organs, kidney, liver and spleen, jejunum, ileum, and the ceca affected up to 28 days post inoculation. However, this was not shown in the present experiment since the filtration organs indicative of systemic infection, namely the liver and spleen and kidney, were not widely affected (5.00%; Table 4.1). Differences in incidence between the two studies could result from the age of the bird i.e. laying hens versus two- week old broilers and the isolate used. In another study, a poor immune response was observed in laying hens challenged with 1×10^3 CFU of SE and low titers of IgM antibody production in birds challenged with 1×10^8 (Chart et al., 1992). Though antibody levels of birds in our study were not measured, 15% percent incidence within the bursa and thymus samples (Figure 4.6) imply that an immune response may have been triggered in some of the birds or may indicate presence of the bacteria within these tissues.

Experimental subcutaneous inoculation of *Salmonella* in poultry is most often useful in determining vaccine efficacy (Desin et al., 2014). However, very few studies, if any at all, fail to investigate the subcutaneous route as a viable mode of *Salmonella* transmission within poultry. Infection may occur if birds are scratched with *Salmonella*- contaminated materials and frequency of scratching can be increased with birds kept at higher stocking densities (Elfadil et al., 1995). Despite this, subcutaneous inoculation has been shown to cause localized infection (Chadwick, 2017). The overall incidence of was 2.2% with 30% of the birds affected, shown in Figure 4.2 and Figure 4.1, respectively, which is comparable to birds changed through the cloacal and oral routes ($P > 0.05$).

Samples greatly affected by subcutaneous inoculation are the skin (14.29%) and bursa and thymus (9.09%); however, organs such as the liver and spleen (3.03%), trachea (1.02%), abdominal cavity (1.01%), bone marrow (1.00%), and ceca (1.00%), were affected but with low incidence (Figure 4.7). Incidence in the skin area can be attributed to the administration of the bacteria in that area for the inoculation, which was also the sample collection point. Attempts of the body to clear the bacteria could have provoked an immune response in a few of the birds, leading to colonization within the bursa and thymus tissues (Dunkley et al., 2009). Yet, low or no incidence found within the other tissues indicate that once inoculated subcutaneously, the bacteria primarily stayed confined to the site of inoculation. This localization effect is caused by the lack of available blood vessels found within the skin that are necessary to transport the organism throughout the body to cause a systemic infection.

The five previously discussed inoculations (i.e. oral, cloacal, intratracheal, ocular, and subcutaneous) represent ways *Salmonella* can entire the body. Traditionally, the oral route has been the most commonly investigated route. However, the data presented imply that infection

with other routes may have a greater potential effect on individual samples. Therefore, it is important to determine the effect on tissues based on what has been shown from the routes and possible implications.

Once ingestion has occurred, the bacteria must adhere to favorable colonization sites through cell receptors found in the intestinal mucin (Craven and Williams, 1997). The ceca are often regarded as the standard for *Salmonella* recovery in the body due to the favorable conditions provided to the bacteria in this area, such as nutrient availability (Kallapura et al., 2014a; Josefiak et al., 2004; Corrier et al., 1999; Hargis et al., 1995; Snoeyenbos et al., 1982; Fanelli et al., 1971). Presence in these tissues without producing inherent clinical signs of infection contribute to the commensal nature of this organism within chickens (Andino and Hanning, 2015). Therefore, incidence within these tissues is unsurprising. Though, in three of the five inoculations cecal incidence was not the greatest (subcutaneous: 1.00%; cloacal: 8.00%; intratracheal: 18.00%; Table 4.1). Cloacal samples are often taken due to shedding of *Salmonella*, which may be increased in stressed birds (Van Hoorebeke et al., 2009). However, cloacal swabs may not accurately reflect *Salmonella* in birds that may not be actively shedding the bacteria i.e. *Salmonella* carriers (Van Hoorebeke et al., 2009; Van Immerseel et al., 2004). This observation was found in the present experiments with the SH incidence found within the ceca unparalleled to incidence observed with cloacal swabs.

Aside from the ceca, Hargis et al. (1995) identified the crop as a major source of *Salmonella* contamination at processing because overall contamination of the crop was about three and a half times greater than incidence within the ceca in commercial birds (52% vs. 14.6%). Birds left without feed prior to processing to reduce fecal contamination can cause birds to peck at the litter in search of dropped feed (Corrier et al., 1999). Though beneficial for

reduction of cecal contamination, the amount of colonization of the crop increases in with increases in the pH, promoting growth and invasion of pathogens within this tissue (Kieronczyk et al., 2016). Moreover, rupturing of the crop could result in contamination of the carcass, which has been found to be 86 times more likely than rupturing of the ceca (Hargis et al., 1995). However, though not significantly different ($P > 0.05$), numerical differences between incidence of SH colonization within the ceca and the crop indicate that crop samples could be used in conjunction with ceca samples to detect the presence of *Salmonella*.

The skin of the bird can be utilized as an indication of external *Salmonella* contamination. Prior to processing, birds may sit in contaminated material or transportation crates containing fecal material may contaminate the birds (Rigby and Petit, 1980). Upon arrival at the processing facility, bacteria already present on the skin may lodge themselves into the feather follicles making them practically inaccessible to antimicrobial rinsing (Lillard, 1989b). Inoculations with the greatest incidence of SH in the skin are birds challenged through the ocular, subcutaneous, and intratracheal routes with 16.00, 14.29, and 10.00%, respectively (Table 4.1). Aside from the subcutaneous route causing incidence resulting from the inoculations, the incidence in the skin from the other routes could result from invasion or presence on the outside of the bird. Wu et al. (2014) found that 21.4% of the 300 neck skins evaluated from *Salmonella*- confirmed breeders were positive. Differences could be attributed to sampling which led to the inclusion of more invasive serotypes such as Enteritidis and Typhimurium. Food safety concerns also arise from *Salmonella* in skin because it is added to non- mechanically separated (non- MSC) ground chicken to obtain a higher fat percentage (Park et al., 2017). Depending on the country, methods of detection within the processing facility may

be slightly different, though both ways are designed to detect these external *Salmonella* (Cox et al., 2010).

The trachea, similar to that of the crop, is also viable indicator of *Salmonella* presence but with emphasis on transmission through the respiratory route (Kallapura et al., 2014a; Kallapura et al., 2014b). As expected, the birds challenged through the intratracheal route have the greatest incidence within the trachea, which was higher than cecal incidence. This is similar to the intratracheal study conducted by Kallapura et al. (2014a) with birds challenged at all inoculation doses with ST. In birds challenged with SE, the opposite effect was noticed in which the cecal incidence was higher than that of the trachea, similar to all other inoculations in the present study. Thus, tracheal samples collected from birds challenged through aerosol would be reflective of *Salmonella* presence in the body; however, in all others the trachea could be collected in conjunction with the ceca to observe an effect on the respiratory system.

The lung tissue and air sacs could be areas affected through exposure from contaminated aerosols. Smaller particles present in the environment, passing down into the lung could take residence and colonize (Chinivasagam et al., 2009). In broiler chicks, lungs of infected birds challenged by oral, respiratory, and intratracheal routes were 18.00, 34.00, and 16.00%, respectively (Cox et al., 1996). However, birds in our experiments were not widely affected by the SH challenge, with the exception of birds challenged through the intratracheal route ($P < 0.05$), and therefore may not be a significant area of recovery in the birds. *Salmonella* infected air sacs could be an indication of systemic infection because of the placement of these organs throughout the body, but significant SH recovery within these organs were not observed ($P > 0.05$)

Salmonella present within the filtration organs of the chicken, namely the liver, spleen, and kidney indicate that the bacteria has penetrated the mucosal epithelium, facilitated by triggering inflammation of these tissues (Bohez et al., 2007). The evolved mechanism of survival intracellularly in macrophages allow evasion from humoral and complement- mediated attacks, sheer- stress induced clearance, and access to host cell nutrients (Ribet and Cosart, 2015). Once penetration has occurred, bacteria, toxins, and nutrients travel to the liver via the hepatic portal vein, leading to circulation throughout the body through the bloodstream and can reach other organs such as the kidney and spleen. Food safety implications can also occur from the consumption of these organs. There is a market for chicken liver because of the versatility in its preparation, but when undercooked or contaminated it poses a threat to food safety (Lanier et al., 2018). The consumption of kidneys as part of whole chicken legs or consumed through whole carcass ground MSC also pose a threat to food safety. Although food safety implications could occur, generally incidence within these organs were not high. Additionally, incidence within the filtration organs were not widely affected by the inoculation route ($P > 0.05$). Therefore, the lack of presence within these organs between the routes indicate that the bacteria was not invasive.

Salmonella contamination of intact muscle such as the breast and thigh most often result from spillage of ingesta from the crop and digesta from the intestines and ceca onto the outside of the carcass (Buncic and Sofos, 2012). SH incidence within the thigh tissue was not significantly different between each of the inoculations ($P > 0.05$). However, differences were highlighted between breast tissue samples ($P < 0.05$). Leach et al. (1999) observed intact muscle contamination attributed to septicemia only in birds challenged through aerosol inoculation and not through oral inoculation. However, this was not consistent between both of the routes that are utilized to simulate aerosol inoculation. In other words, incidence within the thigh samples of

both the ocular and intratracheal routes did not parallel incidence within the breast samples in the same routes.

The spinal cord and the bone marrow both represent organs not typically regarded as an area known to house resident *Salmonella* cells. However, inclusion of these organs into ground chicken products from the backs and necks of MSC carcass make *Salmonella* presence a probable food safety concern (Alali et al., 2016). Recovery in the spinal cord was not significantly different between the inoculation routes ($P > 0.05$), which imply that the spinal cord may not be a significant area of recovery within this tissue. On the contrary, statistical differences were noticed in the bone marrow ($P < 0.05$) and was greatest in birds challenged through the intratracheal route ($P < 0.05$). Previous research conducted found incidence in the bone marrow as high as 20% in orally infected birds (Kaseem et al., 2012) and as low as 0.7% in commercial flocks (Wu et al., 2014). The bone marrow of birds acts as site for new cell production and also is a lymphatic organ that produces new lymphocytes (Campbell, 1967). The incidence of SH within the bone marrow corresponds ($P > 0.05$) to other organs in the body that imply a systemic infection has occurred, namely the liver and the spleen.

Anatomically, the "bursa of Fabricius" is the lymphoid organ located near the cloaca. In young birds, the B lymphoyctes in the bursa are destined for use in humoral or antibody-mediated immunity (Ribatti, 2017). The thymus is a lymphoid organ located in the neck region of the bird and is the site of maturation and differentiation of T lymphocytes which are involved with cell- mediated immunity. Incidence within these tissues indicate that an immune response has been triggered, though antibody levels were not measured in this experiment. The greatest incidence of SH in these tissues occurred in birds challenged through the cloacal route (20.20%; Table 4.1), which can be attributed to anatomy; however, it was similar to birds challenged

through the ocular (15.00%), intratracheal (12.12%), and subcutaneous routes (9.09%) ($P > 0.05$). As an intracellular pathogen of macrophages, detection of the *Salmonella* in the body is mediated through T- cells, though Berndt and Methner (2004) revealed the participation of both humoral and cell- mediated immunity occurred for successful clearance of ST in five- day old orally infected chicks.

Conclusion:

Introduction of SH can occur at any point during rearing including within the hatchery, during grow-out, and prior to entry into the processing facility. Portals of entry into the body including through the mouth, cloaca, respiratory route (nares, eye, and mouth), and skin can lead to differences in colonization of tissues when challenged. Though likely birds in a commercial setting birds could be exposed through many routes at varying times, our results imply that when challenged at day 14 the ocular route produces the greatest overall incidence in birds and produced relatively high incidence in the ceca. Therefore, strategies to further evaluate contamination through the aerosol route should be further investigated.

	Oral (%)	Cloacal (%)	Ocular (%)	Intra-tracheal (%)	Subcuta-neous (%)	P-value (P ≤ 0.05)
ABDOMNIAL CAVITY	0.00	0.00	1.00	2.00	1.01	0.479
BONE MARROW	0.00^{b}	1.00^{b}	2.04^{b}	8.16^{a}	1.00^{b}	**0.001**
CLOACA	0.00^{b}	1.00^{b}	23.00^{a}	14.14^{a}	0.00^{b}	**<0.001**
LUNG	0.00^{b}	0.00^{b}	1.00^{ab}	4.00^{a}	0.00^{b}	**0.017**
BREAST	1.01^{ab}	2.00^{ab}	5.00^{a}	0.00^{b}	0.00^{b}	**0.028**
BURSA & THYMUS	5.00^{b}	20.20^{a}	15.00^{ab}	12.12^{ab}	9.09^{ab}	**0.029**
CECA	6.00^{bc}	8.00^{bc}	50.51^{a}	18.00^{b}	1.00^{c}	**<0.001**
CROP	5.00^{bc}	3.00^{bc}	37.00^{a}	14.00^{b}	0.00^{c}	**<0.001**
KIDNEY	2.00	6.00	5.00	5.00	0.00	0.14
LIVER & SPLEEN	3.00	6.00	5.00	10.00	3.03	0.169
SKIN	0.00^{b}	4.00^{b}	16.00^{a}	10.10^{ab}	14.29^{a}	**<0.001**
SPINAL CORD	0.00	3.06	3.00	0.00	0.00	0.215
THIGH	1.00^{ab}	3.00^{ab}	2.00^{ab}	6.00^{a}	0.00^{c}	0.061
TRACHEA	4.08^{bc}	6.00^{bc}	14.00^{b}	42.42^{a}	1.02^{c}	**<0.001**

Table 4.1 Comparison of *S.* Heidelberg recovery between the five inoculation routes

* indicates swab samples instead of tissue samples

Data were analyzed using a GLM and **bold** values indicate significant differences at $P \leq 0.05$.

$^{(a\text{-}c)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation

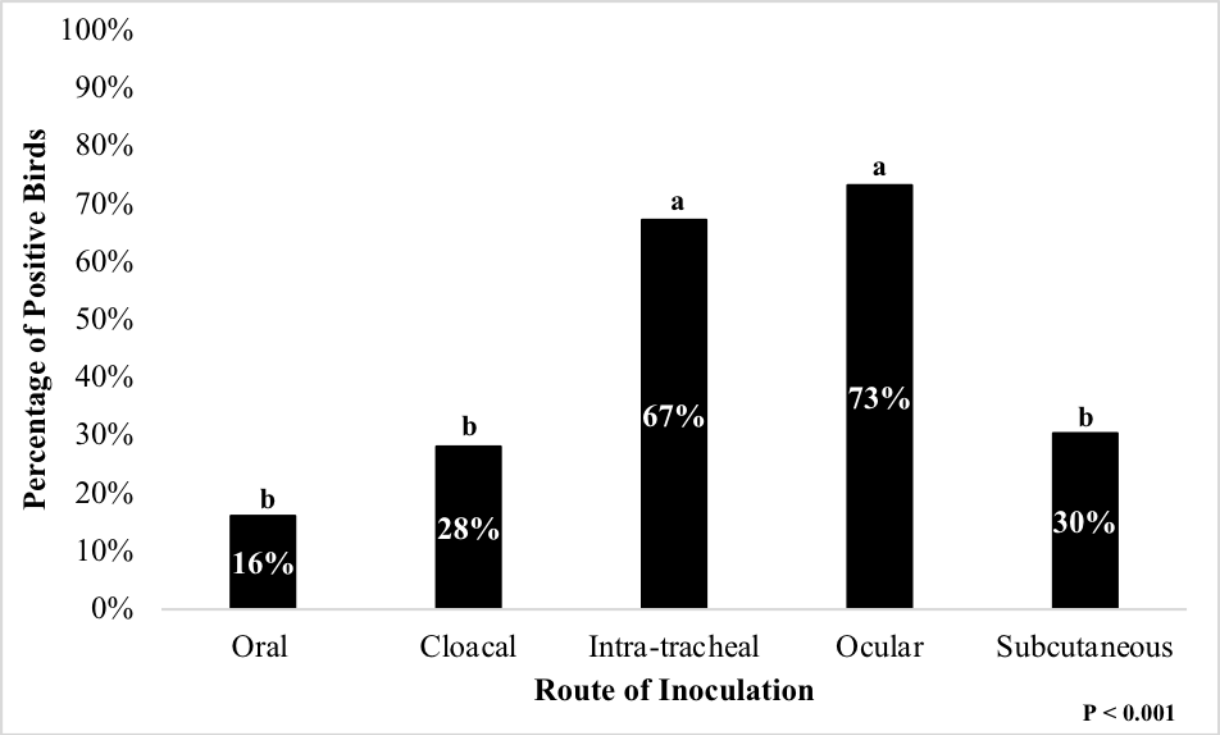

Figure 4.1 Comparison of the percentage of SH positive birds for each route of inoculation

Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$.

$^{(a\text{-}b)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation

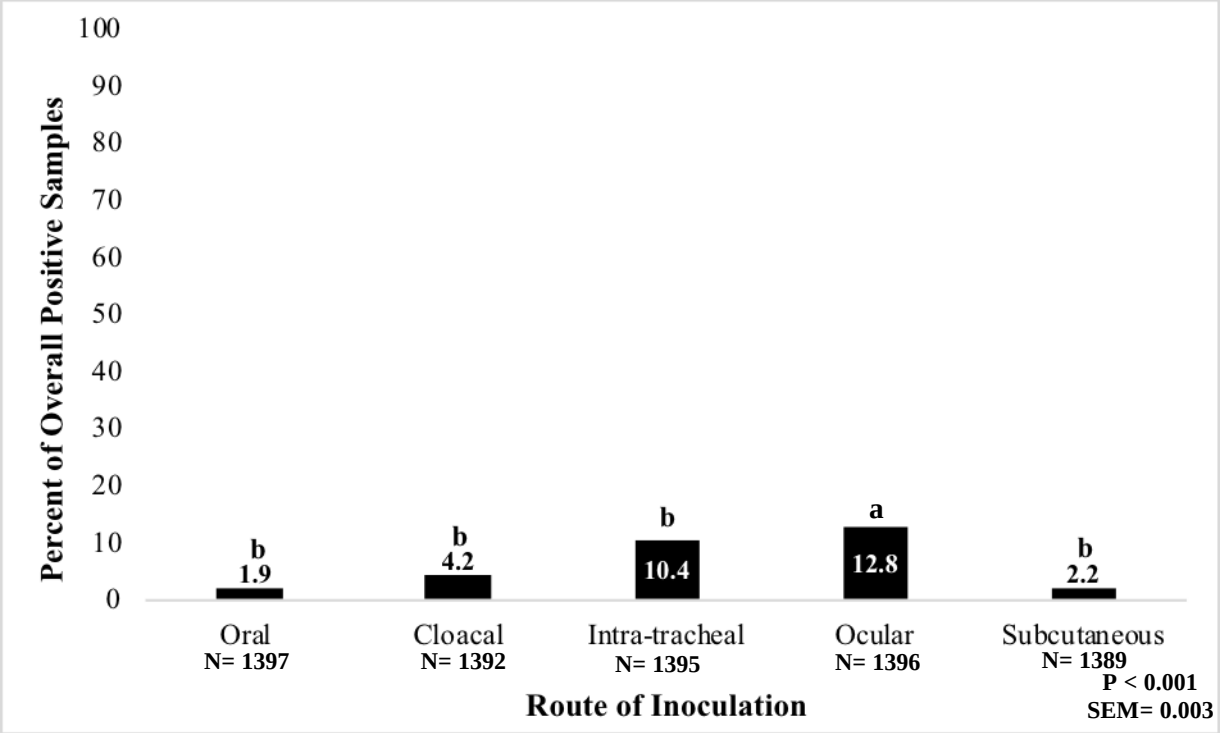

Figure 4.2: Overall percent incidence of positive samples by inoculation route

N= number of samples collected for each of the routes. Variation between routes occurred as a result of missed samples during necropsy

Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$.

$^{(a\text{-}b)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation

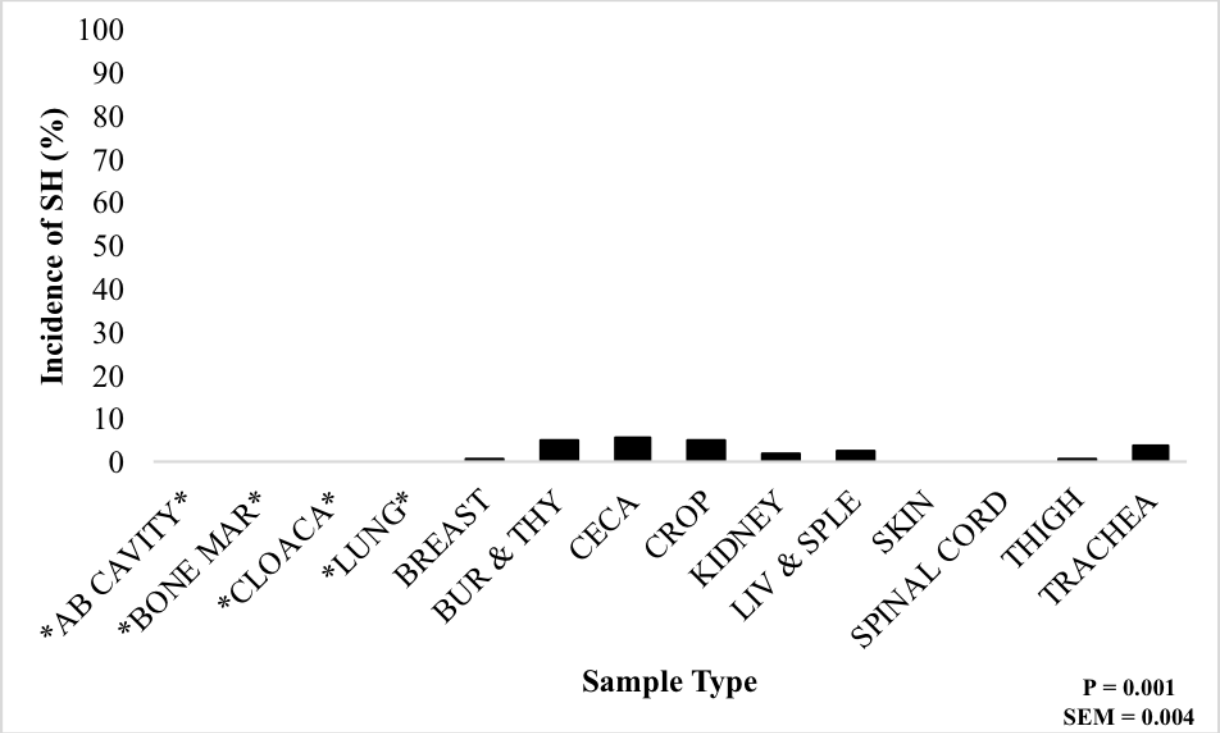

Figure 4.3: Comparison of incidence between the 14 sample types for oral inoculation

Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$.

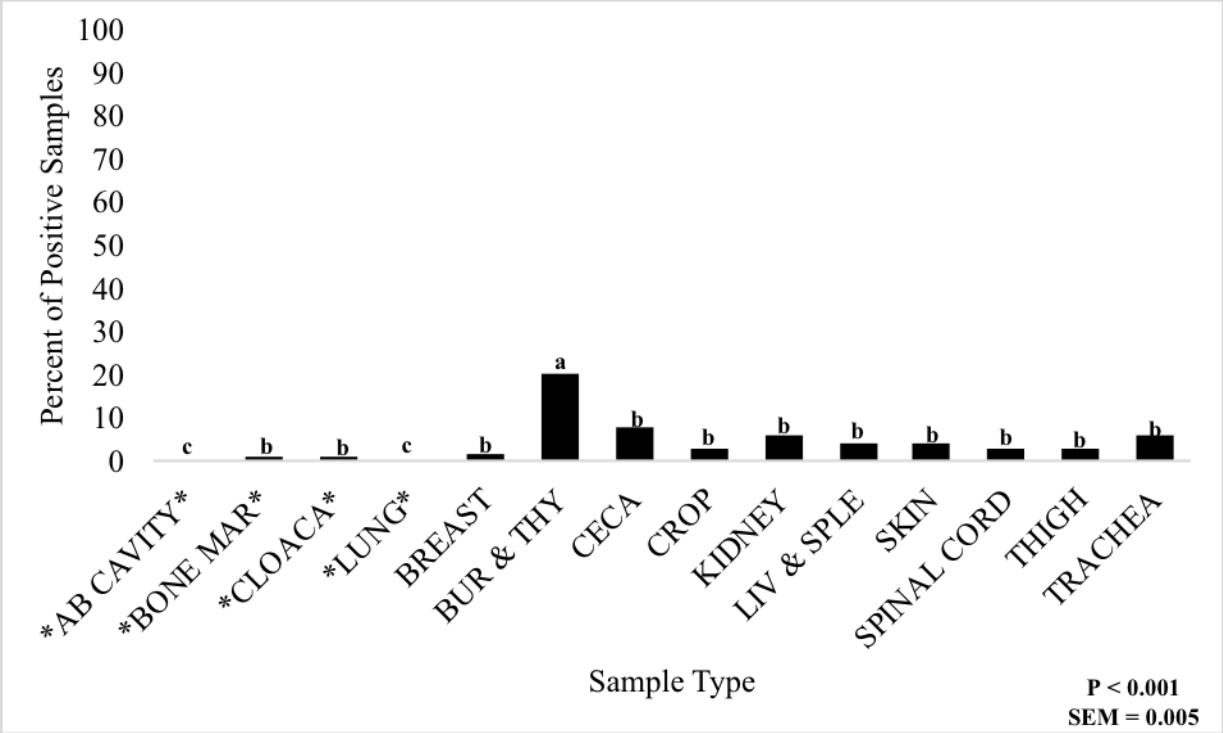

Figure 4.4: Comparison of incidence between the 14 sample types for cloacal inoculation Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$. $^{(a\text{-}e)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation.

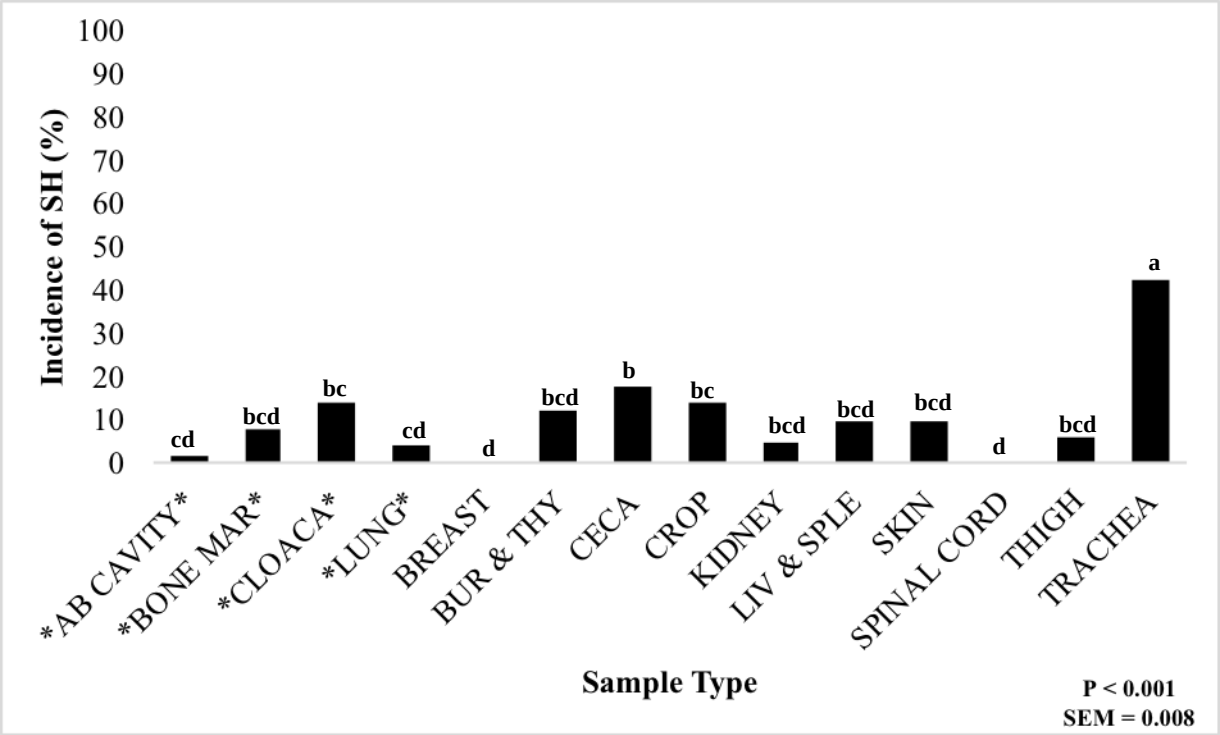

Figure 4.5: Comparison of incidence between the 14 sample types for intratracheal inoculation Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$. $^{(a\text{-}d)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation.

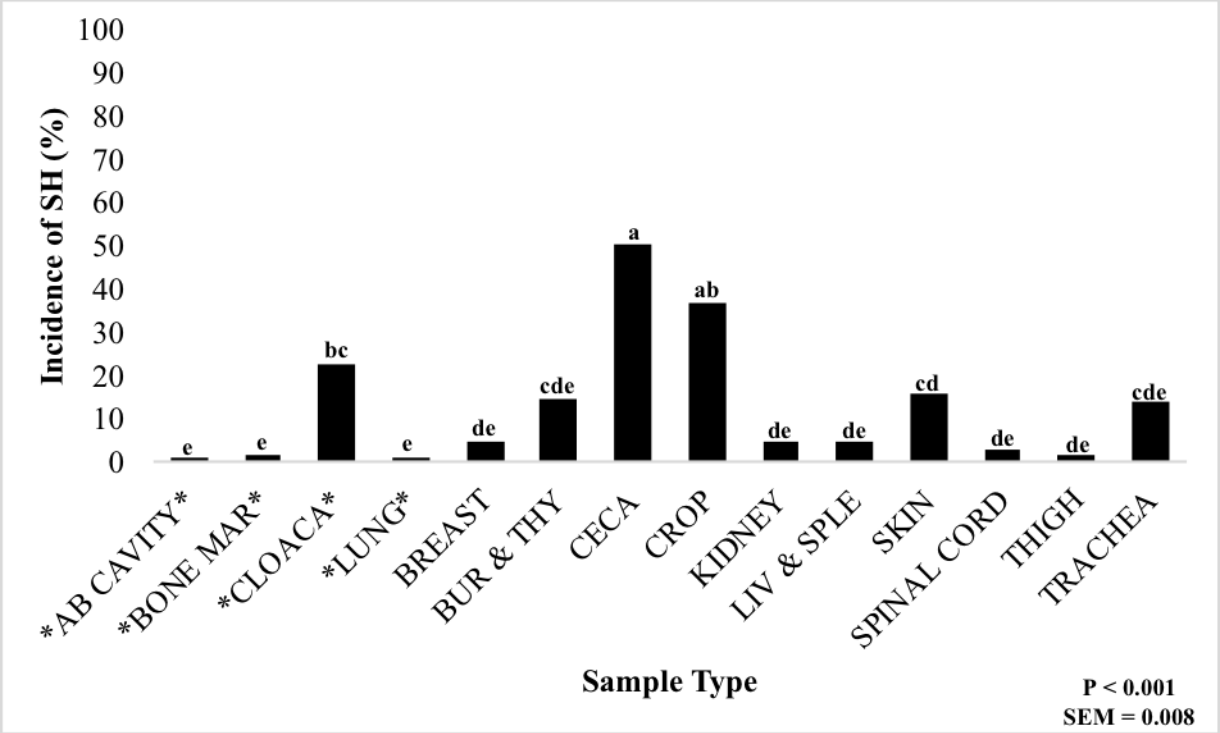

Figure 4.6: Comparison of incidence between the 14 sample types for ocular inoculation Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$. $^{(a\text{-}e)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation.

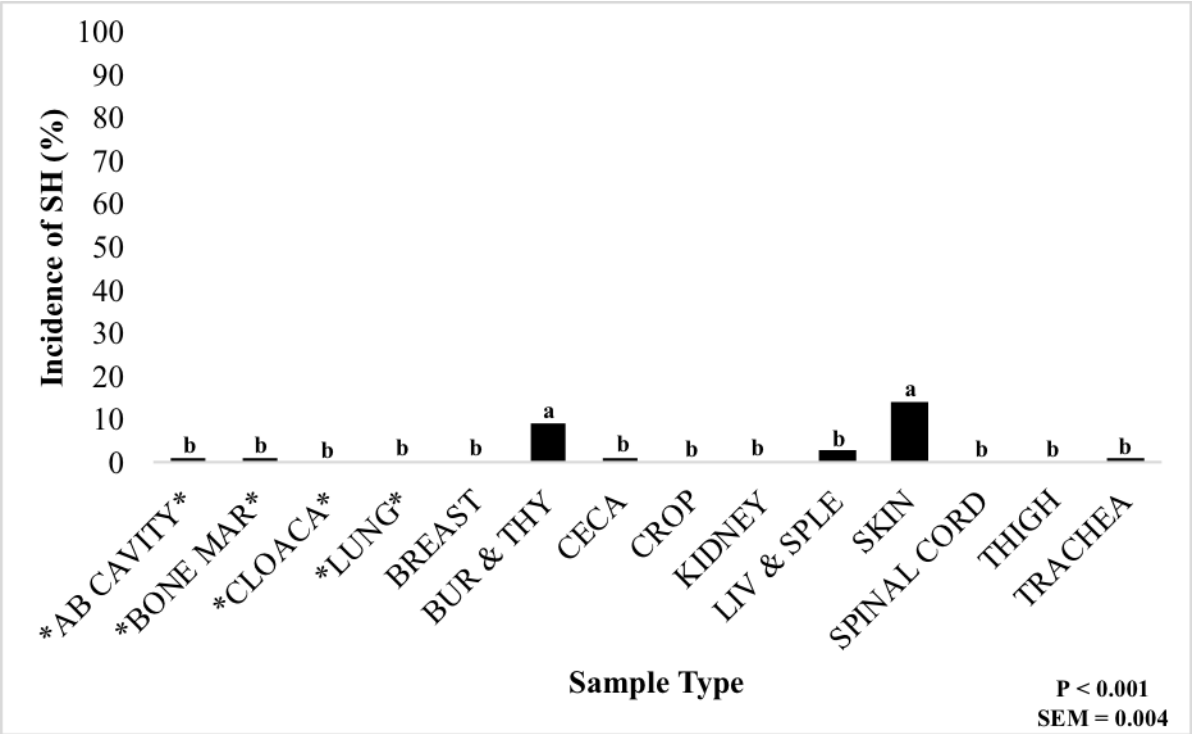

Figure 4.7: Comparison of incidence between the 14 sample types for subcutaneous inoculation Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$. $^{(a\text{-}b)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation.

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Chapter 5.0 A Comparison of Sites Colonized in Broilers Challenged Through Feed Administration with *Salmonella* Enteritidis and *Salmonella* Heidelberg at Day 14

Introduction:

The Center for Disease Control and Prevention (CDC) estimates foodborne illness accounts for 48 million cases, 128,000 hospitalizations, and 3,000 deaths annually (Scallan et al., 2011). It is also estimated that over one million cases, 23,000 hospitalizations, and 450 deaths occur as a result of foodborne *Salmonella* (Scallan et al., 2011). Among these, *Salmonella* Enteritidis (SE) and *Salmonella* Heidelberg (SH) are among the top most frequently reported serovars. For instance, epidemiological data from Hendriksen et al. (2011) revealed SE to be the most commonly isolated serovar in all regions of the world, with the exception of Oceania and North America. In this same study, SH was ranked in the 4th position amongst the North American serovars. Additionally, the Laboratory- based Enteric Disease Surveillance (LEDS) places SE and SH as 1st and 12th, respectively, of the most commonly reported *Salmonella* serovars (CDC, 2016).

When compared to other serovars, SE is regarded as one of the dominating serovars associated with contaminated poultry and egg consumption (Guard- Petter, 2001). It has been hypothesized that the decline of the avian- adapted serovars, *S.* Pullorum and *S.* Gallinarum, opened a favorable ecological niche for an antigenically similar serovar to proliferate i.e. SE (Baumler et al., 2000). As such, suggested differences between SE and others contributing to the increased pathogenicity of this serovar are alterations in the expression of extremes in flagellation, cellular division, swarm cell differentiation, and high cell density growth (Guard-Petter, 2001). However, one of the largest *Salmonella* outbreaks in recent history occurred as a

result of SH- contaminated chicken linked to a single poultry company (Gieraltowski et al., 2016). Additionally, SH has a greater tendency to cause invasive infections within the host when compared to SE (Vugia et al., 2004). Therefore, a comparison of both serovars is warranted because of their strong association between poultry and human illness (Gast, 2007).

At any point along the poultry production continuum, *Salmonella* can infect live broilers and the susceptibility of these birds may be influenced by the factors outlined by Bailey (1987). These factors include the age of the bird at the time of infection, ability of *Salmonella* to survive the gastric barrier passage, competition from other organisms, ability of the organism to locate a hospitable attachment site, broiler diet, bird health, physiological and disease status, environmental stresses (e.g. temperature, stocking density, etc.), medications administered, and host genetics. Both SE and SH, have been shown to colonize the reproductive tissue of hens or could penetrate the eggshell immediately following lay (Gast et al., 2004). Further dissemination could occur throughout the hatchery when chicks are most susceptible to colonization as a result of an immature immune system and the lack of competing gut microflora (Cox et al., 1996). Exposure during the grow- out period can increase the likelihood of infections because of the close proximity in which the birds are reared. This may lead to easy horizontal transmission through contact with feed, water, litter, feces, fluff/ feathers, dust, insects, rodents, shared equipment, personnel, and other contaminated fomites (Poppe, 2000).

Feed can act as either a direct, original contamination of ingredients prior to feed processing, or indirect, contamination of processed feed from the feed mill or rearing facility, vector of *Salmonella* to birds (Maciorowski et al., 2004). The advantages of pelleting feed have been widely recognized including the uniformity of feed, increased feed consumption and thus increase in body weight gain, but also the pelleting process destroys pathogenic organisms such

as *Salmonella* that may be present in the feed (Enberg et al., 2004; Veldman et al., 1995). However, studies have demonstrated *Salmonella* presence in pelleted feeds, possibly the result of post- processing contamination from dust or other vectors (Jones and Richardson, 2004; Veldman et al., 1995).

Another issue associated with *Salmonella* in feed is the uneven distribution of bacterial cells, which make monitoring and control extremely difficult (Maciorowski et al., 2004). Even if found in small numbers, *Salmonella* can persist in feed for extended periods of time due in part to its ubiquity in the environment. One such example is a study that demonstrated *S.* Typhimurium could be isolated from artificially inoculated feed for 495 days when held at 25ºC (William and Benson, 1978). Though *Salmonella* cannot grow in feed, the low water activity found within feed has no apparent effect on the survivability of *Salmonella* because one study revealed that bacterial survivability was greater at a water activity of 0.43 than 0.75 (Juven et al., 1984).

With no active bacterial replication occurring in the feed, feed acts as a vehicle to transport *Salmonella* into the body. The protein and lipids found within feed can act as buffer to protect the microorganisms present from acidic conditions in the upper gastrointestinal tract (GIT; Ha et al., 1998). Once successful entry is made into the body, *Salmonella* can readily colonize favorable sites found within the small intestine and ceca and also further disseminate into the body through the blood. As a result, the ceca, has been a standard recovery site of *Salmonella* but other samples such as the crop, liver, and spleen are also frequently investigated have been shown as areas of *Salmonella* recovery within birds.

Though highly variable, presence of *Salmonella* in feed can create food safety concerns as shown by Shirota et al. (2001) demonstrating the link between SE in feed and SE isolated

from egg contents. With such sporadic and highly variable *Salmonella* presence in feed, highlighting the impact of *Salmonella* from feed in indicator organs and organs with direct safety implications such as those consumed as cut- up parts or utilized for ground chicken product would be beneficial (Alali et al., 2016). Andino et al. (2014) found survivability of *Salmonella* in feed to be dependent on the servovar and strain, which necessitates a comparison of the serovar on tissue colonization in broilers. A previous study by Chadwick (2017) demonstrated that though both SE and SH systemically affected birds challenged on day-of-hatch, SE was the dominate serovar. Therefore, the objective of these experiments was to determine the effect of SE or SH on colonization within tissues when introduced to the birds through feed administration at d 14.

Materials and Methods:

Isolation of Bacteria and Use of Cultures

Naladixic acid and novobiocin resistant *Salmonella enterica* subspecies *enterica* serovar Enteritidis (SE) and *Salmonella enterica* subspecies *enterica* serovar Heidelberg (SH) isolates were utilized for the experiment. The isolates used were stored in a -80ºC freezer were placed onto a plate of Tryptic Soy Agar II containing 5 % Sheep Blood (BBL™, Beckon, Dickinson, and Company, Sparks, MD). They were then incubated for 18-24 h at 37ºC. The isolates were confirmed as *Salmonella* using slide agglutination with *Difco*™ *Salmonella* O Antiserum Poly A-I & Vi (Beckon, Dickinson, and Company, Sparks, MD).. The isolates were then plated onto Xylose Lysine Tergitol- 4 Agar (XLT4; Criterion™, Hardy Diagnostics, Santa Maria, CA) containing naladixic acid (100 μg/mL; Alfa Aesar, Wand Hill, MA) and novobiocin (15 μg/mL; Alfa Aesar, Wand Hill, MA) (NN), for confirmation, and incubated for 18-24 h at 37ºC. A colony from the XLT4 + NN plate was selected and used to inoculate 50 mL of Brain Heart Infusion Broth (BHIB; Hardy Diagnostics, Santa Maria, CA). The BHIB was placed into a

shaking incubator for 18-24 h with 200 revolutions per minute (RPM) at 37ºC. Following incubation, serial dilutions were made from the stock solution to determine the colony forming units per milliliter (CFU/ mL), which was approximately 10^9 CFU/ mL.

Broiler and Farm Management

Broilers used for the trial were housed at the Auburn University Poultry Research Farm in Auburn, AL. A total of 150 chicks for each experiment (150 chicks x 2 serovars = 300 total) were sourced from a commercial broiler hatchery. For both experiments, chicks used were randomly allocated into five floor pens (25 birds/ pen/experiment), with excess birds placed into an unchallenged pen.. These experiments were not run at the same time (SE: October 2017 and SH: August 2018). The birds were reared using standard conditions (stocking density, photoperiod, light intensity, temperature, and ventilation) appropriate for the age of the bird for the duration of the trial. The experiments ran for a total of 34 to 36 d (SE) or 39 to 41 d (SH), depending on when the birds were set and the time necessary to perform the necropsies.

Route of Inoculation

Prior to inoculation via feed administration, the birds were feed a standard corn- soy starter diet. To create the *Salmonella*- containing feed, 500 µL was removed from an overnight *Salmonella* culture and grown in BHIB and placed into 20 mL of sterile water. This was calculated to produce a *Salmonella* concentration in the feed of 1 x 10^4 CFU/g of feed. To ensure equal distribution of the *Salmonella*- containing feed, the inoculum was hand-mixed into 1.5 kg of feed removed from 45.35 kg of standard corn-soy grower feed. The hand- mixed feed and standard feed were then combined into a batch mixer (Twin Shell Dry Blender) and allowed to mix for seven minutes. This process was repeated twice to obtain 90.7 kg of feed. The mixed feed was then further divided into 15.87 kg bags. The birds were given the feed and allowed to consume it in its entirety before being put back onto non-contaminated feed (~6-7 days).

Samples of the feed were collected to determine *Salmonella* presence. Following inoculation, the birds were fed a standard corn-soy grower and finisher feed.

Sampling

To maintain aseptic conditions during necropsy, all instruments (stainless steel round-end forceps, stainless-steel scissors, and stainless-steel poultry shears) were initially dipped into water, then 10% bleach, and finally 70% ethanol, respectively, before and after each tissue collection. In addition, the instruments were cleaned and sanitized as mentioned above when contact was made with surfaces other than the appropriate tissue. The sanitizing agents and water were replaced intermittently throughout the necropsy. Cutting boards used for necropsy were washed with water, scrubbed, and sprayed with 70% ethanol following sampling of each bird. All tissues samples were collected into a sterile Whirl-Pak™ bag (*Nasco®)*; 20 mL of Buffered Peptone Water (BPW; HiMedia®, HiMedia Laboratories Pvt. Ltd, Mumbai, India) was then added to each bag and stomached onsite for one minute. The swab samples were collected using a sterile cotton swab **(**Puritan®, Puritan Medical Products Company LLC, Guilford, ME) and then placed into five mL of BPW tubes.

Samplings were performed between 20-22 (34- 36 d of the experiment) or 25-27 (39-41 d experiment) days post inoculation (DPI). Randomly selected broilers (n=100, 20 broilers/pen x 5 pens/experiment) were used for necropsy. Each bird was given a unique identifier to differentiate during necropsy. The birds were euthanized using a carbon dioxide asphyxiation. Once confirmed dead, cloaca swabs were collected using a swab and this was placed into five mL of BPW. The birds were then dipped into a quaternary ammonia immersion solution from the base of the neck near the shoulders down to the feet sanitize and remove excess dirt, feathers, and fecal material from the body.

Necropsies were carried out utilizing a two-person sampling system for the following 14 tissue and swab samples collected: abdominal cavity swab (ab cavity), bone marrow swab (bone mar), cloaca swab (cloaca), lung swab (lung), breast, bursa and thymus (bur and thy), ceca, crop, kidney, liver and spleen (liv and sple), skin, spinal cord, thigh, and trachea. Person A was responsible for all the samples pertaining to the head and leg: skin, trachea, thymus (pooled with bursa), and spinal cord and leg: thigh meat and bone marrow swab. Person B was responsible for all the samples pertaining to the body: crop, breast meat, abdominal cavity swab, lung swab, liver and spleen (pooled), ceca, bursa (pooled with thymus), and kidney.

Person A:

Following immersion into the quaternary ammonia solution, neck feathers were plucked to the base of the head to expose the neck skin. Using the shears, the neck of the bird was cut off, near the base of the neck being careful to avoid cutting the crop. The bird was allowed to bleed out into kill cones. At which point, one of the legs was also removed for further sample collection. Approximately 5 cm^2 of neck skin (representative skin sample) was removed from the pre-plucked neck section and placed into a bag. To access the thymus, the fold of skin previously cut from the neck skin was then cut upwards, parallel to the side of the neck to expose the thymus. A portion of the thymus, approximately 3-4 cm^2, was removed and placed into the bag with the bursa (pooled samples). Following removal of the thymus, all remaining skin attached to the neck was removed. The bird was laid flat against the side of the cutting board to expose the trachea. Once exposed, the trachea was then pulled away from the neck and approximately 3-4 cm^2 was removed and placed into a bag. With the head held flat against the cutting board, the neck was cut upwards using the shears, perpendicular to the beak to expose the spinal cord. Approximately 2-3 cm^2 of the spinal cord was removed and placed into a bag.

To sample the thigh meat, skin overlaying the muscle was peeled back with connective tissue also cut to free skin and feathers, as they could act as a potential source of contamination. Approximately 3-4 cm^2 of the thigh meat was removed and placed into the bag. To access the bone marrow, the femoral head was cut at an angle using the shears. A swab was then used to dig into the bone marrow and then placed into a BPW tube.

Person B:

Once bled, the skin at the cranial portion of the body was cut open to expose the crop. Approximately 3-4 cm^2 of the crop was taken from the bird and placed into a bag, with excess feed and/or water removed using the back of the scissors. With the forceps, the skin overlaying the breast tissue was peeled back with connective tissue also cut along the way to free the skin and feathers away from the body, as they could act as a potential source of contamination to the rest of the body. Approximately 3-4 cm^2 of the breast tissue was removed and placed into a bag. The ribs of the bird were cut open using the shears and an abdominal swab was taken and placed into a BPW tube. Using a swab, a section of the lung was stabbed and placed into a BPW tube.

To remove the liver and spleen, a lobe of the liver was drawn away from the body and approximately 3-4 cm^2 was removed and placed into a bag. To access the spleen, the intestines were pulled away from the body cavity and inverted to expose the spleen, with caution not to pull the intestines completely out of the body cavity, as this could be a potential source of contamination. The base of the spleen was grabbed and the entire organ was removed and placed into a bag. The ceca tonsils, proximal to the spleen, were exposed following unraveling the intestine. One of the cecal tonsils were cut at the cecal neck to avoid leakage of its contents into the body cavity and approximately 5-6 cm^2 was cut, removed, and placed into the bag. Once removed, the intestines were then completely pulled away from the body cavity to expose the

bursa. Approximately all of the bursa was removed and placed into a bag. The kidney presented a greater challenge to remove, as they are so delicate, difficult to remove, and incorrect removal could result in shredding of the organ. Therefore, removal of the kidney occurred using the scissors to dig underneath the organ to remove a substantial section; it was then placed into the bag. Following sampling of challenged birds, five birds were randomly selected from unchallenged pens and cloaca swabs were taken to determine horizontal transmission to unchallenged birds.

Microbiological analysis

All the macerated tissue samples were stored at 4ºC until further analysis. The samples were incubated for 18-24 hours at 37ºC in 20 mL BPW. Following pre-enrichment, one mL of each sample was placed into five mL of Tetrathionate Brilliant Green Broth tubes (TTB; HiMedia®, HiMedia Laboratories Pvt. Ltd, Mumbai, India) and incubated for 48- 72 h at 37ºC. Enriched samples were then streaked onto XLT4+ NN plates using one µL disposable loops (VWR International, LLC, Radnor, PA) and incubated for 48 h at 37ºC. From those plates, isolated colonies demonstrating typical *Salmonella* colonies were slide agglutinated with *Difco*™ *Salmonella* O Antiserum Poly A- I & Vi (Beckon, Dickinson, and Company, Sparks, MD). The presence of SE or SH was indicative of a score of one (positive), whereas a score of zero (negative) was indicative of SE or SH absence.

Data Analysis

The data were compiled onto a spreadsheet for statistical analyses. All statistical analyses were conducted using IBM® SPSS® software version 22. Data pertaining to a percentage of birds positive for SE or SH overall and percentage of samples positive overall for the challenge (calculated by dividing the total number of positive samples by the total number of samples

collected for each inoculation) were analyzed using a two-sample independent t- test with significance declared at $P \leq 0.05$. Data pertaining to a comparison of differences in incidence among each sample types for SE and SH were analyzed using a General Linear Model (GLM). Significant differences were reported at $P \leq 0.05$, and if applicable, means were separated using Tukey's HSD.

Results:

A summary of the percent incidence for all fourteen tissue and swab samples following feed administration of these two bacteria on d 14 is provided in Table 5.1. For the lung swab, breast, kidney, spinal cord, and trachea samples, no significant differences were observed between the two serovars ($P > 0.05$). However, for all other samples in which incidence occurred, significant differences were observed between the two serotypes ($P < 0.05$). Figure 5.1 illustrates the percentage of positive. Nine percent (9%) of birds challenged with SH were positive, whereas 68% of birds challenged with SE were positive, obvious numerical and highly significant differences were observed between the two serovars ($P < 0.05$).

Highly significant differences were observed between birds challenged with SH (0.7%) and SE (13.1%) for the overall percent incidence of positive samples ($P < 0.05$; Figure 5.2). Significant differences were observed between the sample incidence within the group of birds challenged with the same serovar (Figures 5.3-5.4). In birds challenged with SH (Figure 5.3), the greatest recovery occurred in the trachea, crop, and bursa and thymus with 2.02, 2.02, and 2.00% incidence, respectively. However, no significant differences in incidence were observed between the different sample types ($P > 0.05$). Recovery in birds challenged with SE is represented in Figure 5.4 and incidence in the ceca samples (50.00%) were significantly higher than the remaining thirteen samples. The samples with the next highest incidence were the cloaca swab (31.71%), liver and spleen (29.27%), and bursa and thymus samples (21.95%) with no

significant differences in incidence observed between the samples ($P > 0.05$). There were no significant differences in incidence between the crop (13.58%) and skin (14.81%) samples with the remaining samples such as the kidney (6.10%), trachea (4.88%), bone marrow swabs (4.88%), lung swabs (2.47%), spinal cord (1.22%), abdominal cavity swabs (0.00%) and thigh (0.00%) samples ($P > 0.05$).

Discussion:

Feed can act as a vehicle to transport *Salmonella* present in the feed into the body. Uneven distribution of the bacteria in feed make *Salmonella* challenges through feed administration very difficult (e.g. dry vs. wet inoculum; Alali and Horface, 2016). Another factor influencing *Salmonella* survivability in feed is the serovar used (Andino et al., 2014). Andino et al. (2014) found the recovery of SH greater than wild- type SE in artificially inoculated feed from between holding periods of four to seven days. Despite this, the present study revealed SH recovery in all tissues was significantly lower compared to recovery in birds challenged with SE ($P < 0.05$). In addition to differences in incidence of positive samples, significant differences were revealed in the percentage of SH positive birds was significantly lower than percentage of SE positive birds ($P < 0.05$). These differences may be attributed to survivability of SH in feed since a pilot study conducted to determine presence of the organism through direct plating for six days revealed SH could only be detected for two days as opposed to four days for SE (data not shown). In the present experiment, pathogenicity of SE may have been greater than that of SH influencing the survivability of the organism through the gastric barrier, invasion, and colonization the host (Guard- Petter, 2001).

The proteins and lipids found within the feed may protect *Salmonella* from the enzymes in the upper gastrointestinal tract needed for proper digestion and absorption (Ha et al., 1998). Feed may therefore increase the viability of these cells that would be otherwise destroyed from

the extremely acidic conditions. Subsequent successful movement through the GIT can lead to colonization and result in attachment to favorable colonization sites, identified in the intestinal mucin, found in the small intestine and ceca (Craven and Williams, 1997). In most cases, chickens are largely unaffected by *Salmonella* presence and can become carriers without any apparent clinical signs (Andino and Hanning, 2015). Nutrient availability within the ceca cause the bacteria to persist in these tissues, which create a prime area of colonization (Josefiak et al., 2004). In birds challenged with the SE, incidence within the ceca was 50.00% (Table 5.1). As it compares with previous research, several studies with a wide variety of experimental objectives have evaluated *Salmonella* presence within the ceca with the following recovery rates: 100% (Snoeyenbos et al., 1982), 95.1% (Cox et al., 1996), 81.9% (Fanelli et al., 1971), 66.3% (Byrd et al., 2001), 65% (Cox et al., 2007), 63% (Barnhart et al., 1999), 41.66% (Kallapura et al., 2014), and 36.7% (Ramirez et al., 1997). Differences in the results may be attributed to the age of the birds, culturing technique, and time from challenge to sampling. The lack of SH presence in the birds indicate that survivability within the feed was generally poor and therefore did not translate into colonization of the ceca.

In times of stress, such as transport, these organisms can be intermittently shed from the body, which can result with the presence on the skin (Lillard, 1989a; Rigby and Petit, 1980). Occasionally, the bird remains a carrier and will not actively shed the bacteria (Van Hoorebeke et al., 2009). In such cases, cloacal swabs will falsely represent the actual presence of *Salmonella* in birds similar to the incidence between the cloaca (31.71%) and the ceca (50.00%) in birds challenged with SE (Van Hoorebeke et al., 2009; Van Immerseel et al., 2004). Therefore, the use of cloacal swabs for *Salmonella* detection should be used in conjunction with other samples to account for these cases.

The crop is an organ unique to birds, allowing for rapid consumption of feed and swift departure to escape predation (Kieronczyk et al., 2016). As feed sits in this storage organ, the decreased pH present reduces microbial colonization from pathogens found within the feed (Corrier et al., 1999). However, a common practice of the poultry industry to reduce microbial contamination from cecal rupturing requires the removal of feed prior to transport, known as feed withdrawal. Feed withdrawal may cause the birds to peck at the litter in search of dropped feed, which can increase the intake of feces- contaminated feed into the body. The lack of feed moving through the crop increases the pH within the organ and thus increases the likelihood of this organ to colonization (Kieronczyk et al., 2016; Byrd et al., 2001; Humphrey et al., 1993). As such, persistence of *Salmonella* in the crop tissue can contaminate its contents and, if ruptured, can contaminate the outside of the carcass (Hargis et al., 1995). Hargis et al. (1995) found the rupturing of crops were 86 times more likely to occur than cecal rupturing and colonization in the crop was greater than colonization within the ceca. However, this was not demonstrated in the present experiment with SE challenged birds (crop: 13.58 %; ceca: 50%; Table 5.1).

The incidence in crops (Figure 5.4) of birds challenged with SE (13.58%) were most comparable to results from Barnhart et al. (1999) with 13.8% and Ramirez et al. (1997) with 18.9% crop incidence. Although birds in the present study were not subjected to a feed withdrawal period, incidence in the crop was also most comparable to birds subjected to feed withdrawal after eight hours (Corrier et al., 1999). In contrast, incidence within the crops of birds challenged with SH (2.02%; Figure 5.3) was comparable with 1.9% crop incidence as reported by Corrier et al. (1999). Generally, exposure of contaminated feed to birds for extended periods of time should increase crop incidence from the continuous exposure (Chadwick, 2017).

However, this was not observed in the present study in birds challenged with SE or with SH. In the case of SH, these results imply a lack of viability of the organism within the feed.

Food safety concerns arise with *Salmonella* presence on chicken skin because of its inclusion in ground chicken to obtain higher fat percentages (Park et al., 2017). Evaluation of *Salmonella* on the skin of the bird is important because contamination of the skin is often the result of organisms picked up from the environment. Transportation of birds to processing can increase stress and excretion rates of *Salmonella*- containing feces, which can contaminate processing crates and other birds (Rigby and Petit, 1980). Once lodged into the crevices of the skin, these organisms are protected from the antimicrobials used for microbial reduction (Lillard, 1989a). The sampling of skin in our experiments were used to detect external *Salmonella*; however, in the European Union excised neck skin is used for monitoring for the presence of *Salmonella*, synonymous to whole carcass rinses in the US (Cox et al., 2010). In the present experiments, *Salmonella* recovery on the skin of birds challenged with SE (Table 5.1) did not reflect recovery within the ceca and cloaca ($P < 0.05$). Differences in incidence in the skin between the SE (14.81%) and SH (1.02%; Table 5.1) challenged birds reflect shedding from the body leading to presence on the skin. However, incidence on the skin of experimentally challenged birds in the present study were lower than incidence found in commercial birds (Wu et al., 2014).

Filtration organs of the body, namely the liver, spleen, and kidneys, are organs responsible for blood filtration to remove toxins, pathogenic organisms, and waste products (John, 1994). *Salmonella* present in these tissues indicate that the bacteria has evaded clearance in the intestine, penetrated the mucosa of the gastrointestinal tract, triggering inflammation of these tissues, leading to uptake of the bacterium by phagocytic cells (Bohez et al., 2007). Once

internalized, macrophages offer protection from humoral and complement- mediated attack, clearance by the host, and accessibility of host cell nutrients (Ribet and Cossart, 2015). These organisms gain entry into the bloodstream through the hepatic portal vein, aiding in their circulation throughout the body. General lack of SH presence in birds challenged with this serovar can be attributed to the inability of the organism to establish presence in the blood causing further dissemination. Contrastingly, as shown in Table 5.1 birds challenged with SE were present within the liver and spleen (29.27%) was significantly higher than SH presence (1.00%; $P < 0.05$), but presence within the kidney was not significantly different between serovars ($P > 0.05$). With the ability of *Salmonella* to take residence within the liver tissue, food safety concerns arise from liver that may be contaminated or improperly cooked (Lanier et al., 2018).

Bone marrow in birds, similar to that of other vertebrates, are sites involved in the production of new cells or hematopoiesis but are also important secondary lymphoid organs involved in the generation of lymphocytes (Campbell, 1967). Therefore, *Salmonella* presence found within these tissues would be the result of systemic infection within the bird. Though systemic infections resulting from *Salmonella* infections do occur in birds, limited research has evaluated *Salmonella* presence in this tissue (Wu et al., 2014; Kaseem et al., 2012; Velaudapillai, 1964). Low recovery of SH in the bone marrow of birds challenged with this serovar is a direct reflection in the inability of the organism to invade the bloodstream, which is also apparent in filtration organ incidence (i.e. kidney and liver and spleen). Conversely, incidence found within SE infected birds reveal that the organism causes systemic infection. Previous research conducted with commercial flocks found very low incidence, 0.7% (Wu et al., 2014) and 0.8% (Velaudapillai, 1964), in bone marrow samples. In this research, it was observed that bone

marrow incidence in SE challenged birds was 4.88% (Table 5.1). These differences in incidence may be attributed to the dose of SE given to birds, whereas the other two aforementioned studies were taken from the processing the processing plant. Though not commonly found within these tissues, *Salmonella* presence would have food safety implications since backs and necks are ground into ground chicken product for use in mechanically separated chicken (MSC; Alali et al., 2016).

Primary lymphoid organs of the chicken are the "bursa of Fabricius" or bursa and the thymus (Cooper et al., 1965). Together, the bursa and thymus are involved in humoral and cellular immunity of chickens, respectively (Ribatti, 2017). The bursal folds are open to the lumen of the cloaca and B lymphocytes produced within these tissues are destined for use in humoral immunity (Ribatti, 2017). Additionally, though the bursa atrophies in older birds, it plays an important role in antibody production (Glick et al., 1956). The thymus is an organ clustered in the neck region of the bird where T lymphocytes are matured and differentiated for cell- mediated immunity (Ribatti, 2017). Berndt and Methner (2004) demonstrated the participation of both humoral and cellular immunity in orally- inoculated five- day old chicks. When compared to the previously mentioned study, response measurements such as antibody levels were not taken. However, the higher incidence of SE found within these tissues could possibly indicate that this organism is more likely to elicit an immune response under the given conditions, leading to increased uptake by the macrophages.

Salmonella was not recovered from the abdominal cavity or thigh in any of the experiments. In the lung, breast, kidney, spinal cord, and trachea significant differences in incidence were not observed between the serovars ($P > 0.05$). It can be concluded that these tissues are not prime areas for *Salmonella* recovery during feed challenge. However, in ground

chicken product, tissues such as the spinal cord and kidney are commonly ground for MSC, which increases the surface area of the tissue potentially leading to contamination throughout the product (Park et al., 2017; Alali et al., 2016).

Conclusion:

Introduction of SH and SE to birds during the grow- out can occur through many points but feed offers a consistent entry point into the body. Once ingested these organisms may colonize typical areas such as the crop and ceca but also further disseminate into tissues that have direct food safety implication. Our results imply that challenge at d 14 through feed administration with SE or SH leads to a more significant effect in SE challenged birds with a greater percentage of positive birds and a greater effect in tissue incidence. Differences can be attributed to greater survivability and pathogenicity of SE. Therefore, more effective strategies geared towards monitoring and control of this pathogen in feed should be further evaluated.

	SH (%)	SE (%)	P-value (P ≤ 0.05)
ABDOMINAL CAVITY	0.00	0.00	1.0[1]
BONE MARROW	0.00	4.88	**0.028**
CLOACA	1.01	31.71	**<0.001**
LUNG	0.00	2.47	0.117
BREAST	0.00	1.27	0.271
BURSA & THYMUS	2.00	21.95	**<0.001**
CECA	0.00	50.00	**<0.001**
CROP	2.02	13.58	**0.003**
KIDNEY	1.00	6.10	0.056
LIVER & SPLEEN	1.00	29.27	**<0.001**
SKIN	1.02	14.81	**<0.001**
SPINAL CORD	0.00	1.22	0.271
THIGH	0.00	0.00	1.0[1]
TRACHEA	2.02	4.88	0.288

Table 5.1 Comparison of *S.* Heidelberg and *S.* Enteritidis recovery between the serovars

* indicates swab samples instead of tissue samples

Bold values indicate significant differences at P ≤ 0.05.

[1] used to identify no positive samples were collected for these sites

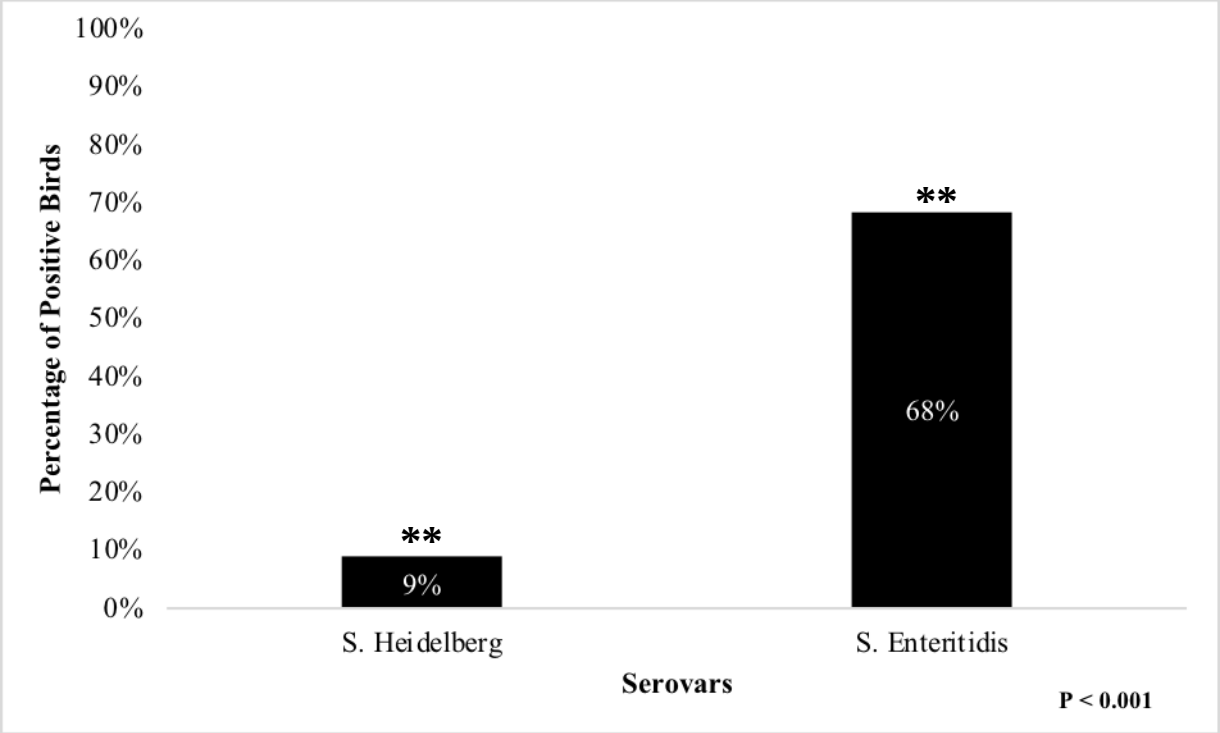

Figure 5.1 Comparison of the percentage of SH or SE positive birds

** indicates significant differences between routes at $P \leq 0.05$.

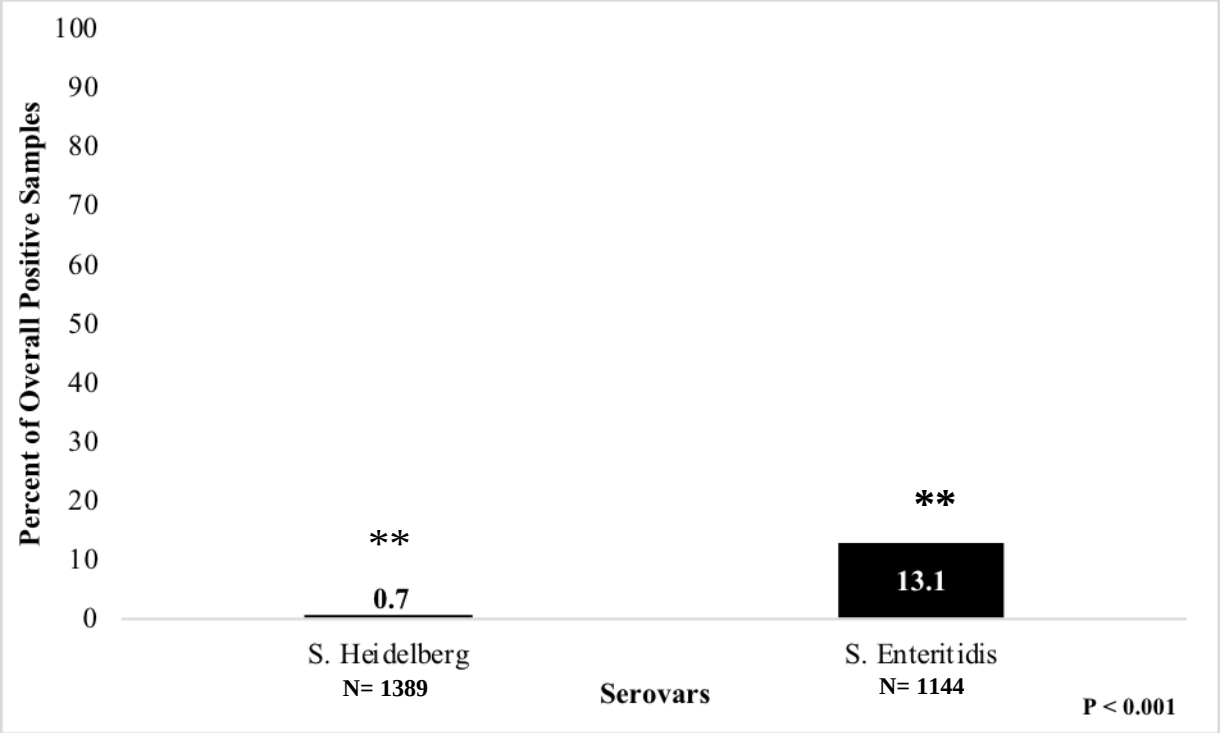

Figure 5.2: Overall percent incidence of positive samples for each serovar

N= number of samples collected for each of the routes. Variation between routes occurred as a result of missed samples during necropsy or the exclusion of data.

Data were analyzed using an independent t-test.

** indicates significant differences between routes at $P \leq 0.05$.

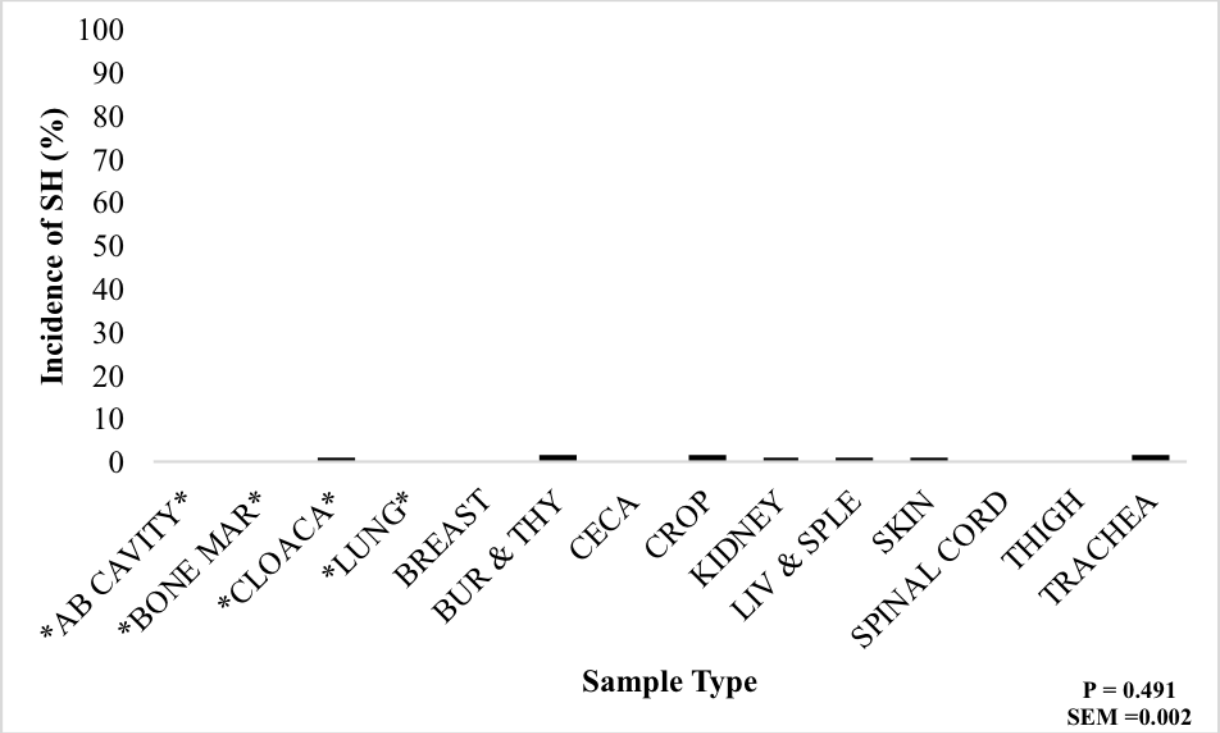

Figure 5.3: Comparison of incidence between the 14 sample types for birds challenged with *S.* Heidelberg

Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$.

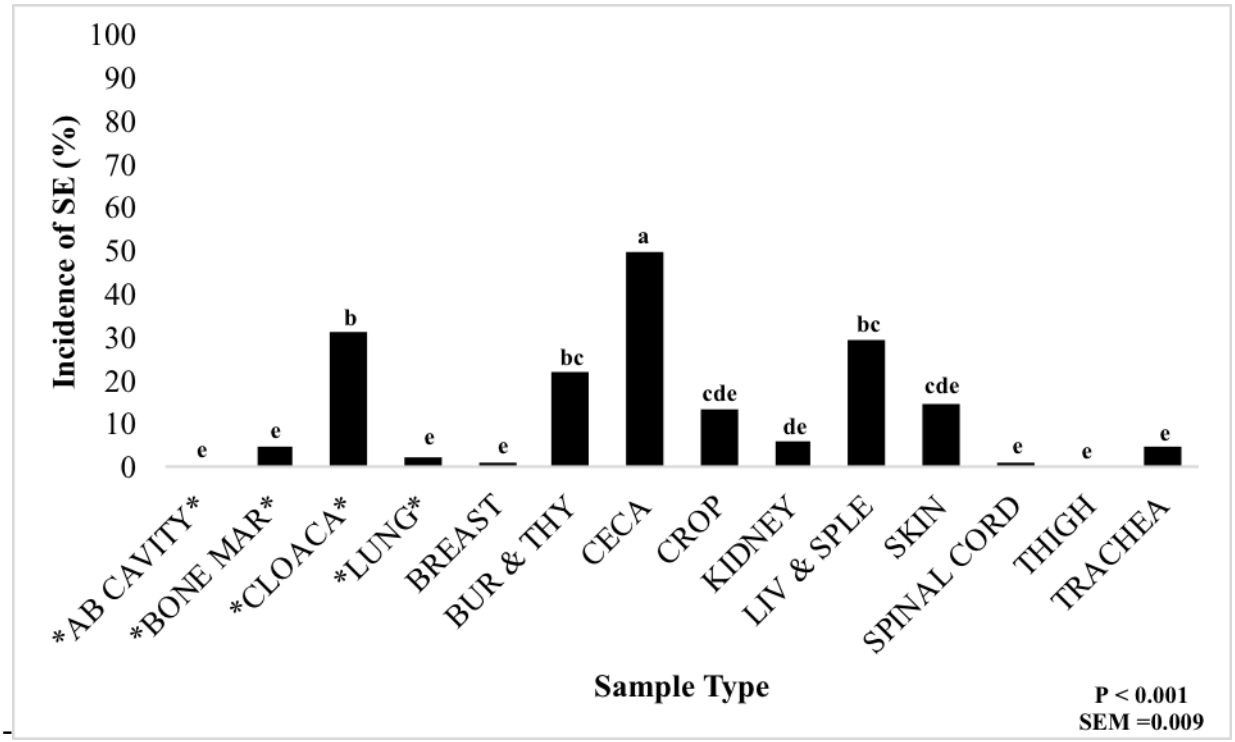

Figure 5.4: Comparison of incidence between the 14 sample types for birds challenged with *S.* Enteritdis

Data were analyzed using a GLM and differences in incidence were reported at $P \leq 0.05$. $^{(a\text{-}e)}$ indicates significant differences between routes at $P \leq 0.05$ by Tukey's HSD means separation

Chapter 6.0 Summary and Conclusion:

Poultry has become increasingly more important as an animal protein and is currently the second most consumed animal protein in the United States (Daniel et al., 2011). With increases in consumption, the likelihood of exposure with organisms such as Non- Typhoidal *Salmonella* may also be increased. Infection with these organisms, collectively known as salmonellosis, may result in large financial and health burden (Alali and Horface, 2016). Of these organisms, SE and SH are among the top five associated with human illness and whole and ground chicken meat (CDC, 2016; USDA- FSIS, 2014b). Understanding transmission of *Salmonella* is quite complex. Contamination of birds could occur at any point along the poultry production continuum and be easily transmitted throughout the various stages. It is widely known that a large *Salmonella* presence in birds coming into the processing facility may be exacerbated during processing with automated equipment and lead to further cross-contamination of non- infected birds. Additionally, once these organisms have become firmly attached to the skin and processing equipment, the antimicrobial activity of physical and chemical decontamination methods used may be reduced (Lillard, 1989a). Therefore, an identification of suitable mitigation can only be attained through an evaluation of *Salmonella* contamination of birds during the preharvest period.

As an enteric pathogen, the oral route has been the most commonly investigated route of infection (Kallapura et al., 2014a). However, previous research conducted by Chadwick (2017) in newly hatched chicks highlighted the importance of investigating other inoculation routes by

also including an evaluation of other tissues that may be affected by *Salmonella* challenge. Thus, an evaluation of *Salmonella* challenge was warranted in older birds, as infection during rearing could occur at any age. In the first set of experiments (oral, cloacal, intra- tracheal, ocular, and subcutaneous routes) with SE, the ocular route produced the greatest effect on the bird population and incidence among the samples. A similar effect from the ocular route was observed in the second set of experiments with SH. The ocular route was performed to simulate infection through aerosol transmission. Aerosol transmission of *Salmonella* in commercial broiler production could occur as large volumes of air are moved throughout the house by negative pressure to remove ammonia, CO_2 and dust present (Ritz et al., 2006). Contaminated dust particles may make contact with the eye (conjunctiva), nares, and mouth and are either swallowed and/ or trapped within the respiratory system (Chinivasagam et al. 2009). In this case, the bird would receive a combined effect of the bacteria affecting both the gastrointestinal tract and the respiratory tract (Kallapura et al., 2014a). Though the intratracheal route did not produce a greater effect on birds and samples compared to the ocular route, a similar impact was observed. With necropsies occurring approximately 21 days post inoculation, higher incidence in tissues of birds challenged through the ocular and intratracheal routes indicate that the organisms are persisting for longer periods of time (Okamura et al., 2005; Mensah and Brain, 1982).

Chadwick (2017) observed differences in colonization of day- old chicks when continuously fed SE or SH, therefore further analysis in older birds feed for a shorter period of time was necessary. In the broiler house, feed has been identified as one of the most important vectors for *Salmonella* introduction into the house. *Salmonella* presence in feed is difficult to detect as a result of uneven distribution of cells or damaged and injured cells that make microbial isolation difficult (Alali and Horface, 2016). In the third experiment, feed administration with SE

or SH, revealed SE had higher recovery in the bird population and recovery within the collected tissues. Differences between the two serovars may be attributed to differences in pathogenicity but also survivability of the organism in feed (Andino et al., 2014).

The ceca are traditionally used as standard organs for *Salmonella* recovery as a result of persistence of the organism within these tissues (Kallapura et al., 2014a; Josefiak et al., 2004; Snoeyenbos et al., 1982; Fanelli et al., 1971). Generally, in all experiments, incidence within the ceca was most often greater than incidence within other organs. However, in some cases incidence in other tissues was significantly greater than the ceca (i.e. bursa and thymus: subcutaneous and cloacal routes, skin: subcutaneous route, and trachea (SH only): intratracheal route). Depending on the route, incidence within the ceca was not significantly different ($P > 0.05$) from incidence within other sites (i.e. crop, skin, trachea, cloaca swab) indicating others could be used in conjunction with the ceca.

Despite the lack of widely available supporting research in broilers, routes used to mimic aerosol transmission in our experiments imposed the greatest effect on the birds in under experimental conditions. Additionally, these birds were challenged with a specified dose at day 14. However, infection in naturally infected flocks could occur at any age, with varying amounts of *Salmonella*, and quite possibly through one or more routes. Results imply that *Salmonella* colonization in tissues may be affected by the route of inoculation. Differences in incidence between birds challenged with SH and those challenged with SE can be attributed to pathogenicity and survivability of the organisms. However, it is important to note that lab strains resistant to novobiocin and nalidixic acid were used as the challenge isolate and wild type strains may not have had the same impact in the birds. Successful management of *Salmonella* in birds, can only be attained through continuous monitoring and mitigation strategies geared towards

reducing or eliminating initial introduction of the pathogen into the environment. Furthermore, more focus on minimizing aerosolization is necessary.

Chapter 7.0 Thesis References

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