SALMONELLA HARBORAGE SITES IN INFECTED POULTRY AND INFLUENCE OF COCCIDIOSIS ON THE COURSE OF *SALMONELLA* INFECTION

by

CLAIRE-SOPHIE RIMET

(Under the Direction of Monique S. França)

ABSTRACT

Salmonella is one of the leading causes of foodborne illness worldwide. The first objective of this research was to identify *Salmonella* harborage sites in tissues present in ground poultry. Experimental infection of turkeys revealed *Salmonella* Heidelberg primarily located on epidermal keratin, indicating that skin may significantly contribute to contamination of ground turkey. The second objective was to investigate the effect of intestinal inflammation caused by *Eimeria* on cecal colonization and systemic spread of *S.* Typhimurium wild-type and mutant strains. Coinfection with low dose of *Eimeria* did not increase intestinal inflammation and total *Salmonella* prevalence in ceca, liver, and drumstick compared to single infection with *S.* Typhimurium strains. Independently of *Eimeria* coinfection, deficiency in tetrathionate reductase did not impair cecal colonization and systemic spread of *S.* Typhimurium. *Salmonella* Pathogenicity Island-2 mutation had a detrimental effect on cecal colonization whereas deficiency in *Salmonella* Pathogenicity Island-1 impaired dissemination of *S.* Typhimurium to liver.

INDEX WORDS: *Salmonella* Heidelberg, *Salmonella* Typhimurium, Skin, Feather follicle, Ground poultry, *Eimeria*, Tetrathionate respiration, *Salmonella* Pathogenicity Island-1, *Salmonella* Pathogenicity Island-2

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DEDICATION

Dedicated to Benjamin and to my family for their unconditional love and endless support throughout my life.

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CHAPTER 1

INTRODUCTION

It is estimated that nontyphoidal *Salmonella* causes approximately 1.03 million illnesses, 19,336 hospitalizations, and 378 fatal cases per year in the United States (Scallan et al., 2011). Poultry products are one of the leading sources of foodborne *Salmonella* outbreaks (CDC, 2016). *Salmonella* contamination of poultry meat mainly occurs by cross-contamination with fecal material from positive birds during carcass processing (Carrasco et al., 2012). However, *Salmonella* is capable of invading intestinal epithelium of infected birds and can spread into internal organs such as muscle and bone (Velaudapillai, 1964; Kassem et al., 2012; Wu et al., 2014; Cui et al., 2015; França et al., 2016). Active invasion of the intestinal mucosa is mediated by the *Salmonella* Pathogenicity Island-1 (SPI-1) (Francis et al., 1993; Galán, 1996; Libby et al., 2004; Velge et al., 2012). After invasion, *Salmonella* survival within host phagocytic cells is mediated by the *Salmonella* Pathogenicity Island-2 (SPI-2), which allows systemic spread of live bacteria through blood vessels and lymphatics (Gulig, 1987; Jones and Falkow, 1996; Vazquez-Torres et al., 2000; Pullinger et al., 2007; Ibarra and Steele-Mortimer, 2009). In chickens, *Salmonella* intestinal colonization and systemic spread can be enhanced by concomitant infection with coccidia, an ubiquitous intestinal disease caused by protozoan parasites of the genus *Eimeria* (Stephens et al., 1964; Stephens and Vestal, 1966; Arakawa et al., 1981; Takimoto et al., 1984; Morishima et al., 1984; Fukata et al., 1987).

We hypothesized that *Salmonella* Heidelberg strains isolated from foodborne outbreaks could colonize muscle of experimentally inoculated turkeys. Furthermore, we hypothesized that intestinal inflammation induced by coinfection with *Eimeria* spp. could enhance *Salmonella* cecal colonization and systemic spread.

The overall aim of this research was to contribute to a better understanding about the role of invasive bacteria in *Salmonella* contamination of ground poultry. In the first study, we inoculated one-day-old commercial turkeys with *Salmonella* Heidelberg strains isolated from foodborne outbreaks to determine *Salmonella* prevalence and harborage sites in tissues used to make ground poultry. In the second study, we experimentally infected chickens to investigate the effect of intestinal inflammation induced by *Eimeria* spp. in cecal colonization and systemic spread of wild-type and mutant strains of *S*. Typhimurium.

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CHAPTER 2

LITERATURE REVIEW

GENERAL CHARACTERISTICS

Named after an American veterinarian, Daniel E. Salmon, *Salmonella* was first isolated from pigs by Salmon and Smith in 1885 (Grimont et al., 2000). *Salmonella* is a genus of the family Enterobacteriaceae (Koneman et al., 1992). *Salmonella* are rod-shaped, Gram-negative, facultative anaerobic, non-spore forming bacteria ranging from 0.7 to 1.5 micrometers wide by 2.0 to 5.0 micrometers long (Grimont et al., 2000; Gast, 2013). The majority of *Salmonella* serovars are motile bacteria possessing up to 10 peritrichous flagella on their surface, except for Gallinarum and Pullorum serotypes, which are not able to move (Libby et al., 2004; Van Asten and Van Dijk, 2005). Species in the genus *Salmonella* are medically important pathogens for both humans and animals (ICMSF, 1996; Sanderson and Nair, 2013).

CLASSIFICATION AND SEROTYPING

The genus *Salmonella* is divided into two species, *enterica* and *bongori* (Brenner et al., 2000). The species *Salmonella enterica* is further sub-divided into six subspecies designated either by taxonomic names or Roman numerals (Table 2.1). *S. enterica* subsp. *enterica* (I) represents the majority (59.4%) of the *Salmonella* serotypes and is mostly isolated from humans and warm-blooded animals (Brenner et al., 2000; Grimont and Weill, 2007) (Table 2.1). Serotypes in *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp.

diarizonae (IIIb), *S. enterica* subsp. *houtenae* (IV), *S. enterica* subsp. *indica* (VI) are usually found in cold-blooded animals and in the environment (Brenner et al., 2000) (Table 2.1). The Kauffman-White scheme, first published in 1934, further divides *Salmonella* subtypes into serotypes based on serological reactions to somatic lipopolysaccharide (O), flagellar (H), and capsular (Vi) antigens (Lignières, 1934; Grimont and Weill, 2007; Sanderson and Nair, 2013). The antigenic formulae of *Salmonella* serotypes are defined and maintained by the World Health Organization (WHO) and new serotypes are listed in updates of the Kauffman-White scheme (Brenner et al., 2000; Grimont and Weill, 2007). In the 9th edition of the Antigenic Formulae of *Salmonella* Serotypes in 2007, 2,579 serotypes were identified (Grimont and Weill, 2007). Serotyping of *Salmonella* isolates is generally accomplished using agglutination tests with batteries of specific antisera (Gast, 2013).

GROWTH CHARACTERISTICS

Growth of *Salmonella* cells depends on several factors such as temperature, pH, and water activity (ICMSF, 1996). Most *Salmonella* species grow optimally at 37°C, but growth has been recorded from temperature just above 5°C up to 47°C (Adams and Moss, 2008; Ray and Bhunia, 2014). *Salmonella* can grow within a pH range of 3.8 to 9.5 with an optimum pH comprised between 7 and 7.5 (ICMSF, 1996). Water activity significantly affects *Salmonella* multiplication; the lower water activity limit for *Salmonella* growth is 0.94 (ICMSF, 1996). As members of the Enterobacteriaceae family, *Salmonella* are catalase-positive, oxidase-negative bacteria that ferment glucose and reduce nitrates to nitrites (Janda and Abbott, 2006). Most *Salmonella* species are indole and urease negative, they do not ferment lactose (Koneman et al., 1992; ICMSF, 1996). *Salmonella* can reduce tetrathionate into hydrogen sulfide (H_2S) (Barrett

and Clark, 1987). These biochemical characteristics are exploited for differential and selective media formulation.

EPIDEMIOLOGY

The distribution of *Salmonella* serotypes in meat-type and egg-type poultry flocks varies considerably overtime and geographically (Gast, 2013; Shivaprasad et al., 2013). Baseline surveys conducted in the European Union (EU) in 2005-2006 revealed that prevalence of *Salmonella*-positive flocks was 23.7% in broilers and 30.7% in commercial turkeys (EFSA, 2007b, 2008). *Salmonella* prevalence varied considerably amongst the EU member states; from 0% to 68.2% in broiler flocks and from 0% to 78.5% in turkey flocks (EFSA, 2007b, 2008). The prevalence of flocks positive for *S*. Enteritidis and/or *S*. Typhimurium was 11.0% in broilers (EFSA, 2007b) and 3.8% in commercial turkeys (EFSA, 2008). In Canada, a national survey conducted between 1990 and 1991 has shown that *Salmonella* was recovered from environmental samples in 86.7% of the flocks (Irwin et al., 1994). In the United States, Byrd et al., 1999 described a 42% *Salmonella* prevalence in broiler houses sampled between 1995 and 1996. In 2014, in the United States, the most common serovars isolated from non-clinical samples (flock monitoring program, environmental sources, food samples) were *S*. Seftenberg, *S*. Mbandaka, *S*. Kentucky, *S*. Enteritidis, and *S*. Typhimurium in chicken flocks and *S*. Seftenberg, *S*. Anatum, *S*. Hadar, *S*. Muenster, and *S*. Agona in turkey flocks (Morningstar-Shaw et al., 2015). The lack of data describing *Salmonella* prevalence at the flock level in the United States may be explained by the regulatory approach to food-borne pathogen control in the U.S. poultry industry, which is primary focused on processing plants (USDA-FSIS, 2016). In laying hens, samples collected between 2004 and 2005 in the European Union demonstrated that 30.8% of the

holdings were positive for *Salmonella* whereas 20.4% were detected positive for *S*. Enteritidis and/or *S*. Typhimurium (EFSA, 2007a). Alike the observations made in meat-type production, *Salmonella* prevalence in laying hen facilities varied considerably among the EU member states (EFSA, 2007a).

PUBLIC HEALTH SIGNIFICANCE

Salmonella enterica is considered the second most common cause of foodborne disease in the United States (Scallan et al., 2011). Every year, nontyphoidal *Salmonella* causes an estimated 1.03 million illnesses including 19,336 hospitalizations and 378 fatal cases (Scallan et al., 2011). In people, salmonellosis is characterized by diarrhea, fever, and abdominal cramps occurring between 12 and 72 hours after ingestion and illness usually lasts from 4 to 7 days (CDC, 2016b). Most individuals recover without any treatment; however, elderly, infants, and immunocompromised people are more susceptible to develop severe salmonellosis and patients may need to be hospitalized (CDC, 2016b). In 2015, 149 *Salmonella* foodborne outbreaks and 3,944 illnesses were reported to the CDC (CDC, 2016a). The same year, the CDC Foodborne Disease Active Surveillance Network (FoodNet) reported 7,719 laboratory-confirmed salmonellosis, which represents an incidence of 15.75 per 100,000 persons (CDC, 2015). The incidence of laboratory-confirmed *Salmonella* cases remained close to 15.0 cases per 100,000 people in the last 20 years in the United States (CDC and NCEZID, 2016); however, the objective of the Office of Disease Prevention and Health Promotion is to reduce infections caused by *Salmonella* to 11.4 per 100,000 people by the year 2020 (ODPHP, 2017). In 2015, the most common serotypes associated with human salmonellosis were *S*. Enteritidis (18.0%), *S*. Newport (10.7%), and *S*. Typhimurium (9.8%) (CDC, 2015). That year, *S*. Heidelberg was

isolated from 2.0% of the laboratory confirmed cases of *Salmonella* infection (CDC, 2015). According to the Centers for Diseases Control and Prevention, consumption of chicken meat was the number 1 source of *Salmonella* outbreaks in the United States in 2013, 2014, and 2015 (CDC, 2016a). In 2013, *Salmonella* prevalence was 4.6 times higher in ground chicken than in chicken carcasses (18.0% versus 3.9%) and 6.5 times higher in ground turkey than in turkey carcasses (15.0% versus 3.2%) (USDA-FSIS, 2015a). This year, *S.* Heidelberg was among the six most prevalent serotypes detected from chicken carcasses and raw ground chicken (USDA-FSIS, 2015a). Between 2011 and 2014, *S.* Heidelberg was implicated in 5 major human foodborne outbreaks linked to poultry products, which caused 1,103 illnesses, 342 hospitalizations and 1 death. Among these outbreaks, one was linked to contamination of ground turkey with a multidrug resistant strain of *S.* Heidelberg that led to the recall of approximately 36 million pounds of finished product (CDC, 2016c).

SALMONELLA CONTAMINATION OF CARCASSES DURING PROCESSING

Transmission of *Salmonella* from poultry farms to the processing plant can occur through dirty crates, trucks, and the catching and pickup crews (Cox and Pavic, 2010; Russel, 2012). Poultry transport containers not thoroughly cleaned between flocks may play a role in cross contamination before birds enter the processing plant (Corry et al., 2002; Ramesh et al., 2003). A linear relationship between the percentage of *Salmonella*-positive live-haul trailers entering the processing plant and the percentage of ground turkey samples detected positive for *Salmonella* has been recently demonstrated (Evans et al., 2015). Moreover, in broilers, parameters such as low rainfall, temperature greater than or equal to 0°C during transport, and prolonged waiting time (≥ 4 hours) prior to slaughter can contribute to a higher proportion of positive carcasses at

the processing plant (Arsenault et al., 2007a). In turkeys, closure of truck lateral curtains during transportation to the slaughterhouse and low wind speed during transportation were associated with an increase in the proportion of *Salmonella*-positive carcasses (Arsenault et al., 2007b).

In the processing plant, cross contamination between *Salmonella* contaminated and non-contaminated birds can occur during scalding, de-feathering, evisceration, and chilling (Lillard, 1989; Fries, 2002; Shelly McKee, 2012; Russel, 2012). During scalding, birds are exposed to a common bath, which can promote spread of *Salmonella* cells from positive to negative carcasses (Russel, 2012). In an experimental study, *Salmonella* present in scalding bath and on chicken skin were able to survive the temperature (50 to 60°C) and the chlorination (10 to 50 ppm) of the scalding water (Yang et al., 2001). During de-feathering, *Salmonella* can be transferred from feathers to carcass skin (Nde et al., 2007) and cross contamination between carcasses can occur via aerosols (Allen et al., 2003), direct contact (Allen et al., 2003), and contact with contaminated picker fingers (Nde et al., 2007). Evisceration is a coordinated series of highly automated operations during which rupture of crop or intestine can lead to cross contamination of carcasses with *Salmonella* (Sarlin et al., 1998; Sams, 2000; Byrd et al., 2002). One to 5% of all broiler chickens produced in the United States must be reprocessed due to disease or contamination with fecal material or ingesta (Russel, 2012). Chilling of carcasses can be made by immersion in cold water, or by exposure to cold air to achieve the temperature of 4°C or less as soon as possible after the evisceration step (Sams, 2000). In the United States, most processing plants use water chilling, whereas air chilling is the most commonly used method in Europe (Russel, 2012). Various studies have described immersion chilling as a major site for *Salmonella* contamination between flocks (James et al., 1992; Sarlin et al., 1998).

During further carcass processing, *Salmonella* is able to adhere to stainless steel (Hood and Zottola, 1997) and can form biofilm on plastic surfaces (Stepanović et al., 2004), which contribute to cross contamination of cut and processed products.

POTENTIAL SOURCES OF *SALMONELLA* IN GROUND POULTRY

Ground poultry is made from grinded deboned, skin-on or skinless parts, such as drumsticks and thighs (USDA-FSIS, 2011). Skin is added to ground meat for its fat content (USDA-FSIS, 2011).

Salmonella external contamination of ground poultry components

In turkeys, *Salmonella* has been detected in 47% of the breast feather swabs collected prior to de-feathering (Nde et al., 2007). In chickens, prevalence of *Salmonella* on feathers was comprised between 52.5 and 75% before scalding (Kotula and Pandya, 1995; Cason et al., 2007). *Salmonella* cells can enter the open feather follicle during scalding and become entrapped in it as carcasses cool down (Cui et al., 2015). *Salmonella* cells lodged in crevices and within feather follicles are protected from carcass rinses and from chemicals treatments, when they are used alone or in combination with sonification (Lillard, 1989, 1993; Yang et al., 2001; Lee et al., 2014). Some studies have shown that 63 to 71% of turkey carcasses were positive for *Salmonella* after de-feathering (Clouser et al., 1995; Nde et al., 2007). *Salmonella* has been detected in 42% of the skin samples in turkeys after evisceration (Cui et al., 2015). After chilling, a recent study demonstrated that *Salmonella* prevalence varied among turkey skin parts; *Salmonella* was detected in 13.7%, 19.7%, and 25% of drumstick skin, thigh skin and wing skin samples, respectively (Peng et al., 2016). In chickens, *Salmonella* has been detected in 21.4% of neck skin

collected after chilling (Wu et al., 2014). Skin parts used as a source of fat in ground poultry meat can be a source of *Salmonella* contamination in the final product.

Internalized *Salmonella* in ground poultry components

Bone particles may enter into the composition of ground products but the amount is regulated and only 130 mg calcium per 100 g of product is allowed (USDA-FSIS, 2015b). During processing, bones may crack and release bone marrow; the presence of *Salmonella* in bones may represent a potential source of *Salmonella* in ground poultry (Cui et al., 2015; Peng et al., 2016). *Salmonella* has been detected in 9.3% of the turkey drumstick bone samples (Cui et al., 2015). In other studies, 0.8% of the chicken drumsticks collected at the processing plant were positive for *Salmonella* (Velaudapillai, 1964; Wu et al., 2014). In experimental challenges, *Salmonella* has been detected in 20% of bone samples from chickens inoculated with *S.* Enteritidis (Kassem et al., 2012) or *S.* Heidelberg (França et al., 2016).

S. Heidelberg and *S.* Typhimurium have been detected in 20% of drumstick muscle samples from 42-days-old specific-pathogen-free chickens experimentally inoculated at day of age (França et al., 2016). *Salmonella* present in lymphatics of drumstick muscles may contribute to contamination of ground poultry (França et al., 2016). In cattle and swine, *Salmonella* present within the lymph nodes has been described as a potential source of contamination of ground meat (Vieira-Pinto et al., 2005; Arthur et al., 2008; Garrido et al., 2014). Birds lack lymph nodes but have lymphoid nodules (Oláh et al., 2014). These rudimentary lymphoid structures are associated with lymphatics vessels running along with the femoral, popliteal, posterior tibial and wing veins (Oláh et al., 2014). Lymphoid nodules present in drumstick may serve as potential harborage sites for *Salmonella* in poultry (França et al., 2016).

PATHOGENESIS AND VIRULENCE FACTORS

Salmonella can cause acute or chronic infections, named salmonellosis, in domestic fowl (Shivaprasad et al., 2013). Four major *Salmonella* infections are important in poultry. Pullorum disease and fowl typhoid are caused by two non-motile host-adapted serovars, *Salmonella enterica* serovar Pullorum and *Salmonella enterica* serovar Gallinarum, respectively. Paratyphoid infections are caused by motile *Salmonella* serovars, and arizonosis is caused by *Salmonella enterica* subsp. *arizonae* (Jones, 2013). Newly hatched birds are highly susceptible to *Salmonella* infection but this susceptibility rapidly decreases overtime (Gast, 2013). In older birds, *S.* Typhimurium usually colonizes the gastrointestinal tracts without causing overt clinical signs, leading to asymptomatic carriers and eventual persistent shedders (Gast, 2013).

Salmonella commonly enters the host through fecal-oral transmission (Gast, 2013). Once ingested, *Salmonella* has to encounter the acidic pH of the upper gastrointestinal tract, which induces the expression of an acid tolerance response (Foster and Hall, 1990). Within the small intestine, bacteria cells are exposed to peristalsis, bile salts, enzymes, intestinal mucus, antimicrobial peptides, and secretory IgA (Khan, 2014). Within the lumen of the gastrointestinal tract, the mucous layer present on the epithelial cells acts as a physical barrier against the invasion of pathogens (Lillehoj et al., 2004). Fimbriae present on the surface of *Salmonella* cells facilitate the attachment to the intestinal mucosa (Darwin and Miller, 1999; Van Asten and Van Dijk, 2005). Once attached to the intestinal host cells, *Salmonella* internalization can occur through phagocytic uptake by professional phagocytic cells (M-cells and dendritic cells) or by active invasion of the intestinal mucosa using a type III secretion system encoded by the *Salmonella* Pathogenicity Island-1 (T3SS-1) (Libby et al., 2004; Velge et al., 2012). The T3SS-1 is a multi-protein complex which acts as a secretion apparatus to inject effector proteins such as

AvrA, SIPABCD, SopE, SopE2, SopB, and SopD into the host cell cytoplasm (Francis et al., 1993; Galán, 1996). Effector proteins induce rearrangement of the actin cytoskeleton resulting in membrane ruffles, epithelial uptake, and internalization of the bacteria (Francis et al., 1993; Galán, 1996). The *Salmonella* Pathogenicity Island-1 is a 40–kb region of DNA located at centisome 63 on the *S.* Typhimurium chromosome (Libby et al., 2004). The T3SS-1 plays a role in the stimulation of inflammatory response and pro-inflammatory cytokine production when *Salmonella* enters the epithelial cells (Hardt et al., 1998; Lee et al., 2000; Galán, 2001; Zhou et al., 2001). In chickens, different studies described an intestinal inflammatory response associated with expression of a wide range of cytokines (IL-1β, IL-8, IL-12, IL-18, LITAF, iNOS) and chemokines (K60) shortly after *Salmonella* invasion (Withanage et al., 2004; Berndt et al., 2007; Fasina et al., 2008). In the mouse colitis model, intestinal inflammation induced by *S.* Typhimurium T3SS-1 promotes oxidation of intestinal endogenous thiosulfate into tetrathionate (Winter et al., 2010), an electron acceptor for *Salmonella* anaerobic respiration (Barrett and Clark, 1987). Tetrathionate respiration has been exploited for years in diagnostic laboratory to enhance *Salmonella* isolation from samples containing competing bacteria (Barrett and Clark, 1987). *S.* Typhimurium tetrathionate respiration is encoded by a *ttrRSBCA* gene cluster present on the *Salmonella* Pathogenicity Island-2. *TtrA*, *ttrB*, and *ttrC* are structural genes encoding for tetrathionate reductase whereas TtrR and TtrS are components of a regulatory system required for the transcription of the *ttrBCA* operon (Hensel et al., 1999). In mice, intestinal inflammation enhances tetrathionate formation and promotes growth of *Salmonella* over the competitive microbiota that solely use fermentative metabolism (Winter et al., 2010; Thiennimitr et al., 2011).

Subsequently, *Salmonella* that reach the submucosa can be internalized by macrophages or dendritic cells within a modified phagosome known as the *Salmonella*-containing vacuole (Ibarra and Steele-Mortimer, 2009). A key virulence factor for *Salmonella* survival within phagocytic cells is the type III secretion system encoded by the *Salmonella* Pathogenicity Island-2 (T3SS-2) (Cirillo et al., 1998; Hensel et al., 1998). The *Salmonella* Pathogenicity Island-2 comprises a 40–kb region of DNA located at centisome 30 of the *S.* Typhimurium chromosome (Libby et al., 2004). Expression of SPI-2 has been shown to prevent exposure of *Salmonella*-containing vacuoles to NADPH oxidase in macrophages, which reduces the oxidative stress encountered by bacteria cells and provides a more hospitable environment for survival, replication, and dissemination of *Salmonella* (Vazquez-Torres et al., 2000; Ibarra and Steele-Mortimer, 2009).

In addition to SPI-1 and SPI-2, *Salmonella* have a large number of virulence factors such as virulence plasmids, fimbriae, flagella, and toxins that contribute to invasion and intracellular survival of organisms (Janda and Abbott, 2006; Jones, 2013; Rycroft, 2013).

Virulence plasmids are extrachromosomal DNA elements present only in a few *Salmonella* serotypes, including *S*. Enteritidis and *S*. Typhimurium. Virulence plasmids carry a 8-kb region of five genes designated as *spv* (for *Salmonella* plasmid virulence) (Janda and Abbott, 2006). These genes may play a role in bacterial virulence in *Salmonella* natural host (*S*. Pullorum in chickens, *S*. Enteritidis in mice) (Barrow and Lovell, 1988; Halavatkar and Barrow, 1993). In contrast, the *S*. Enteritidis virulence plasmid, required for full expression of virulence in mice, did not influence bacterial pathogenesis in newly hatched chickens and did not affect *S*. Enteritidis cecal colonization and tissue invasion of orally inoculated adult laying hens (Halavatkar and Barrow, 1993).

Fimbriae, also referred to as pili, are proteinaceous surface organelles located on the outside membrane of *Salmonella* that are 2 to 8 nm wide and 0.5 to 10 µm long (Van Asten and Van Dijk, 2005; Jones, 2013). *Salmonella* serovars contain large number of fimbrial gene sequences, also called fimbrial operons (Thorns, 1995; Humphries et al., 2001). Different combinations of fimbrial operons make *Salmonella* serovars able to express one or more type of fimbriae during their life cycle (Thorns, 1995; Humphries et al., 2001). In *S.* Typhimurium, 13 fimbrial operons have been identified (Jones, 2013). Flagella organelles are responsible for bacterial motility (Jones, 2013). Bacterial flagellum is composed of three main structures: the basal body, the hook, and a filament which can measure 16 to 22 µm in length (Jones, 2013). At least 50 genes and 15 operons are involved in the flagellar apparatus formation (Libby et al., 2004). In poultry, *S.* Enteritidis fimbriae have been suggested to play a role in intestinal colonization (Thiagarajan et al., 1996); whereas other studies demonstrated that fimbriae and flagella were unnecessary for *S.* Enteritidis intestinal colonization in chickens (Rajashekara et al., 2000; Dibb-Fuller and Woodward, 2000). In another study, SEF14 fimbriae alone did not play a role in *S.* Typhimurium colonization and invasion in internal organs of laying hens and specific-pathogen-free chicks (Thorns et al., 1996). Other studies in chicks described that flagella but not fimbriae were essential for *S.* Enteritidis cecal colonization (Allen-Vercoe and Woodward, 1999a) and for *S.* Enteritidis invasion to liver and spleen (Allen-Vercoe et al., 1999). In vitro, *S.* Enteritidis mutant strains deficient for type 1, SEF14, SEF17, and long polar fimbriae adhered to chicken gut as well as the wild-type strain; mutant strains deficient in *fliC*, *motAB*, and *cheA* flagellum loci, however, adhered significantly less than the parent strain (Allen-Vercoe and Woodward, 1999b). *Salmonella* flagellin, the proteinaceous constituent of flagella, is also responsible for triggering the innate immune response in mammals and avian species (Foster and

Berndt, 2013). Flagellin can activate the Toll-Like Receptor 5 (TLR5) present on chicken enterocytes and heterophils, which results in the production of the pro-inflammatory cytokines IL-1β and IL-8 (Kogut et al., 2005; Iqbal et al., 2005; Foster and Berndt, 2013).

The endotoxic property of *Salmonella* is due to lipid A of the lipopolysaccharide (LPS), an essential constituent of the outer membrane of gram-negative bacteria (Rycroft, 2013). The immune response induced by LPS is responsible for the endotoxic effects observed in the host, including noxious inflammation and septic shock (Libby et al., 2004). Liver and spleen lesions were observed in 2-week-old chickens after intravenous administration of *S.* Enteritidis endotoxin (Turnbull and Snoeyenbos, 1974). LPS could also contribute to *Salmonella* persistence in the avian intestinal tract; *S.* Typhimurium lacking components of the LPS structure did not colonize cecal contents as long as the parent strain in chickens inoculated at one day of age (Craven, 1994).

Cytotoxins have been detected in some *Salmonella* serovars such as *S.* Enteritidis (Ashkenazi et al., 1988). Exotoxins such as salmolysin, a hemolytic toxin encoded by *slyA* gene (Libby et al., 1994), and the enterotoxin encoded by the *stn* gene (Prager et al., 1995; Rahman, 1999) have also been identified in *S.* Typhimurium and *S.* Enteritidis serovars (Prager et al., 1995)

CLINICAL SIGNS OF PARATYPHOID INFECTION IN BIRDS

Paratyphoid infection is usually associated with disease only in newly hatched birds and the susceptibility to paratyphoid *Salmonella* rapidly decreases overtime (Gast, 2013). Infection within a few hours of hatching may result in massive intestinal multiplication, in severe systemic disease, and in mortality in birds (Gast and Beard, 1989; Desmidt et al., 1997; Withanage et al.,

2004). In young birds, clinical signs of paratyphoid infection include depression, anorexia, ruffled feathers, blindness, lameness, and diarrhea (Padron, 1990; Desmidt et al., 1997). Birds may be reluctant to move and present growth retardation (Padron, 1990; Desmidt et al., 1997). Although clinical signs associated with paratyphoid infection are not usually seen in adult birds, a study described drop in egg production, depression, anorexia, diarrhea, and mortality in adults laying hens experimentally infected with different *S*. Enteritidis isolates (Shivaprasad et al., 1990).

PATHOLOGY OF PARATYPHOID INFECTION IN BIRDS

Gross lesions

Thickening of the yolk sac wall, panophtalmitis, diphtheritic typhlitis with bloody cecal contents, cecal core, pericarditis, airsaculitis, hepathomegaly, necrotic foci on the liver, splenomegaly, and arthritis have been described in sick birds (Mitrovic, 1956; Padron, 1990; Desmidt et al., 1997; Withanage et al., 2004).

Microscopic lesions

Infiltration of macrophages, heterophils, and plasma cells can be observed within the lamina propria of the duodenum, ileum, and ceca (Desmidt et al., 1997; Withanage et al., 2004). Cecal tonsils may be thickened and edematous (Desmidt et al., 1997; Withanage et al., 2004). In ceca, extensive interstitial oedema, diphtheritic typhlitis with congestion, hemorrhage, and sloughing of epithelial cells have also been observed (Desmidt et al., 1997). In the liver, focal lesions filled with heterophilic infiltration as well as scattered heterophils may be present (Withanage et al., 2004).

DIAGNOSTIC TESTS

Since clinical signs are rarely pathognomonic, final diagnosis of paratyphoid salmonellosis requires isolation and identification of the causative agent (Gast, 2013; Shivaprasad et al., 2013).

Culture

Routine detection of *Salmonella* generally involves cultural methods which consist in a sequence of pre-enrichment, enrichment, selective-differential plating, isolation, and identification (Gast, 2013). Depending on the nature of the sample processed (animal or environmental sample) large assortments of broth and media are proposed for isolation of *Salmonella* (Waltman and Gast, 2008). First, a non-selective pre-enrichment allows recovery of injured *Salmonella* cells (Gast, 2013). Examples of non-selective pre-enrichment broths include buffered peptone water and lactose broth (Waltman and Gast, 2008). Selective enrichment broths such as tetrathionate broth, tetrathionate broth with brilliant green, Müller-Kauffmann, selenite F-broth or Rappaport Vassiliadis malachite green magnesium chloride broth (RV) encourage selective culture of *Salmonella* while reducing the growth of other organisms (Waltman and Gast, 2008; Gast, 2013). Third, differential and selective media plating are used for colonies isolation. Selective solid media include but are not limited to MacConkey bile-salt lactose agar, brilliant green agar (BGA), Taylor's xylose lysin deoxycholate agar (XLD), xylose lysin tergitol 4 (XLT4) (Waltman and Gast, 2008). Finally, pure colonies with classical *Salmonella* phenotype on selective media are subjected to biochemical and serological tests to confirm genus and serotype identities (ICMSF, 1996; Old, 1996; Waltman and Gast, 2008).

Serology

Among serologic tests, the whole blood plate, rapid serum plate, macroscopic tube agglutination, microagglutination, tube agglutination, and microantiglobulin tests are commonly used to detect *S*. Pullorum and *S*. Gallinarum (Waltman and Gast, 2008). Enzyme-linked immunosorbent assays (ELISAs) are available to detect *S*. Enteritidis antibodies (Gast, 2013; Shivaprasad et al., 2013). However, in general, serological tests are less reliable to detect birds with paratyphoid *Salmonella* (Shivaprasad et al., 2013).

Rapid detection methods

Considerable efforts have been done to develop efficient and reliable rapid *Salmonella* detection methods for emergency response (Lee et al., 2015). These tests are mainly used by food emergency response laboratories (Andrews et al., 2007; Lee et al., 2015). Commercially available rapid *Salmonella* detection methods include nucleic acid-based assays such as PCR, and immunology-based assays such as ELISA, latex agglutination tests, immunodiffusion, immunomagnetic separation, and immunochromatography.

Polymerase chain reaction (PCR) assays allow detection of *Salmonella* in feces, environmental samples, eggs and food samples (Cohen et al., 1994; Burkhalter et al., 1995; Gast, 2013; Lee et al., 2015; USDA-APHIS, 2017). Techniques such as ribotyping, multiplex PCR, multilocus sequence typing, and whole genome sequencing have also been developed in the recent years (Gast, 2013; Shivaprasad et al., 2013).

Among the commercially available immunology-based assays, the ELISA "sandwich" assay is the most commonly used for pathogens detection in food (Andrews et al., 2007). An antibody bound to a solid matrix is used to capture a specific antigen and form the antigen-

antibody complex. Presence of *Salmonella* antigens and antigen concentration in the sample can be evaluated measuring the cleavage of a chromogenic substrate by the enzyme linked to a second antibody (Andrews et al., 2007; Lee et al., 2015). In the latex agglutination technique, specific antibodies are coupled to latex particles to form stable agglutination reagents which reacts with *Salmonella* surface antigens to form visible aggregates (Thorns et al., 1994). The immunodiffusion test consists in detection of motile *Salmonella* by the formation of an antigen-antibody complex (Lee et al., 2015). The system unit consists in two connected chambers, an inoculation chamber and a motility chamber (D'aoust and Sewell, 1988). A pre-enrichment step is necessary afterwards the enriched sample is inoculated into a tetrathionate brilliant green broth in the inoculation chamber, whereas specific antibodies are added onto a semi-solid medium within the motility chamber (D'aoust and Sewell, 1988). When *Salmonella* moves from the inoculation chamber to the motility chamber, a white line of precipitation results from an antibody-dependent immobilization of motile *Salmonella* in the semi-solid medium (D'aoust and Sewell, 1988). Antibodies coupled to magnetic particles or beads are used in immunomagnetic separation (IMS) technology to bind targeted molecules. Binded molecules are then remove from the sample matrix when exposed to a magnetic field (Andrews et al., 2007; Warren et al., 2007). Combination of immunomagnetic separation technology with real-time PCR is usually performed for rapid detection of *Salmonella* in food samples (Mercanoglu and Griffiths, 2005). In immunochromatography assays, dip-sticks contain reagents to detect the presence of *Salmonella* in enriched cultures (Brinkman et al., 1995). The bottom part of the stick is dipped into the enriched culture to test. The flow created will take the culture broth across a reagent pad which contains anti-*Salmonella* antibodies coupled to colored latex bead or to colloidal gold. If antigens are present in the sample, *Salmonella* antigen-antibody complexes then migrate up to

the membrane strip to be captured by immobilized anti-*Salmonella* antibodies (Brinkman et al., 1995; Bautista et al., 2002; Andrews et al., 2007). Formation of a colored line on the stick revealed the presence of *Salmonella* (Brinkman et al., 1995; Bautista et al., 2002; Andrews et al., 2007).

Immunohistochemistry and immunofluorescence

Immunohistochemistry is a method based on antigen-antibody recognition for localizing specific antigens in tissues or cells (Taylor and Shi, 2013). Detection systems consist in a variety of antibody tags including fluorescent compounds, enzymes, and metals such as colloid gold (Ramos-Vara, 2005; Taylor and Shi, 2013). Antigen retrieval immunohistochemistry is possible in formalin-fixed paraffin embedded tissues (Taylor and Burns, 1974). Extremely sensitive methods have been developed to detect one or multiple antigens simultaneously (Ramos-Vara, 2005). Direct methods produce quick results but lack sensitivity (Ramos-Vara, 2005). In the two-step enzymatic method primary antibody is unlabeled and binds a specific antigen, an enzyme-conjugated secondary antibody is raised against the primary antibody and, in the presence of enzyme substrate and chromogen, the enzyme activity generates colored deposits at the sites of antibody-antigen binding (Nakane and Pierce, 1967; Taylor and Shi, 2013). The sensibility of indirect techniques is higher than the direct method because the number of labels per primary antibody is higher which results in an increased intensity of the reaction (Ramos-Vara, 2005). Detection systems used in veterinary medicine include avidin-biotin methods, peroxidase-antiperoxidase method, polymeric labeling two-step method, tyramine amplification method, and immune-rolling circle amplification (Ramos-Vara, 2005). The horseradish peroxidase is the most frequently used enzyme in immunohistochemistry staining; it has a good intracellular

penetration, a fast conversion rate of chromogenic substrate into a colored precipitate, and it is easy to conjugate to antibodies (Kalyuzhny, 2016). A chromogenic substrate for horseradish peroxidase is the DAB (3,3'-diamino-benzidine), which generates a brown color (Kalyuzhny, 2016). Immunohistochemistry has been used in research studies to investigate infection sites of *Salmonella* in poultry (Desmidt et al., 1998; Henderson et al., 1999; Berndt et al., 2007).

BIOLUMINESCENCE IMAGING OF *SALMONELLA*

Bioluminescence is defined as the enzymatic production of light by a living organism (Dunlap, 2009). Luciferases are the generic name given to the enzymes that catalyze the highly exergonic bioluminescent reactions in which chemical energy is transformed into light (Dunlap, 2009). The vast majority of bioluminescent organisms are marine dweller, however luciferases have been found in various organisms including cnidarians, mollusks, annelids, arthropods, echinoderms, insects, fish, fungi, dinoflagellate, and bacteria (Greer and Szalay, 2002; Shimomura, 2012). Among bioluminescent organisms found in nature, luminous bacteria are the most abundant and the most widely distributed (Meighen, 1993). Luminescent bacteria are ubiquitous in the marine environment, free or in symbiosis with fishes and squids (Dunlap, 2009). In the terrestrial environment, they are found in infected insects or as mutualistic symbionts of nematodes (Dunlap, 2009). Luminescent bacteria are all Gram-negative, motile, non-spore-forming bacteria; the majority of them are facultative aerobic and are able to use sugars by fermentation (Dunlap, 2009). Most of the marine luminous bacteria belongs to the genera *Photobacterium* and *Vibrio,* whereas terrestrial luminescent bacteria mainly belongs to the *Photorhabdus* genus (previously designated as *Xenorhabdus* genus) (Meighen, 1993; Dunlap, 2009).

Biochemistry of bacterial bioluminescence

Light emission in bacteria is catalyzed by a luciferase enzyme which mediates the oxidation of reduced flavin mononucleotide (FMNH2) and a long-chain aliphatic aldehyde by molecular oxygen (Dunlap, 2009). Intermediate products of the reaction are formed in their electronically excited state, from which emission of photons occurs (Meighen, 1991; Hastings, 1996).

Bacterial luminescence is encoded by the lux operon *luxCDABE*. The *luxA* and *luxB* genes encode, respectively, for the subunits α and β of the heterodimeric bacterial luciferase (Stewart and Williams, 1992). The *luxC*, *luxD*, and *luxE* genes encode for the subunits of a fatty acid reductase which convert fatty acids into the long-chain aldehydes required for the bioluminescent reaction (Meighen, 1993). Addition of long-chain aldehydes to luminous bacteria containing *luxCDE* genes is not required for bioluminescence (Meighen, 1991). However, FMNH2 and oxygen should be supplied to the bacterial luciferase to remain luminescent at constant level (Meighen, 1993).

In some bacterial species, additional *lux* genes have been identified (*luxF*, *luxG*, *luxI*, *luxH*, *luxR*, *luxY*) (Meighen, 1993). Among them, *luxI* and *luxR* are regulatory genes controlling bacterial luminescence and are located immediately upstream the *luxCDABE* operon in *Vibrio fischeri* (Meighen, 1993). *LuxF* gene encodes for a flavoprotein which may play a specific role in bioluminescence of *Photobacterium* species (Meighen, 1993).

Bioluminescence imaging (BLI)

Different types of imaging systems can be used for detection of bioluminescent bacteria. Among them, cooled charge-coupled device camera (cooled CCD) convert photons emitted by

the bioluminescent reaction of bacteria cells into electrons (Contag et al., 1997). The camera accumulates charges representing the number of photons striking each CCD pixel. CCD can detect photons emitted with a wavelength between 400 and 1000 nm (Contag et al., 1997).

Applications of bioluminescence imaging

Bioluminescence imaging (BLI) is not routinely used as a diagnostic method for *Salmonella* detection in samples. However, during the past few years, various studies have used BLI for *Salmonella* detection and enumeration in living animals, tissue samples as well as in food products (Contag et al., 1995; Burns-Guydish et al., 2005; Karsi et al., 2008; Moza et al., 2009; Howe et al., 2010; Özkaya et al., 2012; França et al., 2016). In mice, BLI has been used to monitor the progression of *Salmonella* infection (Contag et al., 1995; Burns-Guydish et al., 2005). In poultry, bioluminescent *Salmonella* have been used to investigate *Salmonella* invasion in chickens tissues (França et al., 2016), *Salmonella* attachment to chicken skin (Karsi et al., 2008), and *Salmonella* penetration into processed chicken fillets (Moza et al., 2009). Compared to standard microbiological methods, BLI allows a real-time visualization and enumeration of live bacterial cells in samples. *In vitro*, Karsi et al., 2008 were able to detect as low as 125 CFU bioluminescent *Salmonella* on chicken skin. The minimum detectable numbers of bioluminescent *Salmonella* in live animals ranged from 1×10^3 to 1×10^6 CFU (Contag et al., 1995; Burns-Guydish et al., 2005). In another study, bioluminescence imaging detected *Salmonella* levels greater than 10^7 CFU/g in ceca with 97.1% sensitivity (França et al., 2016). However the sensitivity rapidly decreased with low *Salmonella* cells; the sensitivity to detect 10^6-10^7 CFU/g and 10^5-10^6 CFU/g of *Salmonella* in ceca was 40% and 28.6%, respectively (França et al., 2016).

PREDISPOSING FACTORS TO *SALMONELLA* INFECTION

Stress

Management and environmental factors may increase the likelihood of *Salmonella* infection in poultry (Gast, 2013). Water deprivation increased the duration of *Salmonella* fecal shedding in 7-week-old birds experimentally inoculated with *S*. Typhimurium (Brownell et al., 1969). Corrier et al., 1999 demonstrated that *Salmonella* incidence in crop contents of broilers increased during pre-slaughter feed withdrawal. Feed withdrawal protocols are also used to induce molting in hens (Holt and Porter, 1992). Physiological stress generated by induced molting in hens is known to increase levels of *Salmonella* in feces (Holt and Porter, 1992; Holt, 1993; Holt et al., 1995). Nakamura et al., 1994 described that short-term exposure to environmental stress, such as introduction of young chickens to the same rearing room as 7-month-old laying hens, or feed and water removal increased *Salmonella* prevalence in cecal droppings of the stressed-laying hens.

Infectious bursal disease

Specific-pathogen-free chickens inoculated at one day of age with infectious bursal disease virus (IBDV), an immunosuppressive virus, and challenged with *S*. Enteritidis at 2 days of age presented significantly higher mortality than birds only infected with *S*. Enteritidis (Phillips and Opitz, 1995). Specific-pathogen-free birds exposed to IBDV at one day of age and subsequently challenged with *S*. Typhimurium also presented increased mortality compared to control birds (Wyeth, 1975).

Coccidiosis

Coccidiosis is an ubiquitous intestinal disease of poultry that causes significant economic losses from mortality, morbidity, reduced weight gain, and impaired feed conversion (McDougald and Fitz-Coy, 2013). Coccidia oocysts are extremely resistant to common disinfectants and elimination of oocysts from poultry house is never complete between flocks (McDougald and Fitz-Coy, 2013). Avian coccidiosis is caused by protozoan parasites of the genus *Eimeria; E*. *acervulina*, *E. necatrix*, *E. maxima*, *E. brunetti*, *E. mitis*, *E. tenella* and *E. praecox* are the seven species commonly described in chickens (McDougald and Fitz-Coy, 2013). Coccidial infection may be classified into clinical or subclinical coccidiosis (Williams, 1999). Tissue damage caused by multiplication of these parasites in intestinal epithelial cells result in nutrient malabsorption, dehydration, blood loss, loss of skin pigmentation, and increased susceptibility to other pathogens (McDougald and Fitz-Coy, 2013). Anticoccidial drugs have been widely administrated in poultry flocks to prevent coccidiosis (Chapman and Jeffers, 2014). In recent years, vaccination with live vaccines is often used as an alternative to chemical treatments for coccidiosis control in flocks (Chapman et al., 2002). Various studies reported an increase in *Salmonella* levels in cecal contents of chickens concurrently infected with *E. tenella* or *E. acervulina* compared to birds infected with *Salmonella* alone (Arakawa et al., 1981; Takimoto et al., 1984; Morishima et al., 1984; Fukata et al., 1987). *E. acervulina* or *E. tenella* challenge also increased *S.* Typhimurium prevalence in ceca of coinfected birds (Arakawa et al., 1981; Takimoto et al., 1984). Furthermore, coccidia coinfection may also enhance systemic dissemination of *Salmonella* in chickens; a significant increase in *S*. Typhimurium prevalence in liver has been described in chickens coinfected with *E. tenella*, *E. necatrix* or *E. maxima* compared to birds infected with *S*. Typhimurium alone (Stephens et al., 1964; Stephens and

Vestal, 1966; Arakawa et al., 1981; Takimoto et al., 1984). Contrary to these findings, Tellez et al., 1994 demonstrated that prevalence of *S*. Enteritidis in internal organs of infected chickens decreased when *E. tenella* inoculum dose increased from 10 to 10^3 sporulated oocysts. An increase in the thickness of the cecal lamina propria induced by infiltration of inflammatory cells was correlated with a decrease in organ invasion by *S*. Enteritidis (Tellez et al., 1994).

TREATMENT AND CONTROL OF *SALMONELLA* INFECTION

Various antibiotics have been used orally, subcutaneously or in feed to prevent or treat paratyphoid infection in poultry (Gast, 2013). However, administration of antibiotics generally fails to eliminate *Salmonella* infection in birds and contributes to antimicrobial resistance and development of *Salmonella* carriers in treated birds (Smith and Tucker, 1975; Williams and Whittemore, 1980; Gast et al., 1988; Manning et al., 1994). Antimicrobial resistance is a growing public health threat (WHO, 2014). In the United States, the national professional association for U.S. veterinarians (AVMA) promotes the responsible use of antibiotics in food-producing animals according to the FDA Guidance for Industry 209 and 213 (AVMA, 2017; FDA, 2017). To be successful, effective prevention and control programs should consider the diversity of sources from which *Salmonella* can be introduced in poultry during live production (Gast, 2013). In a survey conducted by Shelly McKee, 2012, practices identified to have a role in controlling *Salmonella* contamination in flocks included vaccination of breeder flocks, litter management, biosecurity, heat treatment of the feed, and poultry water treatment. Live and inactivated vaccines are used to control *Salmonella* in poultry; the main objectives are to obtain a colonization-inhibition effect conferred by the vaccine strain against field *Salmonella* challenge,

to reduce the levels of *Salmonella* in flocks, and to confer protective immunity to the progeny of vaccinated breeder flocks (Desin et al., 2013).

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Table 2.1. *Salmonella* species and subspecies, their usual habitats, and number of serotypes identified by the year 2007 (Brenner et al., 2000; Grimont and Weill, 2007)

	Salmonella species and subspecies	No. of serotypes within subspecies in 2007	Usual habitat
I	*Salmonella enterica* subsp. *enterica*	1531	Warm-blooded animals
II	*Salmonella enterica* subsp. *salamae*	505	Cold-blooded animals and environment
IIIa	*Salmonella enterica* subsp. *arizonae*	99	
IIIb	*Salmonella enterica* subsp. *diarizonae*	336	
IV	*Salmonella enterica* subsp. *houtenae*	73	
VI	*Salmonella enterica* subsp. *indica*	13	
V	*Salmonella bongori*	22	
	TOTAL	2,579	

CHAPTER 3

SALMONELLA HEIDELBERG HARBORAGE SITES IN EXPERIMENTALLY INFECTED TURKEYS THAT CONTRIBUTE TO CONTAMINATION OF GROUND MEAT[1]

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[1] Claire-Sophie Rimet, John J. Maurer, Ana M. Villegas, Lisa J. Stabler, Kasey K. Johnson, and Monique S. França. To be submitted to Frontiers in Sustainable Food Systems.

Key words:

Salmonella Heidelberg, Harborge sites, Feather follicle, Turkeys, Ground poultry

ABSTRACT

Poultry products, contaminated with *Salmonella enterica* subsp. *enterica* serovar Heidelberg have been implicated in 5 major human salmonellosis outbreaks in the United States between 2011 and 2014. Cross contamination with fecal material during processing is considered the main source of *Salmonella* contamination in poultry products. However, *Salmonella* is capable of invading intestinal epithelium and spreading beyond the gastrointestinal tract via the reticuloendothelial system to the muscle and bone marrow; components commonly used in ground meat. The objective of this study was to identify harborage sites of *Salmonella* in tissues used to make ground poultry products. One-day-old, commercial turkeys were orally inoculated with a pool of five bioluminescently-tagged *S.* Heidelberg isolates, including 4 strains associated with foodborne outbreaks linked to ground poultry. Drumstick muscles with lymphatics were collected from birds at 6 to 7 weeks and 11 weeks of age. *Salmonella* in tissues was evaluated by bioluminescence imaging, culture, or immunohistochemistry. Breast skin and tibiotarsus were collected from *Salmonella*-challenged birds at 11 weeks of age.

All drumstick muscles collected (n = 132) were negative for *S.* Heidelberg by bioluminescence imaging and culture. All tibiotarsus samples collected (n = 93) were culture negative for *S.* Heidelberg. Thirty percent of the breast skins (n = 93) were positive for *S.* Heidelberg when birds were 11 weeks old. *Salmonella* Heidelberg was primarily located on epidermal keratin and within feather follicles.

Including skin in ground meat may significantly contribute to *Salmonella* contamination of ground turkey meat.

INTRODUCTION

Salmonella enterica is considered the second most common cause of foodborne disease in the United States (Scallan et al., 2011).

Poultry species are acknowledged as natural reservoirs for *Salmonella* and an important food vehicle in outbreaks (Shivaprasad et al., 2013). *Salmonella* Heidelberg has been implicated in 5 major human foodborne outbreaks linked to poultry products between 2011 and 2014, which caused 1,103 illnesses, 342 hospitalizations and 1 death (CDC, 2016). Among these outbreaks, one was linked to ground turkey contaminated with a multidrug resistant *S.* Heidelberg strain that led to the recall of approximately 36 million pounds of finished product (CDC, 2016). Identification of potential sources of *Salmonella* contamination in ground turkey is essential for reducing foodborne outbreaks (ODPHP, 2017).

It has been widely established that cross-contamination with fecal material during processing is the main source of *Salmonella* contamination in poultry products (Carrasco et al., 2012). Birds infected with *Salmonella* are generally asymptomatic (Shivaprasad et al., 2013), especially when brought to the meat processing plant. The few *Salmonella* positive birds in the flock can cross-contaminate carcasses during picking, scalding, and de-feathering in processing plants (Lillard, 1989; Shelly McKee, 2012). During evisceration, rupture of crop or intestine can lead to contamination of carcasses with ingesta or fecal material potentially contaminated with *Salmonella* (Sarlin et al., 1998; Sams, 2000; Byrd et al., 2002). When carcasses reach the chiller tank, *Salmonella* cross-contamination can occur between birds or between flocks (James et al., 1992; Sarlin et al., 1998). Ground turkey contains dark meat from drumsticks, thighs, and wings as well as various parts of skin (Cui et al., 2015; Peng et al., 2016). Previous studies reported the presence of *Salmonella* in drumstick bones in turkeys as a potential source of *Salmonella* in

ground turkey components, when bones crack and release bone marrow during processing and deboning (Cui et al., 2015; Peng et al., 2016).

Salmonella invades the intestinal mucosa through uptake by M-cells and dendritic cells or by active invasion of the intestinal epithelium using a type III secretion system encoded by the *Salmonella* Pathogenicity Island-1 (T3SS-1) (Libby et al., 2004; Velge et al., 2012). After invasion, *Salmonella* can survive within phagocytic cells using a type III secretion system encoded by the *Salmonella* Pathogenicity Island-2 (T3SS-2) (Libby et al., 2004) and can disseminate systemically through the lymphatics (Gulig, 1987; Jones and Falkow, 1996; Pullinger et al., 2007). Lymph nodes are harborage sites for *Salmonella* in cattle and swine and they are possible sources of contamination of ground products in these species (Vieira-Pinto et al., 2005; Arthur et al., 2008; Garrido et al., 2014). Birds lack lymph nodes but have lymphoid nodules, also called mural lymph nodes, which are rudimentary structures associated with lymphatics vessels running along with the femoral, popliteal, posterior tibial, and wing veins (Oláh et al., 2014). Because mural lymphoid nodules contain phagocytic cells, these structures serve as potential harborage sites for *Salmonella* in poultry.

Standard microbiological methods required several days for *Salmonella* detection and enumeration. Bioluminescence imaging allows a real-time visualization and enumeration of live bacterial cells in living animals, tissue samples as well as in food products (Contag et al., 1995; Burns-Guydish et al., 2005; Karsi et al., 2008; Moza et al., 2009; Howe et al., 2010; Özkaya et al., 2012; França et al., 2016). According to Karsi et al., 2008 and Howe et al., 2010, the minimum detectable numbers of bioluminescent *Salmonella* in vitro was 125 CFU and 4.16×10^3 CFU, respectively. In live animals, the minimum detectable numbers ranged from 1×10^3 to 1×10^6 CFU (Contag et al., 1995; Burns-Guydish et al., 2005). Bioluminescent *Salmonella* can

be generated by generalized transduction with a bacteriophage carrying the luciferase genes from bioluminescent organisms (Meighen, 1991). This luciferase is constitutively expressed in *Salmonella* resulting in the production of visible light.

The objective of this study was to determine *Salmonella* Heidelberg prevalence and harborage sites in samples of drumstick muscle with lymphatics, bone, and skin after experimental infection of turkeys in order to assess the contribution of these sites to *Salmonella* contamination of ground turkey products.

MATERIALS AND METHODS

Tagging *S.* Heidelberg strains with a bioluminescent marker

S. Heidelberg strain SH380 in addition to four *S.* Heidelberg strains (SH038, SH682, SH198, and SH890) associated with foodborne outbreaks in the United States were used in this trial. *Salmonella* strains were tagged with mini-Tn5-*luxCDABE* transposon as previously described (Burns-Guydish et al., 2005). The transposon was engineered to constitutively express the kanamycin resistance gene and the *luxCDABE* gene, the latter making the transduced *Salmonella* cells strongly luminescent regardless of growth conditions (Burns-Guydish et al., 2005). *S.* Heidelberg strains were streaked on MacConkey agar with kanamycin (50 µg/mL) and incubated at 37°C for 24 hours. Bioluminescent colonies were detected using an IVIS Lumina XR imaging system (Perkin Elmer; Greenville, SC).

Preparation of *S.* Heidelberg strain cocktail for oral animal challenge.

For each strain, a single bioluminescent colony was collected and grown static in 5mL Luria-Bertani broth at 37°C for 16 hours, after which the bacterial cell density was estimated

from the optical density $(OD)_{600}$ for the cell suspension (~ 0.798 OD_{600} or 8.7×10^8 cells/ml). *Salmonella* cultures were resuspended in equal volume of buffered saline gelatin (BSG) and mixed together, in equal parts for the five strains, to make the final strain cocktail for oral challenge. The final bacterial count was confirmed by plating 10-fold serial dilutions from the inoculum on MacConkey agar with kanamycin (50 µg/mL). After 24 hours incubation at 37°C, colony forming units (CFU) were determined.

Oral challenge of day old turkeys with bioluminescent *S.* Heidelberg cocktail

One-day-old turkey hens (Nicholas) were obtained from a commercial breeder company and placed in four colony houses at the Poultry Diagnostic and Research Center, College of Veterinary Medicine, The University of Georgia, GA, USA. Parent flocks had been vaccinated at 15 and 25 weeks of age with an autogenous *Salmonella* Hadar vaccine. The progeny received a single dose of gentamicin at the hatchery. Fecal samples and chick paper were tested prior to inoculation to confirm that the day old birds were *Salmonella* free. Turkey poults were placed on pine shavings litter and brooded following standard temperature regimens. All birds were given access to water and feed containing coccidiostat (Amprolium 125 ppm) *ad libitum.* Birds were observed for clinical signs twice a day.

One hundred and thirty-three, one-day-old turkey poults were reared in 3 colony houses at the College of Veterinary Medicine, The University of Georgia, GA, USA. Stocking density ranged from 1.2 to 1.8 ft²/bird until week 7; and from 1.9 to 2.1 ft²/bird between week 7 and 11. At placement, turkey poults were orally inoculated with a 0.1 mL inoculum containing 4.4 x 10^7 CFU of the pooled, bioluminescent *Salmonella* strains (SH380-lux, SH038-lux, SH682-lux, SH198-lux and SH-890-lux). Twenty, uninfected control birds were placed in a fourth colony

house and orally inoculated with 0.1 mL sterile buffered saline gelatin (BSG) broth. Oral inoculations were performed using a pipette tip placed in the oral cavity of the birds.

Thirty-nine birds from the *Salmonella* Heidelberg-challenged group and six birds from the control group were euthanized by carbon dioxide followed by neck dislocation between 6 and 7 weeks of age to reduce bird density in the colony houses. The legs of the turkeys were disinfected by dipping the half lower part of the carcass in a 0.08% sodium hypochlorite solution for 5 minutes. Drumstick muscle samples with lymphatics were aseptically collected for bioluminescence imaging and *Salmonella* culture. At 11 weeks of age, all remaining ninety-three birds from the *Salmonella* Heidelberg-challenged group and fourteen birds from the control group were euthanized by carbon dioxide followed by jugular exsanguination. Feathered breast skin was aseptically collected for *Salmonella* culture and the half lower part of the carcass (legs and hip) was disinfected by soaking it in a 0.08% sodium hypochlorite solution for 5 minutes. Drumstick muscle samples with lymphatics were aseptically collected for bioluminescence imaging and *Salmonella* culture. Drumsticks were aseptically collected from the opposite leg and dissected to remove muscle and cartilage from the bone (tibiotarsus). Decontamination of the tibiotarsus surface with 70% ethanol solution for 5 minutes was performed prior to bacteriology in bone marrow samples. Cecal droppings present on litter were collected from all 4 colony houses from weeks 2 to 10 post inoculation for estimating *Salmonella* prevalence and abundance.

Animal experiments were conducted under strict adherence to Institutional Animal Care and Use Committee (IACUC) guidelines at The University of Georgia, GA, USA.

Bioluminescence imaging

The presence of bioluminescent *Salmonella* was monitored using an IVIS Lumina XR imaging system (Perkin Elmer; Greenville, SC) equipped with a cooled charge-coupled device (CCD) camera. Data acquisition and analyses were performed using the Living Imaging Software (Perkin Elmer; Greenville, SC). A greyscale reference image was taken first. Three-minutes images of light (emitted photons) transmitted through the tissues were taken in the dark. After photon collection, a pseudocolor representation of light intensity (red, most intense; blue, less intense) was overlaid to the greyscale image of the tissue surface.

Detection of *S.* Heidelberg in tissues

Tissues were placed in sterile 18 oz. Nasco Whirl-PAK bags (Nasco; Fort Atkinson, WI) and 10 mL of tetrathionate brilliant green (TBG) broth with iodine was added to each sample. Muscle with lymphatics and skin samples were homogenized with a stomacher (Stomacher80 Seward; England) for 1 minute. Bone samples were gently mixed for approximately 1 minute. Tissues homogenates were then incubated at 42°C for 24 hours. A loopful of TBG enrichment (1 µL) was streaked on MacConkey agar plates with kanamycin (50 µg/mL). Plates were incubated at 37°C for 24 hours and bioluminescent *Salmonella* were detected using the IVIS Lumina XR imaging system.

Enumeration of *S.* Heidelberg in cecal droppings

Cecal droppings were placed in sterile 50 mL, conical centrifuge tubes (Thermo Scientific; Rochester, NY), weighed, and an equal volume to weight of BSG was added to each cecal sample. Samples were vortexed thoroughly, serially diluted 10-fold in BSG (final dilution

10^{-7}) for enumeration on MacConkey agar plates with kanamycin (50 μg/mL). Plates were incubated at 37°C for 24 hours and bioluminescent *Salmonella* were detected and enumerated (CFU/g cecal droppings) by bioluminescence imaging. Enrichment was performed to detect low levels of *Salmonella* in cecal samples. Briefly, 10 mL of TBG broth with iodine were added to cecal samples followed by incubation at 37°C for 24 hours. A loopful of the TBG enrichment was streaked on MacConkey agar plates with kanamycin (50 μg/mL). Plates were incubated at 37°C for 24 hours and bioluminescent *Salmonella* were detected by bioluminescence imaging.

Immunohistochemistry

Tissues, positive for *Salmonella* by culture, were processed for immunohistochemistry. Briefly, breast skin samples were placed in 10% buffered formalin, embedded in paraffin, and sectioned at 4 μm. Tissue sections were deparaffinized in xylene and hydrated in decreasing alcohol solutions. Antigen retrieval was performed in citrate buffer at pH 6.0 with the use of a steamer. Sections were washed with distilled water and the endogenous peroxidase activity was blocked using a 3% H_2O_2 (or Bloxall) solution for 10 minutes. After 10 minutes incubation, the sections were washed with PBS and incubated for 1 hour at room temperature with the primary antibody Rabbit anti-*Salmonella* 0 serogroup B (BD; Franklin Lakes, NJ) at 1:500 antibody concentrations. The sections were washed with PBS to remove any unbound primary antibody and then treated with Protein Block solution (Dako Agilent Pathology Solutions; Carpinteria, CA) for 10 minutes. After 10 minutes incubation, the sections were washed with PBS and then incubated for 1 hour with the secondary antibody Rabbit-on-Farma horseradish peroxidase polymer (Biocare Medical; Concord, CA). After washing steps, the slides were stained for 10 minutes with 3, 3'-diaminobenzidine (Vector Labs; Burlingame, CA) and counterstained with

Mayer's hematoxilin. Sections of intestine from chickens infected with *Salmonella* Typhimurium from a previous study were used as positive control samples. Sections of intestine incubated with rabbit antiserum instead of primary antibodies were used as negative controls. The slides were examined using a bright field microscope.

RESULTS

S. Heidelberg prevalence and harborage sites in turkeys

All muscle samples collected between 6 and 7 weeks of age and at 11 weeks of age were negative for *S.* Heidelberg by bioluminescence imaging and culture. Similarly, tibiotarsus samples were all *Salmonella* negative for birds at 11 weeks of age. Only breast skin samples were *S*. Heidelberg positive; 30.1% prevalence in birds at 11 weeks following oral inoculation (Table 3.1). Immunohistochemistry revealed that *Salmonella* group B-positive bacterial cells were localized on epidermal keratin in all 28-skin samples which were positive by bacteriology (Figure 3.1.B). Bacteria cells were mostly organized in clusters. High numbers of *Salmonella* cells were observed entrapped within skin folds (Figure 3.1.C). *Salmonella* group B-positive bacterial cells were also observed within feather follicles in 10.7% of birds culture-positive for *Salmonella* (Figure 3.1.D). Interestingly, *Salmonella* group B-positive bacterial cells were also observed within epidermal ulcers on breast skin samples in two birds at 11 weeks of age; these bacteria cells were surrounded by necrotic material within a serocellular crust (Figure 3.1.E). In one of these two birds, *Salmonella* group B-positive bacterial cells were also observed within a dermal blood vessel, as free cells and in the cytoplasm of monocytes (Figure 3.1.F).

S. Heidelberg cecal shedding in turkeys

Salmonella Heidelberg abundance in cecal droppings was the highest in week 3 post-inoculation (4.4 $\log_{10}$ CFU/g). Between week 2 and 4 post-inoculation, the prevalence of *Salmonella* in cecal droppings collected was 100%. *Salmonella* counts subsequently decreased thereafter, with mean *Salmonella* counts of 1.3 $\log_{10}$ CFU/g at 10 weeks of age (Figure 3. 2).

DISCUSSION

Colonization dynamics of combination of different *Salmonella* strains were first described more than 60 years ago (Meynell, 1957). After an oral challenge of a mixed inoculum of two *S.* Typhimurium strains in mice, Meynell, 1957 demonstrated that these organisms caused fatal infection independently. The conclusion drawn was that the probability of any given bacterium in a mixed inoculum to initiate an infection is entirely determined by its ability to invade the intestinal mucosa. In the present study, we used a combination of five *Salmonella* Heidelberg strains isolated from foodborne outbreaks in the United States in order to determine whether some strains were able to disseminate systemically and infect tissues used as ground turkey components. Newly hatched birds are highly susceptible to *Salmonella* infection, but this susceptibility decreases overtime (Gast, 2013). Infection within a few hours of hatching, as can occur in hatcheries, may result in massive intestinal multiplication and in severe systemic disease in birds (Gast and Beard, 1989; Desmidt et al., 1997; Withanage et al., 2004). For this reason, and for the purpose of this study, turkey poults were inoculated at day-of-age.

Despite numerous measures implemented in processing plants to reduce *Salmonella* contamination in ground products, *Salmonella* prevalence in ground turkey still oscillates between 11 and 20% since the last decade with no noticeable decrease in the last few years

(USDA-FSIS, 2015a). Understanding the origins of *Salmonella* in ground turkey components is necessary to reduce bacterial contamination in final products and to prevent foodborne salmonellosis.

Bone-in and boneless meat from poultry parts, such as drumsticks and thighs, generally go into making ground poultry meat (USDA-FSIS, 2011). Skin in natural proportion is included for its fat content (USDA-FSIS, 2011). The amount of bone particles in meat that enter into the composition of ground products is regulated and only 130 mg calcium per 100 g of product is allowed (USDA-FSIS, 2015b). In the literature, variable *Salmonella* prevalence has been described in turkey or chicken bone. Cui et al., 2015 observed a 21.3% *Salmonella* prevalence in turkey bones collected after the evisceration step in processing plant from *Salmonella* targeted flocks. Other studies described a 0.8% prevalence of *Salmonella* in chicken drumsticks collected at the processing plant (Velaudapillai, 1964; Wu et al., 2014). In experimental challenges, *Salmonella* was detected in 20% of bone samples in 35 days old specific-pathogen-free (SPF) chickens orally inoculated at day of age with 2 x 10^5 CFU of *Salmonella* Enteritidis (Kassem et al., 2012) or with 1 x 10^8 CFU of *Salmonella* Heidelberg (França et al., 2016). There are very limited data on *Salmonella* presence inside the lymphatics of poultry species. Franca et al. 2016 described a 20% prevalence of *Salmonella* Heidelberg and *Salmonella* Typhimurium in lymphatics associated to the posterior tibial vein in 42-days-old SPF chickens inoculated at day of age. In the present study, all samples of drumstick muscle with lymphatics and bone were detected negative for *Salmonella*.

Our results show that S*almonella* Heidelberg in skin of infected turkeys, rather than internalized in muscle and bone, might significantly contribute to contamination of ground turkey as 30.1% of the turkey breast skin samples were culture-positive. Previous studies have

investigated *Salmonella* prevalence in turkey skin (Nde et al., 2007; Cui et al., 2015). A 47% *Salmonella* prevalence was detected by Nde et al., 2007 from turkey breast feathers swabbed prior to de-feathering at the processing plant. Another study described a 86.7% prevalence in turkey neck skins after evisceration in S*almonella*-targeted flocks (Cui et al., 2015). However little is known about *Salmonella* prevalence in turkey skin before entering the processing plant. Turkeys can shed *Salmonella* asymptomatically for a long period of time after infection (i.e. 10 weeks in this study) raising the risk of bacterial introduction and dissemination into the processing plant. During transport, breast skin feathers are routinely in contact with fecal material and can be contaminated if birds are shedding *Salmonella*. Evans et al., 2015 demonstrated a linear relationship between the percentage of *Salmonella*-positive live-haul trailers entering the processing plant and the percentage of *Salmonella*-positive ground turkey samples. Reducing the prevalence of *Salmonella* on poultry skin before entering the processing plant appears to be a major factor to limit risks of cross contamination and bacterial spread during processing.

We revealed by immunohistochemistry that *Salmonella* cells were mainly observed organized in clusters on the epidermal keratin. A large number of non-fimbrial and fimbrial adhesive structures are responsible for auto-aggregation of bacteria and adhesion to surfaces (Wagner and Hensel, 2011). It has been recently demonstrated that specific flagellin subunit (subunit fliC), and flagellar motor were necessary for optimal attachment on chicken skin surfaces (Salehi et al., 2016). Expression of the adhesive structures also depends on environmental factors such as temperature, oxygen, and nutrient availability (Collinson et al., 1993; Maurer et al., 1998; Gerstel and Römling, 2001). Ambient temperature and the lack of nutrients on keratinized epithelium may contribute to thin aggregative fimbriae formation,

leading to bacterial aggregates on birds skin (Collinson et al., 1993; Maurer et al., 1998; Gerstel and Römling, 2001).

Salmonella cells were also visualized within the lumen of feather follicles by immunohistochemistry. Previous studies used scanning electron microscopy, confocal scanning laser microscopy, and fluorescent antibody staining to demonstrate penetration and attachment of *Salmonella* into feather follicles and skin folds of scalded, de-feathered skins inoculated with *Salmonella* cells (Kim and Doores, 1993a; b, Kim et al., 1993, 1996). To the best of our knowledge, this study is the first that describes *Salmonella* cells within the lumen of feather follicles of infected turkeys. *Salmonella* cells lodged in crevices and within feather follicles are protected from rinses and chemicals treatments (Lillard, 1989; Kim et al., 1996; Lee et al., 2014). The USDA-approved chemicals can reduce *Salmonella* loads in scalding and chilling baths but they are not efficient in eliminating *Salmonella* entrapped into the skin (Lillard, 1989; Yang et al., 2001; Lee et al., 2014). Similar observations were made with combination of sonification and chemical treatments on poultry carcasses (Lillard, 1993; Lee et al., 2014). Entrapped *Salmonella* cells may be released once skin parts reach the grinder and this may contribute to contamination of ground poultry when ground skin is mixed to other ground components. This may explain why *Salmonella* prevalence is significantly higher in ground turkey than on raw turkey carcasses (19.9% versus 1.7% in 2014) (USDA-FSIS, 2015a). In this study we demonstrated that *Salmonella* presence in skin of infected turkeys may significantly contribute to contamination of raw end-product. Exclusion of skin may be the best option for reducing *Salmonella* contamination in ground turkey.

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Table 3.1. *Salmonella* Heidelberg prevalence in samples of drumstick muscle with lymphatics, tibiotarsus, and breast skin in turkeys orally inoculated at day of age with 4.4×10^7 CFU of a *S.* Heidelberg cocktail

Weeks post inoculation	*Salmonella* Heidelberg-positive samples/total (%)			
	Direct BLI[a]	*Salmonella* culture[b]		
	Drumstick muscle with lymphatics	Drumstick muscle with lymphatics	Tibiotarsus	Breast skin
6-7 weeks	0/39 (0%)	0/39 (0%)	—[c]	—[c]
11 weeks	0/93 (0%)	0/93 (0%)	0/93 (0%)	28/93 (30.1%)

[a] Samples were tested by bioluminescence imaging (BLI) using an IVIS Lumina XR imaging system

[b] Samples were enriched in tetrathionate brilliant green broth with iodine and subsequently streaked onto MacConkey agar plates with kanamycin. *Salmonella* was detected on MacConkey agar by bioluminescence imaging. Samples were considered positive if any bioluminescent colony was observed on MacConkey agar plates after enrichment

[c] —, Not done.

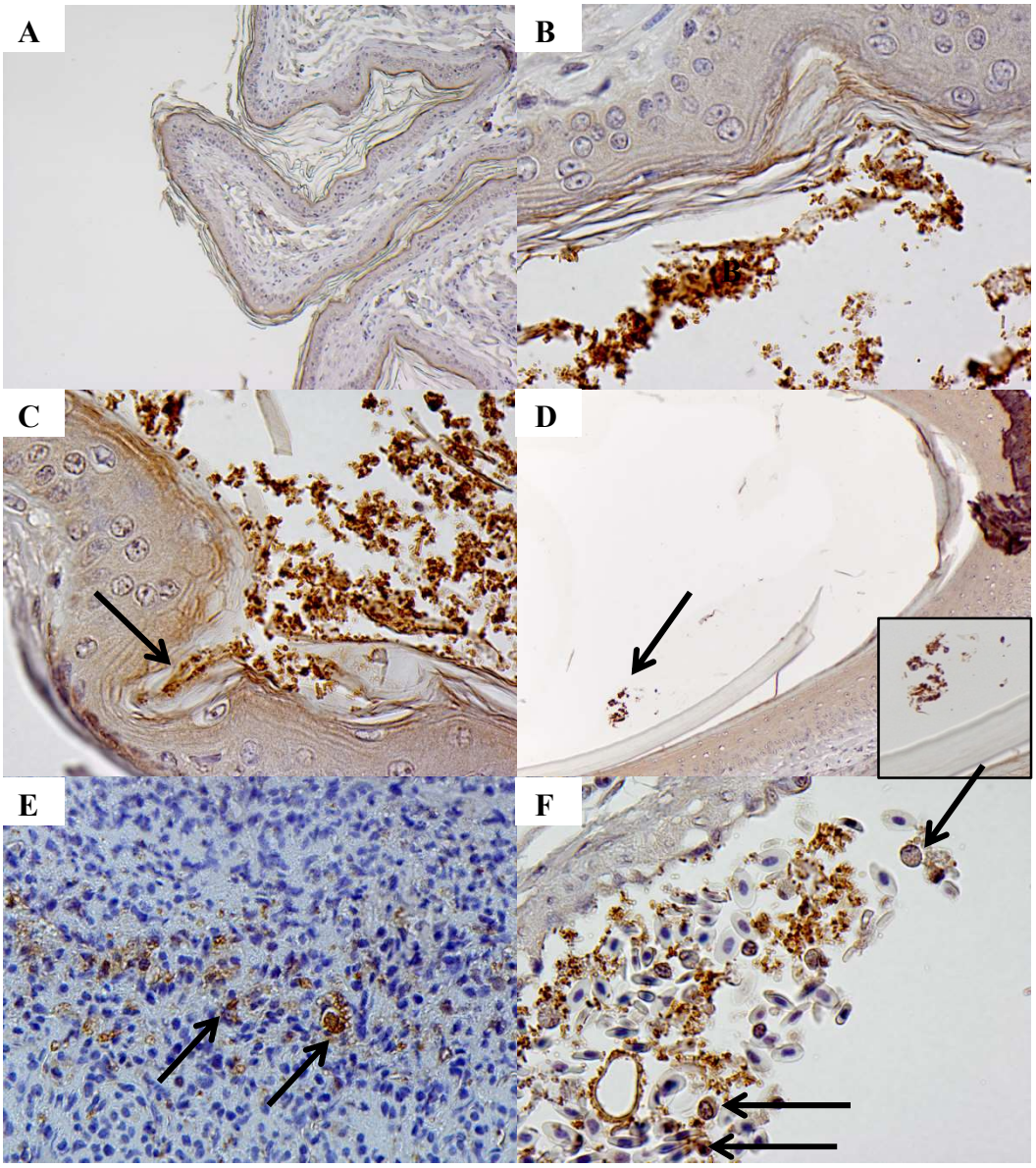

Figure 3.1. Localization of *Salmonella* Heidelberg harborage sites by immunohistochemical staining in skin samples from turkeys orally inoculated at day of age.

A. Sham-inoculated group, integument and epidermal keratin, 200 × magnification. Absence of *Salmonella* group B-antibody positive bacteria **B.** *Salmonella* group B-antibody positive bacteria cells in clusters on epidermal keratin, 1000 × magnification. **C.** *Salmonella* group B-antibody positive bacteria cells in clusters on epidermal keratin and within a fold in the skin (arrow), 1000 × magnification. **D.** *Salmonella* group B-antibody positive bacteria cells within a feather follicle

(arrow), 200 × and 400 x (square) magnification. **E.** *Salmonella* group B-antibody positive bacteria (arrows) within serocellular crust (scab) of an ulcerated skin sample, 1000 × magnification. **F.** *Salmonella* group B-antibody positive bacteria cells within a blood vessel, free or in cytoplasm of monocyte-like cells (arrows), 1000 × magnification.

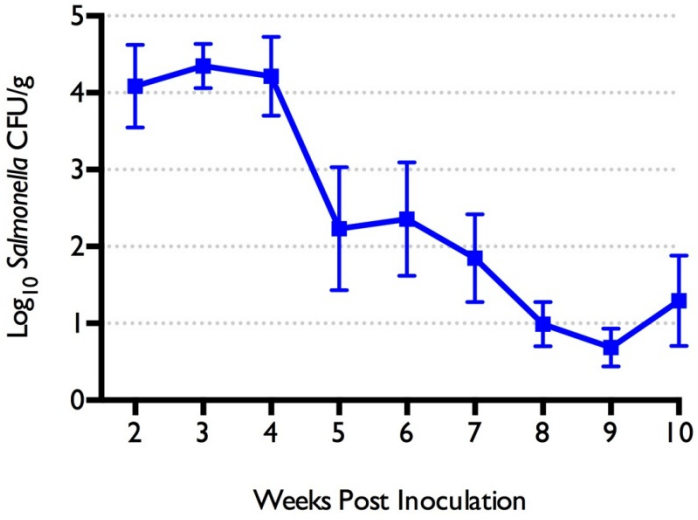

Figure 3.2. *Salmonella* cecal shedding (Log_{10} CFU/g) in turkeys experimentally infected with bioluminescent *S*. Heidelberg at one day of age

CHAPTER 4

THE ROLE OF GENES ENCODING FOR TETRATHIONATE RESPIRATION, SPI-1, AND SPI-2 ON CECAL COLONIZATION AND SYSTEMIC SPREAD OF *SALMONELLA* TYPHIMURIUM IN CHICKENS, WITH OR WITHOUT *EIMERIA* COINFECTION[1]

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Salmonella Typhimurium, Tetrathionate reductase, Salmonella Pathogenicity Island-1 (SPI-1), Salmonella Pathogenicity Island-2 (SPI-2), Eimeria, Intestinal inflammation

ABSTRACT

Intestinal infiltration of inflammatory cells may provide a growth advantage for *Salmonella* and may enhance its systemic spread in chickens. Our objectives were to evaluate intestinal inflammation induced by *Eimeria* spp. and *S.* Typhimurium and determine the fitness of *S.* Typhimurium strains deficient in tetrathionate reductase, SPI-1 (*Salmonella* Pathogenicity Island-1), and SPI-2 (*Salmonella* Pathogenicity Island-2) for cecal colonization and dissemination in tissues.

One-day-old specific-pathogen-free (SPF) chickens were orally inoculated with a sham inoculum or with a total of 4×10^2 oocysts of *Eimeria* spp. (*E. tenella, E. acervulina, E.maxima* and *E.mitis*). Five days later, birds were orally administrated with *S.* Typhimurium wild-type strain in equal combination with *S.* Typhimurium deficient in tetrathionate reductase, SPI-1, or SPI-2 (total oral dose: 3.5 to 4.0×10^8 CFU/bird). Ceca, liver, and drumstick were collected at 3, 7, 14, and 42 days post *Salmonella* infection for bacteriology. Intestinal inflammation was scored by histology.

Significant intestinal inflammation was observed between challenged and control groups. However, there were no significant differences in intestinal inflammation between groups coinfected with *Eimeria* spp. and groups challenged with *S.* Typhimurium alone. Mutation in tetrathionate reductase genes did not impair *S.* Typhimurium cecal colonization and systemic spread in chickens. Deficiency in SPI-2 had a detrimental effect on *S.* Typhimurium cecal colonization whereas deficiency in SPI-1 had a detrimental effect only on *S.* Typhimurium dissemination to the liver, regardless of coccidia coinfection. Low dose of *Eimeria* spp. coinfection did not increase *S.* Typhimurium prevalence in ceca, liver, and drumstick of infected chickens.

INTRODUCTION

Nontyphoidal *Salmonella* cause an estimated 1.03 million illnesses annually in the United States, including 19,336 hospitalizations and 378 fatal cases (Scallan et al., 2011). According to the Centers for Diseases Control and Prevention, consumption of chicken was the most common source of *Salmonella* outbreaks in the United States in 2013 and 2014 (CDC, 2016). Based on the 2016 Foodborne Disease Active Surveillance Network data (FoodNet), *Salmonella enterica* spp. *enterica* serovar Typhimurium was the third most prevalent serovar isolated from laboratory-confirmed cases of salmonellosis (Marder et al., 2017).

Poultry species are acknowledged as natural reservoirs for *Salmonella* spp. and food vehicles for salmonellosis in humans (Shivaprasad et al., 2013). *Salmonella* present in chicken intestinal tract may invade the intestinal mucosa through uptake by M-cells and dendritic cells or by active invasion of the intestinal epithelium using a type III secretion system encoded by the *Salmonella* Pathogenicity Island-1 (T3SS-1) (Libby et al., 2004; Velge et al., 2012). The T3SS-1 expressed by *Salmonella* is a multi-protein complex which acts as a secretion apparatus to inject effector proteins into the host cell cytoplasm, which induce the rearrangement of the actin cytoskeleton and results in membrane ruffles, bacterial uptake, and internalization (Francis et al., 1993; Galán, 1996). *Salmonella* Pathogenicity Island-1 is a 40–kb region of DNA found in all *Salmonella* species, located at centisome 63 on the *Salmonella* Typhimurium chromosome (Libby et al., 2004). The T3SS-1 plays a role in the stimulation of inflammatory response and pro-inflammatory cytokine production when *Salmonella* enters the epithelial cells (Hardt et al., 1998; Lee et al., 2000; Galán, 2001; Zhou et al., 2001; Berndt et al., 2007). After invasion, a second type III secretion system, encoded by the *Salmonella* Pathogenicity Island-2 (T3SS-2), contributes to intracellular survival and replication of S*almonella* within phagocytic cells (Cirillo

et al., 1998; Hensel et al., 1998). SPI-2 comprises a 40-kb region of DNA located at centisome 30 of the *Salmonella* Typhimurium chromosome (Libby et al., 2004). Expression of SPI-2 has been shown to reduce the oxidative stress encountered by bacteria within phagocytic cells, which results in a more hospitable environment for *Salmonella* survival, replication, and dissemination (Vazquez-Torres et al., 2000 ; Ibarra and Steele-Mortimer, 2009). Phagocytic cells containing live *Salmonella* can spread through blood vessels and lymphatics and reach systemic organs (Gulig, 1987; Jones and Falkow, 1996; Pullinger et al., 2007).

Coccidiosis is an ubiquitous intestinal disease of poultry caused by different *Eimeria* spp. Numerous studies described that coccidia coinfection enhances *Salmonella* intestinal colonization and systemic spread in chicken (Stephens et al., 1964; Stephens and Vestal, 1966; Arakawa et al., 1981; Takimoto et al., 1984; Morishima et al., 1984; Fukata et al., 1987). *Eimeria* replication, as well as expression of the T3SS-1 by *Salmonella*, induce infiltration of inflammatory cells in the chicken intestine (Qureshi et al., 1993; Vervelde et al., 1996; Allen, 1997a; b; Lillehoj and Li, 2004; Georgieva et al., 2006). Oxidative mechanisms induced by inflammatory cells promote oxidation of endogenous thiosulfate present in the intestinal lumen into tetrathionate (Winter et al., 2010), an electron acceptor that supports anaerobic respiration in *S.* Typhimurium (Barrett and Clark, 1987). In mice, intestinal inflammation induced by SPI-1 promotes tetrathionate formation and enhances the growth of *S.* Typhimurium over the competitive microbiota in the lumen of the intestine (Winter et al., 2010; Winter and Bäumler, 2011). Furthermore, infiltration of inflammatory cells induced by *Eimeria* spp. (Lillehoj, 1998; Yun et al., 2000) could enhance *Salmonella* uptake from the intestinal mucosa and its dissemination to systemic organs. We hypothesized that intestinal inflammation induced by *Eimeria* spp. could enhance *S.* Typhimurium cecal colonization and its systemic dissemination in

chickens. Our objective was to evaluate intestinal inflammation induced by *Eimeria* spp. and *S.* Typhimurium challenges. We also wanted to determine the fitness of *S.* Typhimurium strains deficient in tetrathionate reductase genes, SPI-1, and SPI-2 for cecal colonization and dissemination in tissues, in the presence or absence of *Eimeria* coinfection.

MATERIALS AND METHODS

Bacterial strains

S. Typhimurium strain TT26179 deficient for *ttrRSBCA* gene cluster was generously provided by Dr. Andreas J. Bäumler, University of California, CA, USA. The *ttrRSBCA* region was replaced with a chloramphenicol resistance cassette (Winter et al., 2010). This mutation was then moved to *S.* Typhimurium SL1344 strain by P22 phage-mediated transduction to create the *S.* Typhimurium SL1344 Δ*ttrRSBCA*. *S.* Typhimurium strains SL1344 deficient for SPI-1 and SPI-2 regions were generously provided by Dr. James W. Wilson, Villanova University, PA, USA, and contained a chloramphenicol resistance marker at the site of SPI-1 and SPI-2 deletions (Wilson and Nickerson, 2006; Wilson et al., 2007). Genes deleted were from *invH* through *sitDCBA* in the SPI-1 region and from *orf70-319-242* through *ssaVNOPQRSTU* in the SPI-2 region (Wilson and Nickerson, 2006; Wilson et al., 2007). *S.* Typhimurium SL1344 with a rifampicin resistance marker was used as *S.* Typhimurium SL1344 wild-type strain. Antibiotics were used at the indicated concentrations; chloramphenicol, 25 µg/mL; rifampicin, 64 µg/mL.

Preparation of *S.* Typhimurium strains inocula for oral animal challenge

S. Typhimurium SL1344 Δ*ttrRSBCA*, ΔSPI-1, ΔSPI-2, and wild-type strains were grown static, separately, in 20mL Luria-Bertani broth at 37°C for 16 hours. The bacterial cell density

was estimated for each strain from the optical density $(OD)_{600}$ of the cell suspensions (~0.5 OD_{600} or 4.0×10^8 cells/mL). Equal volumes of culture of strains to be co-administrated were mixed together, pelleted, and resuspended in 5mL buffered saline gelatin (BSG) to make the strain combinations for oral challenge. The final bacterial count was confirmed by plating 10-fold serial dilutions from each inoculum on XLT4 agar (Difco; Sparks, MD, USA) with chloramphenicol (25 µg/mL) and on XLT4 agar with rifampicin (64 µg/mL). After 24 hours incubation at 37°C, colony forming units (CFU) were determined for each inoculum.

***Eimeria* spp. inoculum**

The *Eimeria* spp. inoculum was generously provided by Lorraine Fuller, Poultry Science Department, College of Agricultural and Environmental Sciences, The University of Georgia, GA, USA. The inoculum was tested by nested-polymerase chain reaction (nested-PCR) for the presence of all seven chicken *Eimeria* species: *E. acervulina, E. necatrix, E. maxima, E. mitis, E. praecox, E. brunetti,* and *E. tenella*. Oocysts were homogenized using ceramic beads in a FastPrep-24™ 5G Instrument (Thomas Scientific; Swedesboro, NJ, USA) at 6 m/s for 20 seconds. DNA from the oocysts was extracted using DNAzol (ThermoFisher Scientific; Waltham, Massachusetts, USA), as per the DNAzol protocol. Following extraction, the DNA was purified using the PowerClean Pro DNA Clean-Up Kit (Qiagen; Germantown, MD, USA). One microliter of DNA was utilized in a nested-PCR procedure designed to detect all major *Eimeria* spp. PCR products were electrophoresed on a 1% agarose submarine gel in Tris–Acetate–EDTA buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA) with ethidium bromide at ~80 V for approximately 45 minutes. Positive bands were excised and DNA purified the Zymoclean Gel DNA recovery kit (Zymo Research; Irvine, CA, USA). Approximately 100

µg of DNA was utilized as template for each nested PCR. The PCR thermoprofile was used as follows for the internal transcribed spacer 1 primers: initial heat activation of polymerase at 95°C for 5 minutes; 35 cycles of denaturation at 95°C for 30 seconds annealing at 50–58°C for 30 seconds and extension at 72°C for 60 seconds and a final extension at 72°C for 3 minutes. The PCR thermoprofile was used as follows for the cytochrome C oxidase 1 primers: initial heat activation of polymerase at 96°C for 10 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 48–64°C for 30 seconds and extension at 72°C for 60 seconds and a final extension at 72°C for 10 minutes. Our inoculum was PCR-positive for *E.tenella*, *E. mitis, E. acervulina,* and *E. maxima* species.

The inoculum was diluted in a saturated sodium chloride solution (366g of NaCl per liter of deionized water) in a 1 to 10 ratio and transferred to a McMaster counting chamber (Chalex Corporation; Portland, OR, USA) for enumeration. *Eimeria* spp. oocysts were differentiated by morphology and size using a bright field microscope (Conway and McKenzie, 2007a) and counted using the formula (number of oocysts) × (10) × (6.67) (Conway and McKenzie, 2007b). The inoculum contained a total of 4×10^2 oocysts with approximately 3×10^2 oocysts of *E. tenella*, 4×10^1 oocysts of *E. acervulina*, 4×10^1 oocysts of *E. mitis*, and 2×10^1 oocysts of *E. maxima* per 0.1 mL inoculation dose.

Oral challenges of specific-pathogen-free, White Leghorn chickens

One-day-old specific-pathogen-free (SPF) White Leghorn chickens were housed in biosafety level 2 (BSL-2) Horsfall isolator units at the Poultry Diagnostic and Research Center, College of Veterinary Medicine, The University of Georgia, GA, USA. Paper was placed on wire-bottom isolators to allow fecal-oral transmission of *Salmonella* Typhimurium strains and

Eimeria spp. Two hundred and forty, one-day-old SPF chickens were randomly divided into 7 treatment groups and 1 control group. Bird density was 12 to 14 birds per isolator unit for the first week of age, 9 to 13 birds per isolator during the second week, 6 to 8 birds per isolator from weeks 3 to 5, and finally 4 to 5 birds per isolator from week 6 until the end of the study. All birds were given *ad libitum* access to water and to an unmedicated feed and were observed for clinical signs twice a day.

Control and treatment groups consisted of 30 chickens each. Chicks were orally inoculated at one day of age either with 0.1 mL of sterile buffered saline gelatin (groups 1, 3, 5, 7) or with 4×10^2 oocysts/0.1 mL of *Eimeria* spp. (*E. tenella, E. acervulina, E.maxima and E.mitis*) (groups 2, 4, 6, 8). Chicks received the inoculum before their placement into the isolator units. Five days later, challenged groups (groups 3 to 8) were orally administered with 3.5-4 $\times$ 10^8 CFU/0.1 mL of a mixture containing *S.* Typhimurium mutant and wild-type strains (Table 4.1). Three inocula were made from an equal amount of *S.* Typhimurium SL1344 mutant strain (Δ*ttrRSBA* or ΔSPI-1 or ΔSPI-2) and *S.* Typhimurium SL1344 wild-type strain. Treatment groups consisted of birds infected either with *S.* Typhimurium strains (groups 3, 5, 7) or coinfected with *Eimeria* spp. and *S.* Typhimurium strains (groups 4, 6, 8) (Table 4.1). *Eimeria* spp. were inoculated by crop-gavage. Oral challenges of *S.* Typhimurium strains were performed using a pipette tip placed in the oral cavity of the birds. Experiments were conducted under strict adherence to Institutional Animal Care and Use Committee (IACUC) guidelines at The University of Georgia, GA, USA.

Before inoculations, on days 1 and 5, 3 birds per group were euthanized by carbon dioxide followed by cervical dislocation. Ileum and ceca were aseptically collected and divided into two equal parts. One part was placed into 10% buffered formalin for intestinal inflammation

scoring by histology. The other part was tested by culture for *Salmonella*. Three birds per group were euthanized by carbon dioxide followed by cervical dislocation on days 3, 7, 14 and 42 post *Salmonella* inoculation. The body of the chickens was sprayed with 70% ethanol prior to necropsy. Ceca with terminal ileum, drumstick (muscle with tibiotarsus bone), and liver samples were aseptically collected and placed individually into 18 oz. Nasco Whirl-PAK bags for *Salmonella* culture. Samples of ileum and ceca were also placed into 10% buffered formalin for intestinal inflammation scoring by histology.

Enumeration of *Eimeria* spp. oocysts

Within each group, 4 to 5 fecal droppings were collected daily from day 4 to day 21, and on days 32 and 38 after *Eimeria* spp. inoculation. Fecal droppings were placed into 18 oz. Nasco Whirl-PAK bags for *Eimeria* spp. oocysts count. Oocysts present in feces were enumerated using the McMaster chamber counting method as previously described by Conway and McKenzie, 2007b. Fecal droppings were weighed and diluted 10-fold in deionized water. After 24 to 48 hours at 4°C, fecal solutions were homogenized and then filtered through a double layer of grade-40 cheesecloth. Fifteen milliliters conical centrifuge tubes (Thermo Scientific; Rochester, NY, USA) were filled with filtered fecal homogenate and centrifuged for 5 minutes at 302 *g* at room temperature to pellet *Eimeria* spp. oocysts. After centrifugation, the supernatant was discarded and pelleted oocysts were resuspended into a 15 ml saturated sodium chloride solution (366g of NaCl per liter of deionized water), homogenized, and transferred to a McMaster counting chamber (Chalex Corporation; Portland, OR, USA). Oocysts were counted using a bright field microscope and numbers of oocysts per gram of fecal material were calculated for each group as (number of oocysts) × (10) × (6.67) (Conway and McKenzie, 2007b).

Enumeration of *S.* Typhimurium strains in ceca

Ceca with terminal ileum samples were placed into sterile 18 oz. Nasco Whirl-PAK bags, weighed, and an equal volume of BSG was added into each bag. Samples were homogenized by pressing the bags between a porcelain mortar and a pestle until obtaining a fluid mixture. Samples were then serially diluted 10-fold in BSG (final dilution 10^{-9}) for enumeration on XLT4 agar with chloramphenicol (25 µg/mL) and on XLT4 agar with rifampicin (64 µg/mL). Plates were incubated at 37°C for 24 hours and *S.* Typhimurium mutant and wild-type strains were detected and enumerated (CFU/g cecal content).

Detection of *S.* Typhimurium strains in tissues

Ceca with terminal ileum, liver, and drumstick samples placed in sterile 18 oz. Nasco Whirl-PAK bags were weighed and buffered peptone water (BPW) (Difco; Sparks, MD, USA) was added into each sample in a 1 to 10 ratio. Ceca and liver samples were homogenized with a stomacher (Stomacher80 Seward; Worthing, United Kingdom) for 1 minute. Drumstick samples were manually macerated through the bag. Tissue homogenates were incubated at 37°C for 24 hours. After incubation, 0.1 mL of BPW cultures was transferred into a 10 mL glass tube of Rappaport-Vassiliadis R10 broth (Difco; Sparks, MD, USA). Tubes were incubated at 42°C for 24 hours. A loopful of the Rappaport-Vassiliadis R10 enrichment (10 µL) was streaked on XLT4 agar with chloramphenicol (25 µg/mL) and on XLT4 agar with rifampicin (64 µg/mL). Plates were incubated at 37°C for 24 hours and *S.* Typhimurium mutants and wild-type strains were detected.

Histology and intestinal inflammation scoring

Formalin-fixed samples of ileum and ceca were embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin. Three sections of intestine were blindly examined from each bird using a bright field microscope. Intestinal inflammation scores were attributed as follow; 0: no inflammation or inflammation within normal limits, 1: mild inflammation, 2: moderate inflammation, 3: severe inflammation (Figure 4.1). Inflammation scoring was based on the severity of the expansion of the lamina propria and mucosa by infiltration of inflammatory cells such as heterophils, lymphocytes, and macrophages. Mucosal and submucosal changes such as ulceration, necrosis, and presence of *Eimeria* spp. organisms were also evaluated.

Statistical Analysis

Bacterial numbers were logarithmically transformed before statistical analysis. The constant 1 was attributed to culture-negative samples before log-transformation. A two-way ANOVA was used to determine whether intestinal inflammation scores differed significantly between groups. *S.* Typhimurium abundance statistical comparisons between mutant and wild-type strains were analyzed using a multilevel mixed-effects model with the Kenward-Roger degrees of freedom method and with a random effect on birds. Pairwise comparisons were made using the Bonferroni procedure. The Mc Nemar's test with all time-points combined was used to compare *S. Typhimurium* mutant and wild-type strains prevalence in organs. Overall *S. Typhimurium* prevalence statistical comparisons between groups were determined using Fisher's exact test. Statistical differences at probability values below 0.05 were considered significant. All tests were performed using Stata/SE 14.2 software (StataCorp, College Station, TX, USA).

RESULTS

Eimeria spp. oocyst shedding

In all groups infected with *Eimeria* spp., the first cycle of oocyst shedding was observed between days 7 and 10 post coccidia challenge. During the first cycle, birds were shedding 13,000 to 44,000 oocysts per gram of feces. The second cycle occurred on days 16 and 17 with birds shedding between 13,000 and 59,000 oocysts per gram of feces. *Salmonella* culture and intestinal inflammation scoring were performed during the first peak of *Eimeria* oocyst shedding, on day 3 post *Salmonella* infection. Next, samples were collected between the first and the second cycle of oocyst shedding, on day 7 post *Salmonella* infection. Finally, samples were collected after the second replication cycle, on days 14 and 42 post *Salmonella* infection.

Intestinal inflammation

Over the length of the study, intestinal inflammation scores were significantly higher in challenged groups (groups 2 to 8) compared to the control group (group 1) (Figure 4.2 and Figure 4.3.A). However, coinfection with *Eimeria* spp. (groups 4, 6, 8) did not significantly increase intestinal inflammation scores compared to infection with *S.* Typhimurium strains alone (groups 3, 5, 7) (Figures 4.3.B, 4.3.C, 4.3.D). In all treatment groups, intestinal inflammation was mild to moderate. Lymphocytes and macrophages were the predominant inflammatory cells observed within the lamina propria of infected birds in all groups.

S. Typhimurium cecal colonization

Abundance of *S.* Typhimurium *ttrRSBCA* mutant strain and abundance of *S.* Typhimurium wild-type strain were not statistically different in ceca on days 3 and 7 post

Salmonella infection, in the presence or absence of *Eimeria* coinfection (Figures 4.4.A and 4.4.B). Interestingly, 14 days post *Salmonella* challenge, *S.* Typhimurium counts in ceca were significantly higher for the *ttrRSBCA* mutant strain than for the wild-type strain, regardless of *Eimeria* coinfection (Figures 4.4.A and 4.4.B). On day 42 post *Salmonella* infection, ceca samples were all culture-negative for *S.* Typhimurium wild-type strain. Only the tetrathionate reductase mutant strain was recovered from ceca at that time-point, in the presence of *Eimeria* coinfection.

Over the length of the study, *Salmonella* numbers in ceca were lower for the SPI-1 mutant strain compared to the wild-type strain, only in presence of *Eimeria* coinfection (Figure 4.4.D). In the absence of *Eimeria* coinfection, abundance of *S.* Typhimurium SPI-1 mutant and wild-type strains were not statistically different in ceca, at any time point during the study (Figure 4.4 C).

Over the length of the study, *S.* Typhimurium SPI-2 mutant strain was significantly less abundant in ceca than the wild-type strain, in the presence or absence of *Eimeria* coinfection (Figures 4.4.E and 4.4.F).

Effect of *Eimeria* spp. coinfection on overall *S.* Typhimurium prevalence in ceca

Overall *Salmonella* prevalence in ceca was determined within groups by calculating percentage of birds with culture-positive ceca, regardless of *S.* Typhimurium strain. Over the length of the study, overall *Salmonella* prevalence in ceca was not statistically different between groups only infected with *S.* Typhimurium strains and groups coinfected with *Eimeria* spp. (*P*-values > 0.05) (Figure 4.5).

S. Typhimurium systemic dissemination

Salmonella prevalence in liver and drumstick was determined for each strain within groups by calculating percentage of birds with culture-positive samples for *S.* Typhimurium mutants and wild-type strains. Over the length of the study, prevalence of *S.* Typhimurium Δ*ttrRSBCA* mutant strain and prevalence of wild-type strain were not statistically different in liver and drumstick samples (P-values > 0.05) (Figures 4.6.A, 4.6.B). These results were independent of *Eimeria* coinfection (Figures 4.6.A, 4.6.B).

SPI-1 and SPI-2 mutant strains were recovered at a lower rate from liver samples in comparison to the wild-type strain, regardless of *Eimeria* coinfection (Figure 4.6.C). However, overall differences in *Salmonella* prevalence between SPI-2 mutant and wild-type strain were not statistically different in liver of birds coinfected with *Eimeria* spp. (P-value > 0.05) (Figure 4.6.D). In groups infected with ΔSPI-1/WT or ΔSPI-2/WT, *S.* Typhimurium wild-type strain as well as strains deficient in SPI-1 and SPI-2 were not recovered from drumstick samples, at any time point during the experiment, regardless of *Eimeria* coinfection.

Effect of *Eimeria* spp. coinfection on overall *S.* Typhimurium systemic dissemination

Overall *Salmonella* prevalence in liver and drumstick was determined within groups by calculating percentage of birds with culture-positive samples for *S.* Typhimurium, regardless of strain. Over the length of the study, overall *Salmonella* prevalence in liver (Figures 4.7.A, 4.7.C, 4.7.D) and in drumstick (Figure 4.7.B) were not statistically different between birds infected only with *S.* Typhimurium strains and birds coinfected with *Eimeria* spp.

DISCUSSION

Coccidia are worldwide distributed and elimination of oocysts from the poultry house is never complete between flocks (McDougald and Fitz-Coy, 2013). In the present study, chickens were inoculated with *Eimeria* spp. at one day of age in order to mimic field challenges that commercial birds face when newly placed on farms (McDougald and Fitz-Coy, 2013). One-day-old birds were challenged with a low dose of *Eimeria* spp. (4×10^2 oocysts) to allow protozoan replication and stimulation of an inflammatory response without inducing mortality. Because prepatent periods of *Eimeria* spp. generally range from 4 to 5 days (Allen and Fetterer, 2002), birds were infected with *Salmonella* at 5 days post *Eimeria* spp. challenge, when intestinal epithelial cells have been damaged by protozoan replication (Lillehoj and Lillehoj, 2000; Allen and Fetterer, 2002). Petrone et al., 2002 demonstrated that birds inoculated with 1×10^4 *E. tenella* oocysts had higher numbers of mucosal and submucosal heterophils in ceca on days 5 and 7 post infection than control birds and birds inoculated with *S*. Enteritidis.

Our results show that *Salmonella* Typhimurium infection as well as *Eimeria* spp. both induced mild to moderate intestinal inflammation in chickens. However, the low dose of *Eimeria* spp. (4×10^2 oocysts) used in our study did not significantly increase intestinal inflammation as compared to chickens only infected with *S.* Typhimurium strains.

We have evaluated the role of genes encoding for tetrathionate reductase, SPI-1, and SPI-2 for *Salmonella* cecal colonization and dissemination in tissues, in the presence or absence of *Eimeria* coinfection. Deletion of the *ttrRSBCA* genes cluster did not impair *S.* Typhimurium cecal colonization in chickens, regardless of *Eimeria* coinfection. In the mouse colitis model, numbers of *S*. Typhimurium *ttrA* mutant strain in colon were significantly lower than the wild-type strain (Winter et al., 2010). In this study, mice infected with *S*. Typhimurium had a severe

intestinal inflammation with infiltration of polymorphonuclear leukocytes (PMN) and a significant increase in the levels of neutrophil chemoattractant messenger RNA (Winter et al., 2010). In the present study, however, intestinal inflammation was mild to moderate in birds infected with *S*. Typhimurium strains alone or coinfected with *Eimeria* spp. and the predominant inflammatory cells observed within the intestinal mucosa were mononuclear leukocytes. On day 14 post *Salmonella* infection, we observed that deficiency in the tetrathionate reductase cluster conferred a growth advantage over the parent strain for *Salmonella* cecal colonization, regardless of *Eimeria* coinfection. On day 42 post *Salmonella* infection, in the presence of *Eimeria* coinfection, the tetrathionate reductase mutant strain excluded the wild-type strain in ceca samples. Previous studies described that *S.* Typhimurium *ttrS* and *ttrB* mutant strains were able to exclude the parent strain in ceca of newly hatched chicks (Harvey et al., 2011). Other works, however, showed no effect of *ttrR* and *ttrS* mutation on fecal excretion of *S*. Typhimurium in chickens over a period of 4 weeks post challenge (Barrow et al., 2015). Our results show that *ttrRSBCA* does not significantly contribute to systemic spread of *S*. Typhimurium in chickens, regardless of *Eimeria* coinfection. Contribution of the tetrathionate reductase to *S*. Typhimurium virulence in chickens in still not fully understood (Harvey et al., 2011; Barrow et al., 2015). Barrow et al., 2015 demonstrated that *ttrR* and *ttrS* mutants had reduced morbidity compared to the *S*. Typhimurium parent strain. In another study, *ttrA* deletion did not produce any attenuation in the virulence of *S*. Typhimurium in infected chickens (Harvey et al., 2011). These findings were confirmed by re-isolation of the mutant strains from liver of diseased birds (Harvey et al., 2011; Barrow et al., 2015).

Our results show that SPI-2 significantly contributed to *S.* Typhimurium cecal colonization in chickens, in the presence or absence of *Eimeria* coinfection. The role of SPI-1 in

colonization of chicken ceca was, however, more questionable. *S*. Typhimurium deficient in SPI-1 was significantly less abundant than the wild-type strain in birds coinfected with *Eimeria* spp. but not in birds only infected with *Salmonella*. Contribution of SPI-1 and SPI-2 in systemic spread and intracellular survival is well documented (Galán, 1996; Cirillo et al., 1998; Hensel et al., 1998; Velge et al., 2012); whereas the role of these genes in the colonization of the chicken gastrointestinal tract is unclear (Jones et al., 2007; Desin et al., 2013). Some works have evaluated the effect of SPI-1 and SPI-2 deletion in *Salmonella* cecal colonization in birds challenged at 1 week of age (Dieye et al., 2009; Desin et al., 2013). Dieye et al., 2009 demonstrated that SPI-1 but not SPI-2 contributed to *S*. Typhimurium cecal colonization; whereas Desin et al., 2013 showed that SPI-1 deletion did not significantly affect *S.* Enteritidis gastro-intestinal colonization in chickens. Other studies have identified individual genes within SPI-1 and SPI-2 that contribute to *Salmonella* colonization of chicken ceca (Turner et al., 1998; Morgan et al., 2004; Bohez et al., 2006; Jones et al., 2007). Morgan et al., 2004 showed that single mutation of *prgK* or *sicA* genes within the SPI-1 region and *ssaQ* mutation within the SPI-2 region induced a reduction in *S.* Typhimurium cecal colonization in chickens. Turner et al., 1998 described that the *sipC* gene of SPI-1 was required for intestinal colonization in chickens; whereas Morgan et al., 2004 demonstrated that mutation of this same gene did not reduce *S*. Typhimurium cecal colonization ability in infected birds. *HilA*, a transcriptional activator that regulates the expression of SPI-1, was required for long-term cecal shedding of *S*. Enteritidis in birds challenged at one day of age (Bohez et al., 2006). In a recent study, *ssaU* gene in SPI-2 was involved in intestinal colonization in 1-week-old chickens, but not in chickens inoculated at day of age (Jones et al., 2007). *S.* Typhimurium SPI-1 and SPI-2 mutant strains used in the present study encompassed the deletion for *prgK*, *sicA*, *sip C*, *hila* genes within SPI-1 as well as the

deletion for *ssaO* and *ssaU* genes within SPI-2 (Wilson and Nickerson, 2006; Wilson et al., 2007).

The role of SPI-1 in *S*. Typhimurium systemic dissemination is clearly demonstrated by differences in *Salmonella* prevalence in liver between SPI-1 mutant strains and the parent strain. These results were independent of *Eimeria* coinfection. In the present study, the role of SPI-2 in systemic dissemination was less obvious. *S*. Typhimurium deficient in SPI-2 was recovered at a lower rate than the parent strain but the difference observed was not statistically significant in birds coinfected with *Eimeria* spp. This result was likely due to a small sample size (3 birds per group at each time point). Dieye et al., 2009 previously showed that SPI-1 and SPI-2 deletion both impaired *S*. Typhimurium splenic colonization in chicken; with SPI-1 contributing more than SPI-2 in systemic colonization. Mutation of *invA*, *invB*, *invC* genes of the SPI-1 region can reduce the colonization of ileal wall and spleen by *S*. Typhimurium after oral inoculation of 1-day-old chickens (Porter and Curtiss III, 1997). Moreover, Jones et al., 2007 demonstrated that *spaS* gene of SPI-1 and *ssaU* genes of SPI-2 played a major role in S. Typhimurium systemic infection in 1-week-old chickens. *S*. Typhimurium SPI-1 and SPI-2 mutant strains used in the present study encompassed the deletions for *invA*, *invB*, *inv, spaS* genes within the SPI-1 region and the deletion for *ssaU* gene wihtin the SPI-2 region (Wilson and Nickerson, 2006; Wilson et al., 2007). In birds inoculated with ΔSPI-1/WT and ΔSPI-2/WT, all drumstick samples were culture negative for *Salmonella*. These results may be due to a small sample size (3 birds per group at each time point); in the literature, *Salmonella* prevalence was 0.8% in drumstick bones of chickens collected at the processing plant (Velaudapillai, 1964; Wu et al., 2014). In experimental challenges, 20% of drumstick bones and 20% of drumstick muscles were detected

positive with *Salmonella* in 35 days old SPF chickens inoculated at day of age (França et al., 2016).

We also evaluated the role of *Eimeria* coinfection in overall *S.* Typhimurium cecal colonization and systemic spread. Previous works described that an oral challenge with 1×10^6 sporulated oocysts of *E. acervulina* (Takimoto et al., 1984) or 5×10^4 sporulated oocysts of *E. tenella* (Arakawa et al., 1981) increased *S.* Typhimurium prevalence in ceca of coinfected bird compared to birds inoculated with *Salmonella* only. Our results showed that a low *Eimeria* challenge dose (4×10^2 oocysts per bird) did not increase overall *S.* Typhimurium prevalence in ceca, liver, and drumstick of infected birds. Other studies described an increase in *S.* Typhimurium prevalence in liver of chickens coinfected with *E. tenella* (Arakawa et al., 1981; Takimoto et al., 1984), *E. necatrix* (Stephens et al., 1964; Stephens and Vestal, 1966), or *E. maxima* (Takimoto et al., 1984) compared to birds only infected with *S.* Typhimurium. However, these works have been conducted with higher challenge doses of *Eimeria* spp. (2×10^4 to 1×10^6 sporulated oocysts per bird) compared to the inoculum dose used in the present study (4×10^2 oocysts per bird). The effect of low dose of *E. tenella* infection on *Salmonella* invasion in systemic organs has been studied by Tellez et al., 1994. The authors reported that, in a dose-related manner, an increase in the inoculum dose from 10 to 1×10^3 *E. tenella* sporulated oocysts resulted in a significant decrease in *S.* Enteritidis prevalence in liver and spleen of infected chickens (Tellez et al., 1994). The decrease in organ invasion was significantly correlated with an increase of the cecal lamina propria thickness induced by infiltration of inflammatory cells (Tellez et al., 1994).

In summary, deletion of *S.* Typhimurium tetrathionate reductase genes did not impair *Salmonella* cecal colonization and systemic spread in chickens. Instead, our results show that

deletion of *ttrRSBCA* may prolong cecal colonization of *S.* Typhimurium. Deficiency in SPI-2 had a detrimental effect on *S.* Typhimurium cecal colonization whereas deficiency in SPI-1 impaired *S.* Typhimurium dissemination to liver, and these effects were independent of *Eimeria* coinfection. Low dose of *Eimeria* spp. did not increase *S.* Typhimurium prevalence in ceca and did not enhance *Salmonella* dissemination in liver and drumstick of infected chickens.

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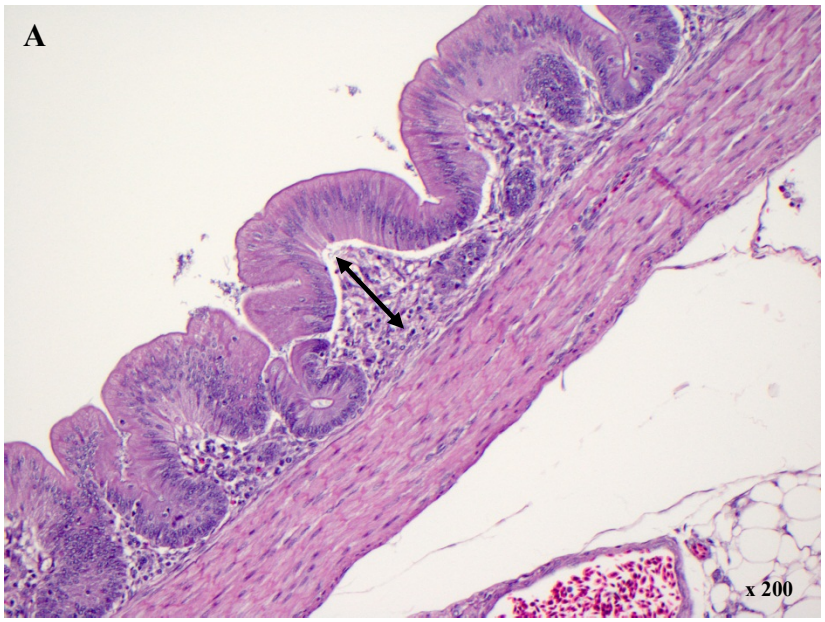
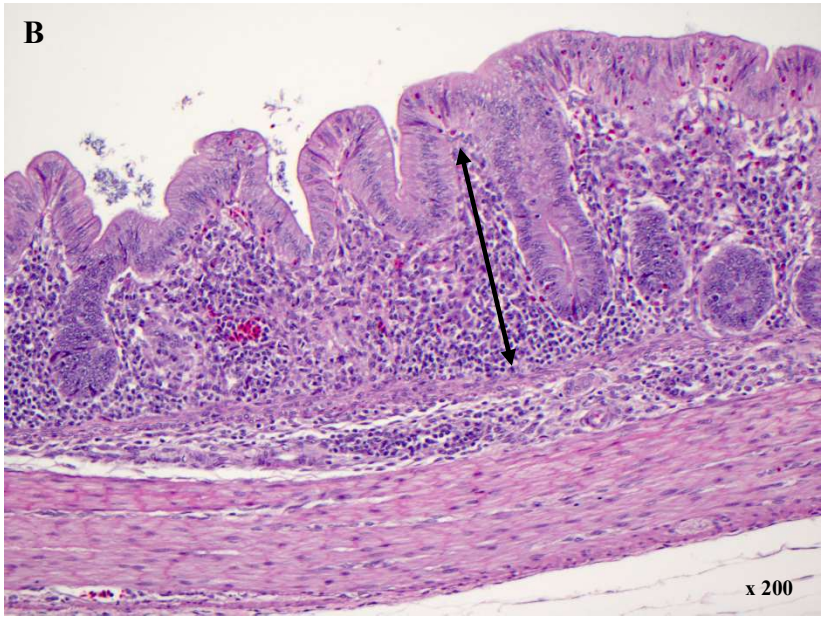
Table 4.1. Oral challenge of SPF chickens. *Eimeria* spp. inoculum contained 4 × 10^2 oocysts/0.1 mL of *E. tenella, E. acervulina, E.maxima and E.mitis*. S. Typhimurium SL1344 *ΔttrRSBCA* /wild-type strains inoculum contained 3.5 x 10^8 CFU/0.1 mL, *S.* Typhimurium SL1344 ΔSPI-1 / wild-type strains inoculum contained 4.0 x 10^8 CFU/0.1 mL, *S.* Typhimurium SL1344 ΔSPI-2 / wild-type strains inoculum contained 3.5 x 10^8 CFU/0.1 mL. [1]Wild-type

	Inocula combination 0.1 mL per inoculum – Oral route	
Groups	Day 1	Day 5
G1 (Negative control)	BSG	BSG
G2	*Eimeria* spp.	BSG
G3	BSG	*S.* Typhimurium SL1344 *ΔttrRSBCA*/WT[1]
G4	*Eimeria* spp.	*S.* Typhimurium SL1344 *ΔttrRSBCA*/WT
G5	BSG	*S.* Typhimurium SL1344 ΔSPI-1/ WT
G6	*Eimeria* spp.	*S*. Typhimurium SL1344 ΔSPI-1/ WT
G7	BSG	*S*. Typhimurium SL1344 ΔSPI-2 / WT
G8	*Eimeria* spp.	*S*. Typhimurium SL1344 ΔSPI-2/ WT


A
x 200


B
x 200

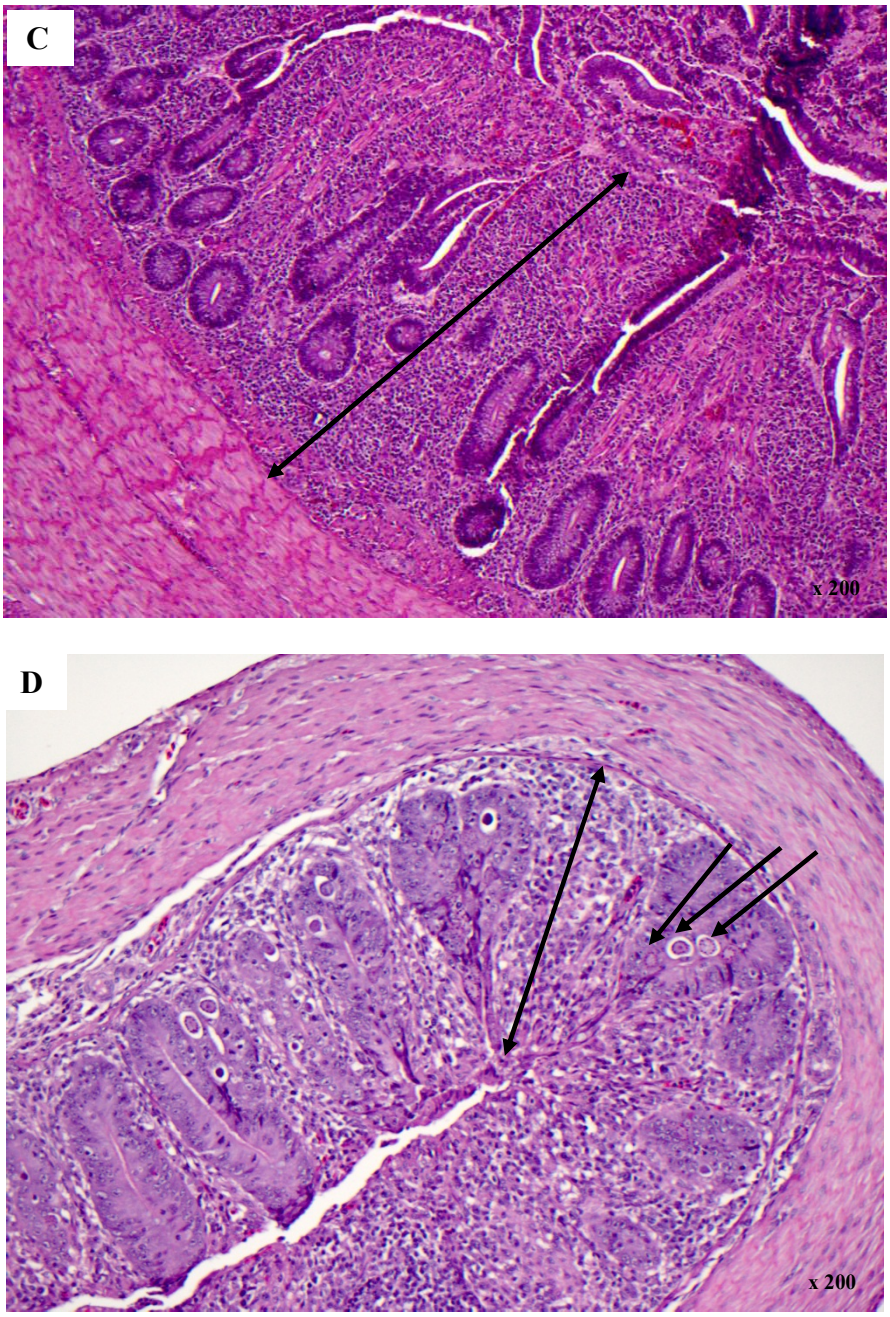
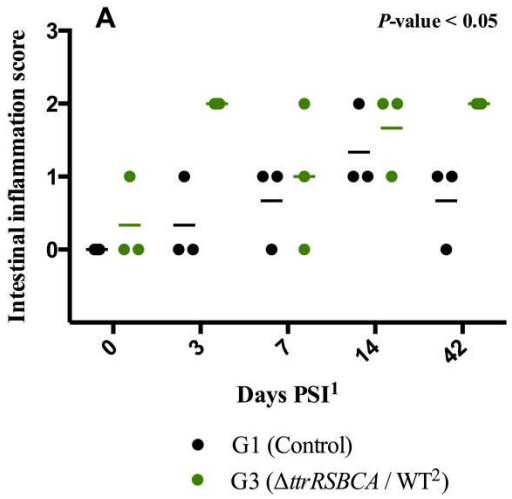
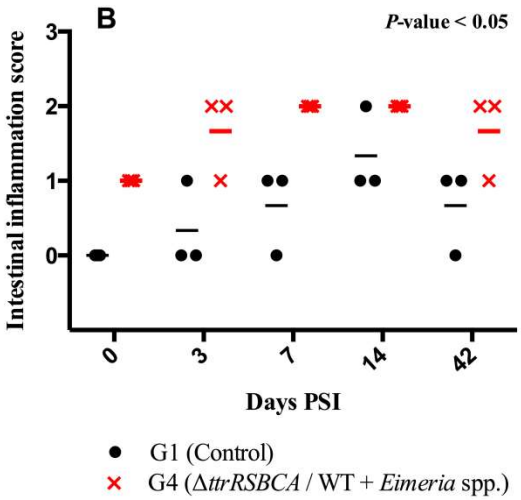
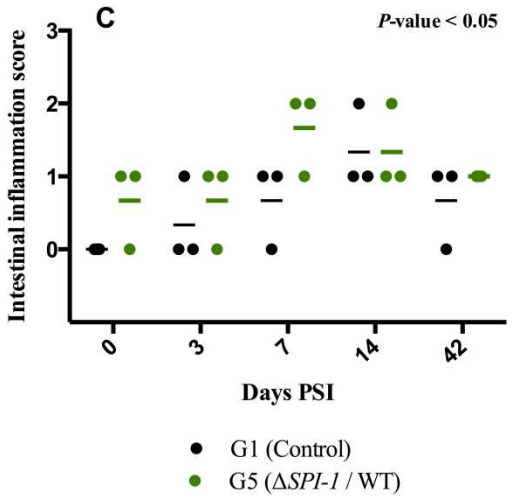


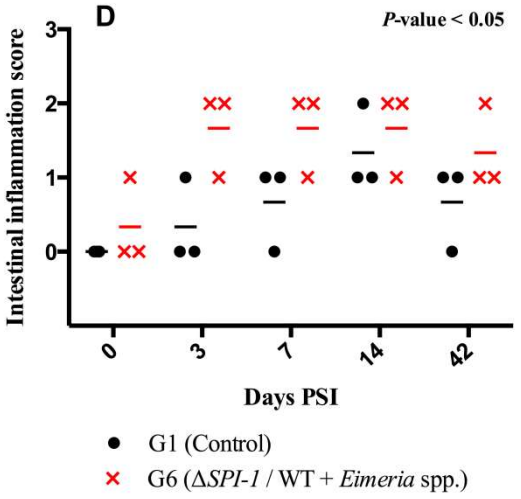
Figure 4.1. Histological scoring system for intestinal inflammation. **(A)** Score 0: no inflammation or intestinal inflammation within the normal limits, 200 x magnification. No or very few isolated inflammatory cells are present within the lamina propria (double-headed arrow). **(B)** Score 1: mild intestinal inflammation, 200 x magnification. Mild infiltration of isolated or clustered inflammatory cells. The lamina propria is less than twice expanded (double-

headed arrow). **(C)** Score 2: moderate intestinal inflammation, 200 x magnification. Moderate infiltration of inflammatory cells within the the intestinal mucosa, usually organized in clusters. The lamina propria is two to three times expanded (double-headed arrow). **(D)** Score 2 with intracellular protozoan in various developmental stages (arrows) associated with lymphocytic and heterophilic infiltration within the lamina propria, 200 x magnification


A
P-value < 0.05
Intestinal inflammation score
0
1
2
3
0
3
7
14
42
Days PSI[1]
G1 (Control)
G3 (ΔttrRSBCA / WT[2])


B
P-value < 0.05
Intestinal inflammation score
0
1
2
3
0
3
7
14
42
Days PSI
G1 (Control)
G4 (ΔttrRSBCA / WT + Eimeria spp.)


C
P-value < 0.05
Intestinal inflammation score
0
1
2
3
0
3
7
14
42
Days PSI
G1 (Control)
G5 (ΔSPI-1 / WT)


D
P-value < 0.05
Intestinal inflammation score
0
1
2
3
0
3
7
14
42
Days PSI
G1 (Control)
G6 (ΔSPI-1 / WT + Eimeria spp.)

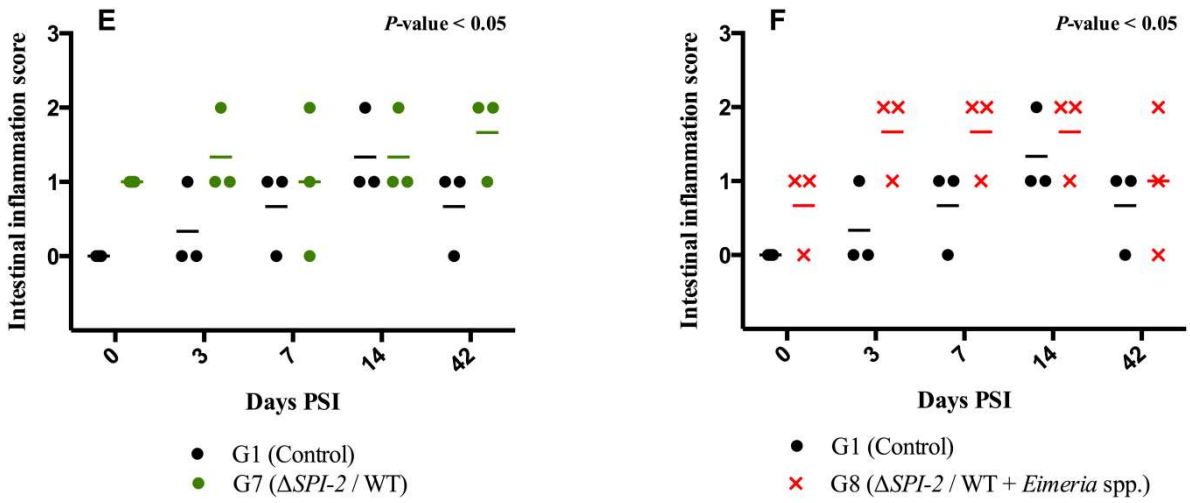


Figure 4.2. Comparison of intestinal inflammation scores between control group and treatment groups. Each point represents lesion score from an individual bird, with mean intestinal lesion score within each group represented by a bar. Over the length of the study, *P*-values < 0.05 show significant differences in intestinal inflammation scores between groups. [1]Days Post *Salmonella* Infection, [2]Wild-type.

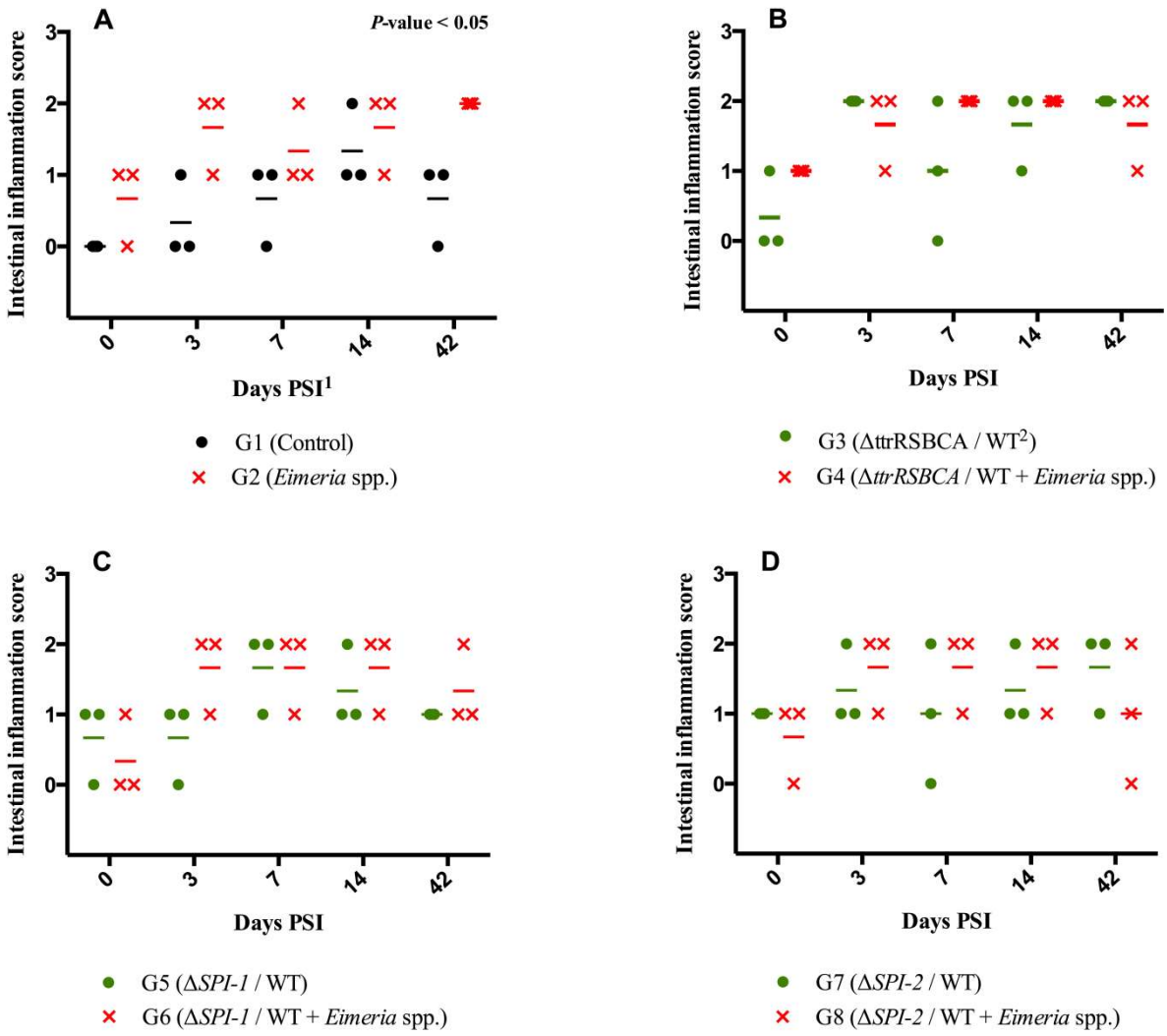


Figure 4.3. Comparison of intestinal inflammation scores between groups challenged with *S.* Typhimurium strains alone and groups coinfected with *Eimeria* spp. **(A)** Comparison of intestinal inflammation scores between control group (G1) and the group only inoculated with *Eimeria* spp. (*E. tenella*, *E. maxima*, *E. acervulina*, and *E. mitis*) (G2); **(B), (C), (D)** Comparison of intestinal inflammation scores between groups inoculated with *S.* Typhimurium (mixture of mutant and wild-type strains) (G3, G5, G7) and groups coinfected with *Eimeria* spp. (G4, G6, G8). Each point represents lesion score from an individual bird, with mean intestinal lesion score represented by a bar. Over the length of the study, *P*-values < 0.05 show significant differences in intestinal inflammation scores between groups. [1]Days Post *Salmonella* Infection, [2]Wild type.

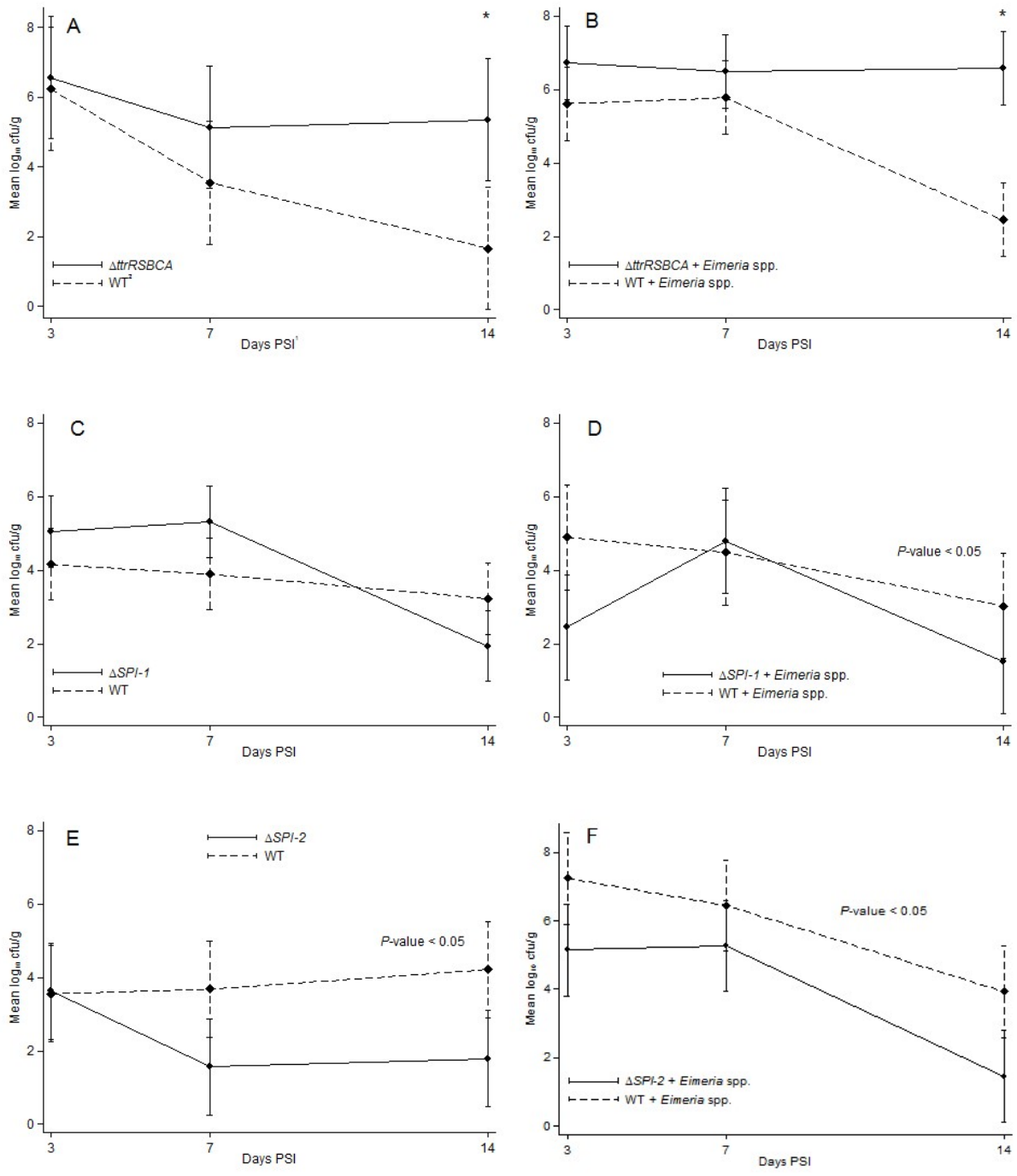
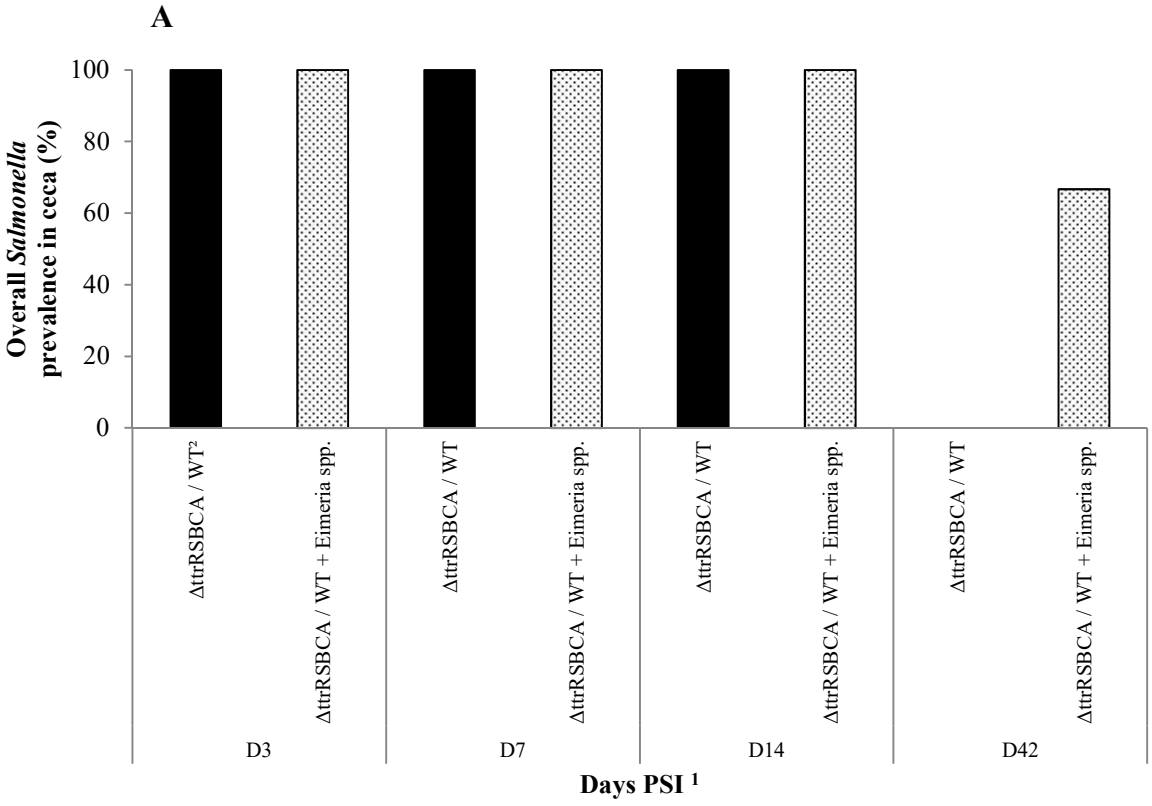
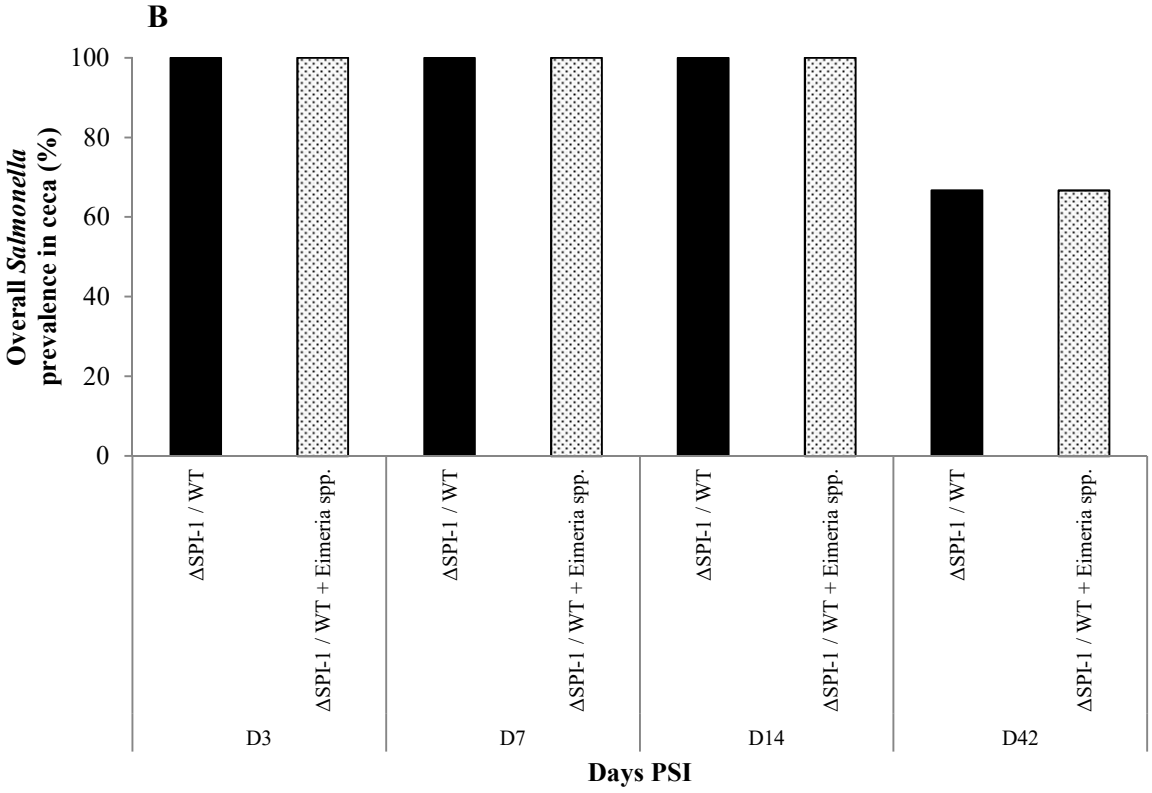


Figure 4.4. Cecal colonization of *S.* Typhimurium mutants and wild-type strains in chickens in the presence or absence of *Eimeria* coinfection. Each point represents mean *Salmonella* $\log_{10}$ CFU/g of cecal content, bars represent 95% interval confidence. **(A)**, **(C)**, **(E)** Birds were inoculated with *S.* Typhimurium mutant and wild-type strains at 5 days of age. **(B)**, **(D)**, **(F)**

Birds were inoculated with *E.tenella*, *E. maxima*, *E. acervulina*, and *E. mitis* at one day of age and with *S.* Typhimurium mutants and wild-type strains at 5 days of age. Over the length of the study, *P*-values < 0.05 show significant differences in *Salmonella* counts between mutant and wild-type strains. At specific time points, asterisks show significant differences in *Salmonella* counts between mutant and wild-type strains. [1]Days Post *Salmonella* Infection, [2]Wild-type.


A
Overall Salmonella prevalence in ceca (%)
100
80
60
40
20
0
ΔttrRSBCA / WT²
ΔttrRSBCA / WT + Eimeria spp.
ΔttrRSBCA / WT
ΔttrRSBCA / WT + Eimeria spp.
ΔttrRSBCA / WT
ΔttrRSBCA / WT + Eimeria spp.
ΔttrRSBCA / WT
ΔttrRSBCA / WT + Eimeria spp.
D3
D7
D14
D42
Days PSI ¹


B
Overall Salmonella prevalence in ceca (%)
100
80
60
40
20
0
ΔSPI-1 / WT
ΔSPI-1 / WT + Eimeria spp.
ΔSPI-1 / WT
ΔSPI-1 / WT + Eimeria spp.
ΔSPI-1 / WT
ΔSPI-1 / WT + Eimeria spp.
ΔSPI-1 / WT
ΔSPI-1 / WT + Eimeria spp.
D3
D7
D14
D42
Days PSI

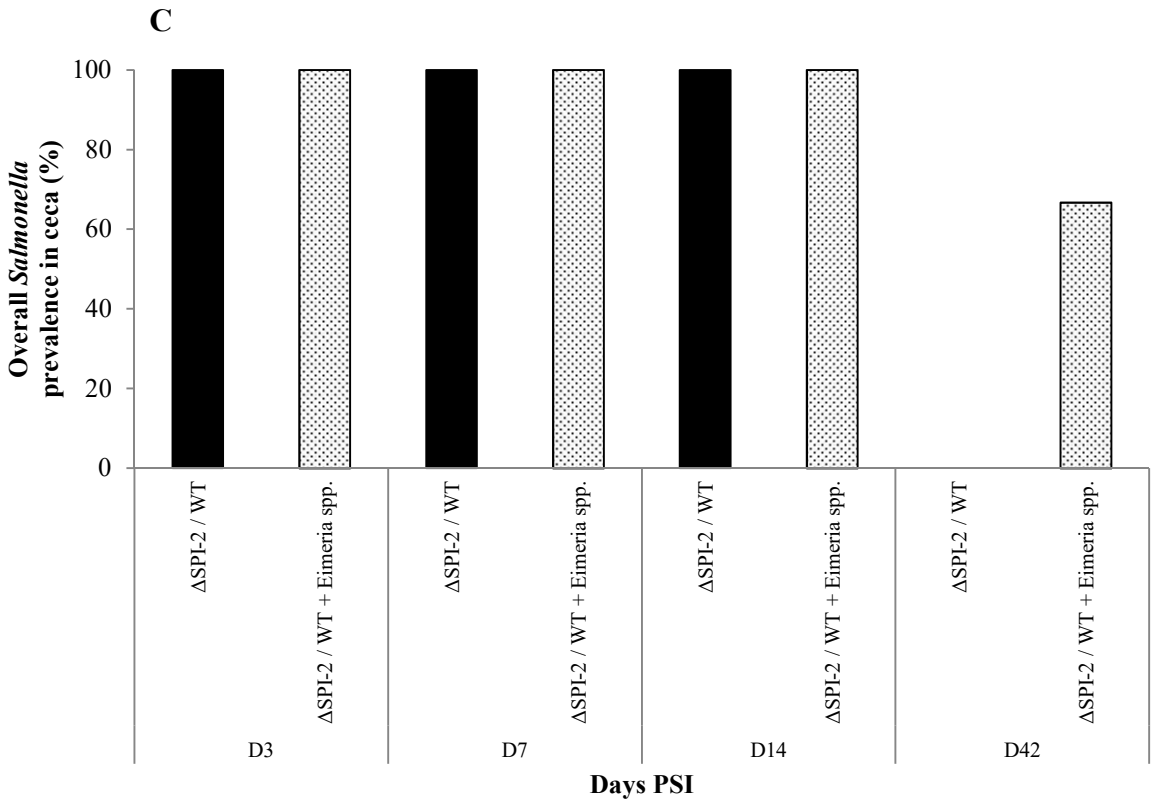
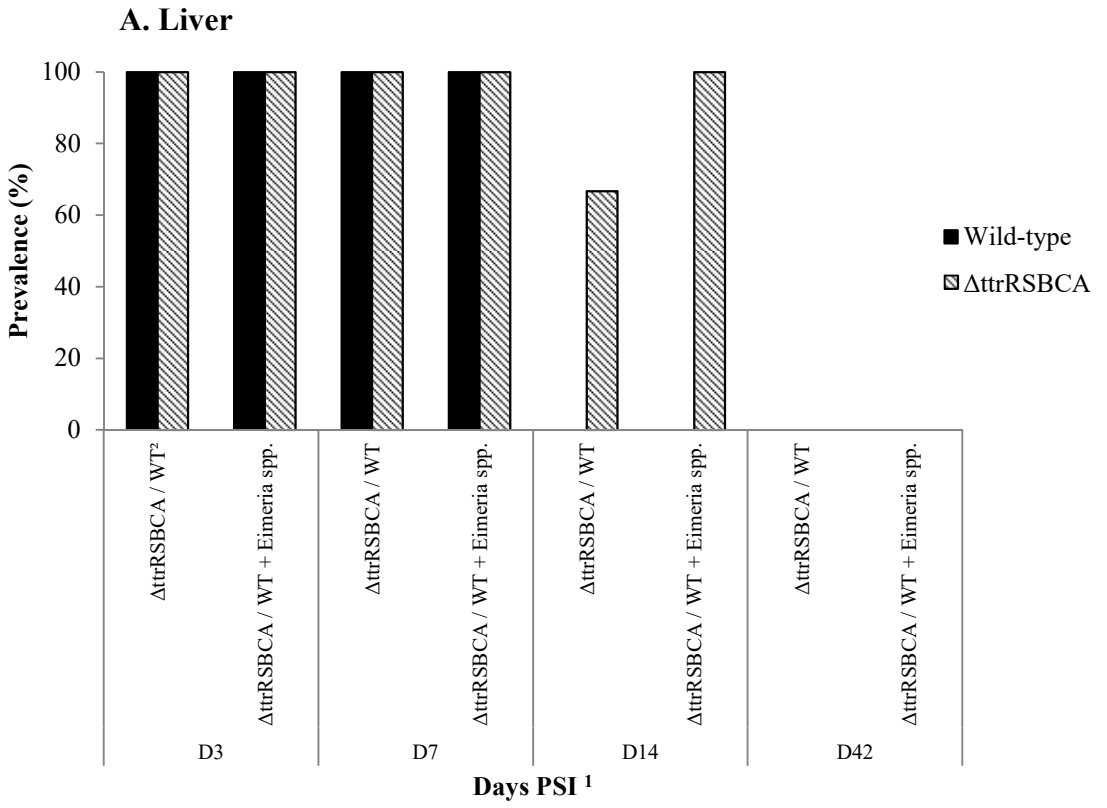
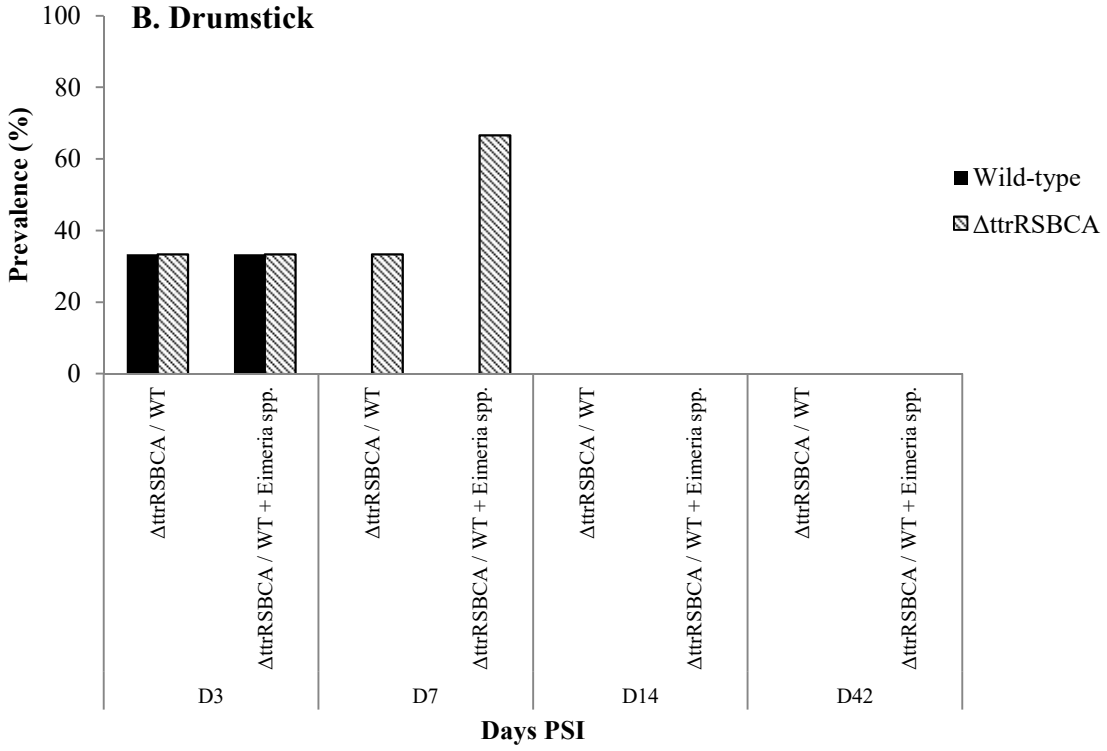
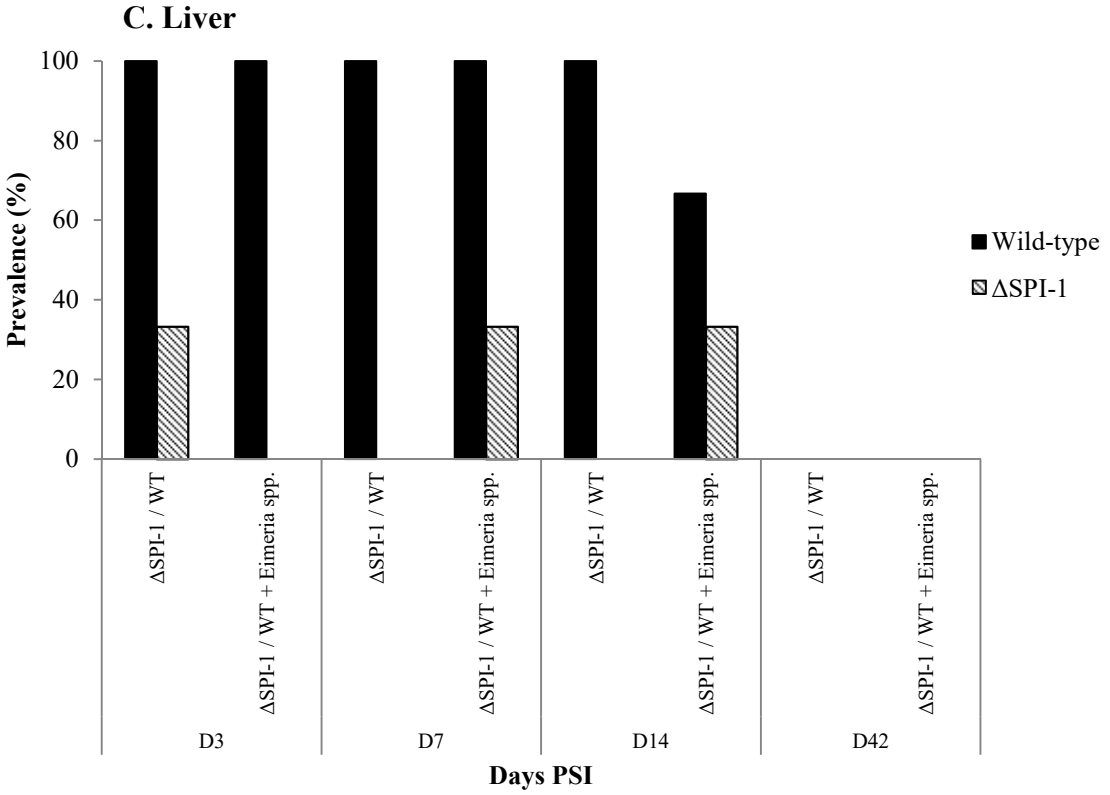


Figure 4.5. Effect of *Eimeria* coinfection on overall *S.* Typhimurium prevalence (mutant and wild-type strains combined) in ceca. [1]Days Post *Salmonella* Infection, [2]Wild-type.


A. Liver
Prevalence (%)
100
80
60
40
20
0
ΔttrRSBCA / WT[2]
ΔttrRSBCA / WT + Eimeria spp.
ΔttrRSBCA / WT
ΔttrRSBCA / WT + Eimeria spp.
ΔttrRSBCA / WT
ΔttrRSBCA / WT + Eimeria spp.
ΔttrRSBCA / WT
ΔttrRSBCA / WT + Eimeria spp.
D3
D7
D14
D42
Days PSI [1]
Wild-type
ΔttrRSBCA


B. Drumstick
Prevalence (%)
100
80
60
40
20
0
ΔttrRSBCA / WT
ΔttrRSBCA / WT + Eimeria spp.
ΔttrRSBCA / WT
ΔttrRSBCA / WT + Eimeria spp.
ΔttrRSBCA / WT
ΔttrRSBCA / WT + Eimeria spp.
ΔttrRSBCA / WT
ΔttrRSBCA / WT + Eimeria spp.
D3
D7
D14
D42
Days PSI
Wild-type
ΔttrRSBCA


C. Liver
Prevalence (%)
100
80
60
40
20
0
Wild-type
ΔSPI-1
ΔSPI-1 / WT
ΔSPI-1 / WT + Eimeria spp.
ΔSPI-1 / WT
ΔSPI-1 / WT + Eimeria spp.
ΔSPI-1 / WT
ΔSPI-1 / WT + Eimeria spp.
ΔSPI-1 / WT
ΔSPI-1 / WT + Eimeria spp.
D3
D7
D14
D42
Days PSI

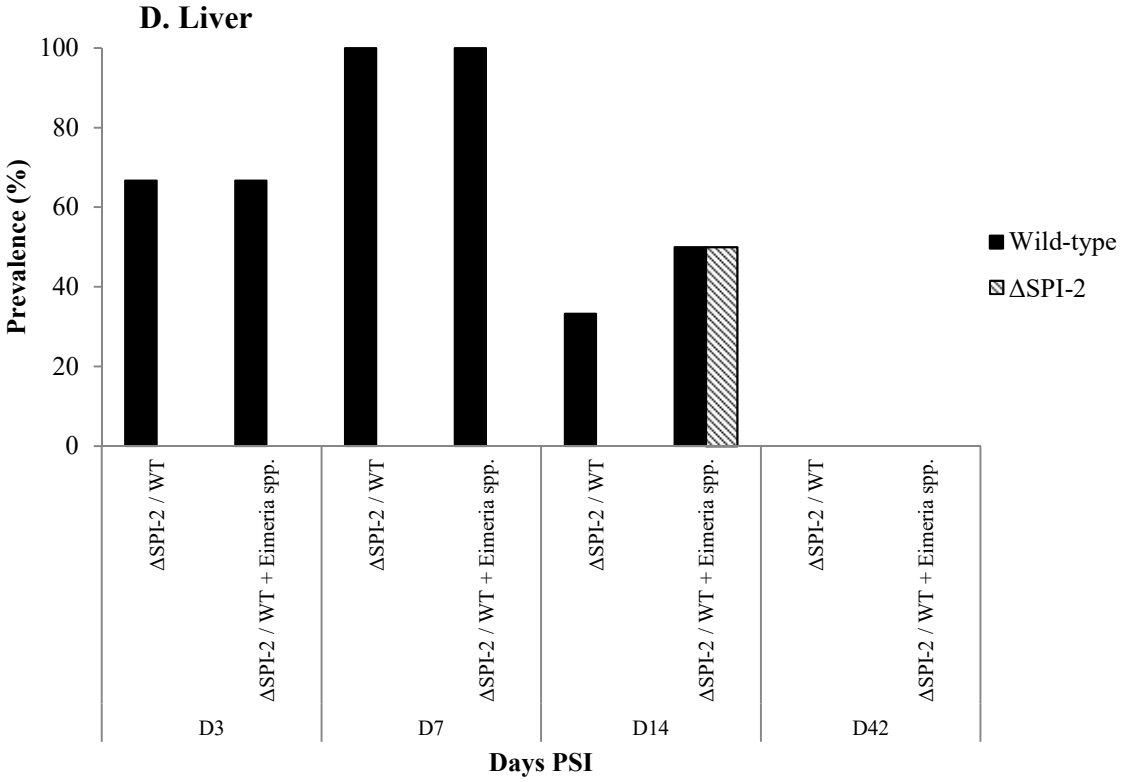
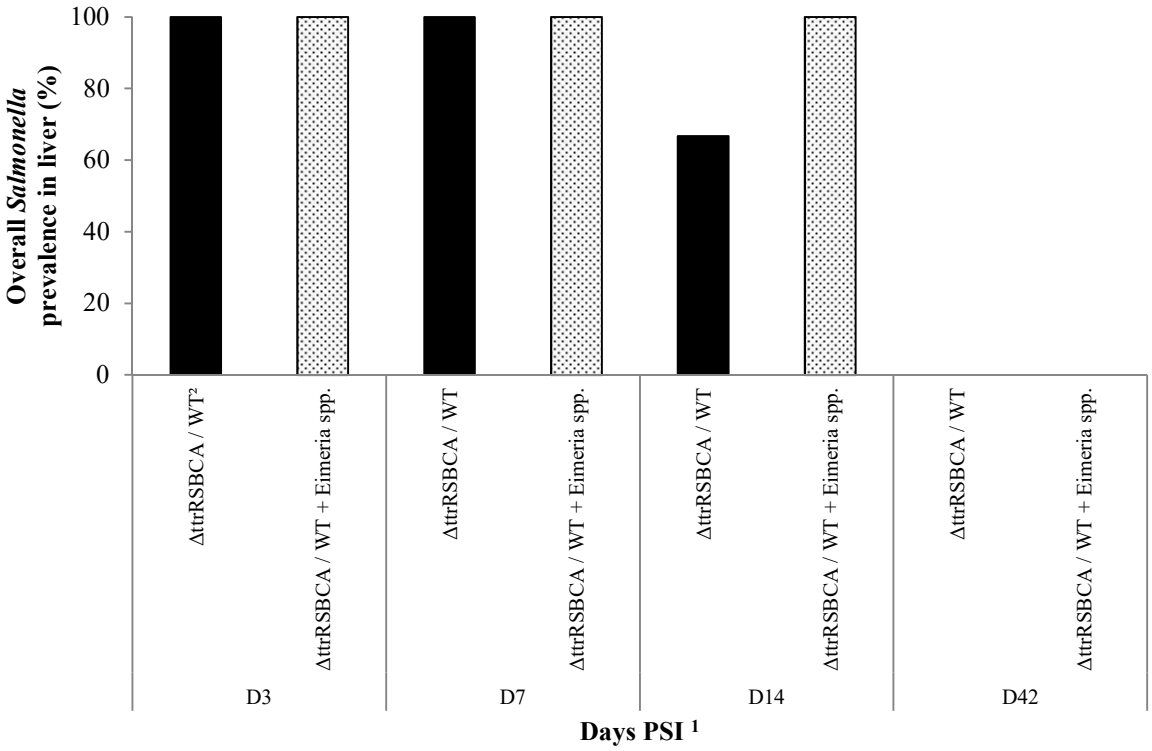
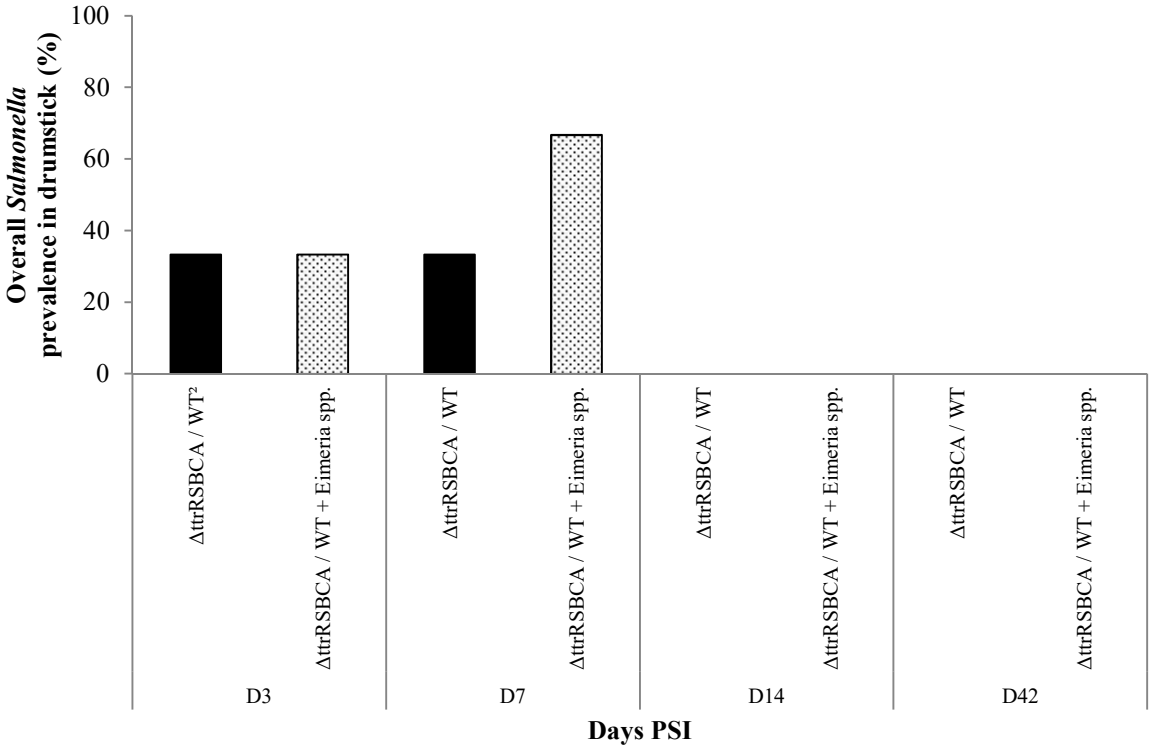


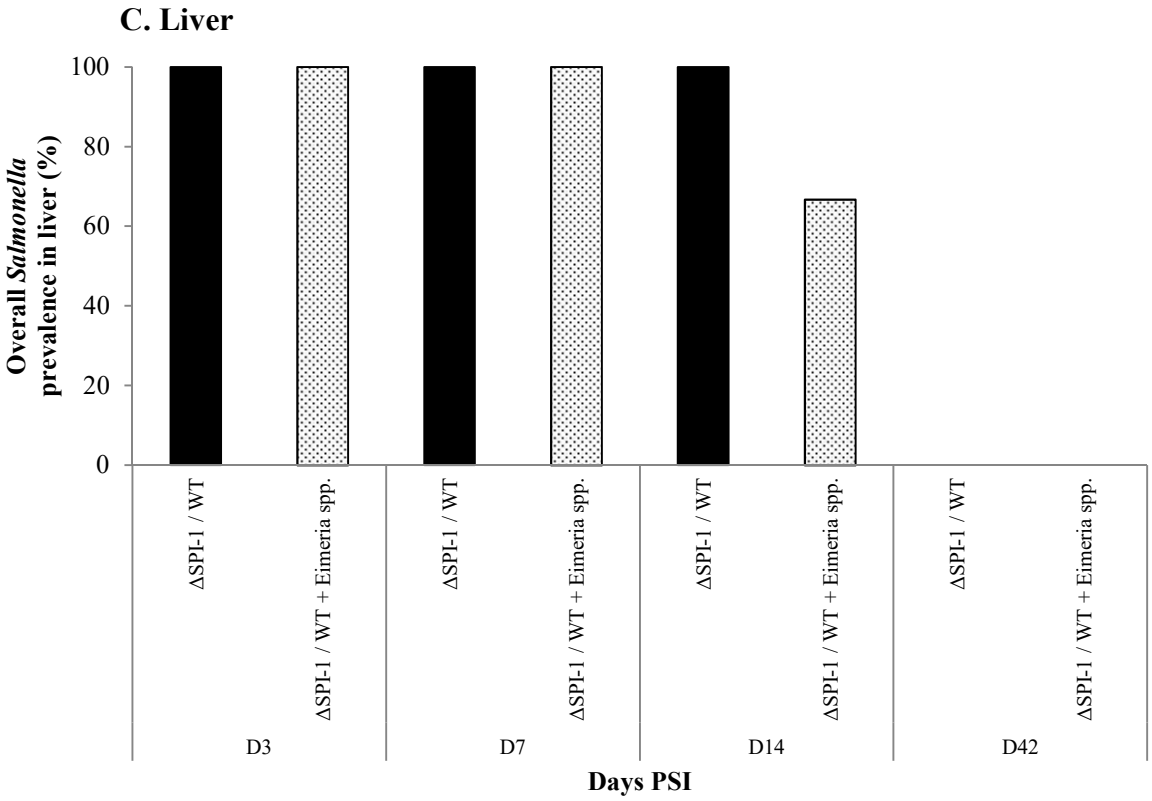
Figure 4.6. Prevalence of *S.* Typhimurium Δ*ttrRSBCA*, ΔSPI-1, and ΔSPI-2 mutants and wild-type strains in liver **(A)**, **(C)**, **(D)**, and drumstick **(D)** samples, in the presence or absence of *Eimeria* coinfection. [1]Days Post *Salmonella* Infection, [2]Wild-type.

A. Liver



B. Drumstick




C. Liver
Overall Salmonella prevalence in liver (%)
100
80
60
40
20
0
ΔSPI-1 / WT
ΔSPI-1 / WT + Eimeria spp.
ΔSPI-1 / WT
ΔSPI-1 / WT + Eimeria spp.
ΔSPI-1 / WT
ΔSPI-1 / WT + Eimeria spp.
ΔSPI-1 / WT
ΔSPI-1 / WT + Eimeria spp.
D3
D7
D14
D42
Days PSI

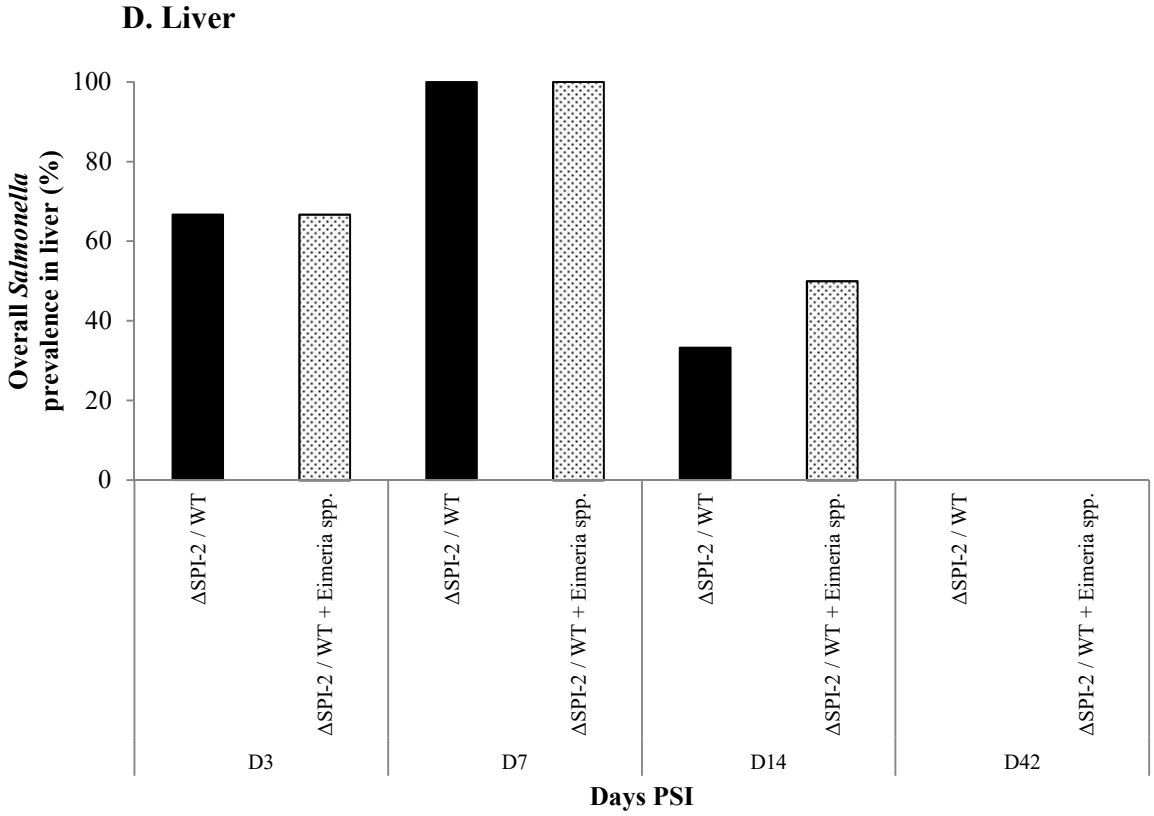


Figure 4.7. Effect of *Eimeria* coinfection on overall *S.* Typhimurium (mutant and wild-type strains combined) prevalence in liver **(A)**, **(C)**, **(D)**, and drumstick **(B)** samples. [1]Days Post *Salmonella* Infection, [2]Wild-type.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Salmonella enterica is estimated as the second most common cause of foodborne outbreaks in the United States. Consumption of poultry meat remains a major source of *Salmonella* infection in humans. Although numerous measures have been implemented to reduce *Salmonella* contamination of carcasses during processing, *Salmonella* incidence rate remains close to 15 cases per 100,000 persons. In poultry flocks, birds that carry *Salmonella* in their intestinal tracts can cross-contaminate carcasses during processing. Moreover internalized *Salmonella* in systemic organs may contribute to contamination of final poultry products. A better understanding of *Salmonella* intestinal colonization and systemic spread in organs of infected birds is needed to reduce bacterial contamination in poultry products and foodborne illnesses.

The first objective of this research was to evaluate *Salmonella* harborage sites and prevalence in drumstick muscle, bone, and skin as components of ground poultry. In this study, we tagged *Salmonella* Heidelberg strains isolated from foodborne outbreaks with bioluminescent markers to visualize infection sites in commercial turkeys inoculated at one day-of-age. Infected turkeys shed *Salmonella* in feces for all 10 weeks. All drumstick muscles collected at 6, 7, and 11 weeks of age and all tibiotarsus collected at 11 weeks of age were negative for *S*. Heidelberg. *Salmonella* prevalence in skin samples was 30.1% at 11 weeks after oral challenge. In skin, *Salmonella* cells were mostly organized in clusters on epidermal keratin. In 10.7% of the culture-

positive skin, *Salmonella* group B-positive bacterial cells were observed within feather follicles. We concluded from this study that long-term cecal shedding of *S.* Heidelberg in turkey may contribute to environmental contamination of the skin by *Salmonella*. Bacteria present within the skin may be a major source of *Salmonella* contamination in ground turkey.

The second objective of this research was to evaluate the role of intestinal inflammation induced by *Eimeria* spp. on cecal colonization and systemic dissemination of wild-type and mutant strains of *S*. Typhimurium deleted for tetrathionate reductase, *Salmonella* Pathogenicity Island-1 (SPI-1), and *Salmonella* Pathogenicity Island-2 (SPI-2). Our results showed that low dose of *Eimeria* spp. induced intestinal inflammation in birds. However, low dose of *Eimeria* spp. coinfection did not significantly increase intestinal inflammation as compared to chickens only infected with *S*. Typhimurium strains. Contrary to previous studies using mice, *S*. Typhimurium tetrathionate reductase deletion did not impair *Salmonella* cecal colonization in infected chickens. Systemic spread to liver and drumstick was not impaired for *S*. Typhimurium strains deficient in tetrathionate respiration, in the presence or absence of *Eimeria* coinfection. In our study, deficiency in SPI-2 has a detrimental effect on S. Typhimurium cecal colonization and deficiency in SPI-1 has a detrimental effect on *S*. Typhimurium systemic spread to liver, regardless of *Eimeria* coinfection. Finally, low dose of *Eimeria* spp. infection did not increase *Salmonella* prevalence in ceca and did not enhance *Salmonella* systemic spread.

This study provides a better understanding of the *Salmonella* pathobiology and harborage sites in live birds and can be used to assist in the comprehension of foodborne outbreaks linked to poultry meat. Our research demonstrates that *Salmonella* internalized in ground turkey components, such as skin, may contribute to *Salmonella* contamination in the end-product. Removal of skin from products used to make ground poultry may be the solution to reduce

foodborne salmonellosis. However, additional research is necessary to further understand bacterial and environmental components that significantly contribute to *Salmonella* intestinal colonization and systemic invasion in commercial birds.