CHARACTERIZATION OF AVIAN IMMUNITY AND *SALMONELLA* INTERVENTION STRATEGIES

A Dissertation

by

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ABSTRACT

Nontyphoidal *Salmonella enterica* infections are a concern for the agricultural and public health sectors. The aims of my research were to develop novel *Salmonella* inhibition strategies and explore the avian immune system. **Study 1:** We hypothesized that adding sodium bisulfate (SBS) to drinking water would decrease *Salmonella* Heidelberg (SH) biofilm on polyvinyl chloride (PVC) and decrease the horizontal transfer of SH in broilers. The PVC coupons were placed in SH inoculated broth and incubated for 6 days. A seeder: contact model was used for horizontal transfer. Treatments consisted of tap water or SBS in water at a pH of 3.5. Biofilm was significantly reduced by SBS when treated for 8 and 24 h. Crop colonization was significantly decreased in trials 1 and 2 in the SBS treatment. Crop pH was significantly decreased in trial 2. Cecal colonization was significantly decreased in trial 1. **Study 2:** We hypothesized that competitive exclusion between *Salmonella* serovars in neonatal broilers would reduce consequent colonization and affect the host immune response. Treatments consisted of a (1) negative control, (2) *Salmonella* Kentucky (SK) only on day 1 (D1), (3) *Salmonella* Typhimurium (ST) or *Salmonella* Enteritidis (SE) only on D1, (4) SK on D1 then ST or SE on day 2 (D2), (5) ST or SE on D1 then SK on D2, and (6) SK and ST or SE concurrently. When a serovar was administered first, colonization by the second serovar was significantly reduced. Significant changes were found in ceca and liver cytokine mRNA expression of IL-1β, IL-6, IL-10, IL-18, and IFN-γ across treatments. **Study 3:** We hypothesized that SK-derived secretome (SKS) grown under *Salmonella* pathogenicity islands-2 inducing, acidic conditions would induce an immune response in chicken macrophages (HD11). The SKS was purified, desalted, and concentrated before administering to HD11 macrophages. Nitric oxide was significantly increased by SKS in HD11 cells compared to the unstimulated cells. Gene expression of

cytokines IL-1β, IL-6, and IFN-γ were significantly increased by SKS compared to unstimulated cells. The overall objectives of these projects were to reduce *Salmonella* and characterize the avian immune response.

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1. INTRODUCTION

Salmonella enterica is a foodborne pathogen that causes an estimated 1.35 million human infections annually in the United States, resulting in $400 million in medical costs (Center for Disease Control, 2019a). Approximately 11% of annual *Salmonella* cases are caused by animal exposure (Hale et al., 2012). Zhao and colleagues (2001) surveyed 59 stores and discovered that chicken (4.2%) had the highest *Salmonella* contamination when compared to beef (1.9%), turkey (2.6%), and pork (3.3%). Per capita consumption of chicken in the United States is 112.5 pounds. As the poultry industry continues to grow, it will require more effective *Salmonella* reduction strategies (United States Department of Agriculture, 2021).

Control of foodborne pathogens, such as *Salmonella*, is regulated by state and federal governments. Food safety is primarily controlled by the Food and Drug Administration (**FDA**) and the United States Department of Agriculture – Food Safety Inspection Service (**USDA-FSIS**; FDA, 2019). The USDA regulates processed eggs which include liquid, frozen or dehydrated products. The FDA passed the Egg Safety Inspection Programs in order to decrease *Salmonella enterica* Enteritidis (**SE**) and prevent contamination of whole shell eggs during production, storage, and transportation by improving biosecurity, pest control, and refrigeration (FDA, 2019). Poultry and egg products are regulated by USDA-FSIS under the Poultry Products Inspection Act (**PPIA**), Federal Meat Inspection Act (**FMIA**), and Egg Products Inspection Act (FDA, 2019). Foodborne pathogens, such as *Campylobacter* and *Salmonella*, are monitored by USDA-FSIS and FDA and follow Hazard Analysis Critical Control Points (**HACCP**), which were implemented as a management system to analyze and control raw material production, procurement, and handling the finished product (FDA, 2017). These programs help decrease

Salmonella, but improvements are necessary in order to achieve total elimination (FSIS-USDA, 2020).

The Veterinary Feed Directive (VFD) mandates only licensed veterinarians can prescribe antibiotics of human health concern to animals (FDA, 2021). This protocol was implemented to reduce antimicrobial resistance in production animals. Drug resistance affects humans, animals, and the environment (plant disease). The CDC (2019b) groups nontyphoidal *Salmonella* as a serious threat due to an increased 200,000 infections of antibiotic-resistant *Salmonella* which has increased by 100,000 cases since the 2013 report. Antimicrobial resistance occurs through mechanisms such as fighting antibiotics with new cell processes, producing destructive proteins or enzymes, altering the target site, or expelling the drug using cell pumps (McManus, 1997; Tenover 2006). Bacteria can also swap resistance genes among other bacteria in the microbiome. These mobile genetic elements are transferred via plasmids, transposons, or bacteriophages. This happens through transduction, conjugation, or transformation (McManus, 1997). Antimicrobial-resistant *Salmonella* could be reduced by implementing improved practices for animal husbandry, vaccination, nutrition, and biosecurity (e.g., infection control).

Salmonella colonization depends on the strain, host immunity, age, and species (Shivaprasad, 2000). The FSIS-USDA (2014) reported 68.6% of chicken, when compared to turkey (6.8%) was contaminated with *Salmonella*. Chai and colleagues (2016) surveyed outbreaks and reported 43% were caused by *Salmonella* contaminated poultry which was the most isolated foodborne pathogen. *Salmonella enterica*, Heidelberg (17%); (SH), Typhimurium (16%); (ST), and SE (17%) were responsible for more than half of the outbreaks (Chai et al., 2016).

Salmonella Enteritidis and ST (CDC, 2020) are in the top three serotypes isolated from humans. *Salmonella* Heidelberg is pathogenic to humans and cattle (USDA, 2018). Gieraltowksi and colleagues (2016) screened 69 strains of SH, and 67% were drug resistant and 35% were multi-drug resistant (MDR). Currently, *Salmonella enterica* Kentucky (SK) is the number one poultry isolate (2007-present). *Salmonella* Enteritidis was the predominant serovar before 1997, and then SH was the leading subtype from 1998-2006 (Foley, 2008). Even though SK is the most prevalent serovar isolated from poultry, it is infrequently associated with human Salmonellosis (0.14%; CDC, 2019). Reducing *Salmonella* by acidified drinking water, competitive exclusion, and vaccine technology are the themes of my research.

1.1. *Salmonella* Background

Salmonella are Gram-negative, facultative anaerobic bacilli (Giannella, 1996). Lipopolysaccharide (**LPS**) comprises the outer membrane of Gram negative bacterial cell walls (Raetz and Whitfield, 2002). The exact structure of LPS differs across bacteria which could explain variances in virulence (Wilkinson, 1996). Under the *Salmonella* genus, there are two species: *bongori* and *enterica (*Brenner et al., 2000). Within *enterica,* there are 6 subspecies: *enterica, salamae, arizonae, diarizonae, houtenae,* and *indica* differentiated biochemically and by genome (Brenner et al., 2000). There are over 2,600 *Salmonella enterica enterica* serovars which can infect a wide range of vertebrate species (Foley et al., 2013). Approximately 100 serotypes are confirmed to cause most human infections (CDC, 2020). *Salmonella* serovars are classified based on flagellar (H), somatic lipopolysaccharide (O), and Vi antigens (Guibourdenche et al., 2010). Serotyping identifies groups of *Salmonella* by agglutination using antibodies to target antigens. *Salmonella* serotypes, or serovars, can be sequenced to identify the strains. *Salmonella enterica* serotypes are divided by their host range (adapted or ubiquitous;

Uzzau et al., 2000). Host-specific serovars include *Salmonella enterica* Gallinarum and Pullorum which cause fowl typhoid and pullorum disease, respectively (Shivaprasad, 2003). Both reportable diseases were eradicated in North America and Europe but are prevalent in developing countries (Barrow, 2011). Broad host range serovars include, but are not limited to, SE, SH, SK, and ST which are able to colonize the gastrointestinal tract (**GIT**) of chickens, cross-contaminate meat, and infect human consumers.

Type I secretion system (T1SS), Type II secretion system (T2SS), Type III secretion system (T3SS), Type IV secretion system (T4SS), and Type VI secretion system (T6SS) assist *Salmonella* invasion and proliferation by transporting virulence factors into host cells (Costa et al., 2015). The T3SS is composed of a transmembrane complex (translocon) that forms in the host cell membrane to transfer effector proteins and was originally found in ST (Kubori et al., 1998; Galan and Wolf-Watz, 2006). Invasiveness of *Salmonella* is dependent on the T3SS which is encoded on *Salmonella* pathogenicity islands (SPI), particularly SPI-1 and SPI-2 (Shea et al., 1996). Virulence genes express *Salmonella* pathogenicity islands through recombination cassettes and increased promoters on chromosomal regions (Marcus et al., 2000; Gal-Mor et al., 2006). Gene cassettes are a recombination specific-site dependent on upstream promoters (Hall and Collis, 1998). The SPI-1 is responsible for initial bacterial penetration of epithelial cells of the intestine, causing membrane ruffling and inflammation, and suppression of pro-inflammatory cytokine expression in macrophages (Finlay et al., 1991; Pavlova et al., 2011). Membrane ruffling is the disruption of the cytoskeleton which increases *Salmonella* uptake (Perrett and Jepson, 2008). The roles of SPI-2 include the growth and survival of *Salmonella* in the host cell which is induced by acidification of the modified phagosome. The *Salmonella*-containing

vacuole is the adaption of macrophages by the SPI-2 secretion system to prevent phagolysosomal fusion inhibiting the antimicrobial activity of lysosomes (Hansen-Wester and Hensel, 2000).

The SPI-2 is necessary for *Salmonella* to colonize the gut and spread systemically. Secretion of SPI-2 proteins is dependent on bacteria translocons (invasion proteins) which are triggered by minimal acidic media at a pH of 5.0-5.8 (Beuzon et al., 1999; Deiwick et al., 1999; Nikolaus et al., 2001; Coombes et al; 2004). Nikolaus and colleagues (2001) identified substrate proteins of the T3SS in the supernatant grown under SPI-2 inducing conditions in minimal media (pH of 5.8). For comparison, the chicken gastrointestinal tract pH ranges from 4.5-6.0 in the crop up to 5.5-7.0 in the ceca (Sturkie, 1976; Hinton et al., 2000a; Hinton et al., 2000b). Secreted proteins SseB, SseC, and SseD were found to be primarily on the surface of *Salmonella*. However, SseC and SseD were located in the cell-associated fraction when SseB was present but were found in the culture supernatant if functional SseB was removed. The protein SseB is located on the bacteria surface and forms part of the translocon of the T3SS encoded by SPI-2 (Yang et al., 2013). Other components of the translocon are the SseC and SseD which are important in order to deliver effector proteins into the host cell membrane (Yu et al., 2002; Yu et al., 2004). Coombes and colleagues (2004) used an *in vitro* secretion assay to further study the translocation of proteins SseB (filament structure), SseC, SseD (pore-forming translocon) during SPI-2 induction using affinity-purified antibodies. The SPI-2 region encodes the regulator protein SsrA, and a mutant deficient in *ssaR* did not secrete proteins SseB or SseD (Bohez et al., 2007). Results showed acidic minimal media is required for the translocation of effector cells to occur, as shown by low amounts of SseB or SseD protein in a Western blot analysis after neutral media growth similar to Nikolaus et al., 2001. Overall, the survival of *Salmonella* depends on SPI-2, because it encodes virulence factors, such as the substrate proteins SseB and SseD, which

are translocated in the host cell by the T3SS which is critically regulated in order to elevate pathogenesis.

Differences in SK's genome could allow for more successful colonization in chickens compared to other serovars. Haley and colleagues (2016) identified phenotypic and genetic differences in SK isolates from cattle and poultry. Rauch and colleagues (2018) also compared SK evolutionary linages, Group I and Group II, in animal-derived SK isolates from Pennsylvania. The groups were separated by polyphyletic lineages which were confirmed by pulse field gel electrophoresis (Vosik et al., 2018). Polyphyly groups are characterized by sharing multiple ancestors with traits that evolved independently (Skejo and Franjevic, 2020). Clustered regularly interspaced short palindromic repeats (**CRISPRs**) are highly variable loci, specific gene location on chromosomes, present in all sequenced bacteria (Grissa, Vergnaud, and Pourcel, 2007). A subtyping protocol, CRISPR-multi-virulence locus sequence typing (**MVLST**), identifies unrelated isolates from virulence genes, *fimH1* and *sseL* combined with CRISPR sequencing (Liu et al., 2011). Isolates identified by CRISPR-MVLST typing overlapped from poultry, human isolates, and cattle in group I. Group II was only found in human clinical isolates. Ultimately, both groups have overlap while only group I is found in poultry and cattle.

Successful colonization and persistence of SK in poultry could be explained physiologically or genetically. Avian pathogenic *Escherichia coli* (**APEC**)-like plasmids were identified in SK (Fricke, 2009). Based on this report, SK has the capability to acquire plasmids encoding MDR and virulence. These virulence factors allow SK to colonize the chicken ceca and persist (Johnson et al., 2010). Significant differences were found in poultry isolate virulence genes in SK when compared to SE and ST (Cheng et al., 2015). A higher expression of *rpos*-regulated genes was in SK compared to ST. Survivability is increased by the stationary-phase

sigma factor *rpoS* regulating stress response and metabolism by initiating transcription (Nickerson and Curtiss, 1997; Dong et al., 2008). *Salmonella* Kentucky has *lpfE* and *stfA* but lacks *saf* and *sef* fimbrial operons which are poultry adapted genes found in SE and ST (Cheng, 2015). Fimbrial genes, such as *lpfE, saf, stfA* and *sefC*, are critical for adhesion to the epithelium during initial invasion (Baumler et al., 1996). The *spv* virulence plasmid marker, *spvC*, was negative in SK poultry isolates. There was a low prevalence of other phage-associated virulence genes, *sopE, grvA, sodC1*, and *sseI*, compared to ST and SE. Further research done by Cheng and colleagues (2015) showed SK was dependent on galactose and *curli* (aggregate fibers) for long-term colonization when compared to ST and SK knockout mutant strains. *Curli* operons are critical for aggregate fibers which allows for biofilm formation (Romling et al., 1998).

In Cheng and colleagues (2015) previously described experiment, SK persisted in broilers until day 36 compared to ST which fell below the detection level by day 15. *Salmonella* Kentucky had an advantage in persisting in chickens when compared to ST; however, when comparing growth rates in minimal media *in vitro*, no differences were measured (2015). Therefore, the advantage appears to be due to inhibition or competition. Joerger and colleagues (2009) found that SK survived in acidic (pH of 5.5) conditions, when compared to ST and SE, was better which implied an environmental advantage in locations such as the chicken ceca since the average pH of adult chicken ceca averages 5.5-7.0 (Sturkie; 1979; Hinton et al., 2000b; Denbow, 2014).

Poultry can contract *Salmonella* through horizontal or vertical transmission (Foley and Lynne, 2008). Vertical transfer occurs through contaminated eggs passed through infected ovaries and oviducts (Kabir, 2010). Horizontal transfer occurs by a fecal to oral route. *Salmonella* Enteritidis can invade the gastrointestinal tract and migrate to the reproductive tract

via phagocytic cells (dendritic cells or monocytes), leading to contaminated eggs (Wigley et al., 2013).

1.2. *Salmonella* biofilm

The Safe Drinking Water Act (**SDWA**) was enacted in the United States in 1974. The Environmental Protection Agency (**EPA**) set the drinking standard to require total coliforms to be 0 cfu/100 mL of water (2021). *Salmonella* is more commonly referred to as a foodborne pathogen; however, it can also be transmitted in drinking or natural water sources (Leclerc et al., 2002; Ashbolt, 2004). It can enter aquatic locations through feces from infected animals via agricultural run-off (Levantesi et al., 2012). Marin and colleagues (2009) surveyed water dispensers and tanks in broiler houses. Incidence of *Salmonella* in water tanks and drinkers was 2.3% and 4.6%, respectively. Furthermore, approximately 50% of *Salmonella* strains isolated were able to produce biofilms. Preventative strategies at the farm level include feed and drinking water additives, diet modifications, antibiotic alternatives, and vaccines (Vandeplas et al., 2010).

Bacteria biofilms are more resilient to disinfectants, immune responses, and environmental factors. Survival of *Salmonella* in non-host settings can be possible due to biofilm formation (Jones and Bradshaw, 1996). Biofilms are adhered bacteria cells on an abiotic or biotic surface in an extracellular matrix (Donlan, 2002). Microbes initially attach to a surface, form micro-colonies followed by maturation and dispersal of the biofilm (Tolker-Nielsen, 2015). Solano and colleagues (2002) compared 204 ST and SE strains ability to form biofilms at room temperature in different environments. Certain strains were unable to form in all environments, in part, due to a lack of cellulose biosynthesis operons *bcs*ABZD and *bcs*EFG as detected in knockout mutant strains. Cellulose did seem to have a role in virulence but is the main component for biofilm formation and survival by SE and ST.

Salmonella strains are able to form biofilms on surfaces commonly found in the poultry industry such as plastic, cement, rubber, glass, and stainless steel (Joseph et al., 2001; Stepanovic et al., 2004; Giaouris and Nychas, 2006). However, growth varies depending on the surface, temperature, medium, and time. De Oliveira and colleagues (2014) analyzed the adhesion of 174 unidentified *Salmonella* previously isolated from poultry breast fillets. Biofilm were grown on PVC, glass, and steel at different temperatures in Petri dishes (2014). Growth was most prominent at 28°C and grew best on PVC or steel but were still weak biofilm producers as determined by optical density (**OD**). Diez-Garcia and colleagues (2012) measured 69 strains from *Salmonella enterica* serovars including Typhimurium (3 strains) and Enteritidis (36) ability to form biofilm on polystyrene. Their results showed that the development of biofilm is serovar dependent based on optical OD readings. Stepanovic and colleagues (2004) used 60 strains of *Salmonella* isolated from humans, animals, and food to compare 24 h biofilm formation on 96-well flat-bottom polystyrene plates. The greatest production of biofilm was in Luria-Bertani broth (**LB**) compared to tryptic soy broth (**TSB**). Agarwhal and colleagues (2011) also found LB biofilm formation was greater than TSB. They then evaluated 151 *Salmonella* strains belonging to 69 serotypes in LB for 24, 48, and 72 hours in a 96-well plate. Only 1 serotype, S. Munster, did not produce any biofilm. Giaouris and colleagues (2005) found that optimal conditions for SE to form a biofilm on stainless steel were 6 days at 20°C. Wang and colleagues (2013) formed SH biofilm on stainless steel surfaces when incubated for 2 d at 20°C. Time, temperature, and media variances all indicate physiological differences in serovars ability to form biofilms.

Biofilms formation and survival are also dependent on genetics. Giaouris and colleagues (2013) compared proteins expressed in *Salmonella* Enteritidis biofilm. Twenty biofilm derived proteins were identified in the following categories based on functions: global regulation and

stress response, nutrient transport, degradation and energy metabolism, detoxification, and DNA metabolism, curli (aggregate fibers) production and murein synthesis. Murein is a peptidoglycan that envelops the cytoplasmic membrane in the periplasm to protect Gram-negative bacteria (Weidel and Pelzer, 1964). Jia and colleagues (2017) established SE biofilm on food grade stainless steel coupons at 20°C for 3 days in TSB at a pH of 6.0 or 7.2 to genetically compare acid response conditions. The genes *invE, sseA, sseE, and sseC* were significantly expressed in acidic biofilm conditions. These genes encode virulence proteins encoded by SPI-1 and 2. As mentioned before, SPI-2 is acid-regulated.

1.3. Efficacy of disinfectants on *Salmonella* biofilm

Disinfectants work on a multitude of surfaces to reduce or eliminate pathogens. Kostaki and colleagues (2012) investigated disinfectant treatments on eight *Salmonella enterica* strain's biofilm on stainless steel coupons. *Salmonella* biofilms were significantly reduced by 2-5 logs after a 6-min exposure of a mixture of hydrogen peroxide (5 ppm) and peracetic acid (5 ppm), peracetic acid (10 ppm), and benzalkonium chloride (10 ppm), and sodium hypochlorite (10 ppm). Chylkova and colleagues (2017) exposed planktonic SH and biofilm to disinfectants. The disinfectants used were used at average FSIS and EPA recommendations at 4°C to mimic chiller conditions for 90 min. Peracetic acid (PAA; 230 ppm) and acidified hypochlorite (aCH; 50 ppm) did not reduce any of the seven SH strains. Cetylpyrinium chloride (CPC; 2,000 ppm) killed all *Salmonella* under the same conditions. Only over FSIS regulatory ranges were aCH (20 - 50 ppm) and PAA (200 - 2,000 ppm) bactericidal. Ban and colleagues (2012) compared the effectiveness of steam and lactic acid treatment on biofilm grown on PVC and stainless steel coupons. Lactic acid (pH of 2.12) and steam applied for 30 s were able to significantly reduce ST biofilm from 6 log cfu to below 1.48 log cfu when compared to an individual treatment.

Corcoran and colleagues (2013) evaluated the efficacy of benzalkonium chloride (0.01%), hypochlorite solution (250 mg/liter), and sodium hydroxide (0.5 M) against ST and SE in two forms. The disinfectants completely inhibited planktonic ST and SE. Biofilm was formed using a reactor on glazed tile, stainless steel, borosilicate glass, polycarbonate plastic, and concrete. Sodium hydroxide eliminated 48 h biofilm; however, no disinfectant completely eradicated, or reduced by $\geq$4 log, 168 h biofilm of ST or SE.

1.4. Effects of acidified water on avian gut physiology and *Salmonella* colonization

Reports demonstrated that *Salmonella* species, such as SH, can colonize crops (Hargis et al., 1995; Corrier et al., 1999). Ishola (2010) challenged laying hens with 1.3×10^4 or 1.3×10^8 cfu/mL of SE to measure challenge-dose dependency from crops. *Salmonella* was recovered up to 5 weeks post challenge from the 10^8 cfu/mL of SE group, whereas it was only recovered from the 10^5 cfu/mL of SE group the first week. Feed-withdrawal can change crop physiology. Hinton and colleagues (1999a) orally challenged 5-week-old broilers with 1.0×10^9 cfu/mL of ST. Five days post infection, crops were processed to measure changes from feed withdrawal. Feed withdrawal increased crop pH from 6.0 to 6.5 from decreased lactic acid bacteria. After a 12 h feed withdrawal, ST numbers were significantly reduced in crops. However, Corrier and colleagues (1999) measured an increase in *Salmonella* incidence in market-age broiler crops after feed withdrawal due to the decrease in host *Lactobacilli*. Ramirez and colleagues (1997) orally gavaged 6-week old broilers with 1×10^8 cfu/mL of SE to measure the effects of feed withdrawal 5 days post-challenge in crops and ceca. *Salmonella* incidence was significantly higher in crops of birds following feed withdrawal.

Crop pH can vary due to differing degrees of fermentation of present bacteria or buffering capacity of the feed ingredients (Lawlor et al., 2005). It can range from below a pH of

5 to greater than a pH of 6. Sturkie reported the pH of crops was 4.51 (Sturkie, 1976). Byrd and colleagues administered ST (10^8 cfu/mL) to 35 day old broilers which were then given 0.5% of lactic acid, formic acid, or acetic acid to measure the effects of acidified drinking water combined with feed withdrawal (2001). After an 8 h feed withdrawal, lactic and formic acid significantly reduced 53% of ST crop incidence to 31% and 36.8%, respectively. Crop pH was significantly decreased from a baseline of 5.77 to 4.79 and 4.80, respectively. In a second experiment, 0.44% lactic acid in the drinking water combined with 10-14 h feed withdrawal significantly reduced ST in the crops. The average crop pH of controls was 5.77 and lactic acid reduced crops to a pH of 4.79.

Acids, such as sodium bisulfate, have been shown to reduce *Salmonella* colonization when added to feed, water, or litter. Payne and colleagues (2002) reported another acid, sodium bisulfate, reduced ST in the litter. Again in 2007, Payne reported a litter pH of <4 was effective in reducing *Salmonella* populations. They compared used turkey litter at a pH of 4, 7, and 9, and altered the pH using sodium hydroxide or hydrochloric acid. Litter samples were inoculated with 10^7 cfu/mL of SH or ST. At a pH of 4, *Salmonella* in the litter was significantly decreased.

1.5. Avian Immune System

The avian immune system has been a hallmark model for research and is divided into the innate and the adaptive/acquired responses. Innate immune responses are characterized, in part, by pathogen recognition receptors (**PRR**) which include Toll-like receptors (**TLR**). The PRRs are located on a host cell's surface or cytoplasm and recognize microbe-associated molecular patterns (**MAMP**) on microbes (Janeway and Medzhitov, 2002; Ausubel, 2005). Examples of critical TLRs against bacteria, such as *Salmonella,* are TLR-2, 4 and 5, which target peptidoglycan, LPS and flagellin, respectively (Leveque et al., 2003; Iqbal et al., 2005).

Poultry gut-associated lymphoid tissues (**GALT**) include the Harderian gland, cecal tonsils, Peyer's patches, Meckel's diverticulum, and lamina propria (Smith et al., 2014). The GALT contains different cells which initiate and mediate immune functions locally and systemically. Non-typhoidal *Salmonella* persists in the GIT of chickens without clinical disease through a process called disease tolerance (Tohidi, 2014). Initially, *Salmonella* are able to penetrate the intestinal mucus layer and attach to the epithelium (Finlay, 2000; Beal, 2006). Mucin is produced by goblet cells in the gut and can prevent pathogen colonization; however mucin also provides nutrients and attachment sites (Wadolkowski et al., 1988). Lysozymes are found in mucosal secretions. They can kill pathogens by damaging the bacterial cell wall (Burns, 1978). Intestinal cells and soluble antimicrobial peptides (**AMP**) begin an innate immune response (Janeway and Medzhitov, 2002; Immerseel, 2002). Avian β-defensins are AMP that are expressed in heterophils and on mucosal surfaces and can recruit adaptive immune cells such as T cells and mast cells (van Dijk et al., 2008). Cathelicidins are AMP that can inhibit the expression of LPS by limiting binding capacity (van Dijk et al., 2011).

Natural killer cells, dendritic cells, granulocytes, monocytes, macrophages, and thrombocytes are innate immune cells that are the first line of defense against pathogens (Juul-Madsen et al., 2014). Heterophils are the avian equivalent to mammalian neutrophils; however, heterophils granules lack myeloperoxidase and produce low amounts of hydrogen peroxide (Daimon and Caxton-Martins, 1976; Wells et al., 1998). Heterophil activity includes phagocytosis, degranulation, oxidative burst, and producing AMP (Genovese et al., 1999). Natural killer cells, macrophages, and TH1 cells produce interferon gamma (**IFN-γ**) which is a pleiotropic cytokine that activates heterophils and macrophages in response to pathogens (Baron 1987; Kogut et al., 2001; Kogut et al., 2005).

Immune cells respond to microbes by releasing cytokines and chemokines which further mediates inflammation (Wigley, 2013; Murphy et al., 2017). Cytokines are small proteins that act as extracellular signals between cells by binding to specific receptors (Kaiser and Stäheli, 2014). Chemokines are chemoattractant proteins which cause cells to migrate to the signal source (Murphy et al., 2017). Pro-inflammatory cytokines are produced by phagocytic or non-immune cells and include IL-1β, IL-6, and IL-12 (Gibson et al., 2014; Al-Khalaifah and Nasser, 2018). Interleukin-1β induces T cells and macrophages to produce other cytokines and chemokines (Weining et al., 1998). An acute phase response is induced by IL-6 which increases synthesis of acute phase protein synthesis and B-cell differentiation to effector antibody producing plasma cells (Kaiser et al., 2000). Acute phase proteins (**APP**) are typically secreted from the liver into plasma in response to inflammation (Gabay and Kushner, 1999). Chicken APP are α1-acid glycoprotein, serum amyloid A, PIT54, ovotransferrin, ceruloplasmin, fibrinogen, fibronectin, mannan binding lectin, and C-reactive protein (O'Reilly and Eckersall, 2014). Macrophage inflammatory protein 1 (**MIP-1**) and IL-8 are chemokines that can be induced in innate immune cells (Kim et al., 2017). Avian innate immune response increased gene expression of inflammatory cytokines, such as IL-1β, IL-6, IL-12 in the ceca and liver (Berndt and Methner, 2001). Macrophages produce IL-18 in response to LPS (Rahman et al., 2012). When IL-18 and IL-12 interact, gamma interferon (**IFN-γ**) is upregulated (Göbel et al., 2003). The influx of cytokines contributes to innate and adaptive immune responses which affect cells locally and systemically (Murphy et al., 2017). Initial response of the innate immune response is critical for inhibiting *Salmonella* colonization.

During antimicrobial host attacks, macrophages express inducible nitric oxide synthase (**iNOS**), exposing *Salmonella* to nitric oxide (**NO**; Burton et al., 2014). The chicken macrophage

cell line, HD11, is used to study bactericidal activity and innate immunity *in vitro* (Babu et al., 2006). Balan and colleagues incubated HD11 cells with SE, SH and ST to compare different serovars effects on cytokine transcriptional changes, persistence, and NO production (2017). A higher persistence of SH at 18 h post infection was measured compared to ST and SE. All serovars increased NO in HD11 cells, which kills or inhibits microorganisms, and elevated expression of pro-inflammatory cytokines IL-1β and IL-6 after 18 h in HD11 cells (Fang, 2004; Balan et al., 2017).

Macrophages, *in vitro*, react differently depending on the serovar. He and colleagues (2012) measured NO production, oxidative burst potential, cell invasion, and survival of HD11 cells when stimulated with ST, SE, SK and SH. Serovars ST and SE did not induce NO, whereas SK and SH did. All serovars reduced oxidative burst potential. Cell invasion was similar among serovars, however, SE had a higher survival indicating a greater resistance to intracellular killing. Van den Biggelarr and colleagues (2020) stimulated HD11 cells with inactivated infectious bronchitis (**IBV**) virus, Newcastle disease virus, and egg drop syndrome virus vaccines to measure NO production and phagocytosis. The vaccines without mineral oil adjuvants caused small amounts of NO production. Out of the five vaccines, the inactivated monovalent IBV not increase phagocytosis. Overall, the vaccines produced different levels of NO production and phagocytosis; however, the immune responses were dependent on an adjuvant due to low immunogenicity.

Immune responses are multi-faceted and require multiple proteins in order to be effective. Macrophages are the first line of defense against invading pathogens. Measuring relative gene expression can be an efficient tool to detect cytokines secreted by macrophages. He and colleagues (2009) stimulated HD11 cells using scavenger receptor ligands (**SR**)-A fucoidan,

poly(G), and poly(I) to show innate immune function of SR in avian macrophages. The SR are proteins which engulf, and phagocytize pathogens on macrophages by binding to bacterial ligands such as cell wall components, DNA, RNA, and other molecules (Peiser et al., 2002). Macrophage HD11 cells were stimulated with SR-A ligands for 24 h and produced significant levels of NO. The SR-A ligands fucoidan, poly(G), and poly(I) significantly upregulated expression of IL-1β, IL-6, and chemokine, MIP-1β, in HD11 cells after 2 h of stimulation (He et al., 2009). Proteins' ability to stimulate immune responses can be effective when creating subunit vaccines.

Salmonella Enteritidis can become systemic when not phagocytized by heterophils (Kogut, 1994). Macrophages, heterophils, and dendritic cells phagocytize *Salmonella* (Sundquist et al., 2004). *Salmonella* can migrate to the spleen and liver through the lymphatic system in mammals (Mastroeni and Menager, 2003). However, chickens lack a sophisticated lymphatic system and encapsulated lymph nodes, so *Salmonella* travels through the bloodstream via phagocytic cells (Kaiser et al., 2006; Gast et al., 2007; He et al., 2012).

The complement system is an important component of the innate immune system and activates adaptive immunity. Complement proteins are synthesized by hepatocytes and macrophages (Carroll, 2004). Phagocytosis, induction of an inflammatory response, increasing B and T cell activity and enhancing cytolysis are all enhanced by complement (Juul-Madsen et al., 2014). Pro-inflammatory cytokines stimulate APP, including C3 which is a key factor in the complement system. There are three antibody-independent pathways activated based on the type of microbial interactions. Antibody-antigen complexes can activate complement, too. All of the pathways (classical, lectin, and alternative) produce C3 convertase which then leads to phagocytosis, lysis or inflammation (Juul-Madsen et al., 2014).

The adaptive immune response includes cell-mediated and humoral immunity. Cell-mediated immunity includes activation of T-cells. The first subset of T cells, CD4+ T helper (Th), recognize major histocompatibility complex (**MHC**) class II molecules and are further divided into Th1, Th2, and Th17 cells (Szabo et al., 2002). The Th1 cells secrete cytokines, such as IFN-γ, critical for defense against intracellular pathogens. Priming chicken heterophils with IFN-γ was shown to increase expression of pro-inflammatory cytokines (IL-1β, IL-6, IL-8) and Th1 cytokine mRNA following stimulation with SE (Kogut et al., 2005). Berndt and colleagues (2007) orally challenged 1 day old chicks with 1-2 x 10^7 cfu/mL of ST or SE. After 2, 4, 7 and 10 days post-infection, there was a significant increase of T cells in the intestinal mucosa which correlated to a significant expression of IFN-γ in the ceca lamina propria. The Th2 cells secrete cytokines important against extracellular pathogens, such as parasites (Allen and Maizels, 2011). The Th17 cells have a general role during inflammation and secrete IL-17 as later discussed. The second subset of T cells are CD8+ cytotoxic T lymphocytes which recognize MHC-I and are important to clear intracellular pathogens, viruses, and detect tumors (Janeway et al., 2001).

A chick's immune system continues to develop post-hatch. Day old chicks were monitored until day 19 to track the development of gut microbiota using 16S rRNA gene terminal restriction fragment length polymorphism analysis (Crhanova, et al., 2011). Birds were orally challenged with 1.0 X 10^6 cfu/mL of SE at various time points. The gut microbiota did not affect ceca colonization during infection. However, it did correlate with immune system maturation with increased resistance in older birds during infection and differences in cytokine expression. Infection induced higher levels of cytokines IL-8 and IL-17. The cytokine IL-17 is produced by activated Th17 cells and is important for intestinal homeostasis (Min and Lillihoj, 2002). Gallinacins are similar in expression profile to IL-17 and were also measured but had no

increased expression during infection. The complexity of the gut microbiota and immune response increased.

The bursa of Fabricius is the location for the development of immature B lymphocytes (Glick, 1994). Protective antibodies can be produced by antigen-specific B-cells (Ratcliffe and Härtle, 2014). Chickens have three immunoglobulin classes (**IgM, IgY, and IgA**) with little to no evidence of equivalents to mammalian IgD or IgE (Ratcliffe and Härtle, 2014). Chicken IgM is the first to be generated during a primary immune response similar to mammals (Ratcliffe et al., 2006). Chicken IgA is in the only secretory Ig isotype (Ratcliffe and Härtle, 2014). Chicken IgY is like mammalian IgG in function but exhibits differing biochemical properties such as a higher molecular weight due to a heavy chain with an extra constant domain, no defined hinge region, and unique oligosaccharide in its overall structure (Warr et al., 1995). Chicken IgY is transported from mother to embryo and is heavily present in the egg yolk (Carlander et al., 1999).

Salmonella colonization will affect metabolism and immune pathways (Kogut et al., 2016). Two important metabolic energy homeostasis enzymes are 5'-adenosine monophosphate-activated serine/threonine protein kinase (**AMPK**; Proszkowiec-Weglarz and Richards, 2008) and mammalian target of rapamycin (**mTOR**). The AMPK is activated by low ATP levels and sends signals via phosphorylation which increases cellular processes. The enzyme mTOR senses nutrients linked to insulin (Punzo et al., 2008). Arsenault and colleagues (2013) challenged five day-old chicks with 1.0×10^5 cfu/mL of ST and collected skeletal muscle samples form 24 h, 96 h, 1 week and 3 weeks post infection. The phosphorylation state was measured using a peptide kinome array. Insulin/mTOR/glucose, fatty acid, and AMPK signaling were significantly

different in birds challenged with ST. These results indicate the importance of characterizing effects of metabolism during a host immune response.

1.6. Competitive exclusion

Competitive exclusion (**CE**), or colonization inhibition, describes how the innate intestinal microbiota inhibits colonization of foreign pathogens by limiting nutrients and space (Revolledo et al., 2009). Direct colonization resistance occurs when commensal host microbiota reduces colonization of intestinal pathogens (Buffie and Pamer 2013). Indirect colonization resistance occurs through microbiota-stimulated host immunity and immune cell interactions. Intestinal inflammation can lead to gut dysbiosis, or imbalances of the composition of microbiota altered from incoming pathogens, immune responses, or diet. However, the loss of microbial density can allow for the proliferation of foreign pathogens. Host microbes promote an enhanced mucosal barrier through the production of mucus, short-chain fatty acids, such as butyrate or acetate, and host antimicrobial peptides from resident Paneth cells (Sassone-Corsi and Raffatellu, 2015).

Probiotics are an antibiotic alternative, competitive exclusion product utilized to reduce pathogenic microbes in poultry production (Lee et al., 2010). Administration of CE products is most effective to young chicks due to delayed development of their intestinal flora (Bolder et al., 1992). Probiotics are alive and naturally occurring microorganisms. Competitive exclusion between *Salmonella enterica* Infantis (SI) and adult chicken gut microflora was identified by Rantala and Nurmi (1973). This experiment is considered the hallmark competitive exclusion experiment. Chicks pre-treated with cultured contents from an adult chicken alimentary tract prevented detectable colonization of SI in ceca. Menconi and colleagues (2011) administered a lactic acid-producing bacteria probiotic (FloraMax) to reduce SH when added to feed. Day-of-

hatch broiler chicks were challenged with 1.0 x 10^5 cfu/mL of SH. After 24 h and 72 h, SH was significantly reduced in the cecal tonsils. Revolledo and colleagues (2009) used a probiotic, Aviguard™, to reduce colonization of ST in cecal contents. Aviguard™ is comprised of intestinal bacteria-freeze-dried products from a healthy chicken. Administration of Aviguard™ combined with an experimental dietary probiotic and abiotic β-glucan significantly reduced ST in chicks after one week.

Intragenus CE has been shown to be effective. Barrow and colleagues challenged day of hatch chicks with 1.0 x 10^9 cfu/mL of a mutant ST followed by 10^5 of virulent ST (1987c). Results showed that colonization was inhibited by the subsequent *Salmonella* strain. Yang and colleagues (2019) administered SE or ST to day of hatch chicks, followed by subsequent infection of ST or SE on day 2 depending on which serovar was administered the previous day. Competitive exclusion was observed between the different serovars in cecal contents. Methner and colleagues (2011) gave day-old chicks 1.0 x10^7 cfu/mL of ST or SE followed by 10^4 of ST or SE on day 1. Ceca were cultured 8-9 days later and the greatest inhibitory potential was measured between isogenic strains. Oral administration of live *Salmonella* to day-old chicks inhibits colonization of other *Salmonella*. These CE mechanisms can be further exploited in vaccines.

1.7. Vaccines

Vaccines are a preventative tool, and efficacy is dependent on levels of colonization, morbidity and mortality rates during experiments (Barrow, 2007). Types of a vaccine include live (low-dose), live-attenuated, killed (inactivated) and subunit. Live-attenuated vaccines can be composed of mutant strains which have deleted genes that are essential for the pathogen to thrive in a host cell (Desin et al., 2014). Killed vaccines are inactivated by methods including formalin,

heat, or acetone. Subunit vaccines are comprised of antigens which are usually proteins, and typically require adjuvants in order to make them immunogenic (Meenakshi et al., 1999).

Attenuated vaccines using knockout mutants have shown the potential to be effective. Bohez and colleagues (2007) evaluated the inhibition of SE by SPI-1 and 2 gene deletion mutants: ΔhilA, ΔsipA, ΔssrA. As previously mentioned, (SPI)-1 *hilA* and *sipA* genes encode for regulatory and effector proteins, respectively in the T3SS. The SPI-2 region encodes the regulator protein SsrA. All the mutant strains fully inhibited the wild type strain *in vitro*. De Cort and colleagues (2013) used day old Ross broiler chicks to study the efficacy and colonization inhibition of a live attenuated SE ΔhilAssrAfliG (a triple mutant) vaccine, administered orally at 10^8 cfu. Birds were then challenged with 1.0 x 10^5 cfu/mL of SE twenty-four hours later. Fecal shedding of SE was significantly decreased in the vaccinated birds ($P < 0.05$). The triple mutant was shed after 21 days and is therefore regarded safe. Overall, there was a significant reduction of fecal shedding and cecal colonization of *Salmonella* Enteritidis and this mutant has potential for controlling an infection by colonization resistance. Kilroy and colleagues (2015) vaccinated chicks with variants of ST, called AviPro *Salmonella* Duo and AviPro *Salmonella* VacT, on the day of hatch and then challenged with ST on day 2 had reduced colonization in ceca on day 7 and 14.

Vaccines offer increased resistance to young chicks against *Salmonella* infection (Mead and Barrow, 1990). Vaccine selection includes clearance from the host at slaughter age, protection, ease of use, and low cost (Bohez et al., 2006). Colonization inhibition mechanisms can create protection against pathogens by limiting attachment to the mucosal lining (Methner, et al., 1999). Inactivated and attenuated vaccines are available for ST and SE but they do not offer complete protection or full cross-serovar protection (Gast 2007). Megan Vac 1™ is an ST

deletion mutant vaccine (Elanco). McReynolds and colleagues (2007) evaluated the combination of Megan Vac 1™ with a competitive exclusion culture in reducing ST in broilers. Cecal incidence in birds receiving both products was significantly reduced.

Salmonella enterica infections are a global burden and are a problem for the agricultural industry and the public health industry. Ferreira and colleagues (2015) developed a subunit vaccine using purified supernatant from ST grown under SPI-2 conditions. As mentioned before, SPI-2 conditions are critical for *Salmonella* survival in macrophages. Under experimental conditions, the vaccine when combined with an adjuvant protected immunized mice. There was a significant decrease of bacteria in the ceca, spleen, and liver. Using a subunit vaccine is considered safer than live attenuated recombinant vaccines which can reverse mutate in the environment (Detmer and Glenting, 2006). A component *Salmonella* vaccine has potential to be effective against colonization.

Wisner and colleagues (2011) subcutaneously vaccinated chickens with 100 µg of total SPI-2 (SseB and SseD) proteins and 30% Emsulsigen D (VSA) on days 14 and 27 of age. The chickens were challenged with 10^{10} cfu/mL of SE on day 34. By day 4 post-challenge, birds given the SPI-2 vaccine had significantly higher IgY serum titers and less cfu/mL of SE in the livers. However, there were no significant differences in incidence or enumeration in ceca and spleens.

Deguchi and colleagues (2009) measured vaccine efficacy using formalin inactivated SE, ST, and SI on 6-week old layers. Four-weeks post-vaccination, chicks were challenged with 2.0 x 10^9 cfu/mL of SE, ST and SH. The vaccine significantly decreased the recovery of SE, ST, and SI from the fecal samples, ceca, and spleens 14 days post infection. The vaccine significantly decreased SH in ceca and feces indicating intragenus protection since SH was not in the vaccine.

Salmonella enterica Kentucky, Heidelberg, Typhimurium and Enteritidis are able to persist in the gastrointestinal tract of poultry. The goals of these studies were to elucidate methods to reduce *Salmonella* colonization. The specific aims were to reduce SH biofilm and SH transmission in broilers, eliminate colonization of SE and ST in chicks with SK, and elicit an immune response in chicken macrophage cells with a SK-derived secretome.

1.8. References

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2. EVALUATION OF SODIUM BISULFATE ON REDUCING *SALMONELLA* HEIDELBERG BIOFILM AND COLONIZATION IN BROILER CROPS AND CECA

2.1. Introduction

Poultry are carriers of *Salmonella enterica* serotypes which can cause salmonellosis in humans (CDC, 2020a). There are over 2,600 *Salmonella* serotypes with less than 100 that cause human disease (CDC, 2020b). The CDC reports drug resistant *Salmonella* are a serious threat because of increased antibiotic resistant infections since 2009 (CDC, 2019). Due to the rise in antimicrobial resistance, demand for antibiotic alternatives has increased. Antibiotic alternatives may include vaccines, probiotics, synbiotics, enzymes, organic/inorganic acids, phytobiotics, and prebiotics (Dhama et al., 2014; Yadav et al., 2016).

Salmonella enterica serovar Heidelberg (**SH**) is one of the top ten serovars associated with human disease and isolated from poultry (FSIS-USDA, 2014). Egg contamination is a concern due to SH being one of few *Salmonella* serotypes able to vertically transfer from hen to chick (Barnhart et al., 1993). A surveillance study from 2002 to 2006, reported 96.6% of SH isolated came from poultry meats (Zhao et al., 2008). *Salmonella* Heidelberg has increasing resistance to common antibiotics, and caused one of the largest multistate foodborne outbreaks from consumption of contaminated chicken (Zhao et al., 2008; Gieraltowski et al., 2016). Antimicrobial resistance continues to be an issue with SH including multi-drug resistant (MDR) strains (Barnhart et al., 1993; Lynne et al., 2009). National Antimicrobial Resistance Monitoring Systems reported SH was MDR 30% of the time in humans (FDA, 2011). In 2014, 9.9% of isolated SH was MDR to five drug classes and 21.1% to 3 or more classes (CDC, 2014). Due to the increased resistance combined with the decrease use of antibiotics, better alternatives are required.

Survival of microbial populations is increased by biofilm formation (Donlan, 2002; Giaouris et al., 2013). *Salmonella enterica* strains are capable of forming biofilms on abiotic surfaces, including ones found in all phases of poultry production (Steenackers et al., 2012; Giaouris et al., 2014). Biofilm forming abilities depend on growth conditions, contact surfaces, and serotypes or strains (Chia et al., 2009). Attachment of biofilms to food contact surfaces (stainless steel, ceramics, glass, or plastic) can lead to cross-contamination of consumer products (Brooks and Flint, 2008; Carrasco et al., 2012; De Oliveira et al., 2014). Reduction of *Salmonella* before processing is important to mitigate cross-contamination (Fries, 2002).

Government agencies regulate disinfectants and sanitizers and work together to standardize effective procedures. The U.S. Environmental Protection Agency (**EPA**) regulates the efficacy, labeling, and handling of disinfectants (CDC, 2016). A disinfectant is a physical process, such as ultraviolet light, or chemical that kills microorganisms (EPA, 2021). The Food and Drug Administration (FDA) and US Department of Agriculture, Food Safety and Inspection Service (FSIS) share mandates regulating concentrations of commonly used disinfectants on surfaces commonly found in the production of meat, poultry and eggs under guidance from the EPA (FDA, 2019). Further control of pathogenic bacterial populations are mandated by USDA-FSIS to follow Hazard Analysis Critical Control Points (**HACCP**; FDA, 2019). While inorganic acids are not EPA registered pesticides, they may also have antimicrobial properties in certain agricultural applications by modifying the microbes in the environment. Methods for protocols may reduce foodborne illness, but improvements are still needed.

Salmonella control includes biosecurity throughout all phases of production. Controlled access, hygienic barriers and pest control can all aide in limiting *Salmonella* (van Immerseel et al., 2009). However, even with biosecurity measures *Salmonella* remains an issue. Chemicals,

such as acidified hypochlorite and peroxyacetic acid (**PAA**), used at the recommended FSIS and EPA ranges were ineffective against seven field strains of *Salmonella* biofilm (Chylkova et al., 2017). Glutaraldehyde, hydrogen peroxide, and formaldehyde at a concentration of 1.0% (vol/vol) were unable to eradicate *Salmonella* on poultry house concrete floors (Marin et al., 2009). Corcoran and colleagues (2014) found sodium hypochlorite (500 mg/L), sodium hydroxide (1 M), and benzalkonium chloride (0.02%) did not eliminate established 48-h or 168-h strains of *Salmonella* Typhimurium (**ST**) or Enteritidis (**SE**) biofilm when treated for 90 min on concrete, glass, steel, polycarbonate, or tile coupons used to simulate food processing environments.

Clean drinking water is important for the health and performance of poultry. Biofilms in potable water systems may host pathogenic bacteria which could be indicated by coliforms. The maximum contaminant level of total coliform bacteria in drinking water is 0 cfu/L per 0.1 L of water in less than 5.0% of 40 samples or more per month (CDC, 2016; EPA, 2021). Maes and colleagues (2019) surveyed broiler farm microbial populations in outside water samples. The total aerobic count ranged from 6 to 300 cfu/mL inside broiler houses (Maes et al., 2019). Mixed-species biofilms can be made up of a combination of *Salmonella* or aerobic species (Schaefer et al., 2013). Schaefer and colleagues (2013) reported *Salmonella* can readily colonize on silicone tubing as established mixed-species biofilms. Broiler and layer farms water drinkers and tanks remain potential hot spots for *Salmonella* (Marin et al., 2009; Levantesi et al., 2012). *Salmonella* contaminated flocks also risk re-infection from contaminated material in the drinkers (van Immerseel et al., 2009).

Sodium bisulfate (**SBS**; $NaHSO_4$), also known as sodium hydrogen sulfate, is categorized by the EPA as a mineral acid salt with antimicrobial properties that dissolves and releases a

hydrogen ion which decreases pH (Sun et al., 2008). When used as a litter acidifier, SBS significantly decreased litter pH from 7.2 to 6.9 and reduced *Escherichia coli* (***E. coli***) by 6-logs after 2 weeks (Pope and Cherry, 2000). A lower litter pH can reduce bacteria that create ammonia gas from uric acid in excreta (Terzich, 1997). *Salmonella* Typhimurium was reduced by 1.3 logs in litter treated with SBS at a concentration of 100 lb/1,000 ft^2 which decreased litter pH from 8.3 to 3.5 (Payne et al., 2002). Chicken drumsticks were inoculated with 10^8 cfu/mL of SE and then treated for 0-3 days with SBS at concentrations of 1%, 2%, 3% (Dittoe et al., 2019). After 3 days, SBS significantly reduced pH from 7.42 to 1.64, 1.45, 1.31 and colonization by 0.92, 1.09, and 1.57 log cfu/g (Dittoe et al., 2019). Micciche and colleagues (2019) found that at a pH of 1.21-1.54, SBS eliminated ST to 0 log cfu/mL in poultry processing reuse water in 5 minutes which was a greater reduction than PAA that reduced ST by 4-5 log cfu/mL at a pH of 4.02. When dog and cat food were treated with SBS at 0.2% and 0.4%, SE was significantly decreased by 2 and 1.6 logs, respectively. (Jeffrey, 2016). *Salmonella* Typhimurium was significantly decreased by 2.7 logs in rendered chicken fat (used for pet food products) by a 6 hour SBS (0.5%) treatment (Dhakal et al., 2019). Versatility of SBS in reducing *Salmonella* across platforms or mediums suggests it's potential.

Water lines can be an initial source of SH biofilm and minimizing colonization would provide cleaner drinkers. Application of SBS in drinkers to prevent biofilm formation and colonization in chicks would improve costs while also reducing overall SH. We hypothesized that SBS at a pH of 3.5 would eliminate SH biofilm on polyvinyl chloride (**PVC**) coupons and reduce horizontal transfer of SH among broiler chicks.

2.2. Materials and Methods

2.2.1. Bacterial strains and growth conditions

Frozen stocks, maintained at -80°C, of *Salmonella* Heidelberg (**SH**) were obtained from USDA-ARS (College Station, TX, USA). Cultures were passaged three times every 8 h in tryptic soy broth (**TSB**; Difco, Sparks, MD, USA) at 37°C. Biofilm cultures were grown in Luria-Bertani broth (**LB**; HiMedia, Mumbai, India). All media was supplemented with novobiocin (25 µg/mL; Alfa Aesar, Haverhill, MA, USA) and nalidixic acid (**LB^{NN}**; 20 µg/mL; MP Biomedicals, LLC, Illkirch, France) to control for extraneous bacteria.

2.2.2. PVC coupons

Coupons (PVC; 2 cm x 5 cm; 1" PVC Schedule 40) were cut with a rotary tool and sanded. Coupons were soaked overnight in Alconox (Alconox, White Plains, NY, USA), rinsed six times with tap water and one time in ddH_2O. The coupons were autoclaved for 15 min at 121°C in water, and then air dried overnight in a biosafety cabinet.

2.2.3. Sodium Bisulfate

Sodium bisulfate (**SBS**; Jones-Hamilton, Co. Walbridge, OH, USA) was prepared in water via manufacturer's instructions. Briefly, 454 g of SBS was mixed into 16 L of water to create a stock solution. The stock solution was titrated into fresh tap water until a pH of 3.5 was obtained.

2.2.4. Biofilm Treatment

Biofilm formation on PVC was evaluated by using methods previously described (Agarwhal et al., 2011; Kostaki et al., 2012). Briefly, sterile coupons were initially inoculated in 1.0 x 10^9 cfu/mL of SH suspension (bacterial attachment step) in 30 mL of LB^{NN} broth for 5 h under static conditions at 37°C. Coupons were then removed using sterile forceps, rinsed with 1 mL of cold PBS to remove loose cells and placed in a new tube with 30 mL of LB^{NN} for 6 days

under static conditions (biofilm formation step) at 37°C. The media was replaced every 48 h. Coupons were rinsed with 1 mL of cold phosphate buffered saline (**PBS**) and air dried for 5 min in a HEPA filtered biological safety cabinet during media replacement. All biofilm work was conducted in a biosafety cabinet. After 6 days, coupons were rinsed, dried and placed in a treatment of 30 mL of SBS at a pH of 3.5 or sterile tap water for 10 min, 8 h, or 24 h at 37°C.

2.2.5. Biofilm analysis

Coupons were sonicated in 30 mL of PBS for 15 min at room temperature using an ultrasonic cleaner (VWR, Radnor, PA, USA) at a fixed frequency of 35 kHZ. Samples were serially diluted into PBS and direct plated onto XLT-4^{NN}. Coupon rinsates were pre-enriched for 24 h in buffered peptone water (**BPW**; Difco), cultured into Rappaport-Vassiliadis broth (**RV**; Hardy Diagnostics, Santa Maria, CA, USA) and struck for incidence. All agar plates were incubated for 24 h at 37°C. Values presented are the averages of six separate experiments (n=6) on different days with triplicate coupon samples per treatment.

2.2.6. Animals and Handling Procedure

Day-of-hatch, male by-product broiler chicks were obtained from a commercial hatchery and placed on clean pine shavings in floor pens. The environment was climate controlled and age-appropriate in disinfected animal biosecurity level 2 rooms according to the primary breeder guidelines (Cobb-Vantress, 2018). Birds were monitored 2-3 times daily to check for morbidity, mortality, temperature and relative humidity. Data loggers (CAS DataLoggers, Chesterland, OH, USA) measured the temperature and relative humidity every 5 min. Pen weights, feed and water intake were measured across all trials to ensure consumption was consistent between treatments. Fresh tap water and SBS water was measured in a graduated cylinder daily. Feed was weighed back when the trial ended to calculate feed consumption. All birds were cared for under

approved Texas A&M University Institutional Animal Care and Use Committee and Institutional Biosafety Committee protocols (IACUC 2019-0171; IBC 2019-073). Each trial was replicated twice at different time points.

2.2.7. Experimental Design and Treatment Groups

Chicks (n=30/pen) were randomly placed across eighteen pens sized 0.9 m by 1.5 m. Pens were assigned to one of two treatment groups with 9 pens (replicates). A balanced unmedicated starter ration and water were provided ad libitum that met or exceeded industry recommendations for nutrition. Upon arrival, a subset of ceca (n=10) were collected for enrichment to verify chicks were *Salmonella* free. Ceca were macerated in BPW and incubated overnight at 37°C. The following day 0.1 mL of pre-enrichment was sub-cultured into RV at 37°C overnight. The enrichment was struck for isolation onto XLT-4 without any antibiotics to screen for wild-type strains. No *Salmonella* were detected.

Pre-seeder birds contaminated clean pine shavings to mimic a commercial broiler barn. Male broiler chicks were randomly selected and placed in groups of 30 chicks per pen. All pre-seeder chicks were orally gavaged with 0.5 mL of 2.0 $x10^7$ cfu/mL of SH upon arrival. On D7, all pre-seeder chicks were orally gavaged a second time with 0.5 mL of 2.0 x 10^8 cfu/mL. Fecal grabs (n=1/pen) were aseptically collected from the litter on D5 post-infection to confirm incidence of SH shedding into the environment. All pre-seeder birds were euthanized on D13 or D14 (based on hatchery schedule) by carbon dioxide (CO_2) asphyxiation. Ceca (n=10/pen) were collected for incidence.

New chicks were randomly placed in groups of 30 chicks per pen onto litter previously contaminated by pre-seeder chicks. Each pen included 10 seeder and 20 contact chicks. There were two treatments. One treatment received tap water. The second treatment received tap water

treated with sodium bisulfate to a pH of 3.5. Each treatment pen was replicated nine times. Seeders were wing banded and orally gavaged with 0.5 mL of 2.0 x 10^8 cfu/mL of SH to mimic horizontal transfer. On day 10, chicks were killed by CO_2 asphyxiation. Ceca were removed from the seeders for incidence (n=5/pen). Crop and ceca samples were aseptically removed from contact birds for enumeration and incidence (n=10/pen). The crop contents were aseptically removed by clamping above and below the crop using Rochester Carmalt forceps (VWR). Crop pH was measured (n=5/pen) by diluting contents 10X in ddH_2O (Trial 1) or directly inserting a Hanna pH probe (Trial 2; Hanna Instruments, Smithfield, RI, USA).

2.2.8. *Salmonella* Challenge

Salmonella Heidelberg was harvested by centrifugation at 600 x *g* for 15 min at 4°C to prepare the bird challenge. The pellet was resuspended in sterile, cold PBS and washed twice prior to challenge. Optical density was measured spectrophotometrically at 625 nm at an absorbance value of 1.30 (SPECTRONIC® 20+ SERIES Spectrophotometers, Thermo Fisher, Waltham, MA, USA) and estimated at 1.0 x 10^9 cfu/mL relative to an established standard curve. Concentration of the challenge stock was confirmed by serial dilution on xylose lysine tergitol-4 (**XLT-4**; Hardy Diagnostics, Santa Maria, CA, USA) agar with added supplement (Difco).

2.2.9. Bacteriological Analysis

Cecal contents were weighed, and approximately 0.25 g of the contents were serially diluted 1:10, 1:100, 1:1,000, and 10,000 in PBS. Crop contents were weighed, and stomached for 30 s in 5 mL of BPW (Stomacher). Crop samples were then serially diluted in PBS (1:10, 1:100, 1:1,000, 1:10,000, 1:10,000). All enrichment samples were pre-cultured in BPW for 24 hours before being sub-cultured into RV. All samples mentioned were cultured onto XLT-4^{NN} at 37°C for 18-24 h.

Colonies exhibiting normal *Salmonella* morphology were periodically confirmed by lysine iron agar (Difco), triple sugar iron agar (Difco) slants and an agglutination assay using *Salmonella* O Poly A-I antiserum (Difco). Samples that were negative from direct plating but positive after RV enrichment were assigned a value of 1.50 $\log_{10}$ *Salmonella* /g cecal contents (Corrier et al., 1994). Crop samples with less than 0.05 g contents were removed.

2.2.10. Statistical Analysis

Statistical analyses were conducted via Student's *t*-test. The mean and SEM were calculated for all treatments. Outliers were removed two standard deviations from the mean. All analyses were considered significant if *P*-value $\leq$ 0.05.

2.3. Results and Discussion

2.3.1. Biofilm

Biofilm production is critical to bacterial persistence (Burmølle et al., 2006). *Salmonella* biofilm on processing surfaces is a food industry concern due to the potential cross-contamination of poultry products (Carrasco et al., 2012). Maharjan and colleagues (2017) found that even with consistent water line cleaning, microbial residue would fluctuate depending on time and location of flocks. Ten minutes were not enough contact time for SBS to significantly decrease SH biofilm (Fig. 2.1A). Sodium bisulfate significantly reduced SH biofilms when applied for 8 h (2.15 log cfu/mL) and 24 h (1.05 log cfu/mL) (Fig. 2.1B and 2.1C). Overall, SH was a poor biofilm former which was similar to previous findings (Wang et al., 2013). Authors believe SH may have decreased at 24 h due to no supplementation of nutrient medium. Sodium bisulfate could be an efficient and safe way to eliminate SH biofilms in water drinkers.

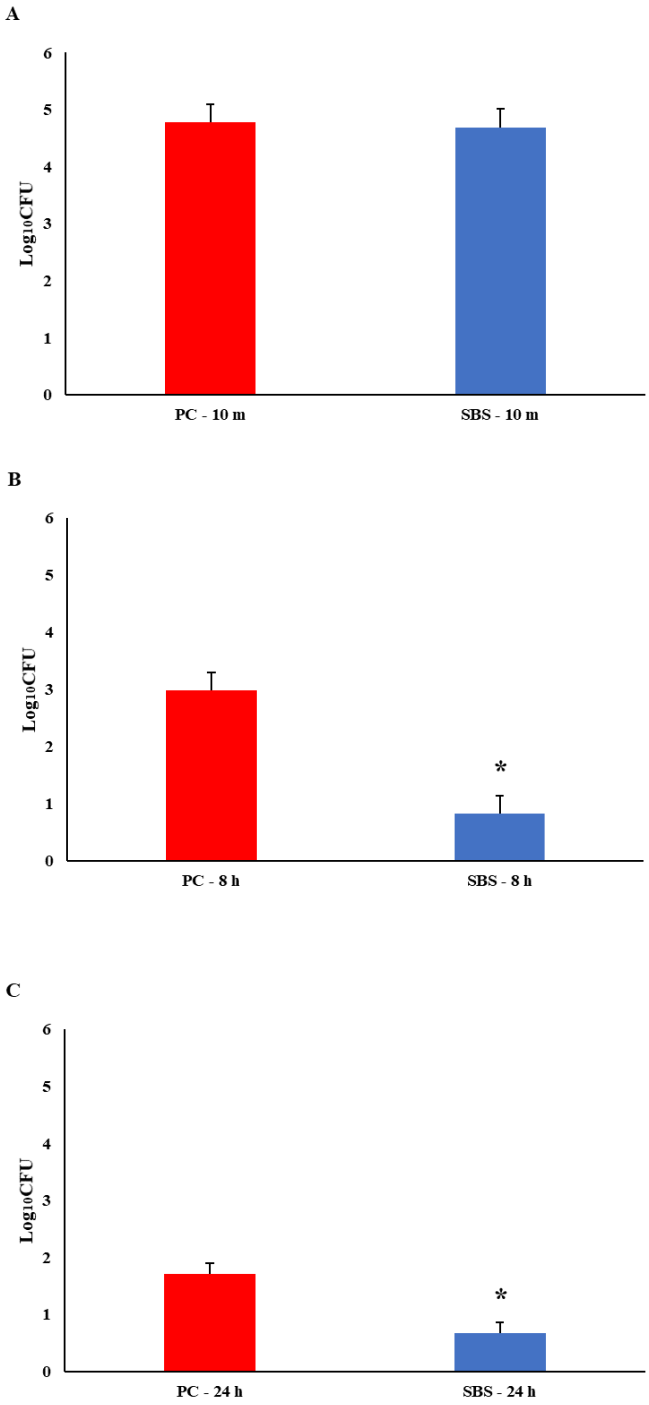

Figure 2.1 *Salmonella* Heidelberg biofilm on PVC coupons (n=6/treatment) were significantly reduced by sodium bisulfate (SBS) at a pH of 3.5 ($P < 0.05$)* when treated for 8 h or 24 h. The positive control was tap water. Values presented are the averages of 6 separate experiments with triplicate coupon samples within each experiment. Coupons were treated for (A) 10 min, (B) 8 h, (C) or 24 h.

2.3.2. Horizontal transfer

There were no differences in pen weights, feed consumption or water consumption across treatments indicating no negative treatment effects of SBS in the water. All incidence of SH in fecal grabs, pre-seeder and seeder ceca were positive (data not shown).

Acidifying the crop is proactive in bacteria inhibition, because it is the second organ in the gastrointestinal tract (Pan and Yu, 2014). Low pH inhibits pathogens, such as *Salmonella*, by acidifying the cell cytoplasm (Lawlor et al., 2005). Ricke (2003) reviewed the importance of analyzing the crop in the initial colonization stages of *Salmonella* Enteritidis. Crop pH was significantly decreased ($p < 0.05$) in the SBS treated group in trial 2 (Table 2). The broiler crop pH can range from below 5 to greater than 6 due to fermentation of feed by host lactobacilli which produces lactic acid (Hinton et al., 2000a; Jozefiak et al., 2006; Rehman et al., 2007; Rynsburger, 2009). The acid binding (buffering) capacity of feed ingredients can, also, affect crop pH (Jozefiak et al., 2006). The crop contains 10^8 to 10^9 cfu/g of primarily Gram-positive facultative anaerobic bacteria such as *Lacotbacillus* (Lawlor et al., 2005). Crop colonization was significantly decreased ($P < 0.05$) in the SBS treated group in trials 1 and 2 (Tables 2.1 and 2.2).

Table 2.1 Trial 1 colonization and incidence of crop and cecal contents and average crop pH.

Treatment	Log_{10} cfu/mL[1]		Enrichment		Crop pH[2]
	Crop	Ceca	Crop	Ceca	
PC	3.40±0.04	4.06±0.11	90/90	90/90	5.30±0.07
SBS	**3.13±0.86***	**3.67±0.12***	90/90	90/90	5.24±0.07

[1]Values are means ± SEMs from 10 birds per pen per treatment with 9 replicate pens. [2]Crops of 5 birds per pen were collected for pH measurements. *$P < 0.05$. PC, positive control; SBS, sodium bisulfate.

Homeostatic pH values are maintained through the bicarbonate cycle (Ruiz-Feria et al, 2011). Hinton and colleagues (2000b) reported the average pH of market-age broiler ceca were 6.2. Cecal colonization was significantly decreased ($P < 0.05$) in trial 1 (Table 2.1). In trial 2, there was no significant reduction of SH in cecal colonization (Table 2.2). We believe this is due to the bicarbonate cycle maintaining homeostatic pH. Other applications of SBS did not see reductions. Harris and colleagues found that *Salmonella* Typhimurium was not significantly decreased by SBS in water in crops and ceca (direct plating and enrichment) in market-age broilers during feed/water withdrawal (Harris et al., 2019). Cochrane and colleagues (2016) treated ST contaminated feed ingredients (feather meal, avian blood meal, porcine meat and bone meal, and poultry by product meal) with 1.0% SBS over a 42-day period and did not see a reduction compared to the control. Line and Bailey (2006) applied SBS to broiler houses before chicks were placed and on week 4 but no significant effects on *Salmonella* prevalence was detected in fecal grabs and drag swabs. When SBS (4.5 kg/t to 9 kg/t) was added to feed, it did not reduce SE in 34 d post infected broilers ceca, feces, spleens or livers when challenged with 2 x 10^5 cfu/mL on d 1 (Kassem et al., 2012).

Table 2.2 Trial 2 colonization and incidence of crop and cecal contents and average crop pH.

Treatment	Log_{10} cfu/mL[1]		Enrichment		Crop pH[2]
	Crop	Ceca	Crop	Ceca	
PC	1.13±0.11	2.01±0.13	55/85	77/90	5.50±0.09
SBS	**0.74±0.01***	2.00±0.14	48/83	72/90	**5.05±0.13***

[1]Values are means ± SEMs from 10 birds per pen per treatment with 9 replicate pens. [2]Crops of 5 birds per pen were collected for pH measurements. *$P < 0.05$. PC, positive control; SBS, sodium bisulfate.

We did not withdraw feed due to the age of the birds and the unlikeliness of the scenario for chicks during brood. Reports have demonstrated that *Salmonella* increases in crops after feed

withdrawal (10% versus 1.9%; Corrier et al., 1999). Researchers speculate that this was caused by consumption of contaminated litter by the birds during the withdrawal period because birds continuously peck and consume excreta in litter (Pan and Yu, 2014). Previous experiments with SBS at a pH of 3.2 in drinking water did not impact *Salmonella* Typhimurium in market-age broilers crops or ceca during feed withdrawal (Harris et al., 2019).

The pH of the water fluctuated during the first 48 h the contact and seeder birds were placed in trial 1 due to uncovered drinkers. The drinkers also leaked which caused a damp and humid environment for the SH to thrive. For trial 2, the drinkers were replaced which lead to dry litter. Garden sprayers were used to add 7 L of water per pen. Interestingly, we saw more differences of SH in the first trial's results due to increased water activity.

Water treatments are important to significantly reduce pathogens on farms (Vandeplas et al., 2010). Prevention of *Salmonella* in water lines can also reduce cross-contamination during production (Fries, 2014). Pope and Cherry (2000) reported SBS used as an antimicrobial agent and litter acidifier reduced the prevalence of *E. coli* and *Salmonella* in broiler houses. Payne and colleagues (2007) reported adjusting turkey litter to a pH of 4.0 with hydrochloric acid was effective in reducing *Salmonella* populations. Inhibition of pH sensitive pathogenic bacteria, such as *Salmonella*, can occur with application of acidifiers at a pH below 5 (Pearlin et al., 2020). The use of SBS as a water acidifier to decrease bacterial incidence would be less expensive than organic acids (Dhakal et al., 2019).

Biofilms and planktonic bacteria respond differently to antimicrobial agents, so the goal of this study was to analyze the differences in zoonotic SH versus as a monospecies biofilm. Sodium bisulfate at a pH of 3.5 was able to reduce *Salmonella* Heidelberg biofilm, ceca and crop colonization in chicks. Effective *Salmonella* control will involve multiple intervention strategies.

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3. COMPETITIVE EXCLUSION OF INTRA-GENUS *SALMONELLA* IN NEONATAL BROILERS[*]

3.1. Introduction

Salmonella enterica is a foodborne pathogen that causes an estimated 1.2 million human infections annually in the United States (Boore et al., 2015). There are over 2,600 *Salmonella enterica* serotypes which can infect a wide range of vertebrate species, but less than 100 serotypes cause the majority of human infections (Foley et al., 2013; FSIS-USDA, 2014). Infected poultry may be asymptomatic of clinical disease but continue to shed zoonotic subspecies into the environment causing gastroenteritis and systemic infections in humans (Acheson and Hohmann, 2001; van Immerseel et al., 2005). *Salmonella enterica* Typhimurium (**ST**), Kentucky (**SK**), and Enteritidis (**SE**) are in the top 5 common isolates found in contaminated chicken (Brenner et al., 2000; van Immerseel et al., 2005; Scallan et al., 2011). Reducing *Salmonella* colonization of poultry would benefit agricultural and public health sectors by decreasing medical costs and lowering cases of human infections.

In 2014 the US Department of Agriculture - Food Safety and Inspection Service determined that SK, SE, and ST were isolated from 60.8%, 13.6%, and 7.7% of young chicken carcasses, respectively (FSIS-USDA, 2014). *Salmonella enterica* serotype Kentucky (SK) is the most prevalent serotype isolated from contaminated poultry carcasses; however, only 0.14% of human clinical disease was reported with this bacterium (CDC, 2016). In 2016, SE and ST were confirmed from 16.8% and 9.8% human Salmonellosis cases, respectively (CDC, 2016). These *Salmonella* are prevalent in poultry; however, only SE and ST are considered a major food safety

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concern when compared to SK (FSIS-USDA, 2014).

Neonatal chicks are more susceptible to foreign bacteria, such as *Salmonella*, due to their lack of mature intestinal microflora (Gong et al., 2008). Initial invasion in the gastrointestinal tract leads to increased expression of chemokines, cytokines and an influx of heterophils and macrophages (Beal et al., 2004; Withanage et al., 2005; Setta et al., 2012). *Salmonella* Typhimurium infection significantly decreased jejunum villus height from the host's inflammatory response due to the influx of heterophils in one day old chicks (Henderson et al., 1999; Shao et al., 2013). *Salmonella* can hide, multiply and survive in macrophages leading to persistence (Haraga et al., 2008). Disease tolerance occurs as *Salmonella* is then able to persist in the gut of chickens without severe clinical signs (Tohidi et al., 2018).

Intestinal direct colonization resistance is the inability of foreign ingested bacteria to colonize due to host bacteria (Lawley and Walker, 2013). Mechanisms can include nutrient competition or active antagonism, such as bacteriocins (van der Waaij et al., 1971; Kamada et al., 2013). Rantala and Nurmi (1973) used mature chicken intestinal bacteria to reduce colonization of *Salmonella* Infantis in chicks. Oral administration of attenuated ST given to one day old chicks has been shown to competitively exclude future colonization of intestinal ST when it is again administered 24 hours later (Barrow et al., 1987a). Methner and colleagues (2011) challenged chicks with various *Salmonella* serotypes and found greater inhibition occurred between isogenic strains. Yang and colleagues (2018) determined intra-genus competitive exclusion occurs between ST and SE when administered 24 h apart in neonatal broilers. All are examples of ways colonization resistance can be used to exploit competitive exclusion in order to reduce *Salmonella* colonization in chicks.

Indirect colonization resistance occurs through microbiota-stimulated host immunity and

immune cell interactions. These include an enhanced mucosal barrier by production of mucus, short-chain fatty acids, such as butyrate or acetate, and host antimicrobial peptides from resident Paneth cells (Sassone-Corsi and Raffatellu, 2015). Characterization of a broiler's immune response during a *Salmonella* infection can be measured by cytokine gene expression. An early localized inflammatory response includes an influx of heterophils and macrophages (Kogut et al., 1994). Interleukin-1β (**IL-1β**) is a pro-inflammatory mediator and expression is increased in response to bacterial, viral, and parasitic infections (Gibson et al., 2014). The expression of other pro-inflammatory cytokines, such as IL-6 enhance protection during ST infection by inducing acute phase protein synthesis and are important in further stimulating a T_H1 host immune responses (Akira, 2000; Schneider et al., 2001). Interleukin-18 is a member of the IL-1 family and is produced by macrophages in response to lipopolysaccharide (Göbel et al., 2003; Rahman et al., 2012). When IL-18 is in the presence of IL-12, gamma interferon (**IFN-γ**) production is upregulated in T_H1 and NK cells (Göbel et al., 2003). Chicken IFN-γ is a macrophage-activating factor and is crucial in response to intracellular pathogens by inducing a cell-mediated T_H1 response (Klasing and Peng, 1987; Janeway, 1992; He et al., 2011). The function of IL-10 is to induce immunoregulatory effects, such as downregulating the production of pro-inflammatory cytokines (Eckmann and Kagnoff, 2001). The cecal tonsils are composed of lymphoid follicles that contain multiple immune cells (Kitagawa et al., 2000). The liver contains immune molecules, such as antigen presentation cells and Kupffer cells, and synthesizes cytokines, chemokines, and acute-phase proteins in response to infection, trauma or stress (Robinson et al., 2016). Acute phase proteins, such as mannan binding lectin, can activate phagocytosis and modulate cytokine expression (O'Reilly and Eckersall, 2014). Therefore, cytokine mRNA expression can be measured in chicken cecal tonsils and livers to measure the host immune

interaction with *Salmonella* as a local or systemic infection (Withanage et al., 2004). The expression of cytokines can correlate with the presence of pathogens in the gut; however, mRNA expression may not correlate with protein synthesis (Withanage et al., 2004).

Salmonella Kentucky (**SK**) is the number one isolated serotype in poultry, but has a low incidence (0.14% cases) of clinical disease in humans (CDC, 2016). Fricke and colleagues (2009) screened *Salmonella enterica* strains for avian pathogenic plasmid uptake from *Escherichia coli* to screen for virulence evolution and host adaptations. Evidence of these plasmids was predominantly found in SK but not in other strains of *Salmonella*, which could give an advantage to SK to cope with stress factors and competition (Fricke et al., 2009). Cheng and colleagues (2015) showed SK persisted until the end of their challenge on day 36 compared to ST colonization which had fallen below the level of detection by day 15. Prevalence of SK in the ceca, over other serotypes, could be further explained at the molecular level, such as increased transcription of regulatory protein, RNA polymerase (Rpos; Cheng et al., 2015). A sigma factor, such as Rpos, initiates transcription in stress response genes, so if it were elevated it would allow for greater proliferation of bacteria (Nickerson and Curtiss, 1997). Since SK is the predominantly isolated serotype, we believe this serotype may be used to exclude and reduce other *Salmonella* as a live vaccine candidate.

We hypothesized that an initial infection by SK can reduce intra-genus serotypes SE and ST by competitive exclusion. Incidence of SK in the poultry industry is increasing (Fricke et al., 2009). Understanding how these serotypes interact is necessary, but there is little information available. Furthermore, we attempted to characterize the host immune response by measuring cytokine gene expression in cecal tonsils and livers during a co-infection.

3.2. Materials and Method

3.2.1. Experimental birds

Day-of-hatch, male broiler chicks obtained from a commercial hatchery were placed on clean pine shavings in floor pens with an environmentally controlled and age-appropriate climate in animal biosecurity level 2 rooms (Cobb-Vantress, 2018a). Chicks were provided ad libitum access to water and a balanced unmedicated starter ration that met or exceeded industry recommendations for nutrition (Cobb-Vantress, 2018b). Upon arrival, chick tray papers were cultured to confirm that the chicks were *Salmonella*-negative. Each cohort of birds were placed in pens sized 3.7 m x 2.7 m. All animal handling procedures were in compliance with the USDA Institutional Animal Care and Use Committee and Institutional Biosafety Committee protocols (IACUC 2018-0423 EX; IBC 2019-073). These trials were replicated twice.

3.2.2. Bacterial strains and growth conditions

All serotyped isolates (Table 3.1) were obtained from USDA-ARS (College Station, TX, USA) and were stored at -80°C. *Salmonella* Kentucky (SK), *Salmonella* Enteritidis (SE), and *Salmonella* Typhimurium (ST) were passaged 3 times every 8 hours in sterile tryptic soy broth (TSB; BD Difco, Sparks, MD) at 37°C. All media were supplemented with novobiocin (25 µg/mL; Alfa Aesar, Haverhill, MA) and nalidixic acid (20 µg/mL; MP Biomedicals, LLC, Illkirch, France) to control for extraneous bacteria. Isolates were selected for resistance for differential plating. The SK isolate was selected for resistance to rifampicin (32 µg/mL; Tokyo Chemical Industry Co., Ltd. Portland, OR). The ST and SE isolates were selected for resistance to gentamicin (50 µg/mL; Corning, Manassas, VA). The culture was harvested by centrifugation at 600 x g for 15 min at 4°C. The cell pellet was resuspended in sterile, cold PBS and washed twice prior to challenge. The culture's optical density was measured spectrophotometrically at

625 nm at an absorbance value of 1.30 (SPECTRONIC® 20+ SERIES Spectrophotometers, Thermo Fisher Scientific, Waltham, MA) and estimated at 1.0 x 10^9 cfu/mL relative to an established standard curve. The concentrations of challenge stocks were confirmed by serial dilution on xylose lysine tergitol-4 (**XLT-4**; Hardy Diagnostics, Santa Maria, CA) agar with added supplement (BD Difco).

Table 3.1 Source of *Salmonella* serotypes.

Bacteria	Strain	Source of Strain	Reference
S. Enteritidis	Primary poultry isolate, #97-11771	National Veterinary Services Laboratory Ames, Iowa	Kogut et al., 1995
S. Kentucky	Broiler field isolate	Southern USA Farm	Byrd et al., 1999
S. Typhimurium	Primary poultry isolate	National Veterinary Services Laboratory Ames, Iowa	Byrd et al., 1998

3.2.3. Experimental design and sample collection

Chick tray papers were pre-enriched in 10 mL of buffered peptone water (BPW; BD Difco) and incubated overnight at 37°C. The following day 0.1 mL of pre-enrichment was sub-cultured into 10 mL of Rappaport-Vassiliadis broth (RV; Hardy Diagnostics) at 37°C overnight. The enrichment was cultured onto XLT-4 without antibiotics and incubated 18-24 h at 37°C to verify that chicks were *Salmonella* free. Chicks were randomly divided into six treatment groups of 30 birds each (Tables 3.2. and 3.3.).

Chicks were killed by carbon dioxide asphyxiation on D3. Ceca, liver, and spleen samples were aseptically removed. Cecal tonsil and liver samples were snap-frozen in liquid nitrogen and stored at –80°C until the total RNAs were isolated.

Table 3.2 Experimental design for trials 1 and 2 (n=30 chicks/treatment).

Treatment	NC	SK	SK→ST	ST	ST→SK	SK+ST
D0	Place chicks					
D1 (Challenge)	PBS	10^4 CFU SK	10^4 CFU SK	10^4 CFU ST	10^4 CFU ST	10^4 CFU SK+ST
D2 (Re-Challenge)	PBS	PBS	10^5 CFU ST	PBS	10^5 CFU SK	PBS
D3 (Kill)	Collect ceca, liver, and spleen tissues.					

All chicks received 0.5 mL of PBS or challenge. NC, negative control; SK, *Salmonella* Kentucky; ST, *Salmonella* Typhimurium.

Table 3.3 Experimental design for trials 3 and 4 (n=30 chicks/treatment).

Treatment	NC	SK	SK→SE	SE	SE→SK	SK+SE
D0	Place chicks					
D1 (Challenge)	PBS	10^4 CFU SK	10^4 CFU SK	10^4 CFU SE	10^4 CFU SE	10^4 CFU SK+SE
D2 (Re-Challenge)	PBS	PBS	10^5 CFU SE	PBS	10^5 CFU SK	PBS
D3 (Kill)	Collect ceca, liver, and spleen tissues.					

All chicks received 0.5 mL of PBS or challenge. NC, negative control; SK, *Salmonella* Kentucky; SE, *Salmonella* Enteritidis.

3.2.4. Bacteriological analysis

There were two sets of XLT-4 plates dependent on the selected antibiotic resistance of each serotype. Samples were sub-cultured onto XLT-4 plates containing novobiocin (25 µg/mL), nalidixic acid (20 µg/mL; XLT-4^{NN}) and either rifampicin (32 µg/mL) or gentamicin (50 µg/mL). All samples were incubated for 18-24 h at 37°C.

Cecal colonization and incidence were measured from 20 chicks per treatment. Livers and spleens were macerated together to measure organ invasion from 20 chicks per treatment.

Cecal contents were weighed, and approximately 0.25 g of the contents were serially diluted to 1:10, 1:100, 1:1,000, and 1:10,000 in PBS and spread plated onto XLT-4^{NN} with either rifampicin or gentamicin. All were cultured in BPW overnight at 37°C before being enriched in RV. The RV cultures were then sub-cultured onto XLT-4^{NN} and incubated 18-24 h at 37°C. Colonies exhibiting normal *Salmonella* morphology were periodically confirmed by lysine iron agar (LIA; BD Difco), triple sugar iron agar (TSIA; BD Difco) slants, and *Salmonella* O Poly A-I antiserum (BD Difco).

3.2.5. RNA isolation and qRT-PCR

Total RNA extraction and cDNA synthesis, and qRT-PCR were previously described (Zhao et al., 2020). Quantification of IL-1β, IL-6, IL-10, and IL-18, and IFN-γ were determined by qRT-PCR using the Applied Biosystems PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific). Primer sequences have been previously reported for all genes except IFN-γ (Iqbal et al., 2005; Hong et al., 2006; Kumar et al., 2009; Khampeerathuch et al., 2018; Markazi et al., 2018). The IFN-γ primers were designed for the current study: (F) 5' CTTGAGAATCCAGCGCAAAG 3' (R) 5' GTTGAGCACAGGAGGTCATA 3'. Each qRT-PCR plate contained target genes and housekeeping gene, β-actin in triplicate and a no-template negative control (Abdul-Careem et al., 2006). The qRT-PCR data was analyzed by the double delta Ct method (Livak and Schmittgen, 2001). The expression of cytokines were calculated as fold change in mRNA levels as compared to the negative control.

3.2.6. Statistical Analysis

Statistical analyses were conducted via a Student's *t*-test for enumeration, gene expression and chi-square for incidence, using JMP Pro 15 (SAS Institute Inc., Cary, NC, U.S.A.). All the data were presented as mean $\pm$ standard error of the mean (SEM). A *P*-value of

< 0.05 was considered significant when compared to the respective positive control. Each trial was replicated twice at different times. Gene expression data were measured from trials 1 and 3.

3.3. Results and Discussion

Colonization resistance is the inability of potentially pathogenic or foreign bacteria to expand due to host microbiota under homeostatic conditions (Sorbara and Pamer, 2019). In trials 1 and 2, competitive exclusion between SK and ST was measured. *Salmonella* was not detected in any of the negative control cultures in trials 1 and 2. As shown in Table 4, ST was not recovered from the cecal contents in birds when SK was administered 1 day before in trial 1. *Salmonella* Kentucky was significantly reduced when ST was administered prior (Table 3.4). *Salmonella* Typhimurium was significantly reduced when *Salmonella* Kentucky was orally administered 24 h before administered and in combination with SK in trials 1 and 2 (Tables 3.4. and 3.5). *Salmonella* Kentucky was recovered from every bird challenged (Tables 3.4 and 3.5).

Table 3.4. Trial 1 colonization and incidence of cecal contents and organ invasion in macerated liver and spleen (L/S).

Treatment[2]	SK Ceca Log_{10}(cfu/g)	SK Ceca Incidence	SK L/S Incidence	ST Ceca Log_{10}(cfu/g)	ST Ceca Incidence	ST L/S Incidence
NC	0.00	0/20	0/20	0.00	0/20	0/20
SK PC	5.36	20/20	2/20	0.00	0/20	0/20
SK→ST	**4.69**[1]	20/20	1/20	**0.00**[1]	0/20	0/20
ST PC	0.00	0/20	0/20	4.90	20/20	3/20
ST→SK	**4.40**[1]	20/20	1/20	5.35	20/20	0/20
SK+ST	5.58	20/20	**5/20**[1]	**3.74**[1]	20/20	2/20

[1] $P < 0.05$ (*P*-values were calculated in comparison to the respective positive control). [2]n=20 samples/treatment. NC, negative control; PC, positive control; SK, *Salmonella* Kentucky; ST, *Salmonella* Typhimurium; L/S, liver and spleen macerated.

Table 3.5 Trial 2 colonization and incidence of cecal contents and organ invasion in macerated liver and spleen (L/S).

Treatment[2]	SK Ceca Log_{10}(cfu/g)	SK Ceca Incidence	SK L/S Incidence	ST Ceca Log_{10}(cfu/g)	ST Ceca Incidence	ST L/S Incidence
NC	0.00	0/20	0/20	0.00	0/20	0/20
SK PC	5.24	20/20	1/20	0.00	0/20	0/20
SK→ST	5.20	20/20	3/20	**2.23**[1]	20/20	0/20
ST PC	0.00	0/20	0/20	4.79	20/20	1/20
ST→SK	**4.60**[1]	20/20	**9/20**[1]	5.02	20/20	**9/20**[1]
SK+ST	**4.79**[1]	20/20	4/20	**3.75**[1]	20/20	2/20

[1] $p < 0.05$ (*p*-values were calculated in comparison to the respective positive control). [2]n=20 samples/treatment. NC, negative control; PC, positive control; SK, *Salmonella* Kentucky; ST, *Salmonella* Typhimurium; L/S, liver and spleen macerated.

Competitive exclusion of *Salmonella* Enteritidis, another prevalent foodborne strain isolated from humans and poultry, and *Salmonella* Kentucky was also compared in trials 3 and 4. *Salmonella* was not detected in any of the negative control cultures in trials 1 and 2. *Salmonella* Enteritidis was significantly reduced when *Salmonella* Kentucky was administered first and when administered in combination with SK in trials 3 and 4 (Tables 3.6 and 3.7). *Salmonella* Kentucky was significantly reduced when challenged by SE 24 hours later and when SE was administered first in Trial 3 (Table 3.6). *Salmonella* Kentucky and Enteritidis were recovered from all respective cecal enrichments from birds challenged (Tables 3.6 and 3.7).

Table 3.6 Trial 3 colonization and incidence of cecal contents and organ invasion in macerated liver and spleen (L/S).

Treatment[2]	SK Ceca Log_{10}(cfu/g)	SK Ceca Incidence	SK L/S Incidence	SE Ceca Log_{10}(cfu/g)	SE Ceca Incidence	SE L/S Incidence
NC	0.00	0/20	0/20	0.00	0/20	0/20
SK PC	5.94	20/20	**7/20[1]**	0.00	0/20	0/20
SK→SE	**5.07[1]**	20/20	2/20	**2.89[1]**	20/20	0/20
SE PC	0.00	0/20	0/20	4.52	20/20	0/20
SE→SK	**3.90[1]**	20/20	0/20	4.21	20/20	0/20
SK+SE	5.40	20/20	2/20	**3.92[1]**	20/20	2/20

[1]$P < 0.05$ (*P*-values were calculated in comparison to the respective positive control). [2]n=20 samples/treatment. NC, negative control; PC, positive control; SK, *Salmonella* Kentucky; SE, *Salmonella* Enteritidis; L/S, liver and spleen macerated.

Table 3.7 Trial 4 colonization and incidence of cecal contents and organ invasion in macerated liver and spleen (L/S).

Treatment[2]	SK Ceca Log_{10}(cfu/g)	SK Ceca Incidence	SK L/S Incidence	SE Ceca Log_{10}(cfu/g)	SE Ceca Incidence	SE L/S Incidence
NC	0.00	0/20	0/20	0.00	0/20	0/20
SK PC	6.74	20/20	3/20	0.00	0/20	0/20
SK→SE	6.62	20/20	1/20	**2.48[1]**	20/20	0/20
SE PC	0.00	0/20	0/20	5.10	20/20	0/20
SE→SK	**3.97[1]**	20/20	2/20	5.20	20/20	0/20
SK+SE	6.33	20/20	1/20	**3.98[1]**	20/20	0/20

[1] $P < 0.05$ (*P*-values were calculated in comparison to the respective positive control). [2]n=20 samples/treatment. NC, negative control; PC, positive control; SK, *Salmonella* Kentucky; SE, *Salmonella* Enteritidis; L/S, liver and spleen macerated.

Research has shown that strains of *Salmonella* can be used to exclude one another through competitive exclusion along with host defense colonization resistance mechanisms.

When ST was administered to day old chicks, it inhibited other *Salmonella* strains from colonizing the alimentary tract (Barrow et al., 1987b). Yang and colleagues (2018) measured competitive exclusion in chicks using SE and ST and presented similar results. Similar to these findings, gnotobiotic pigs were protected from pathogenic ST when administered avirulent *Salmonella* Infantis 24 h before (Foster et al., 2003). As seen in this study, oral administration of *Salmonella* reduces subsequent colonization of an isogenic isolate in neonatal broilers.

Heterophil populations increase in the chick's cecal lamina propria from D2 to D4 post-infection with SE and ST (Henderson et al., 1999; van Immerseel et al., 2002). An influx of avian host defense peptides includes gallinacins, cathelicidins and liver expressed antimicrobial peptides, which are upregulated during infection, with SE or ST (Akbari et al., 2008; Michailidis, 2010; Cuperus et al., 2013). *Salmonella enterica* are facultative intracellular bacteria, which means they can persist in macrophages, travel through the bloodstream and spread systemically (Henderson et al., 1999; Gast, 2007; He et al., 2012). The incidence of SK in liver/spleen macerations was significantly higher in the SK+ST group compared to other treatments in trial 1 (Table 3.4). A significantly higher incidence of SK and ST in liver/spleen macerations was measured in the ST→SK group compared to other treatments (Table 3.5). In trial 3, there was a higher incidence of SK in the livers/spleens compared to the other treatments (Table 3.6). Newly hatched chicks are more susceptible to systemic translocation of *Salmonella* from the gut barrier due to an underdeveloped immune system, immature microflora, and a relatively sterile gut (Gast and Beard, 1989; Turner et al., 1998; Uni et al., 2000; Geyra et al., 2001).

Interleukin-1β is a key mediator during inflammation and induces the production of chemokines, such as IL-8, to attract specific immune cells (Gibson et al., 2014). In trial 1, the mRNA levels of IL-1β were not different in the cecal tonsils (Fig. 3.1A). The liver mRNA IL-1β

levels were significantly higher in the ST treatment compared to the group given ST followed by SK 24 hours later (Fig. 3.1B). In trial 3, the mRNA levels of IL-1β were significantly higher when SK was administered 24 hours prior to SE than the treatments of SK, SE, SE followed by SK, and SK and SE combination in the cecal tonsils (Fig. 3.2A). The elevated expression could be due to the consecutive challenge. There were no changes in the liver (Figure 3.2B). Withanage and colleagues (2004) measured a significant increase of IL-1β levels in cecal tonsils of day-old chicks 6-48 hours post-infection when challenged with 1.0×10^8 CFU of *Salmonella* Typhimurium. Fasina and colleagues (2007) found a significant upregulation in IL-1β mRNA expression in ceca of five and ten days post challenged 4-day old broilers that were gavaged with 7.8×10^6 cfu/mL of ST. Chranova and colleagues (2011) also measured an increase in IL-1β mRNA levels of day-old layer chick ceca when gavaged with 1.0×10^6 CFU of SE. However, changes in IL-1β mRNA levels were not found in the ceca of week-old chicks challenged with 10^8 CFU of ST in Withanage and colleagues' (2005) earlier studies but was found in the liver 24 hours post infection. Different time point measurements and challenge amounts can affect mRNA expression levels which could be the reason why some of our levels contrast with the literature.

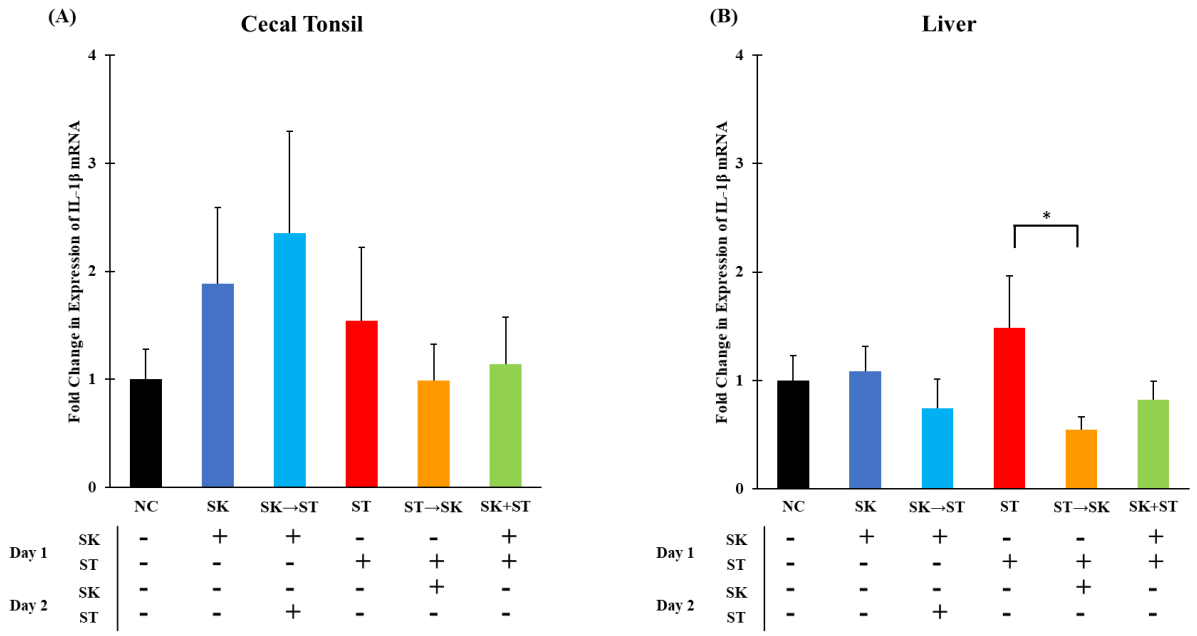

Figure 3.1 The mRNA levels of IL-1β were not different in cecal tonsils but were significantly higher in the ST group compared to the ST→SK treatment in the liver. Relative mRNA expression of IL-1β gene in (A) cecal tonsil and (B) liver was determined by qRT-PCR with normalization to the reference β-Actin mRNA levels. n=5 samples per treatment, except for NC where n=4 samples for cecal tonsils and SK→ST n=4 samples for liver. Asterisk (*) on top of the brackets indicates significant differences at $P < 0.05$.

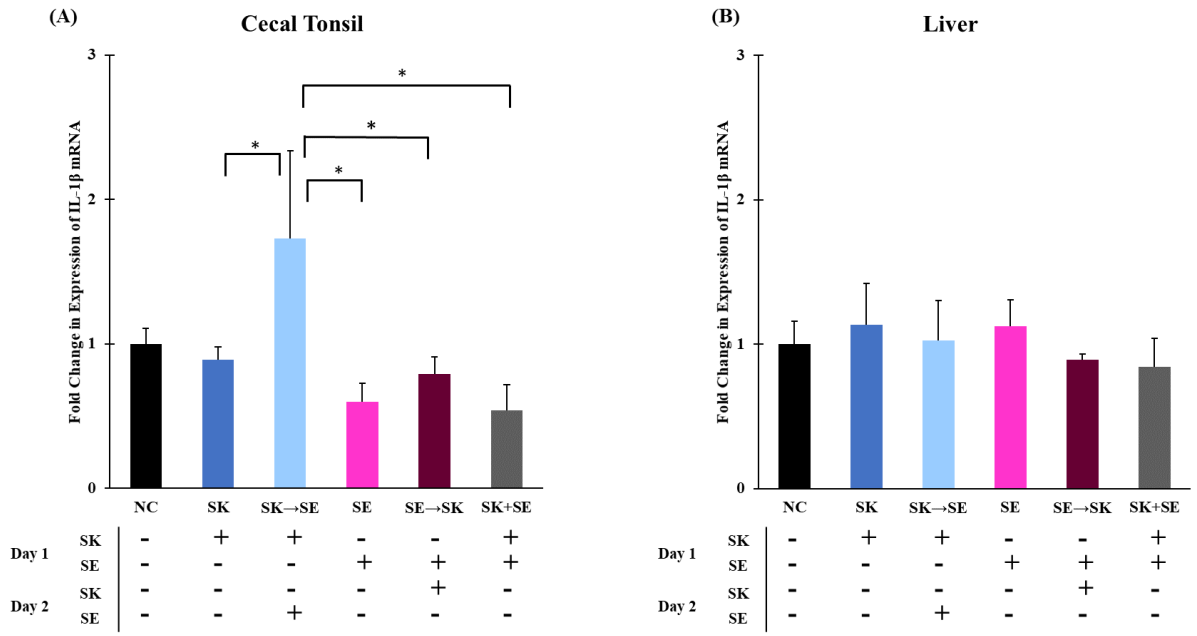

Figure 3.2 The mRNA levels of IL-1β were higher in the SK→SE treatment compared to the SK, SE, SE→SK, and SK+SE treatments in the cecal tonsils, but there were no differences in the liver. Relative mRNA expression of IL-1β gene in (A) cecal tonsil and (B) liver was determined by qRT-PCR with normalization to the reference β-Actin mRNA levels. n=5 samples per treatment, except for SK+SE n=4 samples for cecal tonsil and SK and SK→SE n=4 samples for liver. Asterisk (*) on top of the brackets indicates significant differences at $P < 0.05$.

Another initial pro-inflammatory cytokine, IL-6, induces the synthesis of acute phase proteins, such as mannan binding lectin, from hepatic cells to initiate an innate immune response (Schneider et al., 2001; Nishimichi et al., 2006). The mRNA levels of IL-6 were significantly higher in the ST followed by SK group compared to other treatments in cecal tonsils (Figure 3.3A). An increase in levels could be explained by the consecutive challenge of these two serotypes. There were no significant differences in IL-6 mRNA expression in the liver among the treatment groups (Figure 3.3B). There were no significant changes in IL-6 expression in the cecal tonsils and liver in trial 3 (Figures 3.4A and 3.4B). Changes in IL-6 mRNA levels were not found in the ceca of day-old chicks challenged with 10^8 CFU of ST 6-48 hours post-infection (Withanage et al., 2004). Setta and colleagues (2012) also measured no changes in IL-6 mRNA

levels in the ceca of one day old broilers challenged with 10^9 CFU of SE. In week old chicks challenged with 10^8 cfu of ST, IL-6 was not significantly increased until 21- and 28-days post-infection in the ceca and liver (Withanage et al., 2005). Millet and colleagues (2007) measured an acute phase response to ST lipopolysaccharide in whole blood. We did not observe significantly elevated levels of IL-6 expression in the liver; however, expression does not always correlate to protein function.

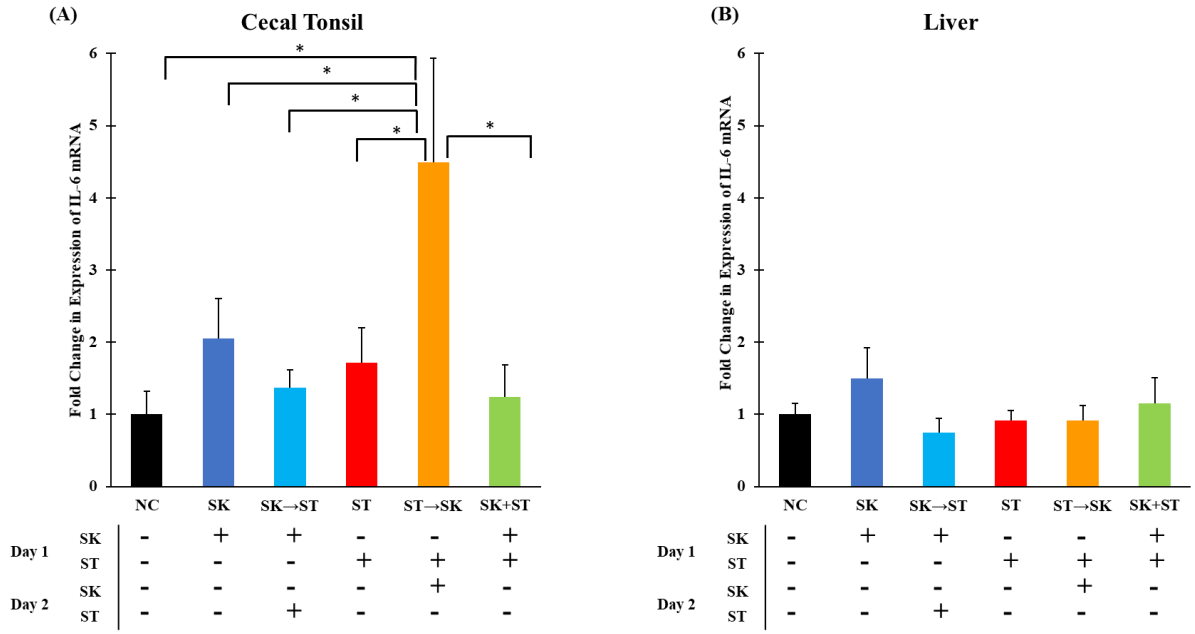

Figure 3.3 The mRNA levels of IL-6 were significantly higher in the ST→SK treatment in the cecal tonsils but were not different in the liver. Relative mRNA expression of IL-6 gene in (A) cecal tonsil and (B) liver was determined by qRT-PCR with normalization to the reference β-Actin mRNA levels. n=5 samples per treatment, except for NC where n=4 samples for cecal tonsils and NC, SK, and ST→SK were n=4 samples for liver. Asterisk (*) on top of the brackets indicates significant differences at $P < 0.05$.

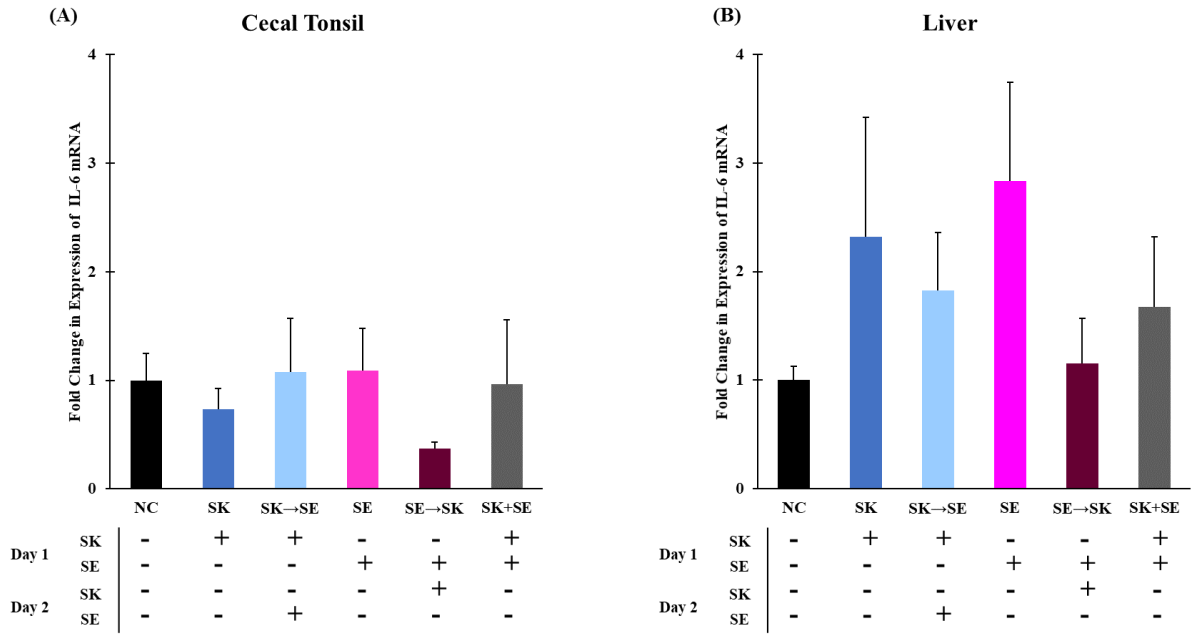

Figure 3.4 The mRNA levels of IL-6 were not different in the cecal tonsils or liver. Relative mRNA expression of IL-6 gene in (A) cecal tonsil and (B) liver was determined by qRT-PCR with normalization to the reference β-Actin mRNA levels. n=5 samples per treatment, except for SE where n=4 samples for cecal tonsils and SK n=4 samples for liver.

Regulatory cytokines, such as IL-10, inhibit the production and secretion of pro-inflammatory cytokines therefore suppressing a T_H1 response (Fiorentino et al., 1991; Rothwell et al., 2004). The mRNA levels of IL-10 were significantly higher in the ST followed by SK treatment than in the SK followed by ST treatment in the cecal tonsils (Fig. 3.5A). The levels of IL-10 were higher in the negative control group than the SK, ST followed by SK, and SK and ST combination treatments in the liver (Fig. 3.5B). The mRNA levels of IL-10 were significantly higher in the SE only treatment than the SE followed by SK treatment in the cecal tonsils (Figure 3.6A). There were no changes in the liver (Fig. 3.6B). The IL-10 levels were downregulated 5 days post ST challenge in the ceca of broiler chicks in Fasina et al., (2007) previously described experiment. Chranova and colleagues (2011) reported mRNA levels for IL-10 were significantly lower in the ceca of the previously mentioned experiment.

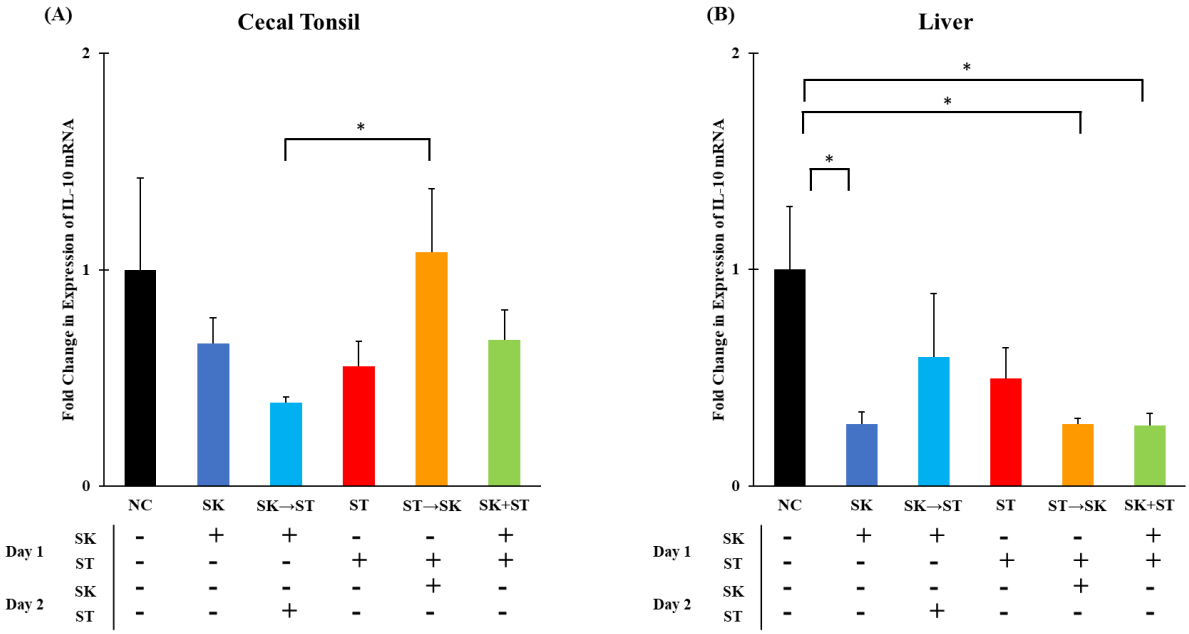

Figure 3.5 The mRNA levels of IL-10 were significantly higher in the ST→SK treatment than in the SK→ST treatment in the cecal tonsils and significantly higher in the NC treatment than the SK, ST→SK, and SK+ST treatments in the liver. Relative mRNA expression of IL-10 gene in (A) cecal tonsil and (B) liver was determined by qRT-PCR with normalization to the reference β-Actin mRNA levels. n=5 samples per treatment, except for NC where n=4 samples for cecal tonsils and livers. Asterisk (*) on top of the brackets indicates significant differences at $P < 0.05$.

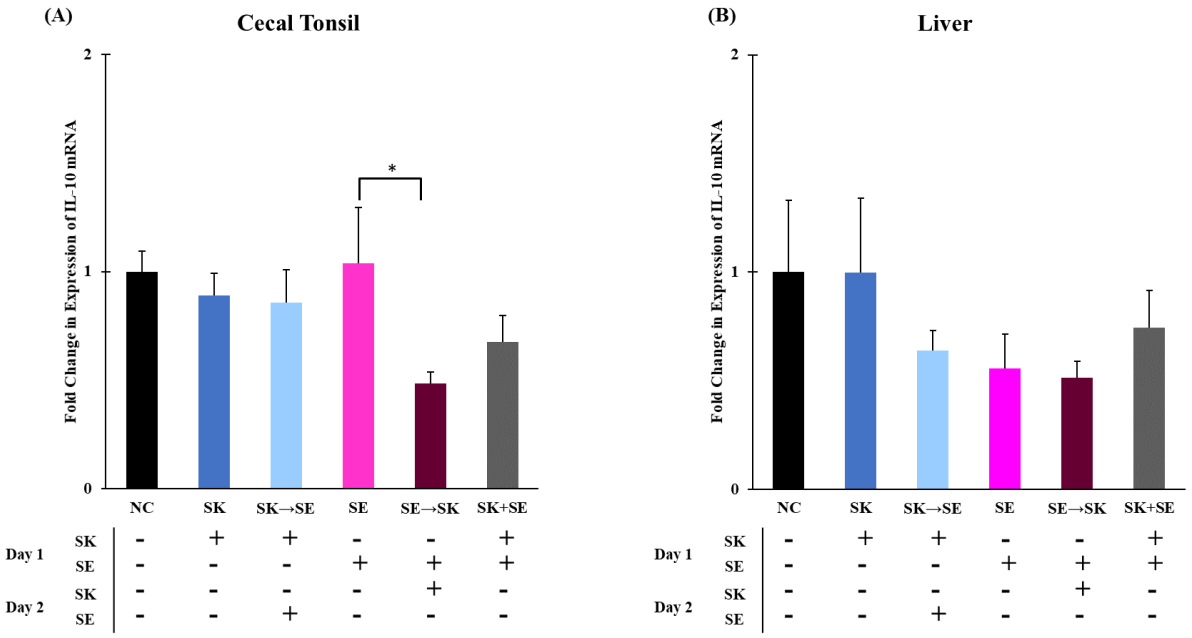

Figure 3.6 The mRNA levels of IL-10 were significantly higher in the SE treatment than the SE→SK treatment in the cecal tonsils, but there were no changes in the liver. Relative mRNA expression of IL-10 gene in (A) cecal tonsil and (B) liver was determined by qRT-PCR with normalization to the reference β-Actin mRNA levels. n=5 samples per treatment except for SK and SE n=4 samples for liver. Asterisk (*) on top of the brackets indicates significant differences at $P < 0.05$.

Chicken IL-18 is produced by macrophages and induces production of IFN-γ which further mediates T_H1 cell development (Göbel et al., 2003). The mRNA levels of IL-18 were significantly higher in the SK followed by ST treatment than in the SK, ST, ST followed by SK, and SK and ST combination treatments in the cecal tonsils (Fig. 3.7A). Levels of IL-18 were significantly higher in the negative control group compared to the ST followed by SK treatment in the liver (Fig. 3.7B). The mRNA levels of IL-18 were significantly higher in the SK followed by SE treatment than the SK only, SE only, SE followed by SK, and SK and SE combination treatments in the cecal tonsils (Fig. 3.8A). There were no changes in the liver (Fig. 3.8B). Berndt and colleagues (2007) measured a significant increase in IL-18 mRNA levels in the ceca of chicks gavaged with 1.0 x 10^7 CFU of SE or ST at a peak 2- and 4 days post-infection. Secretion of IL-18 is important for a later adaptive immune response to produce IFN-γ (Göbel et al., 2003).

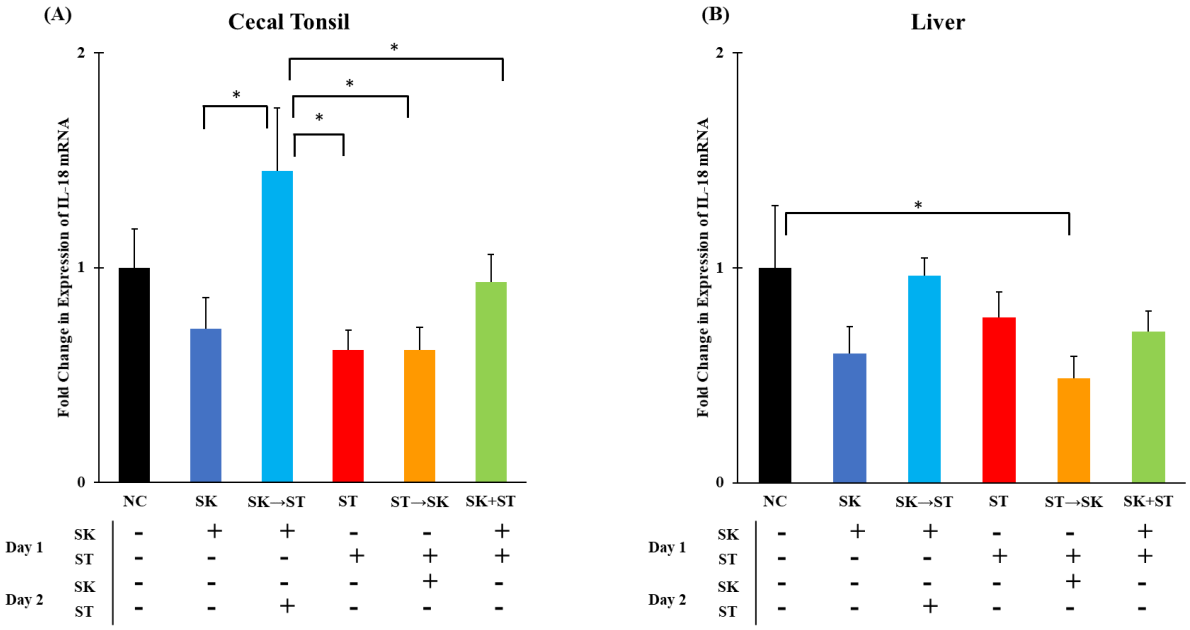

Figure 3.7 The mRNA levels of IL-18 were significantly higher in the SK→ST treatment than in the SK, ST, ST→SK, and SK+ST treatments in the cecal tonsils and were significantly higher in the NC treatment compared to the ST→SK treatment in the liver. Relative mRNA expression of IL-18 gene in (A) cecal tonsil and (B) liver was determined by qRT-PCR with normalization to the reference β-Actin mRNA levels. n=5 samples per treatment, except for NC where n=4 samples for cecal tonsils and SK→ST n=4 samples for liver. Asterisk (*) on top of the brackets indicates significant differences at $P < 0.05$.

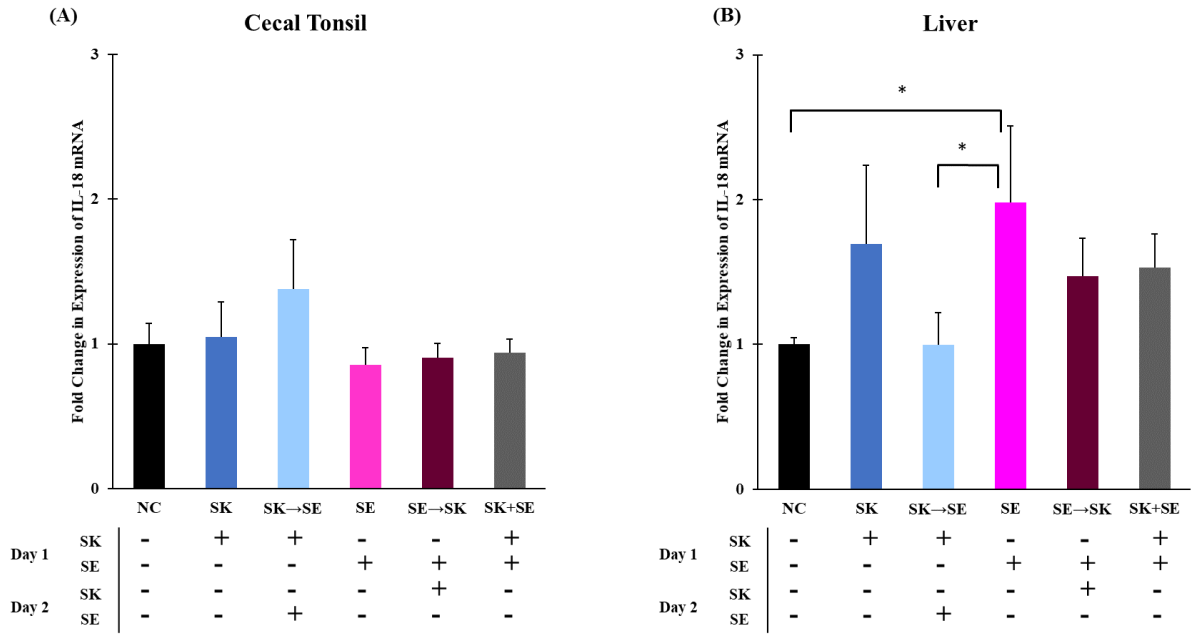

Figure 3.8 The mRNA levels of IL-18 were significantly higher in the SK→SE treatment than the SK, SE, SE→SK, and SK+SE treatments in the cecal tonsils, but there were no changes in the liver. Relative mRNA expression of IL-18 gene in (A) cecal tonsil and (B) liver was determined by qRT-PCR with normalization to the reference β-Actin mRNA levels. n=5 samples per treatment, except for SK→SE and SK+SE where n=4 samples for cecal tonsils and SK n=4 samples for liver. Asterisk (*) on top of the brackets indicates significant differences at $P < 0.05$.

Chicken IFN-γ is primarily produced by T_H1 lymphocytes and natural killer cells and is driven by the production of IL-12 and IL-18 for a later immune response (Schroder et al., 2004; Kogut et al., 2005; Kaiser and Stäheli, 2014). Expression of IFN-γ is critical to the host immune response to intracellular pathogens because it activates macrophages, which increases their ability to kill (Kagaya et al., 1989). There were no significant changes in IFN-γ expression in the cecal tonsils and liver in trial 1 (Fig. 3.9A and 3.9B). The mRNA levels of IFN-γ were not different in the cecal tonsils (Fig. 3.10A). Levels of IFN-γ were significantly higher in the SE→SK group compared to the NC, SK, SK→SE, SE, and SK+SE groups in the liver (Fig. 3.10B). Gamma interferon expression is increased by signals from pro-inflammatory or T_H1 cytokines such as IL-6 and IL-18 in an adaptive immune response (Schroder et al., 2004; Kaiser

and Stäheli, 2014). Berndt and colleagues (2007) measured a significant increase IFN-γ mRNA levels in the ceca up to 4 days post infection.

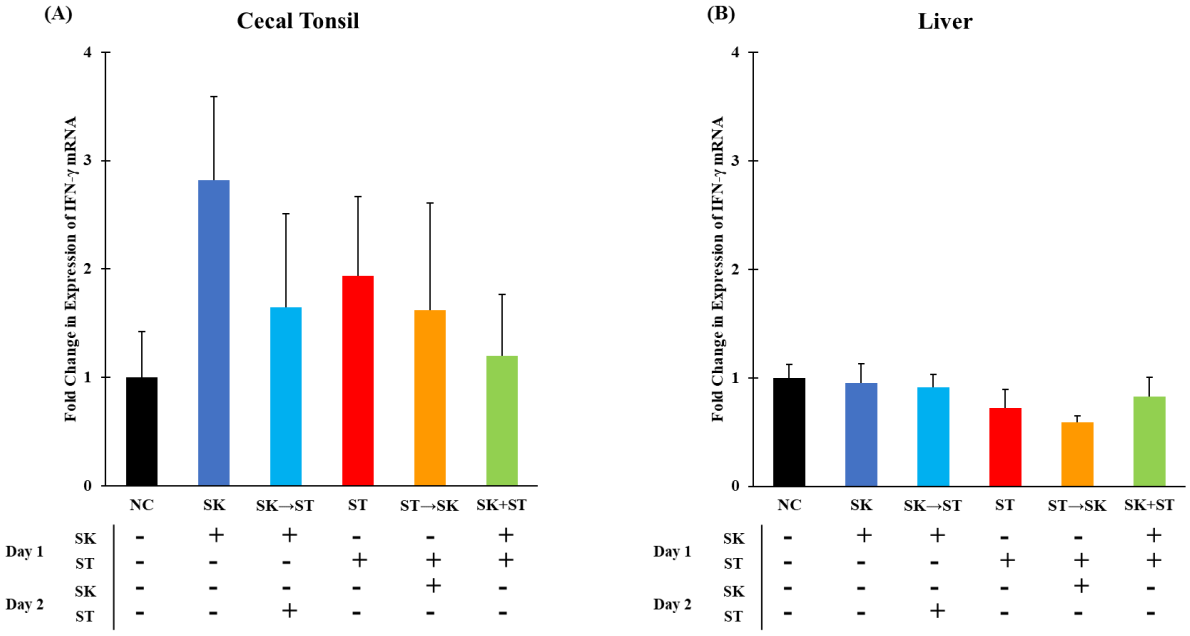

Figure. 3.9 The mRNA levels of IFN-γ were not different in the cecal tonsils or liver. Relative mRNA expression of IFN-γ gene in (A) cecal tonsil and (B) liver was determined by qRT-PCR with normalization to the reference β-Actin mRNA levels. n=5 samples per treatment, except for NC, SK, SK→ST, ST and SK+ST where n=4 samples for cecal tonsils and ST→SK n=4 samples for liver.

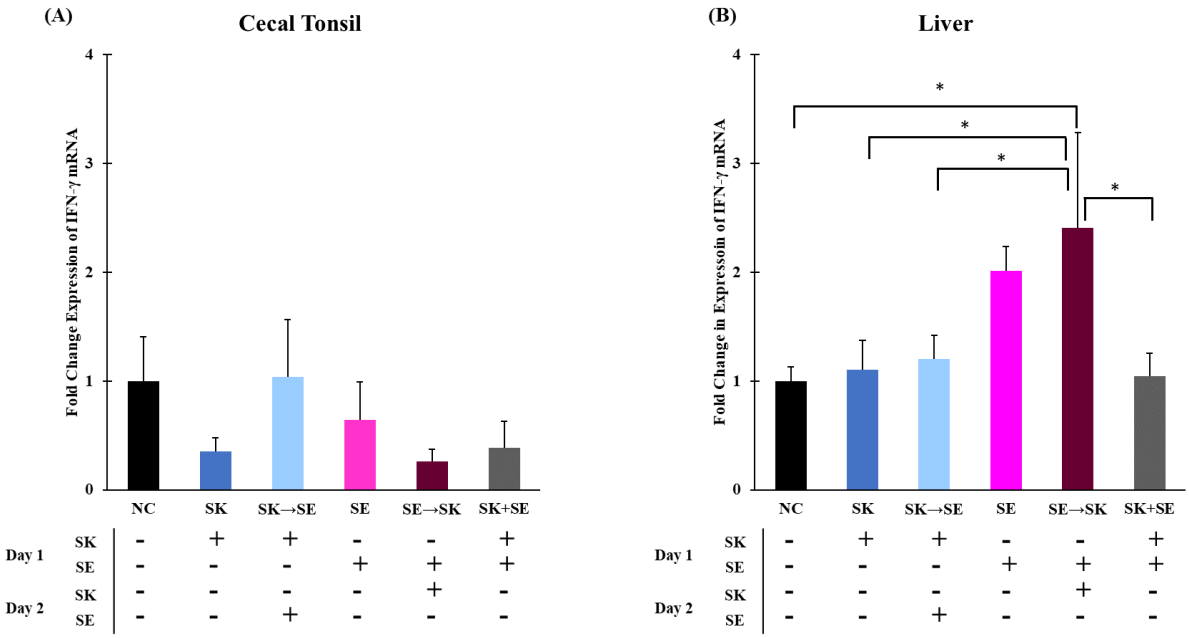

Figure 3.10 The mRNA levels of IFN-γ were not different in the cecal tonsils, but significantly higher in the SE→SK group than the NC, SK, SK→SE, and SK+SE groups in the liver. Relative mRNA expression of IFN-γ gene in (A) cecal tonsil and (B) liver was determined by qRT-PCR with normalization to the reference β-Actin mRNA levels. n=5 samples per treatment, except for NC, SK, SK→ST, ST and SK+ST where n=4 samples for cecal tonsils and ST→SK n=4 samples for liver. Asterisk (*) on top of the brackets indicates significant differences at $P < 0.05$.

Cytokine mRNA expression measured by (qRT)-PCR does not always correlate with protein levels, but it is a sensitive method (Dar et al., 2019). An increase in cytokine expression has been found as early as 12 hours post infection (Kitagawa et al., 2000). Here we measured cytokine mRNA expression 24-48 hours post- infection and the responses could have had an earlier or later expression pattern. Broiler chick ceca are colonized by *Salmonella* quicker than the spleen and liver which could explain the variable results of our mRNA levels (Sorbara and Pamer, 2019). The cecal tonsils are a part of the gut-associated lymphoid tissues and are a local site for immune responses against enteric bacteria (Smith et al., 2014). The liver produces acute phase proteins in response to pro-inflammatory cytokines (Juul-Madsen et al., 2014). There was a lower incidence of *Salmonella* in the liver/spleen macerations, therefore, maybe not all the

livers were affected like the ceca. However, soluble factors from the gastrointestinal tract can affect the liver so that could explain the results we do see (O'Reilly and Eckersall, 2014). Cheeseman and colleagues (2007) measured an increase of IFN-γ in the spleen compared to the ceca in birds challenged with SE, indicating differences in immune gene expression across organs. Withanage and colleagues (2004) found initial cytokine detection was greater in the liver before the spleen indicating a more rapid response. The differences in cytokine gene expression suggest differences in *Salmonella* subspecies' interactions and pathogenesis (Kaiser et al., 2000).

The current research focused on the characterization of an immune response in the cecal tonsils and liver during a concurrent infection. Intestinal cytokine responses are stimulated by foreign or pathogenic bacteria (Buffie and Pamer, 2013) *Salmonella* may not be pathogenic to the avian host, but it can persist and colonize the cecal lumen of chickens, which would allow shedding into the environment (Phalipon and Sansonetti, 1999). As previously mentioned, *Salmonella* Kentucky was isolated from fewer human cases, than ST and SE (CDC, 2016). Yet, SK was isolated from more chicken carcasses than ST and SE combined (FSIS-USDA, 2014). In conclusion, the data presented show that the oral administration of *Salmonella* Kentucky reduced subsequent colonization of Enteritidis and Typhimurium in neonatal broilers. The current study shows expression of cytokines were affected by consecutive challenges indicating immune function could be altered during competitive exclusion. A subunit vaccine exploiting SK's mechanisms to colonize and persist in chickens could benefit public health and agricultural sectors.

3.4. References

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4. AN *IN VITRO* SCREENING OF CHICKEN HD11 MACROPHAGES EXPOSED TO EXTRACELLULAR COMPONENTS SECRETED BY *SALMONELLA* KENTUCKY

4.1. Introduction

Salmonella enterica causes an estimated 1.2 million human foodborne illness cases annually in the United States (CDC, 2019). Approximately 100 out of 2,600 *Salmonella enterica* serotypes are confirmed to cause human infections (CDC, 2020). *Salmonella* colonization depends on strain, host immunity, age, and species (Shivaprasad, 2000). Contaminated poultry is a common source of foodborne human illness (CDC, 2019). The Food and Drug Administration (2011) reported 12% of retail was contaminated with *Salmonella*. The number one recovered poultry isolate was *Salmonella enterica* Kentucky (**SK**); however, it is infrequently associated with human salmonellosis (0.14%; CDC, 2016). Current *Salmonella* prevention includes use of vaccines, enzymes, organic/inorganic acids, synbiotics, probiotics, prebiotics, and phytobiotics (Yadav et al., 2016).

Salmonella's invasiveness depends on the type III secretion system (**T3SS**) which delivers effector proteins into host cells (Kubori et al., 1998; Galán and Wolf-Watz, 2006). The T3SS is encoded on *Salmonella* pathogenicity islands 1 and 2 (**SPI-1 and SPI-2**) which are expressed by virulence genes (Marcus et al., 2000; Gal-Mor et al., 2006). The SPI-1 is responsible for initial bacterial penetration of intestinal epithelial cells, causing membrane ruffling and suppression of pro-inflammatory cytokine expression in macrophages (Finlay et al., 1991; Pavlova et al., 2011). Effector proteins produced by SPI-2 delay phagolysosomal maturation increasing *Salmonella*'s survivability in macrophages (Haraga et al., 2008). *Salmonella* can then persist in the gastrointestinal tract of chickens and spread systemically without long-term signs of clinical disease (Tohidi, 2014).

Macrophages have multiple anti-microbial functions, which include nitric oxide (**NO**) production and secretion of pro-inflammatory cytokines, such as IL-1β and IL-6. The inflammatory response is mediated by IL-1β (Gibson et al., 2014). The acute phase response is further induced by IL-6 that increases acute phase protein synthesis and promotes development of adaptive immunity (Kaiser and Stäheli, 2014). Macrophage inflammatory protein 1 (**MIP-1**) is a chemokine that attracts other immune cells to the site of inflammation (Kim et al., 2017). A cell-mediated TH1 response against intracellular pathogens is induced by interferon gamma (**IFN-γ**) (Kaiser and Stäheli, 2014). Macrophages also produce IL-12 which stimulate immune cells, such as natural killer cells, to secrete IFN-γ, activating macrophages to produce inducible nitric oxide synthase (**iNOS**; Chan et al., 1991). The enzyme iNOS leads to microbicidal NO production when stimulated with intracellular pathogens and IFN-γ (Ding et al., 1988). In response to pathogens, NO generates reactive compounds during oxidation to nitrite, which bind to bacterial DNA, proteins and lipids inhibiting and killing the pathogen (Lancaster, 1992; Schairer et al., 2012).

Vaccine research continues to grow in the animal health industry. One vaccine class includes subunit vaccines, which are composed of antigens, usually proteins, and typically require adjuvants to increase immunogenicity (Desin et al., 2013). Research has shown that *Salmonella* secretes SPI-2 virulence proteins when inoculated in minimal acidic media at a pH of 5.0-5.8 (Deiwick et al., Beuzon et al., 1999; Nikolaus et al., 2001; Coombes et al. 2004). Ferreira and colleagues (2015) developed a subunit vaccine from purified *Salmonella enterica* Typhimurium supernatant grown under SPI-2 inducing conditions. The supernatant was highly abundant in effector proteins and offered protection against subsequent infection in mice when combined with an adjuvant (Ferreira et al., 2015).

The chicken macrophage cell line, HD11, is used to evaluate bactericidal activity and innate immunity *in vitro* (Babu et al., 2006). Due to SPI-2 virulence, we believe the SK secretome would be an ideal vaccine candidate. We stimulated HD11 macrophages with SK-derived secretome to measure nitric oxide production and gene expression of cytokines.

4.2. Materials and Methods

4.2.1. Supernatant Preparation and Purification of *Salmonella* Kentucky

Serotyped poultry isolate *Salmonella* Kentucky (SK) was obtained from USDA-ARS (College Station, TX, USA) and was stored at –80°C. Supernatant was harvested from SK during the log phase of growth under conditions that selectively induce SPI-2 secretion as previously described by Coombes et al. (2004). Briefly, SK was passaged three times every 8 h in sterile Luria-Bertani broth (**LB**; HiMedia, Mumbai, India) supplemented with 25 µg/mL novobiocin (**NOV**; Alfa Aesar, Haverhill, MA, USA) and 20 µg/mL nalidixic acid (**NA**; MP Biomedicals, LLC, Illkirch, France) to control for extraneous bacteria. The culture was pelleted by centrifugation at 600 x *g* for 15 min at 4°C. The cell pellet was washed three times with low phosphate-low magnesium (**LPM**) medium [7.5 mM ammonium sulfate (VWR Chemicals, LLC, Solon, Ohio, USA), 5 mM potassium chloride (Acros Organics, Fair Lawn, NJ, USA), 0.5 mM potassium sulfate (VWR Chemicals, LLC), 337 µM phosphoric acid (Sigma Aldrich, St. Louis, MO, USA), 0.3% (vol/vol) glycerol (Fisher Scientific, Ottawa, ON, CAN), 0.1% casamino acids (MP Biomedicals, LLC), and 80 mM 2-morpholinoethanesulfonic acid monohydrate (MES; VWR Chemicals, LLC)]. The pH of LPM was adjusted to a pH of 5.8 with sodium hydroxide (Fisher Scientific), as described by Coombes et al. (2004) and Niemann et al. (2011). Cells were cultured in LPM for 12-16 h at 37°C.

Salmonella Kentucky supernatant was collected by centrifugation at 600 x *g* for 20 min at

4°C. The SK supernatant was further purified by centrifugation at 3,000 x *g* for 20 min at 4°C. The SKS was passed through a 0.22-µm pore filter and stored at –80°C for future experiments.

4.2.2. Purification of SK Secretome (SKS) from supernatant

Confirmation of the absence of SK in supernatant. The purified supernatant was grown in LB for 24 h at 37°C and then inoculated into Rappaport-Vassiliadis broth (RV; Hardy Diagnositics, Santa Maria, CA, USA) at 37°C overnight. The enrichment was cultured onto xylose lysine tergitol-4 (XLT-4; Hardy Diagnostics) agar with added supplement (BD Difco, Sparks, MD, USA) and incubated for 18-24 h at 37°C to check for sterility. No growth of SK was observed in the SK supernatant.

Purification of SKS. Cell-free SK supernatant was desalted by a Zeba™ Spin Desalting column, 7KDa molecular weight cut off (MWCO; Thermo Fisher Scientific) following the manufacturer's protocol. After desalting, SK supernatant was concentrated using a 10 kDa MWCO protein spin concentrator (Thermo Fisher Scientific) at 3,000 x *g* for 20 min at 4°C. The same volume of LPM medium alone was desalted and concentrated as a medium control. Protein concentration was measured by the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

SDS-PAGE. To evaluate protein presence, SK-derived secretome (**SKS**) was precipitated in 10% (vol/vol) trichloroacetic acid (VWR Chemicals, LLC) at 4°C for 12-16 h. The precipitate was collected by centrifugation and washed twice in ice-cold acetone (VWR Chemicals, LLC) at 3,000 x *g* for 20 min. The pellet was air-dried in a HEPA filtered biosafety cabinet for 30 min and resuspended in PBS. The SKS was suspended in 4X Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) and heated at 100°C for 10 min to denature proteins. The protein was loaded into a Novex™ WedgeWell™ 4-20% Tris-Glycine precast gel (Invitrogen, Carlsbad, CA,

USA) and separated at 100 V for 30 min in Bolt™ MOPS SDS Running Buffer (Invitrogen) in a mini gel tank (Invitrogen) powered by a PowerEase® 300W electrophoresis system (Thermo Fisher Scientific). A 250 kDa molecular weight protein standard (Bio-Rad) was used in the gel to aid in the identification of protein sizes and concentrations. Gel was stained in QC Colloidal Coomassie Stain (Bio-Rad) following the manufacturer's protocol with a detection limit of 3 ng per band.

4.2.3. Heat-killed *Salmonella* Preparation

Salmonella Kentucky was passed three times every 8 h in sterile LB supplemented with NA and NOV. The culture was harvested by centrifugation at 600 x *g* for 15 min at 4°C. The cell pellet was resuspended in sterile, cold PBS and washed twice prior to challenge. The culture's optical density was measured spectrophotometrically at 625 nm at an absorbance value of 1.30 (SPECTRONIC® 20+ SERIES Spectrophotometers, Thermo Fisher Scientific) and estimated at 1.0×10^9 cfu/mL relative to an established standard curve. The concentration was confirmed to be 1.71×10^9 cfu/mL of SK by serial dilution on XLT-4 agar supplemented with NA and NOV. Heat-killed *Salmonella* Kentucky (HKS) was prepared by incubating the suspension in a 75°C water bath for 15 min. The bacterial suspension was confirmed dead by overnight enrichment in RV.

4.2.4. Chicken Macrophage Cell Line HD11

Culture conditions. The MC29 virus-transformed chicken macrophage cell line (HD11; Beug et al., 1979) was provided by Dr. Luc Berghman's research laboratory (Department of Poultry Science, Texas A&M University). Cells were maintained in complete Dulbecco's Modified Eagle Medium (**DMEM**; Gibco, Waltham, MA, USA) containing 5% chicken serum (Gibco), 8% fetal bovine serum (R&D Systems, Minneapolis, MN, USA), 1% antibiotic-

antimycotic (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of amphotericin B; Gibco), 1% Glutamax (Gibco), 1% sodium pyruvate (Gibco) in a 5% CO_2 incubator at 35°C, following routine cell culture procedures.

Treatment. For experiments, HD11 cells were harvested from 75-cm^2 cell culture flasks (Sigma-Aldrich, Saint Louis, MO, USA) when they reached ~85% confluence using trypsin/EDTA (Gibco). Cells were diluted in trypan blue (VWR Chemicals, LLC) and counted using a hemocytometer. Subsequently, the counted cells were seeded at 2.0 x 10^5 cells/mL per well into 96-well (VWR International, Radnor, PA, USA) or 24-well plates (Corning Inc., Corning, NY, USA) for Griess assay or mRNA extractions, respectively, for 16-18 h until ~85% confluence. After incubation, HD11 cells were washed with PBS. The media was replaced with treatments to a final volume of 200 µL/well or 500 µL/well for the Griess assay or to measure relative gene expression, respectively.

Treatments were unstimulated cells (**UC**), heat-killed *Salmonella* Kentucky (**HKS**) as the positive control (**PC**), LPM-medium only (**NC**), and SKS. The HD11 cells were stimulated with 350 µg/well, 700 µg/well, 1,050 µg/well, 1,400 µg/well, or 1,750 µg/well of SKS to determine the optimal concentration of protein quantified by NO production in 96-well plates. Cells were stimulated for 24 h for the Griess assay or 2 h for quantifying gene expression (He et al., 2009). The SKS protein concentration was increased to 4,200 µg/well for the gene expression experiments in 24-well plates to account for the increased volume.

4.2.5. Nitrite Assay

Nitrite oxide is produced by stimulated macrophages and was measured by the Griess assay (Green et al., 1982). After a 24 h incubation, nitrite concentrations in the supernatants were determined as previously described by He et al. (2009). Values presented are the averages of four

separate experiments on different days with triplicate samples per treatment.

4.2.6. Total RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR) were previously described (Zhao et al., 2020). Quantification of IL-1β, IL-6, IFN-γ, and macrophage inflammatory protein (**MIP**)-1 were determined by qRT-PCR using the Applied Biosystems PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific). Primer sequences have been previously reported for all genes (Table 4.1). Each qRT-PCR plate contained a no template negative control, target genes, and housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) in triplicate (Abdul-Careem et al., 2006). The qRT-PCR data were analyzed by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The expression of cytokines was calculated as fold change in mRNA levels as compared to the unstimulated control group. Values presented are the averages of three separate experiments on different days with triplicate samples per treatment.

Table 4.1 Primer pairs used in this study.

Target Gene	Primers	Primer Sequence (5' to 3')	Amplicons (bp)	Reference
IL-1β	Forward	TGGGCATCAAGGGCTACA	244	Hong et al., 2006
	Reverse	TCGGGTTGGTTGGTGATG		
IL-6	Forward	TGTTCGCCTTTCAGACCTACC	123	Khampeerathuch et al., 2018
	Reverse	TTATCACCATCTGCCGGATCG		
IFN-γ	Forward	CTTGAGAATCCAGCGCAAAG	143	Pineda et al., 2021
	Reverse	GTTGAGCACAGGAGGTCATA		
MIP-1	Forward	CCTGCTGCTTGTCCTACG	81	Lin et al., 2020
	Reverse	GGCGGCATTTGCTGCTGG		
GAPDH	Forward	AGAACATCATCCCAGCGT	182	Guo et al., 2016
	Reverse	AGCCTTCACTACCCTCTTG		

4.2.7. Statistical Analysis

Statistical analyses were conducted via a Student's *t*-test using JMP Pro 15 (SAS Institute Inc., Cary, NC, USA). All the data were presented as mean ± standard error of the mean (SEM). A *P*-value of < 0.05 was considered significant when compared to the treatments.

4.3. Results

4.3.1. Characterization of protein presence in SKS

Salmonella Kentucky-derived secretome was characterized by SDS-PAGE to ensure protein presence (Fig. 4.1.). The LPM (NC) casamino acids was incompatible with BCA, so SKS was purified by desalting and centrifugal concentration. The final protein concentration was 35 µg/µL per the BCA kit results. We observed that the SK-derived secretome contained different

sizes of proteins ranging from 20 to 150 KDa with the broadest band being approximately 55 kDa. The NC was confirmed to have no protein in the growth medium (Fig. 4.1.).

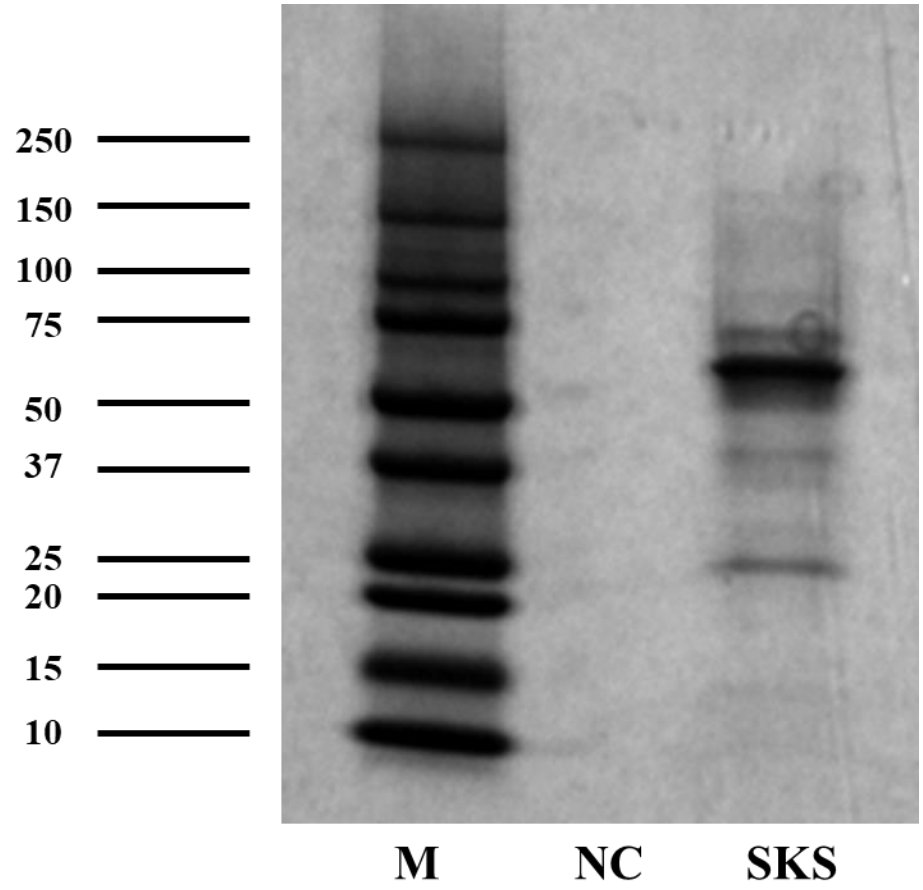

Figure 4.1 Confirmation of protein presence in SKS by SDS-PAGE. Log-phase growth of SKS was verified by direct plating. Equal amounts of filtered supernatant derived from 200 mL of SK or 200 mL of LPM were precipitated in 10% trichloroacetic acid at 4°C for 12-16 h. The precipitation of proteins from LPM only (NC) and SKS were analyzed by 4-20% SDS-PAGE, followed by Coomassie blue stain. M = molecular weight marker in kDa.

4.3.2. Dose Response and Nitric Oxide Production

Protein concentrations were compared to determine the most effective dose. Responses were compared based on NO production in stimulated HD11 cells (Fig. 4.2.A). The concentrations of SKS for each well in a 96-well plate were 350 µg, 700 µg, 1,050 µg, 1,400 µg, or 1,750 µg. Cells treated with 700 µg of SKS had significantly ($P < 0.0001$) increased NO production compared to the UC and the NC (Fig. 4.2.B). Nitric oxide was increased by 8,458-

fold in cells stimulated by SKS which was comparable to the PC (8,694). The NC (4,771-fold) stimulated NO expression but was significantly ($P < 0.05$) different from the PC and the SKS.

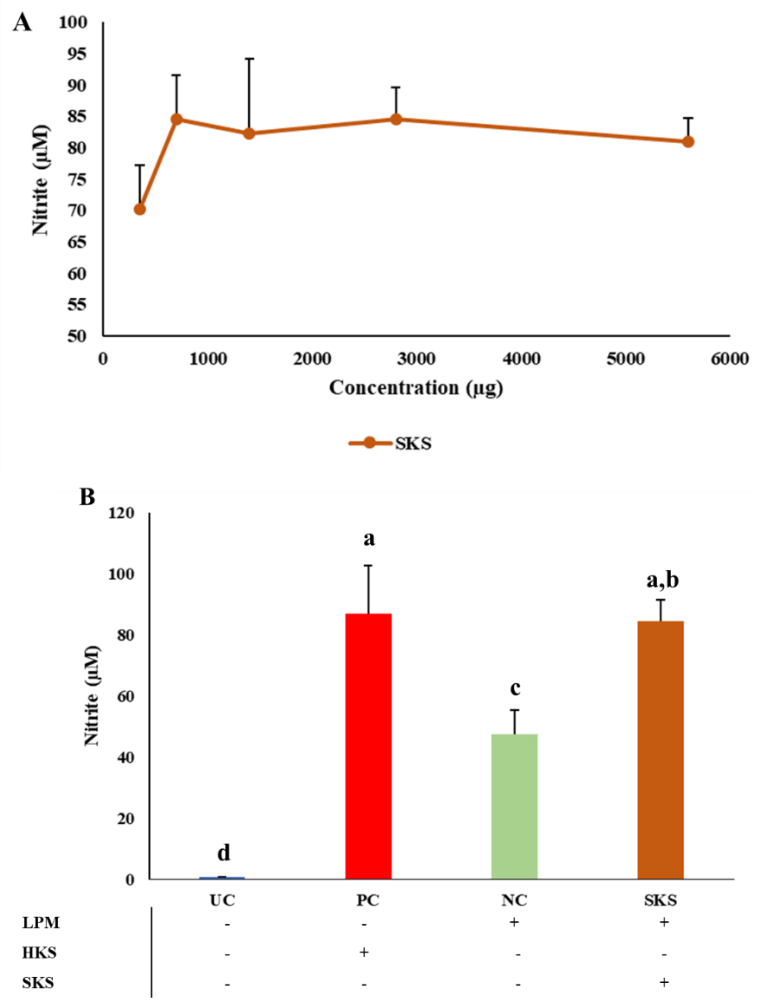

Fig. 4.2 Nitrite production in stimulated HD11 cells. The HD11 cells were incubated with treatments for 24 h at 39°C in a 5% CO_2 humidified incubator. (**A**) SKS dose response per well in a 96-well plate (**B**) Treatments were unstimulated cells (UC), heat-killed *Salmonella* Kentucky (HKS) as the positive control (PC), LPM growth medium as the negative control (NC), and 700 µg/well of *Salmonella* Kentucky-derived secretome in a 96-well plate (SKS). $n = 4$ samples per treatment, with triplicate wells for each experiment. This experiment was replicated four different times. Different letters indicate that the difference between groups is statistically significant ($P < 0.05$).

4.3.3. Relative mRNA Expression

Based on the optimal NO results with the lowest protein volume required, we calculated

the concentration of SKS used for the gene expression experiments to correlate to a 24-well plate, which was 4,200 µg/well. As shown in Fig. 4.3.A, relative IL-1β mRNA levels increased 223-fold ($P = 0.0009$) in the PC treatment compared to the levels in the UC treatment. Compared to UC group, the mRNA levels of IL-1β in NC was 165-fold higher ($P = 0.0107$). The SKS treatment exhibited 171-fold higher IL-1β mRNA expression ($P = 0.0084$) compared to the UC treatment. However, the IL-1β mRNA levels in SKS group showed no significant change compared to the NC group.

Relative IL-6 mRNA levels did not significantly increase in the PC or NC treatments compared to the levels in the UC treatment (Fig. 4.3.B). The SKS treatment elevated IL-6 expression by 2,237-fold ($P = 0.0002$) compared to the UC. The mRNA levels in SKS group were significantly higher ($P = 0.0036$) than the NC group. The SKS treatment exhibited higher ($P = 0.0027$) IFN-γ mRNA expression as compared to the PC.

As shown in Fig. 4.3.C, relative IFN-γ levels did not significantly increase in the PC or NC treatments compared to the UC. Levels of IFN-γ expression significantly increased ($P < 0.001$) 354-fold in the SKS treatment compared to the UC. Expression of INF-γ was significantly increased ($P = 0.0037$) by the SKS treatment compared to PC. The SKS group significantly increased IFN-γ expression compared to NC ($P = 0.0057$).

In addition to cytokines, the mRNA expression of chemokine MIP-1 was measured (Fig. 4.3.D). However, there were no significant changes in the expression of MIP-1 in any of the treatments.

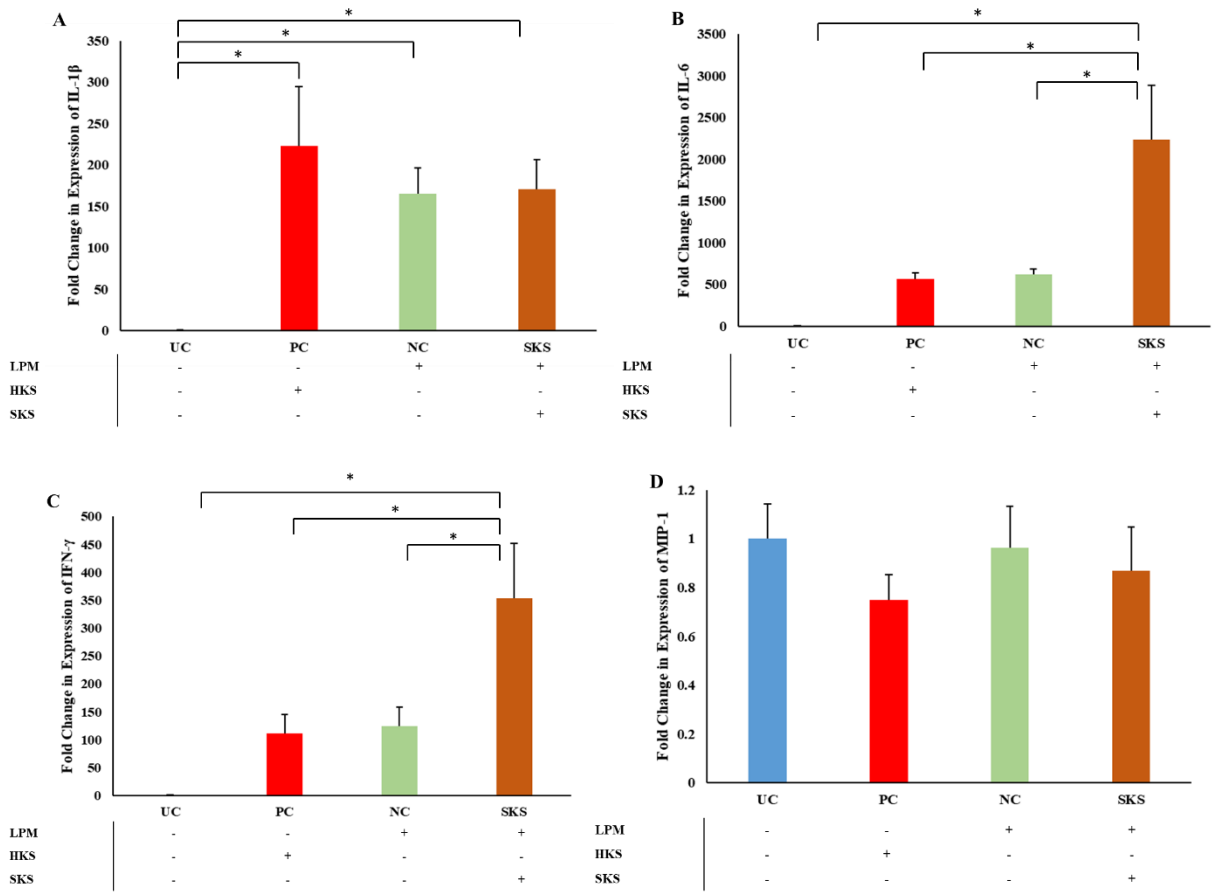

Fig. 4.3 The mRNA levels of HD11 cells. The HD11 cells were incubated with treatments for 24 h at 39°C in a 5% CO_2 humidified incubator. Relative mRNA expression of (**A**) IL-1β gene, (**B**) IL-6, (**C**) IFN-γ, and (**D**) MIP-1 in HD11 cells was determined by quantitative real-time PCR with normalization to the reference GAPDH mRNA levels. $n = 3$ samples per treatment, with triplicate wells for each experiment. Asterisk (*) on the top of the brackets indicates significant differences at $P < 0.05$.

4.4. Discussion

Salmonella secretes multiple effector proteins and outer membrane vesicles into host cells (Niemann et al., 2010). The cell free supernatant is composed of virulence proteins, LPS, bacterial chaperones, and outer membrane vesicles (Niemann et al., 2010; Ferreira et al., 2015). Our goal was to purify the cell-free supernatant of SK and perform an *in vitro* screening for immunogenicity in HD11 macrophages. Deiwick and colleagues (2006) identified proteins in ST with molecular masses between approximately 30-35 kDA. Similarly, Bai and colleagues (2014)

identified supernatant proteins from 26-130 kDA. Both cultured ST in minimal medium conditions and were similar to our SDS-PAGE result with bands ranging from 20-150 kDA.

Macrophages are the first line of defense against intracellular pathogens, such as *Salmonella*. Van den Biggelarr and colleagues (2020) compared inactivated poultry vaccines and found none of them resulted in significant amounts of NO in HD11 cells without an adjuvant. We observed significant production of NO without an adjuvant indicating an elevated immune response. Macrophage production of NO influences expression of IL-1β, IL-6, IFN-γ and chemokines such as MIP-1 (Tripathi, 2007). However, NO alone can also kill and reduce pathogens and is an important immune response.

Cytokines are essential for the immune response to transition from innate to adaptive. The SPI-2 can alter cytokine and chemokine production in HD11 macrophages. Interleukin-1β activates immune cells such as macrophages and T-cells which leads to production of cytokines and chemokines (Weining et al., 1998). Iqbal and colleagues (2005) found the expression of IL-1β was increased in chicken kidney cells and embryonic fibroblast cells when stimulated for 6 h with *Salmonella* Typhimurium. As a key mediator of the innate system, elevated expression of IL-1β is an indication of an inflammatory response. Interleukin-6 activates the adaptive immune response by stimulating T and B cells (Lee et al., 2010). Natural killer and T cells produce IFN-γ along with macrophages (Giansanti et al., 2006). Chicken IFN-γ is a TH1 cytokine and can activate macrophages which increases their ability to kill (Kagaya et al., 1989). Chemokines, such as MIP-1, are produced by macrophages and are important immune regulators. Surprisingly, we did not see changes in MIP-1 expression; however, MIP-1 may have a slower transcription due to requiring an increased cytokine response to activate more cells locally.

The low-phosphate, low magnesium growth medium (NC) may have induced an immune

response due to casamino acids being derived from casein which can stimulate immune cells. The LPM also caused a reaction with BCA before we removed the casamino acids and salts. Cavallo and colleagues (1996) measured increased T cell production in peripheral lymphocytes stimulated with casein, which is a bovine milk-derived protein. The peptide from casein upregulated IL-6 protein in bone marrow-derived macrophages of mice (Sandré et al., 2001). In addition, casein regulated other cytokine production (IL-8, IL-10, and tumor necrotic factor-alpha) in human primary peripheral blood mononuclear cells (Kiewiet et al., 2017). Upregulated nitrite production was also documented in rodent peritoneal macrophages treated with casein (Hrabak et al., 2006). While no proteins were detected in LPM, trace amounts of casein from the casamino acids could have caused the immune responses.

Innate immunity is multi-faceted and relies on several cell types and functions. Cytokines and chemokines are host defenses against antigens, so the change of expression levels during an immune response can indicate vaccine efficacy. In our study, SKS was able to increase production of NO and upregulate the gene expression of IL-1β, IL-6, and IFN-γ which could act on other cells and macrophages to increase an immune response. Future studies could analyze phagocytosis and oxidative burst to characterize an immune response in HD11 macrophages stimulated with SKS.

4.5. References

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5. CONCLUSIONS

Current *Salmonella* intervention strategies are not entirely effective. Due to the rise in antimicrobial resistance, demand for antibiotic alternatives has increased. The objectives of my dissertation were 1) to reduce SH biofilm and SH colonization in broiler crops and ceca using SBS, 2) competitively exclude SE and ST in neonatal chicks with SK and measure gene expression of cytokines, and 3) elicit nitric oxide production and increase gene expression in chicken HD11 cells with a SK-derived secretome.

Salmonella Heidelberg (SH) on contaminated poultry causes economic and health risks to producers and consumers. Clean drinking water is important for maintaining bird health. Biofilms increase microbial survival leading to contamination of poultry products. We hypothesized that sodium bisulfate (SBS) would decrease SH biofilm on polyvinyl chloride (PVC) coupons and decrease horizontal transfer of SH in broilers. Reducing SH biofilm on PVC with SBS could be an efficient way to clean poultry drinkers. We saw no changes in feed, water consumption, mortality, or pen weights in birds; therefore, administering SBS in drinkers could be a safe SH intervention strategy by reducing biofilm or SH colonization.

Host microbiota competitively excludes pathogenic bacteria by limiting space, available nutrients. Immune responses can indirectly mediate colonization resistance by inhibiting pathogens. The changes in cytokine gene expression could indicate differences in *Salmonella* serovars interaction and pathogenesis; however, future experiments should measure protein concentrations. Intragenus *Salmonella* serovars were able to significantly reduce colonization of the subsequent serotype in broiler ceca in all four trials. *Salmonella* Kentucky significantly excluded ST and SE, therefore, we measured SK-derived secretome's effects on HD11 macrophages.

Salmonella enterica Kentucky (SK) is the primary *Salmonella* isolate found in poultry; however, SK is not commonly found in human salmonellosis cases. Bacteria-derived secretomes are composed of proteins that can be immunogenic. We characterized the immunogenicity of a SK-derived secretome in chicken HD11 macrophages. The macrophages significantly produced NO and increased gene expression of cytokines in response to SKS. To our knowledge, this is the first experiment to analyze the SK-derived secretome's immunogenic effects on chicken HD11 macrophages. Future experiments should look at phagocytosis and oxidative burst to further characterize activated macrophages.

Reducing foodborne salmonellosis would benefit the public health and agriculture. These projects demonstrate how current *Salmonella* intervention strategies could be improved utilizing a water acidifier or competitive exclusion. The *in vitro* screening of SKS showed immunogenicity which could have future application as a subunit vaccine.