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Experimental Approaches to Understand and Control Salmonella Infection in Poultry

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Experimental Approaches to Understand and Control *Salmonella* Infection in Poultry

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ABSTRACT

Salmonella is a major foodborne pathogen around the world and chickens are the major reservoir to transmit *Salmonella* into the human food chain. For decreasing the infection of *Salmonella*, we developed six attenuated live vaccines based on *Salmonella* Enteritidis (SE) and Typhimurium (ST) for testing the cross-serovar and cross-serogroup protection from the challenge of *Salmonella* Heidelberg and *Campylobacter jejuni*. One of the constructed vaccine strain showed ability to protect against challenge from *Salmonella* Heidelberg. Even though some preventive approaches are able to decrease *Salmonella* colonization in the gastrointestinal tract of chickens or other farm animals, *Salmonella* transmission mechanisms remain unclear. For analyzing *Salmonella* transmission routes and dynamics, we designed a series of *Salmonella* isogenic barcode-tagged strains by inserting six random nucleotides into a functionally neutral region on the chromosome of *Salmonella* Enteritidis. These barcode-tagged strains can be used as a tool for quantitative tracking of *Salmonella* transmission in the chicken flock by profiling the barcode regions using high-throughput sequencing. The efficiency of this novel approach has been estimated by chicken experiments and can be applied for further studies about *Salmonella* transmission and population dynamics. Due to the increasing pressure of using antibiotics as the growth promoter in the farm animals, probiotics is a suitable alternative to replace antibiotics by providing beneficial effects, such as promotion of animal health, decreased infection by pathogens, and improval of growth performance in poultry. In this study, 90 probiotic candidate strains were isolated and evaluated for decreasing the gut permeability in Caco-2 cell lines. The result from animal experiments indicated that a combined batch culture of 3 selected strains showed significant efficacy in controlling intestinal colonization of ST in neonatal turkey poults.

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CHAPTER ONE

1.1 Introduction

1.1.1 Development of *Salmonella*-based Vaccines

For controlling the infection of *Salmonella*, many kinds of vaccines have been applied in farm animals and humans. Killed whole-cell *Salmonella* vaccines have been studied and applied over the last century (Collins, 1974; Eisenstein *et al.*, 1984b). However, whole-cell killed *Salmonella* vaccines confer partial protection against intestinal colonization, fecal shedding, and egg contamination in chickens (Mastroeni *et al.*, 2001). Even though the protection of whole-cell killed vaccines in chickens was greatly improved by adding strong adjuvants, such as oil emulsions, it still provides less protection than live vaccines (Gast *et al.*, 1992; Barbour *et al.*, 1993). This is probably due to the fact that killed vaccines are able to induce humoral responses, but have a poor ability to induce cell-mediated immunity in the vaccinated animals (Collins, 1974; Bairey, 1978; Aitken *et al.*, 1982). Besides killed vaccines, *Salmonella* bacterial fractions such as detoxified lipopolysaccharide (LPS), outer membrane proteins (OMPs), and O-polysaccharides also have been shown to elicit high antibody responses and induce moderate protection (Ding *et al.*, 1990; Anderson *et al.*, 1991; Meenakshi *et al.*, 1999; Watson *et al.*, 1992; Konadu *et al.*, 2000). Although these inactivated and subunit vaccines are able to elicit high antibody responses, prevent shedding in chickens, and confer some degree of protection against *Salmonella* challenge, there are several live attenuated vaccines that are potentially superior for controlling *Salmonella* in animals and human.

For developing the live attenuated *Salmonella* vaccines, it is critical to choose the target genes for making deletion mutation for virulence attenuation. Attenuation should produce *Salmonella* with reduced ability to grow and survive in the host. Genetic inactivation of too many critical genes

or inappropriate selection of target genes results in failing to colonize in the host and to induce innate or acquired immunity, and finally failing to produce durable protection for the vaccinated subjects (Dilts *et al.*, 2000; Levine *et al.*, 1987; Hohmann *et al.*, 1996). The successful attenuated vaccines that perform well in clinical trials is targeting both mutation of metabolic pathways and virulence determinants (Galen and Curtiss, 2014). When the strategies for attenuation are confirmed by targeting virulence and metabolism, the balance between the safety and immunogenicity should be considered. The reason is that the over-attenuation of metabolic genes may weaken the metabolic fitness of the vaccine strain, resulting in decrease of immunogenicity. In our project, two genes, *aroA* and *htrA*, were deleted for making the attenuated vaccine strain. The gene *aroA* is involved in aromatic amino acid synthesis and biosynthesis of para-aminobenzoic acid (PABA) and 2, 3-dihydrobenzoate (DHB) (Hoiseth and Stocker, 1981). Multiple studies have shown that *Salmonella aroA* mutants are able to produce robust immune responses and then protect animals against lethal challenge (Eisenstein *et al.*, 1984a; Harrison *et al.*, 1997; Killar and Eisenstein, 1985). The gene *htrA* encodes a serine protease that is related to the protection against heat stress and oxidative stress (Pallen and Wren, 1997). Research on *Salmonella* Δ*htrA* mutants indicated decreased survival within macrophages, decreased virulence in mice, but increased protection for the challenge from *Salmonella* Typhimurium (Baumler *et al.*, 1994; Johnson *et al.*, 1991; Pickett *et al.*, 2000). The mutant strain with double deletions of *aroA* and *htrA* genes confer protection against the oral challenge with virulent *Salmonella* by single dose vaccination (Chatfield *et al.*, 1992). These two deletion mutants have been established to be safe in humans and be able to induce anti-flagellar antibodies, as well as cell-mediated responses to particular antigens (Lowe *et al.*, 1999; Tacket *et al.*, 1992).

The live *Salmonella* attenuated vaccine strain is not only used to provide protection from the challenge of *Salmonella*, it is also applicable for delivering heterologous antigens and constructing multivalent vaccines. The attenuated *Salmonella* vaccine strain can be used as a vector to express heterologous antigens from other pathogens, can be delivered into mucosal surfaces for eliciting strong local and systemic immune responses, and finally can provide protection against *Salmonella* as well as other pathogens that are derived from heterologous antigens (Roberts, 1994; Hormaeche, 1995). The efficacy of multivalent attenuated *Salmonella* vaccines requires the sustained expression of heterologous antigens in an immunogenic form and at a sufficient level to induce the strong and lasting immune responses. For achieving these goals, some of the common problems should be solved, such as plasmid instability, toxicity of the foreign antigens for the bacterial host, and incorrect folding of the expressed epitopes (Mastroeni *et al.*, 2001). In this project, the heterologous genes were integrated into the *lamB* region in the *Salmonella* chromosome, resulting in the stabilized expression of the foreign antigens. Briefly, the I-SceI and kanamycin cassette was introduced in the genome of *Salmonella* expressing the Red Recombinase enzymes. Next, this sequence was replaced by the joined DNA fragments, a section of *Campylobacter* Omp18 protein (Cj0113), peptidoglycan associated lipoprotein of *Salmonella* (PAL) and a portion of chicken high mobility group box 1 protein (HGMB1). DNA fragments used for homologous recombination were linear PCR products which consisted of the foreign insertion sequence flanked by homologous sequences of the up-stream and down-stream of *lamB* gene of *Salmonella* encoding outer membrane protein.

1.1.2 Analysis of *Salmonella* Transmission Mechanism

Salmonella is a major foodborne pathogen causing approximately one million foodborne illnesses in the US every year (CDC). Chickens play an important role for transmitting *Salmonella* into the human food chain. Even though many approaches have been developed to reduce *Salmonella* colonization in the chicken gastrointestinal tract, the fundamental understanding of *Salmonella* transmission routes and mechanism within the chicken flock remain unclear. During the past decades, several researchers studied *Salmonella* transmission mechanisms by constructing the marker strains that are antibiotic-resistant or expressing fluorescence genes. These marker strains can be differentiated from the wild-type strains when administered into the chickens for analyzing *Salmonella* transmission mechanisms. However, there is a possibility that the phenotype may be altered in the marker strains, resulting in a change of behavior from the corresponding wild type strains. For analyzing *Salmonella* transmission dynamics within the chicken flock, it is necessary to construct the marker strains that perform in the same manner as the wild-type strains when used for quantitatively tracking within the chicken and environment. In this project, a series of isogenic barcode-tagged strains were constructed by inserting six random nucleotides into a functionally neutral region in the chromosome of *Salmonella* Enteritidis as a tool for quantitative tracking of *Salmonella* transmission within a chicken flock. These *Salmonella* isogenic barcode-tagged strains can be administered in different dose levels at the same time through different routes, which are powerful for analyzing *Salmonella* transmission routes, infection dose levels, and competition between different strains.

1.1.3 Isolation and Evaluation of Probiotic Candidate Strains for Reducing Gut Permeability and Decreasing *Salmonella* Typhimurium Colonization

Due to the increasing pressure against the use of antibiotics as a growth promoter in recent years, there is an increasing demand for seeking alternative products to promote animal health, decrease the infection from pathogens, and improve growth performance in the poultry industry. Probiotics are able to provide beneficial effects on the host when orally administered. The benefits of probiotics have been demonstrated in farm animals and humans by many studies, and therefore probiotics have attracted the interest of the poultry industry and can be used as an alternative to antibiotic growth promoters (Dicks and Botes, 2010). However, the mechanism of these beneficial effects on the hosts remain elusive and appear diverse for different probiotic strains. Therefore, appropriate method is lacking to select effective probiotic strains aiming at different effects on the host. To elucidate this problem, we developed a novel approach to screen a large number of probiotic strains isolated from healthy chicken guts for identifying the probiotic candidate strains with the capability of enhancing the gut barrier functions.

Probiotic strains play an important role in maintaining the gut health and reducing pathogens in poultry. The interaction between the probiotic strains population and gut epithelial cells is also crucial for the gut health. The gut mucosal epithelial cell lining are able to maintain a defensive barrier for controlling the pathogen invasion, as well as allowing the nutrient absorption (Rao and Samak, 2013). Any damage of the mucosal epithelial cell lines is correlated with the decrease of barrier function, which allows translocation of pathogens from the intestinal lumen into the blood stream (Song *et al.*, 2014; Ulluwishewa *et al.*, 2011). The single layer of epithelial cells are joined together by tight junction proteins and form the gut barrier. Many studies have demonstrated that the disorder of epithelial cell lining are related to multiple diseases, such as inflammatory bowel

disease (IBD) and food allergies (Ulluwishewa *et al.*, 2011; Miyauchi *et al.*, 2012). The tight junction proteins are multi-protein complexes and are used for sealing the paracellular space between adjacent epithelial cells and limiting the transportation of pathogens (Miyauchi *et al.*, 2012). Transepithelial electrical resistance (TEER) can be used as the measurement of the intestinal barrier integrity. Some conditions including heat stress and feed restriction have been shown to increase the gut permeability, which is ameliorated by administration of probiotic strains (Song *et al.*, 2014; Vicuna *et al.*, 2015). In this project, we developed a novel approach for screening functional probiotic strains targeting improving the gut integrity and evaluated the efficacy of probiotic candidate strains in turkey poults by using the therapeutic model of *Salmonella* colonization.

1.2 Objectives

The objectives of my research in this dissertation were to develop recombinant *Salmonella* vaccines to provide cross-serovar and cross-serogroup protection, to analyze *Salmonella* transmission mechanisms within the chicken flock, and to identify and evaluate probiotic candidate strains for reducing gut permeability and decreasing *Salmonella* Typhimurium colonization in turkey poults.

Specific objectives for this dissertation are as follows:

1. To develop recombinant *Salmonella* vaccines against the challenge from *Salmonella* Heidelberg for testing of cross-serogroup protection with *S.* Enteritidis vectored vaccine candidates.
2. To quantitative track of *Salmonella* transmission routes using barcode-tagged isogenic strains in chickens.
3. To study the theory of *Salmonella* competitive exclusion by using barcode-tagged isogenic strains.

4. To isolate and evaluate lactic acid bacteria candidates for intestinal epithelial permeability reduction and *Salmonella* Typhimurium colonization in neonatal turkey poults.

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CHAPTER TWO

Evaluation of Recombinant *Salmonella* Vaccines to Provide Cross-Serovar and Cross-Serogroup Protection

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2.1 Abstract

Historically, *Salmonella* vaccines have been either live attenuated or killed bacterin vaccines that fail to offer cross-protection against other serogroups, which limits true risk mitigation and protection of consumers. Subunit recombinant vaccines which possess highly conserved antigens offer potential to provide cross-serogroup protection, and ability to express immune-enhancing molecules that promote uptake and recognition by the immune system. Six *Salmonella* subunit vaccine candidates were developed in either attenuated *S.* Enteritidis (SE) or *S.* Typhimurium (ST) that on the cell surface expresses antigenic epitopes of high mobility group box 1 immune-enhancing sequence (H), peptidoglycan associated lipoprotein (P), and Omp18 protein Cj0113 (C) in different pattern arrangements for evaluation against *S.* Heidelberg (SH) challenge in broilers. In exp. 1, chicks were orally vaccinated with SE-CPH, SE-HCP, SE-CHP, ST-CPH, ST-HCP, or ST-CHP at $1x10^7$ CFU/chick, or saline control on d1 and d14. On d17 all birds were challenged with $6x10^6$ cfu/chick SH, and ceca collected on d23 and d28. On d23 only SE-CPH reduced ($P<0.05$) SH recovery at 0.34 ± 0.23 $\log_{10}$ CFU when compared to control at 1.19 ± 0.26 $\log_{10}$ CFU. On d28, SE-CPH and ST-HCP reduced SH recovery at 0.40 ± 0.40 and 0.51 ± 0.26 $\log_{10}$ CFU, respectively in comparison to control at 1.36 ± 0.23 $\log_{10}$ CFU. For exp. 2, chicks were orally vaccinated with $1x10^8$ CFU/chick SE-CPH, SE-HCP, SE-CHP or saline on d1. At d7 all chicks were orally challenged with $7x10^6$ CFU/chick SH and ceca collected on d28 and d35. Only SE-CPH reduced ($P<0.05$) SH recovery on d28 when compared to control (6.16 ± 0.13 vs. 4.71 ± 0.55 $\log_{10}$ CFU). In exp 3, chicks were vaccinated by spray in a commercial vaccination cabinet with SE-CPH vaccination, $1.6x10^7$ CFU/chick, or saline control. Birds were challenged on d14 with $3x10^7$ CFU/chick SH and ceca collected on d18 and d25. SE-CPH reduced SH recovery ($P<0.05$) on d18, 2.75 ± 0.05 $\log_{10}$ CFU, and d25, 1.89 ± 0.43 $\log_{10}$ CFU, as compared to control

chickens at 5.6±0.37 (d18) and 3.98±0.5 $\log_{10}$ cfu (d25). The results of these experiments suggest that cross-serogroup protection is possible using these SE and ST vectored subunit vaccines.

2.2 Introduction

Cases of salmonellosis are most commonly linked to contaminated eggs and poultry products, which are the leading source of *Salmonella* outbreaks in the United States and Europe. In addition, *Campylobacter* continues to be a food safety concern with over 18 million annual cases of gastroenteritis associated with the pathogen in the United States (Kaakoush et al., 2015). As a result, the poultry industry has sought multiple ante mortem intervention strategies that include increased biosecurity, therapeutic antibiotics, competitive exclusion and probiotics, and vaccination. The two most common types of vaccines used in animal agriculture are killed whole-cell bacterins and live attenuated vaccines, both of which are useful in many applications, but do have limitations (Barrow, 2007). Killed vaccines have been primarily used to protect against systemic infections, and although they have been known to reduce colonization and shedding, the protection provided by these vaccines has only limited ability to stop mucosal colonization (Deguchi et al., 2009; Nakamura et al., 2004). Additionally, they often require injection, which is not practical in the poultry industry due to the large number of animals that would require vaccination.

Live attenuated vaccines have ability to colonize the gut which may provide a mucosal immune response, as well as potentially competitively exclude heterologous strains of *Salmonella* from colonizing the GIT (Barrow, 2007; Holt and Gast, 2004; Van Immerseel et al., 2005). The adaptive immune response elicited by live vaccines is composed of a humoral as well as cell-mediated response similar to that seen in a natural infection (Barrow, 2007; Mastroeni et al., 2001; Van Immerseel et al., 2005). This is in contrast to a killed vaccine, which primarily stimulates a

humoral response. After vaccination, protection against homologous strains of *Salmonella* is almost complete (Chacana and Terzolo, 2006; Ghany et al., 2007), but often little protection is afforded against a challenge by a heterologous serotype (Barrow, 2007; Young et al., 2007). Thus cross-serotype protection, especially against minor serovars for which live attenuated vaccines are not available, has become a desirable aspect of *Salmonella* vaccines.

Subunit vaccines, genetically modified bacteria, containing antigens against multiple pathogens, including attenuated live recombinant bacterial vectored vaccines have become viable options for animal agriculture vaccines as a result of recent advances in the field. Many research programs have modified multiple species of bacteria including *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Typhi, *Escherichia coli*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Bacillus subtilis*, and *Bacillus thuringiensis*, to name a few, to express protein antigens against bacterial, viral, and protozoal pathogens as well as toxins (Arnold et al., 2004; Deguchi et al., 2009; Duc and Cutting, 2003; Kajikawa et al., 2007; Mauriello et al., 2004; Tacket et al., 1997; Wu and Chung, 2007; Yang et al., 1990; Zhang et al., 2006). These vaccines have an advantage over other types of vaccines in that they are able to be delivered directly to a mucosal surface via nasal, ocular, or oral administration, which may enhance mucosal immune response, the primary surface through which most pathogens invade.

Presently, multiple *Salmonella* subunit vaccines were developed to test possibility of providing cross-serogroup protection, plus protection against *Campylobacter* in order to improve vaccination strategies and effectiveness in the poultry industry, by providing an orally effective vaccine. These vaccines were modified to insert highly conserved antigenic epitopes of peptidoglycan associated lipoprotein (PAL) of *Salmonella* and *Campylobacter* Omp18 protein Cj0113 (CJ0113; Layton et al., 2011). Additionally, a portion of chicken high mobility group box

1 protein (HMGB1) was included as an immune enhancing molecule to promote uptake by antigen presenting cells. A challenge with *Salmonella* Heidelberg provided for testing of cross-serogroup protection with *S.* Enteritidis vectored vaccine candidates.

2.3 Materials and Methods

2.3.1 Vaccine Construction

***Overview of mutational strategy*.** The strategy used for construction of the vaccine strains in both *S.* Enteritidis and *S.* Typhimurium backgrounds was previously described (Cox et al., 2007). Briefly, in this two-step mutagenesis process, the I-SceI site/kanamycin resistance (Km^r) cassette was first inserted into the *lamB* gene by Red recombinase-mediated homologous recombination via selection with kanamycin. Then, this mutation was replaced by the linear fragment carrying desired insertion sequence encoding combinations of PAL, CJ0113 and HMGB1 sequences in three different orders (Fig. 1). For the selection of the desired mutant, the linear PCR product carrying the desired insertion sequence was transformed simultaneously with the plasmid, pBC-I-SceI, expressing I-SceI homing endonuclease enzyme used for counter-selection against the strains carrying the I-SceI site (Cox et al. 2007).

***Bacterial Strains and Culture Conditions*.** SE164 strain, which carries I-SceI/Km^r cassette in the loop 9 region in *lamB* gene and also contains plasmid pKD46, was used for construction of the three SE vaccine strains in this study (Cox et al. 2007). For vaccines based on *S.* Typhimurium background, the wild type *S.* Typhimurium 110 (ST) was used first for construction of the attenuated strain with deletion in *aroA* gene, and the I-SceI/Km^r cassette was inserted into the loop 9 region of *lamB* gene via Red recombination in this study as previously described (Cox et al. 2007). Luria-Bertani (LB) media was used for routine growth of *Salmonella* cells, and 2×YT broth (Thermo Fisher Scientific, Cat. #22712-020) for the preparation of electro-competent cells. SOC

media (Invitrogen, Carlsbad, CA, USA) was used for phenotypic expression after electroporation. The following antibiotics were added to the media when appropriate: ampicillin (Amp) at 100 μg/ml, kanamycin (Km) at 50 μg/ml, and chloramphenicol (Cm) at 25 μg/ml.

***Plasmids*.** Plasmids pKD46 used in the present study was described previously (Cox et al., 2007; Datsenko and Wanner, 2000). Plasmid pKD46 encodes Red recombinase enzymes that mediate homologous recombination of incoming linear DNA with chromosomal DNA. This plasmid also contains the ampicillin resistance gene (Amp^r) and has a temperature-sensitive replicon, requiring 30°C for maintenance of the cell. Plasmid pBC-I-SceI expresses the I-SceI enzyme, which cleaves the rare SceI sites of 18 bp long (Cox et al., 2007; Kang et al., 2004). Also, plasmid pBC-I-SceI carries the chloramphenicol resistance (Cm^r). Plasmids pCJ0113, pPAL and pHMGB1, which carry individual epitope sequences CJ0113, PAL, and HMGB1, respectively, were synthesized by GeneScript®. These plasmids were used as templates for PCR amplification of respective DNA fragments.

***PCR*.** All primers used for PCR are listed in Table 1. Typical PCR conditions consisted of approximately 0.1 μg of purified genomic, plasmid or PCR-generated DNA, 10× cloned *Pfu* polymerase buffer, 5 U *Pfu* polymerase (Stratagene La Jolla, CA, USA), 1 mM dNTPs (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), 1.2 μM each primer in a total volume of 50 μL. The DNA engine thermal cycler (Bio-Rad, Hercules, CA, USA) was used with the following amplification conditions: 94°C for 2 minutes; 30 cycles of 94°C sec for 30 sec, 58°C for 60 sec, 72°C for 90 sec per 1 kb; and 72°C for 10 minutes for final extension. Each PCR product was gel-purified (Qiagen, Valencia, CA, USA) and eluted in 20 μL EB buffer for preparation of templates used in overlapping extension PCR. For the linear overlapping PCR product, the product was gel-

purified and eluted in 50 µL EB buffer, ethanol precipitated, and resuspended in 5 µL of ddH_2O for concentration, which then was used for electroporation.

***Transformation*.** Transformation of pKD46 into *S.* Typhimurium was the first step carried out so that Red recombinase enzymes could be used for mediating recombination between a linear PCR product and chromosomal DNA. Plasmid pKD46 was used for transformation into *S.* Typhimurium 110 by electroporation. Briefly, cells were inoculated into 10 mL of 2X YT broth and grown at 37°C overnight. Then 100 µL of overnight culture was re-inoculated into 10 ml fresh 2 × YT broth at 37 °C for 3–4 hours until OD_{600} reached 0.8-1.0. Then, the cells were heated at 50°C for 25 minutes to inactivate host restriction system temporarily. Cells were washed five times in 10% glycerol and resuspended in 60 µl of 10% glycerol. Cells were then electrically pulsed at 2400–2450 kV for 1–6 ms, followed by shaking in SOC medium (500µl) for 2–3 hours at 30°C and plated on LB agar media with ampicillin. *S.* Typhimurium transformants carrying pKD46 were maintained at 30°C. When these transformants were prepared for Red recombination-based homologous recombination through electroporation, all steps were the same except that 15% arabinose was added to induce Red recombinase enzymes one hour prior to washing, and cells did not undergo the 50°C heating step.

Construction of intermediate construct carrying I-SceI/Km^r. Construction of *Salmonella* Enteritidis (SE) mutant strain carrying the I-SceI/Km^r fragment in the loop 9 region of the *lamB* gene and plasmid pKD46 (SE164) was described previously (Cox et al. 2007). The equivalent intermediate construct for *S.* Typhimurium 110 was constructed essentially by the same method. However, prior to insertion of the I-SceI/km^r, the *aroA* gene in *S.* Typhimurium was deleted as described previously (Cox et al. 2007) for virulence attenuation. Briefly, the deletion *aroA* gene was performed by inserting the Km^r gene in place of and thereby deleting *aroA*. This was done by

first by amplifying the *aroA* upstream (primers aroA-1F and aroA-1R), and downstream (aroA-2F and aroA-2R) sequences, which was joined with the Km^r cassette amplified from plasmid pKD4 using primers Kan 3F and Kan 3R (Table 1). This deletion cassette constructed by joining the 3 fragments by overlapping PCR was transformed into *S*. Typhimurium 110 strain, followed by selection with kanamycin. Additionally, the previously described pCP20 was introduced into Δ*aroA* cells to remove the Km^r gene from the *aroA* gene locus (Cox et al. 2007).

Construction of the vaccine strains surface-displaying three epitopes. The first step was to amplify the linear PCR fragments that carry the DNA sequences of the three epitopes PAL (P), CJ0113 (C) and HMGB1 (H) in different orders along with four spacers of serine residues as shown in Fig. 1. The DNA codons for encoding the amino acids for the epitopes as well as serine (TCC) were optimized for the most frequently used codons in *Salmonella* proteins (Burns and Beacham, 1985). The designs and DNA sequence of the primers are shown in Fig. 1 and Table 1, respectively. To simplify the construction steps, the same lamB up and lamB dn fragments were commonly used for construction of all 3 epitope cassettes. The sequence of CJ0113 in the cassette CHP and CPH or HMGB1 in the cassette HCP was amplified to include a region overlapping with the 3' end of the lamB up fragment. The sequence of PAL in the cassette CHP, and HCP or HMGB1 in the cassette CPH was amplified to include a region overlapping with the 5' end of the lamB down fragment. For each cassette three individual fragments were separately amplified from relevant plasmids (pCJ0113, pPAL and pHMGB1), and were joined together by overlapping PCR. Then, each of the overlapping PCR products was joined to the common lamB up and lamB dn fragments by overlapping PCR to obtain the epitope cassettes that consist of lamB up-3 epitopes-lamB dn as shown in Fig. 1.

Genomic replacement of I-SceI/Km^r with three epitope sequences. The resulting epitope cassettes and the plasmid pBC-I-SceI (at a molar ratio of approximately 40:1) were simultaneously electroporated into the competent cells of SE and ST background, which carried the I-SceI/Km^r cassette in loop 9 of the *lamB* gene and plasmid pKD46. Colonies for each recombination mutation were chosen according to the ability to grow on Cm plates, but not on Km plates, due to the replacement of Sce-I-Km^r cassette with the incoming epitope cassette. Finally the vaccine candidates were verified by DNA sequencing using primers lamb-3f and lamb-3r, which are located outside of the loop 9 up and dn regions.

2.3.2 Experimental Design

In all *in vivo* challenge experiments, chickens were raised in floor pens with wood shavings and were separated by solid panel walls to prevent cross-contamination with live vectored vaccines. Broilers were provided feed and water *ad libitum*, and diet was formulated to meet or exceed nutritional requirements. Ambient temperature in the rearing facility was maintained at age-appropriate levels. All experiments were conducted under University of Arkansas Institutional Animal Care and Use Committee approved protocols.

Bacterial cultures. Challenge strain of *Salmonella* Heidelberg (SH) was a field isolate confirmed to serogroup level by agglutination with O factor antiserum (Becton, Dickinson and Co., Sparks, MD) and typed by the National Veterinary Services Laboratories (Ames, IA, USA). Cultures were prepared by growth in tryptic soy broth (TSB, Becton, Dickinson and Co., Sparks, MD) for approximately 8 h each for three passages by transferring 1% of culture to fresh TSB. The cells were washed three times in sterile saline by centrifugation (1,864 × g), and the approximate concentration of the stock solution was determined spectrophotometrically. The stock solution was serially diluted and confirmed by colony counts of three replicate samples plated on XLT4 agar

(Becton, Dickinson and Co., Sparks, MD) plates. For cecal recovery of SH after challenge, birds were killed by CO_2 inhalation and entire ceca aseptically removed. Samples were diluted 1:4 w:v in sterile saline, 100 µL removed for CFU determination by serial plating on XLT4 agar, while the remaining portion was enriched in tetrathionate broth (Becton, Dickinson and Co., Sparks, MD) by overnight incubation at 37 °C, followed by plating on XLT4 agar.

The challenge strain of *Campylobacter jejuni* was a field isolate confirmed by culture techniques and microscopic observations of typical *Campylobacter* morphology. For recovery, approximately 6 cm of the lower ileum was aseptically collected into sterile sample bags, diluted 1:4 w:v in sterile saline, then stomached for 10s, plus a portion of diluted ceca supernatant (above) was serially diluted. Intestinal samples were directly plated onto selective *Campylobacter* chromogenic agar (R-F Labs, Downers Grove, IL). After incubation for 48 h at 42 °C under microaerophilic conditions, colonies consistent with *Campylobacter* were enumerated.

Experiment 1. All six vaccine candidates were tested by oral gavage of approximately $1x10^7$ CFU/chick, 20 chicks per each vaccine, on day of hatch and d14. Three days after boost vaccination (d17 of age), $6x10^6$ CFU/chicken of SH were administered by oral gavage as a challenge dose, and negative control received sterile saline carrier. Recovery of SH was tested 5d (d23 of age) and 10d (d28 of age) post-challenge.

Experiment 2. In this experiment, only the three SE-vectored vaccine candidates, SE-CPH, SE-HCP, and SE-CHP, were tested for protection against SH challenge after a single vaccination. On day of hatch, approximately $1x10^8$ CFU/chick was administered to 20 chicks per vaccine, and the negative control received a sterile saline carrier. On d7 all chicks were challenged with $7x10^6$ CFU/chick of SH by oral gavage, and cecal samples were collected 21d (d28 of age) and 28d (d35 of age) post-challenge. *Campylobacter* recovery was performed at 28d post-challenge only.

Experiment 3. In the final experiment, only SE-CPH vaccine was tested against SH challenge with a spray vaccination instead of oral gavage to simulate conditions likely to be applied in a hatchery setting. On day of hatch 50 chicks, per group, were placed in a cardboard hatchery transport box, blocked off to provide chicks with typical spacing, which was then placed into a commercial vaccine spray chamber that was loosely covered with plastic on each end to prevent escape of sprayed vaccine. Control chicks were sprayed with saline carrier, and an estimated dose of $1.6x10^7$ CFU/chick was sprayed into the cabinet for treated chicks. Particle size of the spray was confirmed by collection of spray onto a microscope slide, and measured at ~6 µm droplets. Birds were challenged with SH on d14 with $3x10^7$ CFU/chick, followed by collection of ceca at 4d (d18 of age), 7d (d21 of age), and 12d (d25 of age) post-challenge.

2.3.3 Statistical Analysis

Numerical data from all experiments were subjected to ANOVA (SAS Institute, 2002). Log_{10} CFU values per sample are expressed as mean ± standard error of mean and deemed significant at $P \leq 0.05$. The data were also subjected to mean separation using Duncan's multiple range test of significance.

2.4 Results

2.4.1 Construction and confirmation of the vaccine strains

After electroporation colonies were chosen as the candidates according to the ability to grow on Cm plates, but not on Km plates. Colony PCR was conducted using candidate colonies with lamb-3f and lamb-3r primers that anneal outside of the lamB up and lamB dn regions. When the expected size of the PCR products of approximately 726bp was confirmed by gel electrophoresis, the PCR product was gel-purified for DNA sequencing. The colonies for which DNA sequencing

data confirmed the insertion of the correct sequence for each epitope cassette in the correct location in the *lamB* loop 9 region were stored at -80ºC with 30% glycerol.

2.4.2 Experiment 1

Five days post SH challenge (DPC), negative control birds tested positive at a mean level of 1.19±0.26 $\log_{10}$ CFU/g of ceca, whereas SE-CPH vaccinated chickens had a recovery level of only 0.34±0.23 $\log_{10}$ CFU/g of ceca which was significantly lower when the groups were directly compared by t-test (Table 2). Levels of SH recovered from ceca of other challenged groups at 5 DPC were 0.69±0.53 $\log_{10}$ CFU/g (SE-HCP), 0.64±0.48 $\log_{10}$ CFU/g (SE-CHP), 3.02±0.57 $\log_{10}$ CFU/g (ST-CPH), 0.94±0.63 $\log_{10}$ CFU/g (ST-HCP), and 0.68±0.28 $\log_{10}$ CFU/g (ST-CHP). Ten DPC, ST-HCP vaccinated group dropped SH level to 0.51±0.26 $\log_{10}$ CFU/g, which was significantly lower than the control group, by direct comparison, at 1.36±0.23 $\log_{10}$ CFU/g. Levels of SH recovery from the SE-CPH rose to 0.40±0.40 $\log_{10}$ CFU/g, which was different from control group ($p=0.0514$) by direct comparison.

2.4.3 Experiment 2

After day of hatch vaccination, chickens were challenged with SH on day, followed by detection of the challenge strain 21 and 28 DPC. At 21 DPC (or Twenty one DPC?), vaccine candidate SE-CPH was the only vaccination group to have significantly lower recovery of SH from the ceca than negative control at 4.71±0.55 vs. 6.16±0.13 $\log_{10}$ cfu/g (Table 3). Though the difference between 21 and 28 DPC was less than 0.1 $\log_{10}$ change for SE-CPH vaccine group, negative control recovery dropped to 5.11±0.59 $\log_{10}$ cfu/g, and the significant difference did not persist. *Camylobacter* recovery was highest in the ceca (Figure 2), with levels ranging from 7.14±0.29 $\log_{10}$ cfu/g after SE-CPH vaccination to 7.70±0.29 $\log_{10}$ cfu/g in the non-vaccinated

control group. Levels of *Campylobacter* recovered in the ileum were much lower with a range of 0.17±0.17 $\log_{10}$ cfu/g recovered from SE-CHP vaccinated birds to 1.05±0.57 $\log_{10}$ cfu/g in SE-HCP vaccinated birds. There were no statistical differences among any of the groups for *Campylobacter* levels.

2.4.4 Experiment 3

Only SE-CPH vaccine candidate was tested in Experiment 3, with a time course study to evaluate persistence of protection. Four and seven DPC, recovery of SH in vaccinated chickens was 2.7±0.0 $\log_{10}$ cfu/g and 1.89±0.41 $\log_{10}$ cfu/g, respectively. Compared to recovery in negative control group, at 5.61±0.36 $\log_{10}$ cfu/g (4 DPC) and 3.98±0.47 $\log_{10}$ cfu/g (7 DPC), a significant reduction was observed (Figure 3). However, by 12 DPC, the difference in cecal SH recovery was reduced to 3.93±0.66 $\log_{10}$ cfu/g in negative control and 3.01±0.92 $\log_{10}$ cfu/g in vaccinated treatment, which was not significantly different.

2.5 Discussion

Construction of all 6 vaccine strains described in study was constructed essentially by the method previously established in our lab (Cox et al. 2007). Once the epitope cassettes of different designs were prepared by overlapping PCR, the construction of the vaccine strains carrying the epitope cassettes was a straightforward process. In the final constructs, the antibiotic resistance cassette (Km^r cassette) was removed and replaced by the desired epitope cassette, making them free of antibiotic markers. Once the final constructs were confirmed at DNA sequence level, the functional expression of each of the epitopes was also confirmed by standard antibody agglutination assays with hyper-immune sera against each of the antigens.

The first vaccine experiment evaluated six different SE and ST vectored vaccines, each containing CJ0113, PAL, and HMGB1 genetic inserts in different patterns, against SH challenge.

Five days after challenge SE-CPH vaccinated chickens had reduced recovery of SH from ceca, with a trend in reduction ten DPC (P=0.0514) and 0.96 Log_{10} CFU/g reduction in cecal recovery (Table 2). Vaccination with ST-HCP also resulted in reduced recovery of SH from cecal tonsils, when compared directly to the negative control group, but only at ten DPC. Given the overall lack of reduction in SH recovery 5 DPC with ST-vectored vaccine candidates, they were not carried forward in subsequent experiments. Though, not all the SE-vectored strains were effective at a significant reduction in SH detected, there was some numerical differences, and all three vaccines were re-evaluated. As suggested previously, this lack of effectiveness could be related to natural folding and expression of the antigen epitopes on the cell surface in a manner that inhibited antigenicity or immunogenicity. Experiment 2 tested only SE vectored vaccine candidates against SH challenge and recovery at 21 and 28 days post challenge. Only SE-CPH resulted in decreased cecal recovery of SH with a 1.45 Log_{10} CFU/g reduction, and no vaccine group had lower recovery by 28 days post infection (Table 3). These results at least partially confirmed results of Experiment 1, in that only SE-CPH was able to provide protection, evidenced by the >96% decrease in SH at 21 days post challenge, and that SE-HCP and CHP were not as robust as providing protection against colonization. This study also evaluated effect of vaccination against *Campylobacter jejuni* challenge, with samples collected from ileum and ceca 28 days post challenge. Contrary to previous studies utilizing CJ0113 antigen (Layton et al., 2011), no statistically significant reduction of *Campylobacter* was observed (Figure 2). However, these results are not entirely inconsistent with Layton et al. (2011) which reported efficacy of an SE vectored CJ0113 vaccine against *C. jejuni* recovery in ileum of chickens. In that report, *C. jejuni* was detected by quantitative PCR techniques with a limit of detection at 1.0 Log_{10} CFU. Thus, had the present study followed the detection techniques, it is unlikely that *C. jejuni* would have been detected in

chickens vaccinated with SE-CHP, but it should be noted that ileum levels of *C. jejuni* in the Negative Control group also would have been low, and were much lower than those reported by Layton and co-workers (2011). Recent changes in culture detection techniques and better understanding of *Campylobacter* infection in poultry, such as ceca as the primary site of infection versus ileum (Bahrndorff et al., 2015; Chaloner et al., 2014; Chintoan-Uta, 2016) likely account for differences between this and the previous report. It should also be noted that the previous research report inserted a CD154 immune-enhancing molecule instead of the HGMB1 molecule used in these studies, which may have accounted for differences.

The final experiment investigated the ability of a single day of hatch vaccination by SE-CPH to reduce levels of SH in ceca at four, seven, and twelve days post-challenge. Early recovery time points, four and seven DPC, had significantly lower levels of SH in vaccinated groups. However, a decline in levels in Negative Control combined with an increase in SE-CPH vaccinated group, negated this difference by 12 DPC (Figure 3. Re-infection of GIT is possible through coprophagic activity in poultry and these data suggest that a single vaccination likely does not provide long term immune protection against re-infection from exposure later in life.

The purpose of testing protection of SE-vectored vaccination against SH was to determine the ability of the vaccine to provide cross-serogroup protection in broilers because 92% of *Salmonella* positive samples from broilers analyzed under the Food Safety and Inspection Service HACCP program in 2014 were represented across three different serogroups, suggesting that a vaccine capable of providing protection across these serogroups would have great impact on reduction of *Salmonella* on poultry products (United Stated Department of Agriculture, 2016). Though *S.* Kentucky was the highest isolated serotype, it is not among serotypes commonly associated with human illness. Thus, SH was selected due to the ability to prove both cross

serotype and serogroup protection among ST or SE vaccinated chickens. These studies showed that vaccination with SE vectored subunit vaccine containing the highly conserved antigen PAL was able to confer protection against SH challenge for up to 21 days post-infection. *Salmonella* Typhimurium vector was able to reduce SH recovery up to 10 days post-infection in the single experiment tested.

Subunit vaccines expressing cross-protective antigens may be useful for the poultry industry due to ability to reduce levels of *Salmonella* across multiple serotypes and serogroups, thus effectively reducing levels of *Salmonella* on animals as they enter processing facilities. The studies presented here were primarily aimed at the ability of a highly conserved antigen, PAL, expressed in a double-attenuated SE (serogroup D) strain to provide protection against a SH (serogroup B) strain, as well as an attenuated ST strain to provide protection against SH. Results indicate that live attenuated vaccination with a serogroup D *Salmonella* Enteritidis subunit vaccines containing antigen PAL and the immune enhancing molecule HMGB1 can reduce cecal levels of serogroup B *Salmonella* Heidelberg, suggesting that cross-serogroup protection against *Salmonella* infection in poultry is achievable.

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2.7 References

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2.8 Figures and tables

Figure 1. Construction of the 3 epitope cassettes using overlapping PCR. A. CPH, B. HCP, and C. CHP.

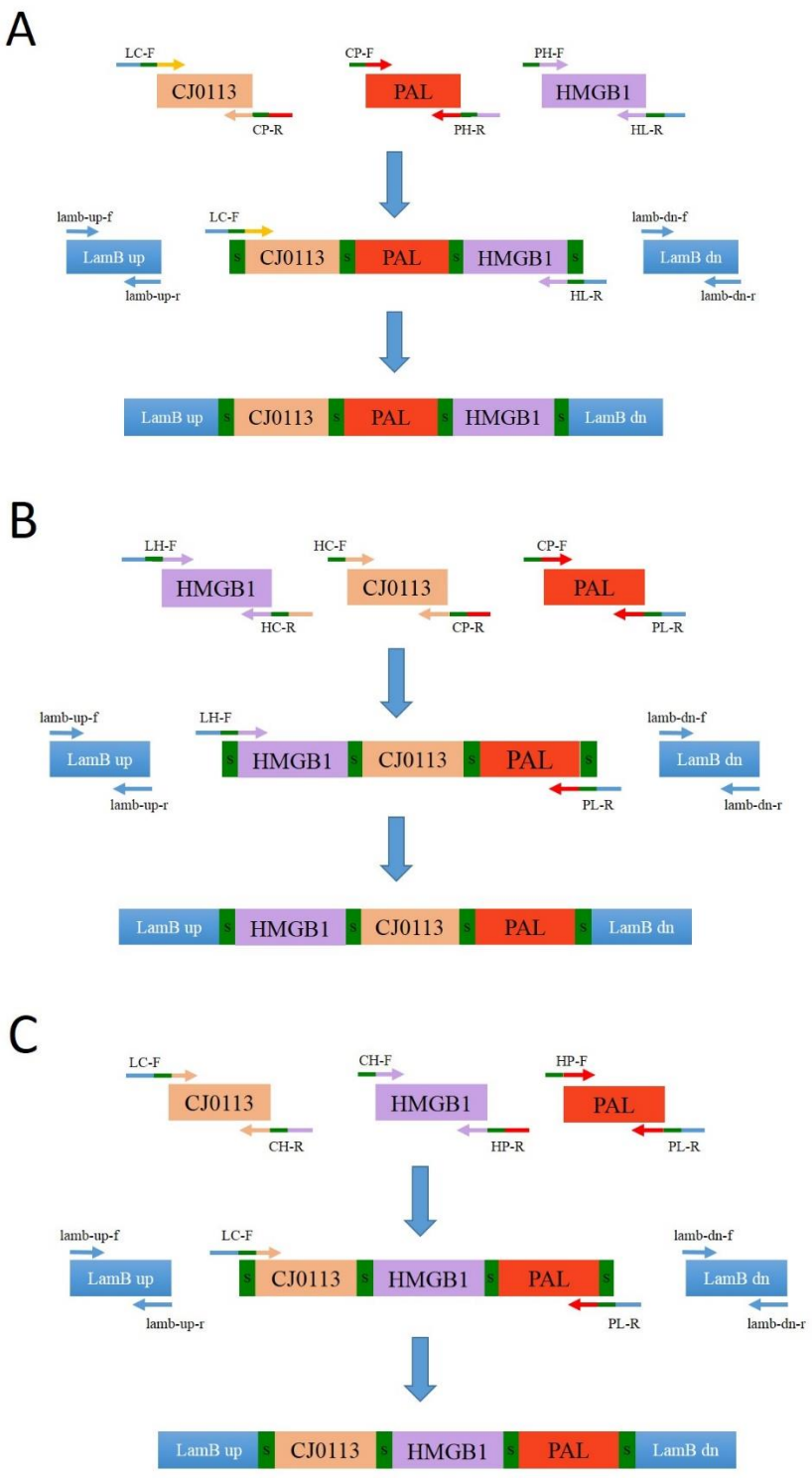


Table 1. Oligonucleotides used in this study

Homology sequence		
lam-up-f	lamB up	TGTACAAGTGGACGCCAATC
lam-up-r		GTTATCGCCGTCTTTGATAT
lam-dn-f	lamB dn	ATTTCCCGTTATGCCGCAGC
lam-dn-r		GTTAAACAGAGGGCGACGAG
lamb-3f	Checking epitope insertion	ATGATGATTACTCTGCGCAA
lamb-3r		CGCTGGTATTTTGCGGTACA
CJ113 + PAL + HMGB1		
LC-F	LamB up + $(Ser)_3$ + CJ0113	AAATGGGGCTATATCAAAGACGGCGATAACTCCTCCTCCG GTGTTTCTATCACCGTTGAA
CP-R	CJ0113 + $(Ser)_3$ + PAL	ACGTTCGTCCGCGTGACCTTCGGAGGAGGACGCCTGGTTG TATTCGTCGG
CP-F	$(Ser)_3$ + PAL	TCCTCCTCC GAAGGTCACGCGGACGAACGT
PH-R	PAL + $(Ser)_3$ + HMGB1	ACGCGGTTTTTTCGGGTCGCCTTTACCCAT GGAGGAGGAA CGTTCACCCAGAGAGATGTTGTATT
PH-F	$(Ser)_3$+ HMGB1	Same as CP-F
HL-R	HMGB1 + $(Ser)_3$ + LamB	GGAGTTAGTCGCTGCGGCATAACGGGAAATGGAGGAGGA GTCTTCTTCTTCTTCTTTTT
HMGB1 + CJ0113 + PAL		
LH-F	LamB + $(Ser)_3$ + HMGB1	AAATGGGGCTATATCAAAGACGGCGATAACTCCTCCTCCA TGGGTAAAGGCGACCCGAAA
HC-R	HMGB1 + $(Ser)_3$ + CJ0113	TTCAACGGTGATAGAAACACCGGAGGAGGAGTCTTCTTCT TCTTCTTTTT
HC-F	$(Ser)_3$ + CJ0113	TCCTCCTCC GGTGTTTCTATCACCGTTGAA
CP-R	CJ0113 + $(Ser)_3$ + PAL	ACGTTCGTCCGCGTGACCTTCGGAGGAGGACGCCTGGTTG T ATTCGTCGG
CP-F	$(Ser)_3$ + PAL	Same as CP-F
PL-R	PAL + $(Ser)_3$ + LamB	GGAGTTAGTCGCTGCGGCATAACGGGAAATGGAGGAGGA ACGTTCACCCAGAGAGATGTTGTATT
CJ113 + HMGB1 + PAL		
LC-F	LamB up + $(Ser)_3$ + CJ0113	AAATGGGGCTATATCAAAGACGGCGATAACTCCTCCTCCG GTGTTTCTATCACCGTTGAA
CH-R	CJ0113 + $(Ser)_3$ + HMGB1	TTTCGGGTCGCCTTTACCCAT GGAGGAGGACGCCTGGTTG TATTCGTCGG
CH-F	$(Ser)_3$ + HMGB1	TCCTCCTCC ATGGGTAAAGGCGACCCGAAA
HP-R	HMGB1 + $(Ser)_3$ + PAL	ACGTTCGTCCGCGTGACCTTC GGAGGAGGAGTCTTCTTCT TCTTCTTTTTTTTTTTAGA
HP-F	$(Ser)_3$ + PAL	Same as CP-F
PL-R	PAL + $(Ser)_3$ + LamB	GGAGTTAGTCGCTGCGGCATAACGGGAAATGGAGGAGGA ACGTTCACCCAGAGAGATGTTGTATT
Deletion of *aroA* gene		
aroA-1F	aroA up	CTGGACGTCTCTCGCTATGG

aroA-1R		TAGGAACTTCGAAGCAGCTCCAGCCTACACATAAAAACCC CACAGACTGG
aroA-2F	aroA dn	GGAATAGGAACTAAGGAGGATATTCATATGGTCTTCTGT TGCGCCAGT
aroA-2R		CTTGCGAGAGTGCCCTAAAG
Kan 3F	Km^r gene in pKD4	GTGTAGGCTGGAGCTGCTTC
Kan 3R		CATATGAATATCCTCCTTAG

Underline indicates the nucleotides corresponding to overlapping regions.

Table 2. Recovery of *Salmonella* Heidelberg from ceca after vaccination by oral gavage on day of hatch and 14 days of age, Experiment 1. On day of hatch and day 14, chickens were vaccinated with respective antigen-vectored vaccines, challenged with $6x10^6$ cfu/chick of *S.* Heidelberg on d17, and ceca were collected for recovery 5 and 10 days post challenge (DPC).

	Log_{10} *S.* Heidelberg (CFU/gram)	
	5 DPC	10 DPC
Negative Control	1.19 ± 0.26^{ab}	1.36 ± 0.23^{a}
SE-CPH	$0.34\pm0.23^{b*}$	$0.40\pm0.40^{a**}$
SE-HCP	0.69 ± 0.53^{b}	1.08 ± 0.49^{a}
SE-CHP	0.64 ± 0.48^{b}	1.10 ± 0.39^{a}
ST-CPH	3.02 ± 0.57^{a}	1.45 ± 0.48^{a}
ST-HCP	0.94 ± 0.63^{b}	$0.51\pm0.26^{a*}$
ST-CHP	0.68 ± 0.28^{b}	1.16 ± 0.45^{a}

[ab]Values with different superscripts are significantly different $P<0.05$

*Different from Negative Control $P<0.05$ by direct comparison

** $P=0.0514$ (SE-CPH vs. Negative Control)

Table 3. Recovery of *Salmonella* Heidelberg from ceca after vaccination by oral gavage on day of hatch, Experiment 2. On day of hatch, chickens were vaccinated with respective antigen-vectored vaccines, challenged with $6x10^6$ cfu/chick of *S*. Heidelberg on d7, and ceca were collected for recovery 21 and 28 days post challenge (DPC).

	Log_{10} *S*. Heidelberg	
	21 DPC	28 DPC
Negative Control	6.16±0.13[a]	5.11±0.59[a]
SE-CPH	4.71±0.55[b]	4.80±0.57[a]
SE-HCP	6.32±0.15[a]	5.16±0.18[a]
SE-CHP	5.94±0.18[a]	5.31±0.19[a]

Figure 2. Recovery of *Campylobacter* from ceca after vaccination by oral gavage on day of hatch, Experiment 2. On day of hatch, chickens were vaccinated with respective antigen-vectored vaccines, challenged with *Campylobacter jejuni*, and ceca and ileum were collected for recovery 28 days post challenge (DPC).

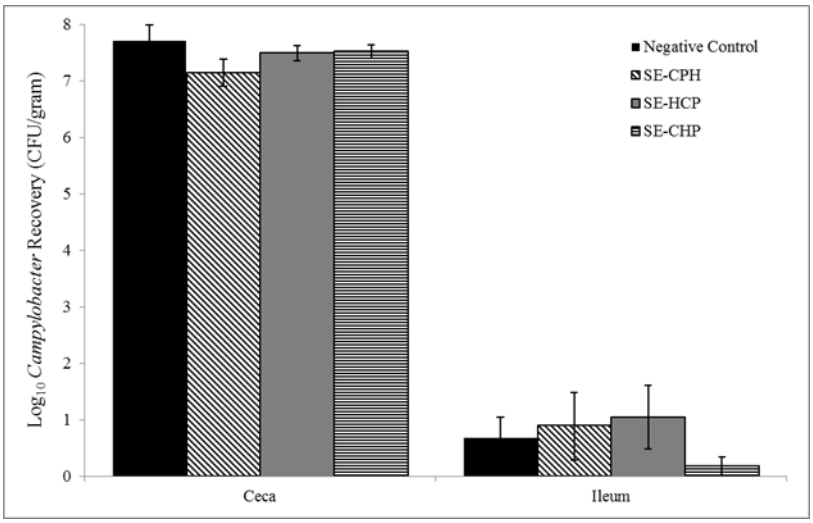
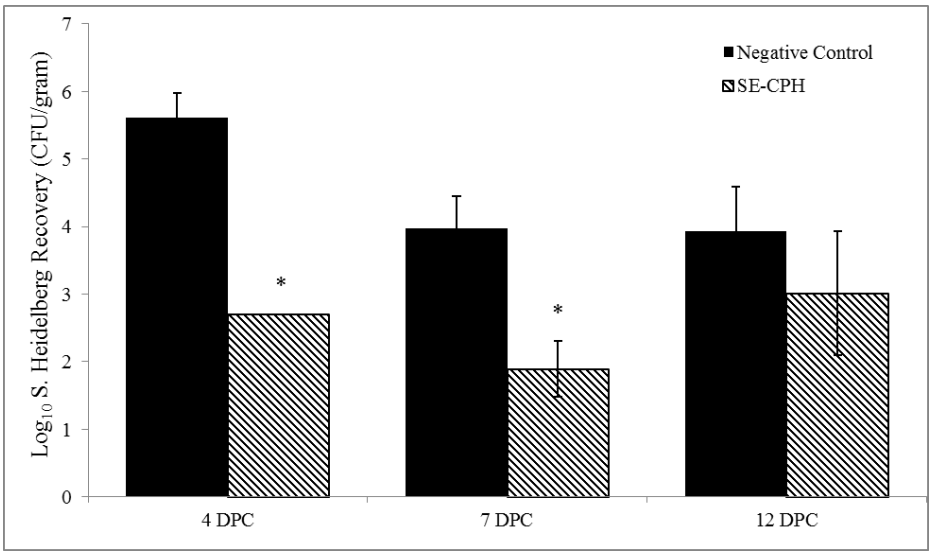


Figure 3. **Recovery of *Salmonella* Heidelberg from ceca after vaccination by spray administration on day of hatch, Experiment 3.** On day of hatch, chickens were vaccinated with SE-CPH vaccine, challenged with $3x10^7$ cfu/chick of S. Heidelberg on d14, and ceca were collected for recovery 4, 7 and 12 days post challenge (DPC).



CHAPTER THREE

Quantitative Tracking of *Salmonella* Enteritidis Transmission Routes Using Barcode-tagged Isogenic Strains in chickens: Proof of Concept Study

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3.1 Abstract

Salmonella is an important foodborne bacterial pathogen, however, a fundamental understanding on *Salmonella* transmission routes within a poultry flock remains unclear. In this study, a series of barcode-tagged strains were constructed by inserting six random nucleotides into a functionally neutral region on the chromosome of *S.* Enteritidis (SE) as a tool for quantitative tracking of *Salmonella* transmission in chickens. Six distinct barcode-tagged strains were used for infection or contamination at either low dose (10^3 CFUs; 3 strains) or high dose (10^5 CFUs; 3 strains) in three independent experiments (Exp 1 oral gavage; Exp 2 contaminated feed; Exp 3 contaminated water). For all chick experiments, cecal and foot-wash samples were collected from a subset of the chickens at Days 7 or/and 14, from which genomic DNA were extracted and used to amplify the barcode regions. After the resulting PCR amplicons were pooled and analyzed by MiSeq sequencing, a total of approximately 1.5 million reads containing the barcode sequences were analyzed to determine the relative frequency of every barcode-tagged strain in each sample. In Exp 1, the high dose of oral infection was correlated with greater dominance of the strains in the ceca of the respective seeder chickens, and also in the contact chickens yet at lesser degrees. When chicks were exposed to contaminated feed (Exp 2) or water (Exp 3), there was no clear patterns of the barcode-tagged strains in relation to the dosage, except that the strains introduced at low dose required a longer time to colonize the ceca with contaminated feed. Most foot-wash samples contained only 1 to 3 strains for the majority of the samples, suggesting potential existence of a unknown mechanism(s) for strain exclusion. These results demonstrated the proof of concept of using barcode-tagged to investigate transmission dynamics of *Salmonella* in chickens in a quantitative manner.

3.2 Introduction

Salmonella species induce bacterial illness and are also one of the leading causes of hospitalization among all the foodborne bacterial pathogens (1, 2). According to the Centers for Disease Control and Prevention (CDC), there is approximately 1.2 to 4 million human *Salmonella* infections in the United States each year (3, 4, 36). There are multiple sources of *Salmonella* infection in humans such as consumption of contaminated food and water, or contact with infected animals (5). Among others, poultry products are a prominent source of human salmonellosis, and the contamination can originate from a multitude of sources during poultry production (6-8). *S.* Enteritidis is considered as one of the most commonly identified serovars in association with human infection in the United States (9). The number of human infections by *S.* Enteritidis continued to increase from the 1980s, and had reached the point where *S.* Enteritidis became the predominant serovar in the 1990's and currently still remains a prominent foodborne disease-causing serovar (10, 34). Therefore, it is critical to not only understand the transmission modes of *S.* Enteritidis in chicken flocks, but to also be able to quantitate their relative contribution of each route to contamination during poultry production. Knowing the quantitative contribution of various transmission routes would be very helpful in designing optimal strategies to minimize the spread of *Salmonella* within a chicken flock via interventions such as vaccines and antimicrobials administered in the feed or drinking water (37, 38).

The transmission of *Salmonella* in a chicken flock involves an initial infection with single or multiple *Salmonella* strains from different sources through oral or tracheal routes (11-15). While the oral route is believed to be the primary infection route of *Salmonella* based on experimental evidence (1, 2), there are indications that airborne transmission is also a possible route (16-19). Once infection occurs the *Salmonella* population disseminates in the host from the entry site and

may colonize the intestinal tract or systemically invade the host tissues (20). Once a host becomes infected locally in the intestinal tract or systemically, *Salmonella* can, in turn, be disseminated to other susceptible hosts (21).

Salmonella, as an enteric pathogen, can be disseminated to poultry flocks through several sources. Drinking water, feed, wildlife or pets, transportation mode, manure or litter can be vehicles contributing to dissemination of *Salmonella* into poultry (41). Water is an important vehicle and can serve as a reservoir for *Salmonella* dissemination. *Salmonella* possesses the capacity to not only survive in the water for a long period of time but the expression of key virulence factors can also be increased when *Salmonella* is exposed to stressors in a water environment (40). *Salmonella* appear to possess the mechanisms to retain viability and successfully survive in the river environments as well. The relationship between the contaminated feed and the occurrence of *Salmonella* in poultry has been substantiated by several studies (44, 45). For the reason of labor and technical simplification, most chicken feed is produced in the farm as milled and blended mash, most of which are not heat-treated or pelleted. The vertical integration nature of the commercial poultry production cycle could impact the risk of introducing pathogens such as *Salmonella* to poultry production as a result of contaminated feed (42, 43).

Quantitative resolution of critical routes for *Salmonella* establishment in chickens requires the ability to track the strains introduced to the flock distinctively using some sort of a recoverable signature. Traditionally, *Salmonella* monitoring has been based on techniques such as introducing foreign elements into the candidate strain to construct marker strains that are antibiotic resistant or express genes for fluorescence proteins (22-24). However, these methods risk introducing phenotypic features into the resulting marker strains that could alter the pathogenicity and physiological status such that the resulting strains no longer behave in exactly same fashion as the

corresponding wild type. For example, green fluorescence proteins have been shown to alter growth physiology, while exposure to nalidixic acid can influence gene expression (25, 26). It is well established that acquisition of antibiotic resistance often entails fitness cost or enhanced fitness of the pathogenic strains in the absence of selection pressure (39).

The objective of the present study was to evaluate the proof of concept of barcode-tagged isogenic strains of *Salmonella* Enteritidis in broiler chickens using different routes of infection. A series of isogenic *S.* Enteritidis strains in which distinct DNA barcodes were inserted in a functionally neutral locus in the genome were constructed and the resulting strains employed to quantitatively track the transmission routes of the respective strains by profiling the barcode-regions using high-throughput sequencing. The advantages of these barcode-tagged strains over previously used marker strains are that each strain can be tracked quantitatively as a distinguishable part of the entire population at high accuracy, allowing for differentiation among multiple barcode-tagged strains as well as discrimination from the environmental *Salmonella* without altering phenotypes or behaviors during infection, colonization and dissemination.

3.3 Materials and Methods

3.3.1 Bacterial Strains and Culture Condition

Salmonella enterica serovar Enteritidis phage type 13A strain, which is a primary poultry isolate, was originally obtained from the USDA National Veterinary Services Laboratory (Ames, IA). The plasmid pKD4 was used as a template to amplify the kanamycin-resistance gene for construction of the barcode-tagged strains. The *Escherichia coli* strain BW25141 carrying pKD4 was inoculated in Luria-Bertani (LB) broth overnight and plasmid pKD4 was extracted with the illustra plasmidPrep Mini Spin Kit (GE Healthcare Life Sciences). The *Salmonella* Enteritidis strain (SE) containing pKD46 that encodes Red recombinase system was used for construction of

barcode-tagged strains via electroporation (46, 47). The plasmid pKD46 contains an ampicillin-resistance gene and is also a temperature-sensitive replicon requiring 30°C for replication of the plasmid in the cell. LB broth was used for cultivation of barcode-tagged strains. Super optimal broth with catabolite repression (SOC) media (Invitrogen, Carlsbad, CA, USA) was used for phenotypic expression of the transformed cells immediately after the electroporation. Appropriate antibiotics were used at the following concentrations when necessary: kanamycin (Km) at 50 μg/ml and ampicillin (Amp) at 100 μg/ml.

3.3.2 Rationale for the Genomic Location Selection

Ideally, the barcode along with the kanamycin resistance gene should be inserted into a functionally neutral genomic locus. Based on Chaudhuri et al. (27) we first searched for two adjacent genes that are not required for intestinal colonization in chickens and are also transcribed toward each other. We determined that SEN1521 and SEN1522 met these conditions, and therefore the intergenic region (141 bp) between these two genes was selected for insertion of a barcode plus the kanamycin resistance gene (Figure 1). When foreign sequences are inserted in the middle of this intergenic region without removing any original genomic sequences, it can be ensured that the insertion would not cause any polar effect on the downstream genes which would minimize, if any, phenotypic change due to the barcode insertion.

3.3.3 Construction of Barcode-tagged strains

All PCR primers are listed in Table 1. The 3' end and downstream regions of the coding genes SEN1521 (232 bp) and SE1522 (267 bp) were amplified from the genomic DNA of *S.* Enteritidis 13A with the primer pairs of T1-F and T1-BC-P1-R, and T3-P2-F and T3-R, respectively (termed, upstream and downstream fragments, respectively) (Figure 1). The T1-BC-P1-R primer contained

a barcode of 6 random nucleotides and the sequence overlapping with 5' end of the Km resistance gene (P1). The T3-P2-F primer contained the sequence overlapping with 3' end of the Km gene (P2). The Km resistance gene (1,496 bp) was amplified from the plasmid pKD4 with the primer pair of P1 and P2. The PCR assays were conducted by combining approximately 0.1 μg of purified genomic DNA or plasmid along with 1 μl of 2.5 U/μl Pfu polymerase (Agilent Technologies), 5μl of 10× cloned Pfu polymerase buffer, 4 μl of 2.5 mM dNTPs (TaKaRa) and 1 μl of 1.2 μM of each primer resulting in a total volume of 50 μL. The DNA Engine ® Thermal Cycler (Bio-Rad, Hercules, CA, USA) was used with the following amplification cycles: 94°C for 2 minutes; 30 cycles of 94°C sec for 30 sec, 58°C for 60 sec, 72°C for 60 sec per 1 kb; and 72°C for 10 minutes for the final extension. Each PCR product was gel-purified and eluted in 25 μl EB buffer for preparation of templates to be used for overlapping extension PCR. Overlapping extension PCR was employed to join the three fragments (upstream fragment plus a barcode + Km resistance gene + downstream fragment) together with the primers T1-F and T3-R (Figure 1). After running the agarose gel for confirmation of the correct size, electroporation was used to introduce the overlapping PCR fragments into *S.* Enteritidis carrying pKD46 plasmid. A number of transformants selected on LB agar plates supplemented with Km were first analyzed by PCR for the presence of the barcode plus kanamycin resistance gene in the correct genomic locus with the primers BC-F and BC-R, and, if positive, analyzed for barcode sequences by Sanger sequencing of the PCR products. Finally, we isolated and confirmed 10 barcode-tagged strains carrying unique barcodes. Six of them were used in this study, and the barcodes in the respective strains were: BC1 (CTCCAA), BC2 (TGTCAT), BC3 (ACGGGC), BC4 (CACCCG), BC5 (CTCATC), and BC6 (GCCGAC).

3.3.4 Chicken Infection Experiments

All animal procedures in this study were conducted in accordance with the protocol approved by the University of Arkansas Institutional Animal Care and Use Committee. In all experiments, day-of-hatch broiler chicks were obtained from Cobb-Vantress (Siloam Springs, AR). To test the utility of the barcode-tagged strains for quantitative tracking of *Salmonella* transmission, we set up three independent experiments as describe below.

3.3.5 Oral Infection into Seeder Chickens Experiment 1.

Six chickens were randomly selected for oral infection with *Salmonella* barcode-tagged strains (referred to as seeder chickens hereafter) on day 1. Three of the chickens (seeder chickens #1 through 3) were orally infected at low dose (10^3 CFUs) with BC1, BC2 and BC3 strains, respectively. The other three chickens (seeder chickens #4 through 6) were orally infected at high dose (10^5 CFUs) with BC4, BC5 and BC6 strains, respectively. The other 10 chickens were not infected with any barcode-tagged strains, and were referred to as contact chickens (# 7 through 16). Seeder and contact chickens were housed together for the 14 days. On the day 7 post-infection three contact chickens (# 7 through 9) were euthanized and cecal contents were removed and stored at -20°C for genomic DNA isolation. Each bird foot was washed thoroughly in 5 ml of PBS buffer in a sterile Ziploc bag, and bacterial cells from the rinse were subsequently harvested via centrifugation at 5, 000 rpm for 10 mins. The bacterial pellets were stored at -20°C and used for genomic DNA isolation. On the day 14 post-hatch, four seeder chickens (chick 1, 4, 5, 6; chick #2 and 3 were not sampled) and four contact chicks (chick 10 through 13) were also euthanized and cecal contents as well as foot wash were collected for DNA isolation as describe previously.

3.3.6 Consumption of Contaminated Feed Experiment 2.

The same six barcode-tagged strains were used to inoculate a balanced antibiotic-free corn/soybean based diet at 2 different levels: at low dose (103CFUs) with BC1, BC2, and BC3 strains, respectively, and at high dose (10^5 CFUs) with BC4, BC5, and BC6 strains, respectively. To minimize the volume of the liquid inoculum, the cell suspension of each barcode-tagged strain was concentrated to contain the target cell number in 1 μl inoculum. Feed (1.36 kg) on the feeder was inoculated with 1 μl of the inoculum for each of the six barcode-tagged strains. Sixteen chickens were allowed to consume this contaminated feed for 48 hours. After two days, the contaminated feed was replaced by *Salmonella*-free feed and water *ad libitum*. On day 7 and 14, two and four chickens were euthanized, respectively. For each euthanized bird, both ceca and foot-wash samples were collected and processed by the same procedures described previously.

3.3.7 Drinking Water Administration Experiment 3.

This experiment was set up essentially in the same way as Experiment 2, except that the six barcode-tagged strains were added to and mixed in 11.36 liters of drinking water. Chickens (n= 16) were allowed to drink *ad libitum* this contaminated water for 48 hours. After two days the contaminated water was replaced with *Salmonella*-free fresh water. On the day 7 and 14 four chicks were euthanized respectively. Cecal and foot-wash samples were collected and processed by the same procedure described previously.

3.3.8 Illumina Sequence Sample Preparation

Genomic DNA was isolated from each sample using QIAamp DNA MiniKit (Qiagen). The concentration of purified DNA was measured by a Qubit®3.0 Fluorometer (ThermoFisher Scientific). Subsequently, the barcode regions in the extracted genomic DNA of each sample were

amplified using the primers BC-F and BC-R (Table 1), and G2 PCR mixture (Promega) with an initial incubation of 2 min at 95°C followed by 35 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C followed by a 10 min extension at 72°C. The PCR products of 191 bp were purified by using a QIAquick PCR purification kit (Qiagen) for use as a template in the next round of PCR. The second step PCR was conducted to attach Illumina-specific sequences along with the combinatorial sample index sequences (6 nt) on both ends using the Illumina Index forward and reverse primers shown in Table 1. A total of 9 Illumina Index forward and 6 Illumina Index reverse primers were used, allowing up to 54 (9 x 6) samples to be sequenced simultaneously. The resulting amplicons of 167 bp were purified by ethanol purification method and were pooled together to generate an amplicon library for MiSeq sequencing with single-end read option via 150 cycles.

3.3.9 Analysis of DNA sequencing results

Custom Perl script was used to perform the following data analysis: First, the barcode regions of 57 bp in the sequence reads from Illumina MiSeq data were extracted. The 12 bp-index sequences were obtained by extracting and combining forward Index sequence (6 bp) and reverse index sequence (6 bp), and used to sort the barcode reads to different samples. The 6 different barcodes were subsequently extracted and used to determine the relative abundance of different barcode-tagged strains in each sample.

3.4 Results and Discussion

3.4.1 Quantitative Profiling of Barcode-tagged Strains

A total of 1,461,014 sequence reads of 150 bp was obtained from the MiSeq sequencing run. The sequence reads were binned into different files according to the combinatorial index sequences corresponding the samples from the three experiments. If any reads did not match perfectly to one

of the original six barcode sequences they were subsequently deleted. Since the read numbers reflect only relative frequency of each barcode-tagged strain in a given sample, the original read numbers were converted to calculate the percentage of each barcode-tagged strain in each sample.

3.4.2 Experiment 1: *Salmonella* Transmission after Oral Infection

The results of the transmission of the SE barcode-tagged strains the cecal content and foot wash of seeder chickens on Day 14 from Experiment 1 are summarized in Table 2. For cecal samples of the seeder bird #1, which was infected with BC1 strain at low dose (10^3 CFUs), the BC1 strain was the predominant colonizer (46.37 %), however, the other strains challenged at a higher dose (10^5 CFUs) were also recovered from cecal content of chicken 1: BC3 (20.40 %), BC4 (29.63 %), and BC5 (3.59 %). These results suggest that a significant mixed infection by different *S.* Enteritidis BC strains could occur when the chick was infected by barcode-tagged strains at low dose and subsequently comingled with other infected chickens. The barcode-tagged strains used in this study are isogenic strains with the identical genome sequence except for the barcode region. Therefore, it is possible that the multiple barcode-tagged strains may be recognized as the same strains from each other and/or by the host, leading to avoidance of the exclusion mechanism(s) observed among different strains as has been described previously in chickens and mammals (29 - 31). In the seeder chickens #4, #5 and #6 infected by respective barcode-tagged strains at high dose, the barcode-tagged strains used for infection were the dominant strains (93.21%, 98.56%, and 99.94%, respectively) in the ceca (Table 2). It appears that barcode-tagged strains introduced at high dose saturated all potential colonization niches, thus impeding colonization by other strains. This phenomenon observed in the chicks infected by a high dose of *Salmonella* is consistent with the colonization-inhibition theory (29, 30). In conclusion, these results suggest that the outcome of cecal colonization in terms of the number of the barcode-tagged strains colonizing the ceca is

dose-dependent, and a high dose beyond a certain threshold level results in dominant colonization by a single strain.

Contamination of feet by dominant barcode-tagged strains occurred for the seeder chickens #1, #4 and #6 (93.57 % of BC6, 99.55 % of BC5, and 78.86 % of BC6, respectively), but they were not necessarily the same strains used for infection of the same chickens (Table 2). For the case of the seeder #5, the foot was contaminated by three strains, BC4, BC5, and BC6 strains (36.77 %, 52.97 %, and 21.10 %, respectively) among which BC5 was the one used for oral infection of the bird. The vast majority of the strains contaminating feet were those used for infection at high dose (BC4, BC5, and BC6), which indicated that high dose of *Salmonella* BCs is widely disseminated in the environment and thus may frequently be isolated from the feet. However, there is no correlation between the orally infected strain and dominant strain occurring on the feet. It is possible that the major strain isolated from the feet is from the environment instead of coming from chick itself.

Figure 2 shows the results of the transmission of the *S.* Enteritidis barcode-tagged strains in the oral infection model in contact chickens. For the contact chickens, almost all (99%) of the barcode-tagged strains colonizing ceca on day 7 were strains administered at high dose, namely BC4-6. However, on day 14, a more diverse set of barcode-tagged strains were detected from the ceca of contact chickens, including a greater proportion of the barcode-tagged strains that were used to infect seeder chickens at low dose (BC1-3). It seems that the contact chickens are more likely to be colonized by the strains initially used for infection at high dose, but they eventually become colonized in the ceca also by the strains originating from the low dose as time progresses (Figure 2). In contrast, foot-wash samples from all contact chickens did not reveal any obvious trends as compared to those observed in cecal samples. On day 7, BC3 strain, which was

administered at low dose, was the only strain (100%) contaminating the foot of the contact bird #7. Conversely the feet of the contact chickens #8 and 9 were colonized mainly by the two strains, BC4 and BC6, which were used for infection at high dose. After the passage of time the barcode-tagged strain populations on the feet of the contact chickens became more diverse on day 14. Comparing the relative abundance between day 7 and 14 indicated that the barcode-tagged strains that were used for infection at low dose increased the chances to contaminate the feet with the exception of BC3, which was not detected on the feet of any bird on day 14.

3.4.3 Experiment 2: *Salmonella* Transmission after Infection through Contaminated Feed

The results of the transmission of the SE barcode-tagged strains in a feed contamination model (Experiment 2) are shown in Figure 3. On day 7 the ceca from the two chickens were colonized mainly by the barcode-tagged strains that were introduced at the higher dose. On day 14 the ceca from the bird #4 and 6 were predominantly colonized by BC3 (91%) and BC2 (94%) (both were introduced at a low dose), respectively, while the bird #5 was exclusively colonized by BC5. On day 14, only bird #3 showed colonization by multiple strains, mostly BC1 (44%) and BC6 (51%) strains. By comparing the combined percentages of the low *versus* high dose strains in the ceca at the day 7 (0 *versus* 100%) and day 14 (60% *versus* 40%), it is apparent that the strains introduced to feed at a low dose eventually colonized the ceca, but it required a much longer period of time when compared to the strains introduced at high dose. Greater diversity of the strains was also detected at the day 14 as compared to the day 7 for the feet samples with the exception of the bird #3 (Figure 3).

3.4.4 Experiment 3: *Salmonella* Transmission after Infection through Contaminated Drinking Water

Figure 4 summarizes the results of the transmission of the SE barcode-tagged strains in water contamination model (Experiment 3). When the chicks were infected through contaminated drinking water, only three barcode-tagged strains (BC2, BC3, and BC6), representing both the strains that had been introduced at low and high dose, were recovered from the ceca on day 7 and 14. Strain BC6 (high dose), which was the predominant cecal colonizer, was also detected as the predominant strain contaminating the feet. Interestingly, BC1 (a low dose challenge strain), even though not detected in the ceca of any chicken at any time, was recovered as the predominant strain in the feet of the chickens (Figure 4). Since only 8 chickens were analyzed out of the total of 16 chickens, BC1 is probably the predominant colonizer in at least one of the remaining chickens that was not used for sample collection.

3.5 Conclusions

Salmonella transmission in chicken flocks have already been the subject of several studies in which the *Salmonella* strains introduced to the flock were identified and quantified by culturing on selective agar plates and confirmed by biochemical and serological methods (48-53). In the studies conducted by De Vylder et al. (50) and Thomas et al. (51, 52) single *Salmonella* Enteritidis strains were used to analyze different aspects of *Salmonella* transmission within the laying hen flocks. These approaches have been useful in understanding the impact of different phage type strains or housing system on the frequency of horizontal transmission (50, 53, 54) or measuring different parameters of *Salmonella* transmission (51). However, detailed picture of the transmission involving interactions among multiple strains or serotypes cannot be investigated using the culture methods, due to the inability to differentiate multiple strains based on the culture methods.

Several investigators have study the persistence of horizontal fecal shedding of *Salmonella* Enteritidis in experimentally infected laying hens housed on different commercial conditions (53, 54). However, these studies are still limited to reflect the complexity of the environmental conditions that *Salmonella* is exposed to during transmission in a poultry farm. The other weakness of culture method approaches is that the isolated strains may be from environment rather than the strain externally introduced as a part of an experimental infection, thus handicapping the ability to differentiate the corresponding strain. Even though the strain might be confirmed as an experimental strain by further characterization, the result can only indicate the presence of the strain and reliable quantification is not possible

In order to quantitatively track the *Salmonella* transmission routes from environment to flock, we constructed a series of barcode-tagged strains, which carry distinct barcode tags that would allow them to be identified and quantified accurately by high-throughput sequencing of the barcode regions. The similar methods of barcode-tagging have been applied to understand the transmission dynamics within the infected hosts for *Salmonella* (32, 33), other pathogenic bacteria (28) and viruses (35). However, to our knowledge this is the first report on the application of the barcode-tagged strains to study transmission dynamics within a population of the host animals. In this study, we used the barcode-tagged strains of *S.* Enteritidis to understand the transmission dynamics of *Salmonella* in a quantitative manner after initial introduction through oral infection, or consumption of contaminated feed or drinking water.

In the current study six barcode-tagged *S.* Enteritidis strains were employed to infect six chickens (seeder chickens) orally in oral infection experiment. In contaminated feed and water study the same six barcode-tagged strains were introduced into feed or water in each isolator. Following the exposure via different routes, the corresponding distributions of the six different

barcode-tagged strains at different colonization sites (ceca and feet) were analyzed at different time points post-infection.

Utilizing PCR and Illumina MiSeq analysis the population structure could be assessed and representative transmission figures could be constructed. The results are important for understanding the patterns of *S.* Enteritidis dissemination in poultry and are revealing by demonstrating that a higher dose of *S.* Enteritidis has a greater opportunity to infect flocks. In addition, the data from this study suggests that colonization-inhibition by competing *Salmonella* is somewhat dosage dependent. Based on qPCR result for quantification of the combined load of all barcode-tagged strains (not shown) it appears that recovery of *S.* Enteritidis barcode-tagged strains introduced orally were not different among the seeder chickens and contact chicks in both cecal and foot-wash samples on day 14. All barcode strains combined in the cecal samples remained stable on days 7 and 14 in Experiment 1, while those from foot-wash samples increased 10 fold in the three experiments after time had elapsed.

To better establish the implications for commercial poultry production settings, larger scale experiments are needed to assess additional environmental and host factors. However, the current experiment demonstrated the proof of concept that the use of barcode-tagged strains is a novel and an effective approach to understand the dynamics of *Salmonella* transmission within a chicken flock and can provide valuable insights for the potential to develop and optimize measures that protect host animals from infection with *Salmonella*. Studies to evaluate and confirm previous work published by our laboratory (14-16, 20) that demonstrate the importance of airborne transmission of *Salmonella versus* oral infection as well as the competitive exclusion concept of *Salmonella versus Salmonella* (55, 56) or cross protection (57-59) using these SE barcode-tagged strains are currently being evaluated.

3.6 Acknowledgements

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3.8 Figures and Tables

Table 1. Oligonucleotides used in this study

Primers for construction of barcode-tagged strains (5'→3')	
T1-F	GCAAGGTTGGTGTCTGTCCT
T1-BC-P1-R	GAAGCAGCTCCAGCCTACACNNNNNNATTATTGTTAATTTATTCTT
P1	GTGTAGGCTGGAGCTGCTTC
P2	ATGGGAATTAGCCATGGTCC
T3-P2-F	GGACCATGGCTAATTCCCATAAAGGTTAAGCAGTGACCCA
T3-R	GTTGATGGACTGGGTTCGTT
BC-F	AGCGTCCTGAAATAATAAAAGAA
BC-R	CGGACTGGCTTTCTACGTGT
Illumina Index Forward Primers (5'→3') *6nt-index sequences are underlined.	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATCACGGCGTCCTGAAATAATAAAAGAATAAA	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATGTGCGTCCTGAAATAATAAAAGAATAAA	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTTAGGCGCGTCCTGAAATAATAAAAGAATAAA	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTGACCAGCGTCCTGAAATAATAAAAGAATAAA	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACATGTGCGTCCTGAAATAATAAAAGAATAAA	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCAATGCGTCCTGAAATAATAAAAGAATAAA	
Illumina Index Reverse Primers (5'→3') *6nt-index sequences are underlined.	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTATCACGGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTCGATGTGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTTTAGGCGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTTGACCAGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTACATGTGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTGCCAATGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTCAGATCGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTACTTGAGAAGCAGCTCCAGCCTACAC	
CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTGATCAGGAAGCAGCTCCAGCCTACAC	

Table 2. Relative abundance of the *Salmonella* Enteritidis (SE) barcode-tagged strains in seeder chickens on Day 14 in the cecal content and foot wash from Experiment 1.

Ceca Content						
	BC1	BC2	BC3	BC4	BC5	BC6
Chicken 1 BC1/ 10^3	46.37 %	0.00 %	20.40%	29.63 %	3.59 %	0.01 %
Chicken 4 BC4/ 10^5	0.04 %	0.004%	0.00%	93.21 %	6.74 %	0.01 %
Chicken 5 BC5/ 10^5	0.04 %	0.01%	0.27%	0.00 %	98.56 %	1.12 %
Chicken 6 BC6/ 10^5	0.03 %	0.00%	0.004%	0.00 %	0.03 %	99.94 %
Foot Wash						
	BC1	BC2	BC3	BC4	BC5	BC6
Chicken 1 BC1/ 10^3	0.03 %	6.37 %	0.00 %	0.00 %	0.02 %	93.57 %
Chicken 4 BC4/ 10^5	0.42 %	0.01 %	0.00 %	0.01 %	99.55 %	0.00 %
Chicken 5 BC5/ 10^5	0.04 %	0.003 %	0.00 %	36.77 %	52.97 %	10.21 %
Chicken 6 BC6/ 10^5	0.03 %	0.005 %	0.00 %	0.002 %	21.10 %	78.86 %

Six chickens were randomly selected for oral infection with *Salmonella* barcode-tagged strains on day 1. Chickens 1 through 3 were orally infected with 10^3 CFUs with BC1, BC2 and BC3 strains, respectively. Chickens 4 through 6 were orally infected with 10^5 CFUs with BC4, BC5 and BC6 strains, respectively. At 14 days post challenge, cecal content or foot wash sample were collected from each chicken and used for isolation of genomic DNA. Following PCR and MiSeq analysis of barcode regions, to the number of the sequence reads corresponding to different barcodes were used to determine the relative abundance (%) of each SE barcode strain from each chick. Chickens 2 and 3, orally gavaged with BC 2 and BC 3 were not sampled in this experiment.

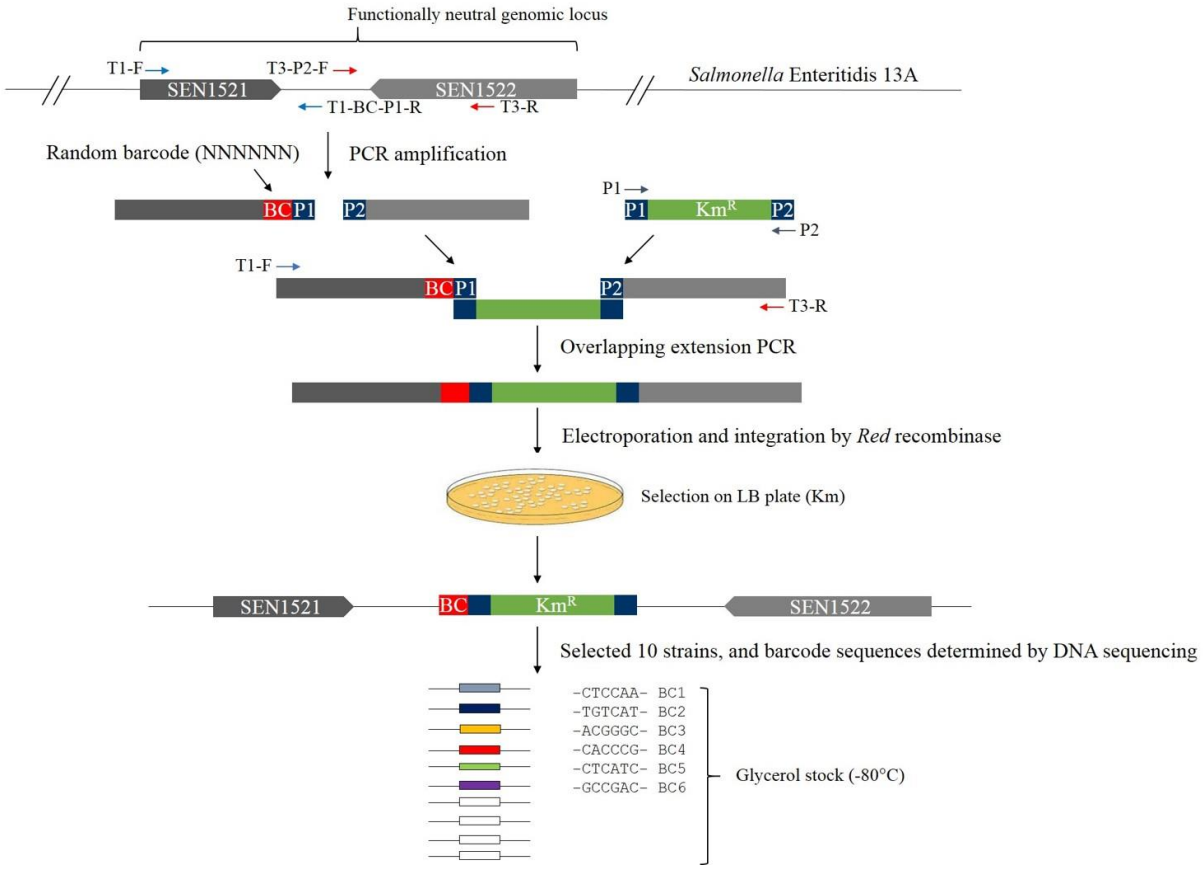

Figure 1: Schematics of the construction of barcode-tagged *Salmonella* Enteritidis 13A strains

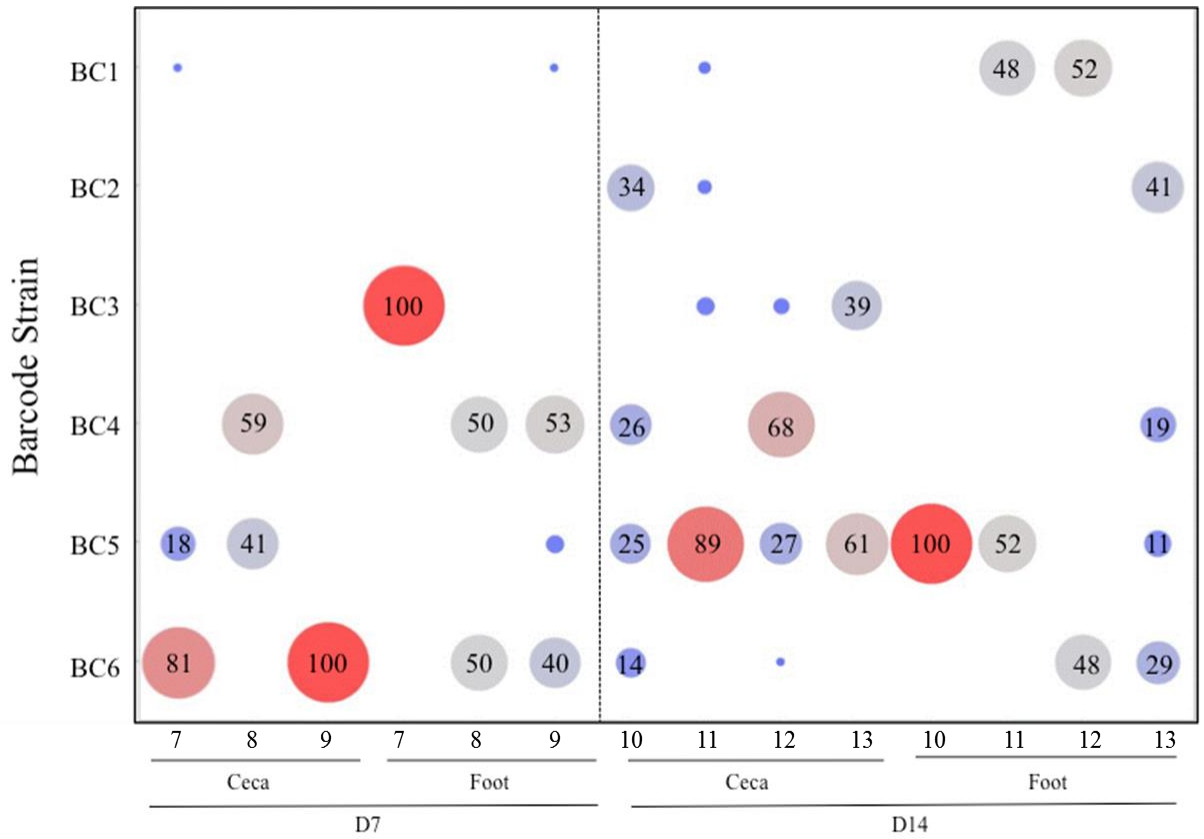

Figure 2. Transmission of the *Salmonella* barcode-tagged strains in contact chickens in oral infection model. In Experiment 1 six seeder chickens were infected by different dose of SE barcode strains (BC1 and BC2 and BC3 are used for infection of three chickens at 10^3 CFUs; BC4 and BC5 and BC6 are used for infection of other three chickens at 10^5 CFUs). Other 10 chickens were roomed together with these six seeder chickens and named as contact chickens. Three contact chickens were euthanized on day 7 and four contact chickens were euthanized on day 14. The cecal tonsil and foot wash samples were collected from each chicken by aseptic technique. X-aixs represents different contact chickens from Experiment 1 and y-axis represents different SE barcode strains. The number in bubble presents the relative abundance of each barcode strain in each chicken. Bigger size and red color means the higher relative abundance, and smaller size and blue color means lower relative abundance.

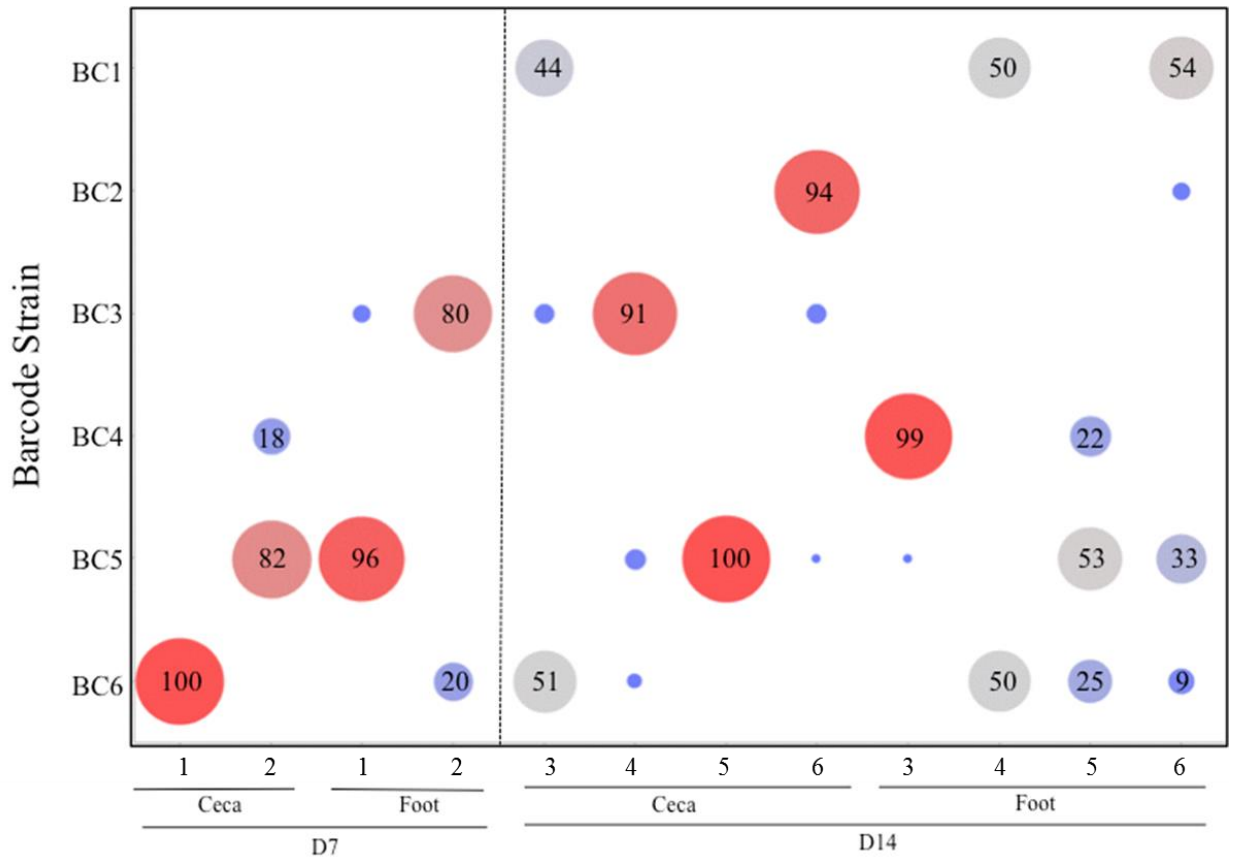

Figure 3. Transmission of the *Salmonella* barcode-tagged strains in feed contamination model. In Experiment 2 the feed was contaminated by 6 SE barcode strains at two doses (BC1 and BC2 and BC3 were introduced into the feed at 10^3 CFUs, BC4 and BC5 and BC6 were at 10^5 CFUs on day 1). Two chickens were euthanized on day 7 and the other four chickens were euthanized on day 14. The cecal and foot wash samples were collected from each chicken by aseptic technique and used for isolation of genomic DNA. Following PCR and MiSeq analysis of barcode regions, to the number of the sequence reads corresponding to different barcodes were used to determine the relative abundance (%) of each SE barcode strain from each sample.X-axis represents different chickens from Experiment 2 and y-axis represents different SE barcode strains. Bigger size and red color means the higher relative abundance, and smaller size and blue color means lower relative abundance.

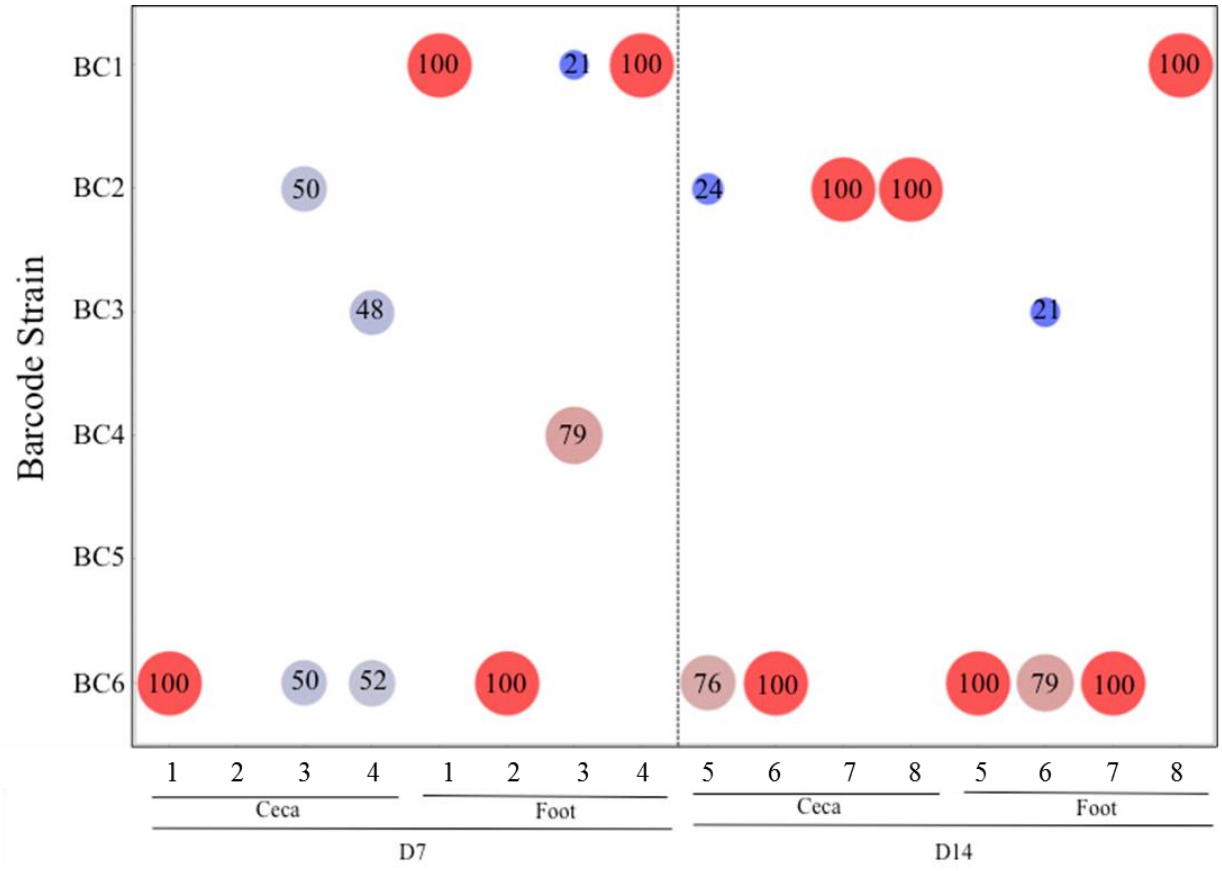

Figure 4. Transmission of the *Salmonella* barcode-tagged strains in water contamination model. In Experiment 3 the water was contaminated by 6 SE barcode strains at two doses (BC1 and BC2 and BC3 were introduced into the water at 10^3 CFUs, BC4 and BC5 and BC6 were at 10^5 CFUs on day 1). Four chickens were euthanized on day 7 and the other four chickens were euthanized on day 14. The cecal and foot wash samples were collected from each chicken by aseptic technique and used for isolation of genomic DNA. Following PCR and MiSeq analysis of barcode regions, to the number of the sequence reads corresponding to different barcodes were used to determine the relative abundance (%) of each SE barcode strain from each sample. X-axis represents different chickens from Experiment 2 and y-axis represents different SE barcode strains. Bigger size and red color means the higher relative abundance, and smaller size and blue color means lower relative abundance.

CHAPTER FOUR

Salmonella excludes *Salmonella* in poultry*:* Confirming an old paradigm using conventional and barcode-tagged isogenic strains

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4.1 Abstract

Salmonella is one of the major foodborne pathogens and chickens play an important role as a reservoir for introducing *Salmonella* from the environment into becoming a public health concern. However, the mechanism of *Salmonella* transmission within the chicken flock is not fully understood, including the competitiveness among *Salmonella* strains when infecting and colonizing chickens. The purpose of the present study was to evaluate the competitive exclusion between *Salmonella spp.* consecutively challenged through the oral route. Different approaches were used to evaluate the competitive exclusion effect including tracking *Salmonella* transmission by wild-type strains or DNA barcode-tagged isogenic strains. Using wild-type strains, chicks were predominantly colonized by the first serovar administered at day one, ranging from 82.5 to 86.7% of recovery. A similar trend was also found by using barcode-tagged strains ranging from 91.3 to 97.8% of recovery of the first barcoded strain administered. However, mixed colonization by two barcoded strains existed when these strains were given on the second day. These results provide quantitative evidence for the competitive exclusion theory that oral administration of *Salmonella* will produce predominant inhibition over the subsequent colonization of ceca by the following *Salmonella* strain administered one day later. Therefore, utilizing barcode-tagged isogenic strains and sequencing by Illumina sequencing can serve as a quantitative method for studying *Salmonella* infection, transmission and colonization in poultry.

4.2 Introduction

As the highest foodborne illness cases caused by *Salmonella*, prevention of *Salmonella* colonization in the gastrointestinal tract (GIT) of chickens is necessary, especially for newly hatched chickens. The reason is that the normal microbiota cannot be fully developed until 6 weeks

of age, which caused chickens to be vulnerable and susceptible of infection by *Salmonella* (Barnes *et al.*, 1972).

The live attenuated *Salmonella* vaccine strain has been identified as an effective approach for controlling the colonization of pathogenic *Salmonella* in the GIT by potentially acting as competitive exclusion (CE) and triggering the immune system (Methner et al 2011). The characteristic of these vaccines should be nonpathogenic, be able to colonize in the GIT, therefore exclude other *Salmonella* pathogenic strains. Dueger et al. (2002) constructed the *Salmonella* Typhimurium vaccine strain by deleting the DNA adenine methylase genes and the vaccine strain are demonstrated to provide cross-serotype protection against oral challenge with homologous and heterologous *Salmonella* serovars in mice and chickens.

These vaccine strains reduce the ability of *Salmonella* colonization in the chickens may be via stimulating cell-mediated immunity (Babu *et al.*, 2004; Lillehoj *et al.*, 2000). Another possibility is that vaccination may alter the microbial composition and diversity in the chicken GIT (Park *et al.*, 2017). Crhanova et al. (2011) also suggested that attenuated *Salmonella* vaccines are able to modify the chicken gut microbiota, enhance the gut immune system maturity, and then increase resistance to infection by pathogenic strains. However, these mechanisms may not be the only reason that a decrease *Salmonella* colonization occurs in the chickens, because the immunity stimulation and microbiota change take a longer time than competitive exclusion mechanism. Methner et al. (1999) proposed that vaccination of chicks at day one age ensures the colonization by the vaccine strain, which produces an inhibitory effect and stimulates the development of an immunological response to the following infection.

Barrow et al. (1987) also demonstrated that oral administration of live strains of foodborne pathogenic *Salmonella* to day-old chickens produced inhibition in the subsequent colonization of

the ceca by a strain of *Salmonella* Typhimurium (ST) given one day later. Remarkably, closely related enterobacteria were unable to induce the same effect (Barrow et al., 1987). More recently, Rabsch et al. (2000), with mathematical models that combined epidemiology and population biology, demonstrated that *Salmonella* Enteritidis (SE) filled the ecological niche vacated by eradication of *Salmonella* Gallinarum (SG) and *Salmonella* Pullorum (SP) from poultry. The theory suggests that SG was able to competitively exclude SE from poultry flocks in the early 20th century, and the elimination of SG and SP in poultry led to an epidemic increase of SE in poultry and human infections in the 80's. The cross-immunity between two serovars is probable. Immunization of chickens with SG protects against colonization by SE, instead of ST, is possible because SG and SE have the same immunodominant O-antigen on their cell surface (17-23 from Rabsch 2000). Holt and Gast 2004 estimated that prior infection of hens with ST or *Salmonella* Muenchen (SM) can reduce the infection by SE, which indicated that cross-serovar protection existed among ST, SM and SE. *In vitro* experiment conducted by Calo et al. (2014) exhibited a decrease growth of ST when *Salmonella* Heidelberg is present in the spent media.

Based on the CE phenomenon that may exist between the same or different serovars, it is important to employ an accurate approach and quantitatively measure CE. For analyzing the CE theory, antibiotic marker strains are widely used as an approach, however, these marker strains do not always behave in exactly same fashion as the corresponding wild type strains (Nutt et al. 2003). Hence, it is critical to use the isogenic strains for studying CE to decrease the bias from different infection strains. We have constructed a series of isogenic SE barcode-tagged strains in which distinct DNA barcodes were inserted in a functionally neutral locus in the genome of SE and the resulting strains can be used to quantitatively track the colonization of the respective strains by profiling the barcode-regions using a high-throughput sequencing method (Yang et al., 2017). The

advantages of these barcode-tagged strains over previously used marker strains are that each strain can be tracked quantitatively as a distinguishable part of the entire population at high accuracy, allowing for differentiation among multiple barcode-tagged strains as well as discrimination from the environmental bacteria or viruses without altering phenotypes or behaviors during infection, colonization and dissemination (Yang et al., 2017)(Varble et al., 2014)(Troy et al., 2013). The purpose of the present study was to evaluate the competitive exclusion of *Salmonella* consecutively or simultaneously challenged with SE or ST in poultry using conventional and barcode-tagged isogenic strains.

4.3 Materials and Methods

4.3.1 Project 1: Recovery of *Salmonella* from Leghorn Chicks Consecutively or Simultaneously Challenged with SE or ST

4.3.1.1 *Salmonella* Cultures

A highly invasive poultry isolate of SE was obtained from the USDA National Veterinary Services Laboratory (Ames, IA 50011). The isolate was selected for resistance to 25 µg/mL novobiocin (Sigma, St. Louis, MO) and 20 µg/mL nalidixic acid (NA, catalog no. N-4382, Sigma). This strain of SE was also found to be naturally sensitive to 10 µg gentamicin (GM10) on antibiotic sensitivity discs. An invasive isolate of ST (ATCC13311) was also selected for resistance to NO and NA for use in these experiments. This ST was resistant to GM10. For the present studies, 100 µL of SE or ST (depending on the trial) from a frozen aliquot was added to 10 mL of tryptic soy broth (TSB, catalog no. 211822, Becton Dickinson, Sparks, MD) and incubated at 37°C for 8 h. This was followed by 3 passages every 8 h into fresh TSB, for a total of 24 h, to ensure log phase growth. Post incubation, bacterial cells were washed 3 times in sterile 0.9% saline by

centrifugation (1,864 × g, 4°C, 15 min), quantified with a spectrophotometer (Spectronic 20D+, Spectronic Instruments Thermo Scientific, Waltham, MA) at 625 nm using an established concentration curve, and diluted in sterile 0.9% saline as per required concentrations (cfu/mL) for the trials. Concentrations of SE or ST were also determined retrospectively by serial dilution and further plating onto brilliant green agar (BGA, catalog no. 70134, Sigma) with NO and NA for enumeration of actual cfu per milliliter used for challenge, as reported below.

4.3.1.2 Experimental Birds

Naïve, day-old single comb white Leghorn male chicks were obtained from a local hatchery and randomly placed in cages (N=10 or 20 chicks/cage) within electrically heated starter batteries located within a modern biological hazard isolation unit on the research farm of the College of Veterinary Medicine, Texas A & M University. Chickens were provided *ad libitum* access to water and a balanced unmedicated corn-soybean diet meeting the nutrition requirements of poultry recommended by the NRC (1994). Adequate body temperature was maintained using heat lamps within the isolators. All animal handling procedures were in compliance with Institutional Animal Care and Use Committee at Texas A & M University. Twelve chickens for each trial were humanely killed and sampled upon arrival at the laboratory. Whole ceca-cecal tonsils (CCT), liver, spleen and yolk sac were aseptically removed from these neonatal chicks, incised, and cultured in 10 mL of tetrathionate enrichment broth (TEB) (Tet, catalog no. 210420, Becton Dickinson, Sparks, MD) and incubated overnight at 37°C. The samples were confirmed negative for *Salmonella* by plating them on to selective BGA with NO and NA.

4.3.1.3 Experimental design

Experiment 1 consisted of two independent trials. In trial 1, on day one, two groups were orally gavaged with 10^4 cfu/mL ST. On day two, two groups were orally gavaged with 10^5 cfu/mL SE. Twenty-four hours post challenge, chickens were euthanized and cultured for *Salmonella* recovery in CCT. Briefly, whole CCT were enriched in tetrathionate enrichment broth (TET, catalog no. 210420, Becton Dickinson, Sparks, MD) and incubated for 24 h at 37°C. Samples were taken from the enriched broth and subcultured on BGA plates containing 20 µg/mL NA and 25 µg/mL NO for approximately 18 hours at 37 °C. Ten isolated colonies from the control groups were taken from each BGA and lined onto Mueller-Hinton plates (Finegold and Baron, 1986). Twenty isolated colonies from the treatment groups were lined onto Mueller-Hinton plates. An antibiotic sensitivity disc of GM10 was placed on each line and the results tabulated 24 hours later as either Resistant=ST or Sensitive=SE. Approximately 20 isolates were randomly selected during these experiments and serogrouped using commercially available antisera to verify accuracy of serovar separation. Trial 2 was performed in the same manner as trial 1 except that SE was used for the first challenge (10^4 cfu/mL) on day one, which was followed by ST challenge (10^5 cfu/mL) on day 2.

Experiment 2 includes independent trial 3 and 4. Trial 3 includes 3 groups. On day one, group 1 (N=20) were orally gavaged with 2×10^4 cfu ST, group 2 (N=20) were orally gavaged with 2×10^4 cfu SE, and group 3 (N=20) were orally gavaged with 2×10^4 cfu SE and 2×10^4 cfu ST simultaneously. Twenty-four hours later, the chicks were euthanized and cultured for *Salmonella* recovery from CCT. Trial 4 was performed in the same manner as trial 3 except that SE and ST were administrated on day 2.

4.3.2 Project 2: Recovery of Barcode-tagged Isogenic Strains with Illumina Sequence PCR in Day-old Broiler Chickens

4.3.2.1 Construction of Barcode-tagged Strains

The method for construction of barcode-tagged strains is described in a previous publication (Yang et al., 2017). Briefly, SE containing pKD46 that encodes Red recombinase system was used for construction of barcode-tagged strains *via* electroporation. Overlapping extension PCR was employed to join the three fragments (upstream fragment plus a 6 nt random barcode + Km resistance gene + downstream fragment) together. After electroporation, the mutants carrying the barcode sequence along with the kanamycin resistance gene inserted into a functionally neutral genomic locus, SEN1521 and SEN1522, were selected and used in this study as previously described in detail (Yang et al. 2017).

4.3.2.2 Bacterial Strains and Culture Condition

SE barcode-tagged isogenic strains were incubated overnight at 37°C in Luria-Bertani (LB) broth with kanamycin (50 µg/ml), and were harvested by centrifuge at 4°C. The cell pellet was washed three times and resuspended in distilled 0.9% saline. A suspension of 10^8 colony forming units (cfu)/ml was obtained by using a spectrophotometer to agjust OD_{625} =0.147. The suspension inocula were subsequently diluted into 10^5 cfu/ml for infecting chicks.

4.3.2.3 Experimental Birds

Day-of-hatch, male broiler chickens were obtained from Cobb-Vantress (Siloam Springs, AR) and placed in floor pens with a controlled age-appropriate environment. Chickens were provided *ad libitum* access to water and a balanced unmedicated corn-soybean diet meeting the nutrition requirements of poultry recommended by the NRC (1994). Adequate body temperature

was maintained using heat lamps within the isolators. All animal handling procedures were in compliance with Institutional Animal Care and Use Committee at the University of Arkansas. Twelve chickens for each trial were euthanized and sampled upon arrival at the laboratory as described in experiment 1.

4.3.2.4 Experimental Design

This experiment was set up to confirm if oral administration with one SE barcode-tagged strain inhibits the colonization by the other SE barcode-tagged strain in the ceca of chickens. A total of 90 day-of-hatch broiler chicks were randomly separated into six groups (N = 15 chicks/group). In Group 1, all chickens were orally gavaged with 10^5 cfu/mL of SE barcode-tagged strain BC1 on day 1 and on day 2 they were gavaged with 0.9% sterile saline. In Group 2, all chickens were orally gavaged with 10^5 cfu/mL of SE barcode-tagged strain BC2 on day 1 and on day 2 they were gavaged with sterile saline. In Group 3, all chickens were orally gavaged with 0.9% sterile saline on day 1 and on day 2 they were gavaged with 10^5 cfu/mL of SE barcode-tagged strain BC1. In Group 4, all chickens were orally gavaged with 0.9% sterile saline on day 1 and on day 2 they were gavaged with 10^5 cfu/mL of SE barcode-tagged strain BC2. In Group 5, all chickens were orally gavaged with 10^5 cfu/mL of SE barcode-tagged strain BC1 on day 1 and on day 2 they were gavaged with 10^5 cfu/mL of SE barcode-tagged strain BC2. In Group 6, all chickens were orally gavaged with 10^5 cfu/mL of SE barcode-tagged strain BC2 on day 1 and on day 2 they were gavaged with 10^5 cfu/mL of SE barcode-tagged strain BC1. On day three, 12 chickens from each group were euthanized. Cecal contents and liver/spleen were collected, smashed, and suspended in 0.9% saline in 1:4 ratio in sterile bags. One mL of suspension from each bag was collected for genomic DNA isolation.

4.3.2.5 Illumina Sequence Sample Preparation and Analysis of DNA Sequencing results

These procedures are the same as Yang et al. (2017). Briefly, genomic DNA were isolated from each sample, from which the barcode regions were amplified first by PCR. The PCR products were purified and used as the template in the next round PCR. The second step PCR was conducted to attach Illumina-specific sequences along with the combinatorial sample index sequences (6 nt) on both ends using the Illumina index forward and reverse primers. The resulting amplicons were isolated by the ethanol purification method and were pooled together to generate an amplicon library for MiSeq sequencing. Custom Perl script was used to perform the Illumina Miseq data analysis.

4.3.2.6 Statistical Analysis

The enrichment data were expressed as positive/total chickens (%) and the percent recovery of *Salmonella* was compared using the chi-squared test of independence, testing all possible combinations to determine the significance ($P \leq 0.001$) for these studies (Zar, 1984). Barcode % recovery data within experimental groups were subjected to one way ANOVA (SAS Institute, 2002). Barcode % recoveries were expressed as means ± SEM and deemed significant if $P \leq 0.001$. The data were also subjected to mean separation using Tukey's multiple range test significance.

4.4 Results

4.4.1 Recovery of *Salmonella* from Leghorn Chicks Consecutively or Simultaneously Challenged with SE or ST

The recovery of *Salmonella* in CCT from consecutive different *Salmonella* serovar challenges in day-old Leghorn chicks in trial 1 and trial 2 of Experiment 1 are shown in Table 1.

In both trials, each serovar was recovered from CCT following single administration respectively. However, when ST was administered on day one at 10^4 cfu, followed by the consecutive challenge of SE twenty-four hours later at 10^5 cfu, the birds were predominantly colonized by ST (81.6 %), the first serovar administered ($P < 0.001$). In no case was SE alone, the second serovar administered, isolated from any bird. Those colony isolates, which were serotyped as SE, came only from birds with mixed infections. In addition, these colony isolates were significantly lower for SE recovery (2.91 %) as compared to 100 % in the SE controls chickens. Similar effects were observed in trial 2, when chickens were challenged with SE on day one at 10^4 cfu followed by the consecutive oral challenge of ST on day two at 10^5 cfu. In this trial, SE, the first serovar administered, dominated (82.5 %) in those birds cultured. Colonies isolated as ST from CCT were significantly lower at 3% respectively as compared to serotyped ST controls at 100%.

Two simultaneous *Salmonella* serovar challenges were performed in experiment 2. On trial 3, chicks were challenged with 10^4 cfu of ST and 10^4 cfu of SE on day one. Ninety percent of the *Salmonella*-culture positive chicks exhibited mixed infections in CCT. Colony isolated from those chicks that received both serovars simultaneously exhibited a mixed isolate profile in the CCT. In trial 4, simultaneous *Salmonella* serovar challenges were performed on day 2 at 10^4 cfu. Overall results in CCT were similar as seen in trial 3, with mixed *Salmonella* serovars recovered.

4.4.2 Recovery of Barcode-tagged Isogenic Strains with Illumina Sequence PCR in Day-old Broiler Chickens

The results of the percentage *Salmonella* barcode strains recovered from cecal samples enumerated from Illumina sequence data in day-old broiler chickens in Experiment 2 are summarized in Table 3. A total of 3,138,578 sequence reads of 167 bp was obtained from the MiSeq sequencing run. The sequence reads were binned into different files according to the

combinatorial index sequences corresponding the samples from the four experiments. If any reads did not match perfectly to one of the original six barcode sequences they were subsequently deleted. Since the read numbers reflect only relative frequency of each barcode-tagged strain in a given sample, the original read numbers were converted to calculate the percentage of each barcode-tagged strain in each sample. Oral gavage of SE barcode-tagged strain BC1 on day one followed by saline on day two in group 1 resulted in 97.77 % BC1 recovery. Similarly, in group 2, administration of SE barcode-tagged strain BC2 on day one followed by saline on day two in group 2 resulted in 97.77 % BC2 recovery. However, oral gavage of barcode 1 on day one and barcode 2 on day two in group 5 resulted in 91.25 % barcode 1 recovery while only 0.5 % of barcode 2 was recovered. The opposite effect was observed when barcode 2 was administered first with 84.25 % recovery of barcode 2 and only 6 % of barcode 1 recovered from CCT. However, both SE barcode-tagged strains could be isolated from ceca in the chicks from group 3 and 4, even though only one single SE barcode-tagged strain was introduced on day two (Table 3). Figure 1 shows the percentage of each barcode-tagged strain in each cecal sample and figure 2 shows the percentage of each barcode-tagged strain in each liver/spleen sample.

4.5 Discussion

It has been estimated that foodborne infections in the US cause over seventy six million illnesses and are responsible for five thousand fatalities each year (Allos et al., 2004). In addition, the annual economic losses attributed to the four most common enteropathogens (*Salmonella spp.*, *Campylobacter* spp., *E. coli* and *Shigella*) have been estimated to reach $ 7 billion dollars (Archer and Kvenberg, 1985). Hence, understanding the mechanisms of pathogenesis of enteropathogens such as *Salmonella*, is still critical in order to find alternative methods to antibiotics that can eliminate or reduce these pathogens from poultry products (Mead et al., 1999).

Several investigators have also shown a significant protection from the second *Salmonella* challenge with sequential administration of *Salmonella* serovars in mice (Collins, 1968) or chicks (Barrow et al., 1990)(Barrow and Page, 2000)(Berchieri and Barrow, 1990). Early studies by (Howard, 1961; Collins, 1968a) indicated that prior infection with ST could protect mice from the intravenous challenge of another *Salmonella* serovar. Similar investigations have demonstrated that intravenous challenge of SG caused protection against subsequent intravenous challenge with SE (Collins et al., 1966). In their study, Collins et al. (1966) demonstrated that SG was able to persist in the tissues therefore protecting against SE challenge. However, SP, an antigenically similar organism, was unable to establish within the tissues which apparently allowed SE to colonize. In addition, live attenuated *Salmonella* vaccines provide protection from subsequent *Salmonella* challenges, without the virulence factor, within 4 weeks of vaccination (Copper et al., 1992) (Dougan et al., 2011)(Berchieri and Barrow, 1990). Similarly, the results of the present study confirm those by Barrow et al. (1987) in which day-old chicks which received sequential *Salmonella* challenge resulted in an almost exclusive infection within 24 hours. Hence, regardless of route of challenge, experimental animal or time between challenges, sequential *Salmonella* challenges allow chickens to become refractory to the second *Salmonella* serovar administered. The mechanism for resistance to sequential challenges has not been completely elucidated. Our results suggest that *Salmonella*-infected chicks become refractory to a second challenge serovar within 24 hours. These data confirm previous reports of rapid induction of resistance to consecutive *Salmonella* challenge.

However, several investigators have evaluated *Salmonella* transmission in commercial poultry flocks using conventional bacteriology and serological methods (Gast et al., 2014)(Grant et al., 2008)(Thomas et al., 2011). Even though these studies have helped in understanding the

impact of different phage type strains or housing system on the frequency of horizontal transmission, comprehensive understanding of the transmission and pathogenesis involving interactions among multiple serovar strains cannot be examined using the traditional culture methods. In the present study, the use of two barcode-tagged SE were used to investigate transmission dynamics of *Salmonella* in chickens in a quantitative manner. These strains have served as an initial conceptional proof to quantitatively track the *Salmonella* transmission routes from environment to flock, since they carry distinct barcode tags that would allow them to be identified and quantified accurately by high-throughput sequencing of the barcode regions (Yang et al., 2017). In summary, utilizing isogenic barcode-tagged strains, the population structure can be quantified to evaluate the patterns of SE infection and dissemination in chickens, and determine whether infection of neonatal chickens with one *Salmonella* strain, excludes the infection of a second strain (cross protection). In order to better comprehend the implications of barcode-tagged strains for commercial poultry, larger scale experiments are needed to assess additional environmental and host factors. Nevertheless, the current experiment demonstrated that the use of barcode-tagged strains is an original and an effective method to understand the dynamics of *Salmonella* infection, which provides valuable opportunities to develop and improve measures that protect poultry flocks from infection with *Salmonella*. Studies to evaluate and confirm previous work published by our laboratory (Kallapura et al., 2014) that demonstrate the importance of airborne transmission of *Salmonella versus* oral infection using these SE barcode-tagged strains are currently being evaluated.

4.6 References

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4.7 Tables and Figures

Table 1. Consecutive *Salmonella* serovar challenges in day-old chicks following per os administration of *Salmonella* Enteritidis or *Salmonella* Typhimurium in trial 1 and trial 2 of Experiment 2.

Treatment	Day	Challenge Dose (cfu)	No. *Salmonella* Culture Positive Birds/Total			No. *Salmonella* Isolates/Total	
			ST recovery CCT	SE recovery CCT	Both CCT	ST recovery CCT	SE recovery CCT
Trial 1							
ST Control	1	10^4	30/30 (100 %)	0/30 (0 %)	0/30 (0 %)	300/300 (100 %)	0/300 (0 %)
SE Control	2	10^5	0/30 (0 %)	22/30 (73.3 %)	0/30 (0 %)	0/220 (0 %)	220/220 (100 %)
ST and SE Principals	1, 2	10^4, 10^5	49/60 (81.66 %)	0/60 (0 %)	11/60 (18.33 %)	1165/1200 (97.08 %)	35/1200 (2.91 %)
Trial 2							
SE Control	1	10^4	0/20 (0 %)	20/20 (100 %)	0/20 (0 %)	0/200 (0 %)	200/200 (100 %)
ST Control	2	10^5	16/20 (80 %)	0/20 (0 %)	0/20 (0 %)	160/160 (100 %)	0/160 (0 %)
SE and ST Principals	1, 2	10^4, 10^5	0/30 (0 %)	33/40 (82.5 %)	5/40 (12.5 %)	19/760 (2.50 %)	741/760 (97.50 %)

Table 2. Simultaneous *Salmonella* serovar challenges in day-old chicks following per os administration of *Salmonella* Enteritidis or *Salmonella* Typhimurium in trial 3 and trial 4 of Experiment 2.

			No. *Salmonella* Culture Positive Birds/Total			No. *Salmonella* Isolates/Total	
Treatment	Day	Challenge Dose (cfu)	ST recovery CCT	SE recovery CCT	Both CCT	ST recovery CCT	SE recovery CCT
Trial 3							
ST Control	1	10^4	20/20 (100%)	0/20 (0%)	0/20 (0%)	200/200 (100 %)	0/200 (0 %)
SE Control	1	10^4	0/20 (0%)	20/20 (100%)	0/20 (0%)	0/200 (0 %)	200/200 (100 %)
ST and SE Principals	1, 1	10^4, 10^4	3/40 (8%)	1/40 (3%)	36/40 (90%)	502/791 (63%)*	289/791 (37%)*
Trial 4							
SE Control	2	10^5	16/20 (80%)	0/20 (0%)	0/20 (0%)	153/153 (100 %)	0/153 (0 %)
ST Control	2	10^5	0/20 (0%)	10/20 (50%)	0/20 (0%)	0/100 (0 %)	100/100 (100 %)
SE and ST Principals	2, 2	10^5, 10^5	4/40 (10%)	2/40 (5%)	14/40 (35%)	670/781 (86 %)*	111/781 (14 %)*

*Asterisk within rows indicates significant difference at $P < 0.001$

Table 3. Percentage *Salmonella* barcode strains recovered from cecal sample enumerated from ilumina sequence data in day-old broiler chickens. Experiment 2.

Experimental Groups	Day	Challenge Dose (cfu)	Barcode % Recovery from Ceca
Group 1			
Barcode 1	1	10^5	BC1=97.77 ± 0.52 [a]
Saline	2	Saline	BC2=0.0 ± 0.0 [b]
Group 2			
Barcode 2	1	10^5	BC1=3.17 ± 2.04 [b]
Saline	2	Saline	BC2=97.77 ± 10.67 [a]
Group 3			
Saline	1	Saline	
Barcode 1	2	10^5	
Group 4			
Saline	1	Saline	
Barcode 2	2	10^5	
Group 5			
Barcode 1	1	10^5	91.25 ± 4.85 [a]
Barcode 2	2	10^5	0.50 ± 0.35 [b]
Group 6			
Barcode 2	1	10^5	84.25 ± 6.36 [a]
Barcode 2	2	10^5	6.00 ± 3.43 [b]

Data expressed as Mean ± SE; n = 12 chickens

[a,b] Values within treatment group (row) with different superscripts differ significantly ($P < 0.001$).

Figure 1: Percentage *Salmonella* Barcode-tagged Strains Recovered from Cecal Sample Enumerated from Ilumina Sequence Data

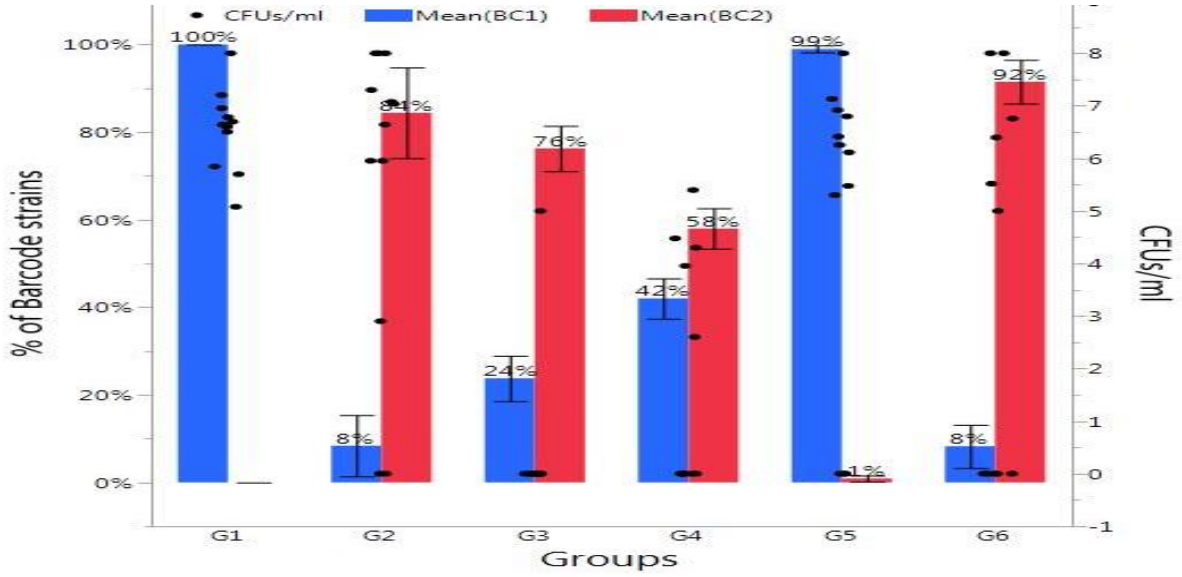
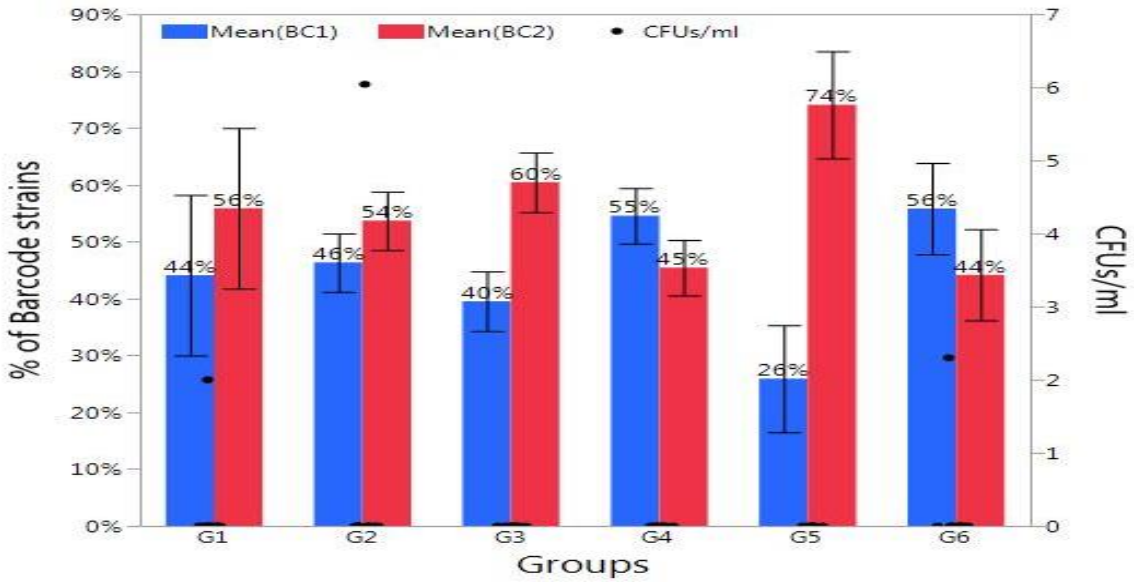


Figure 2: Percentage *Salmonella* Barcode-tagged Strains Recovered from Liver/Spleen Sample Enumerated from Ilumina Sequence Dat



CHAPTER FIVE

Characterization and evaluation of lactic acid bacteria candidates for reduction of intestinal epithelial permeability and *Salmonella* Typhimurium colonization in neonatal turkey poults

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5.1 Abstract

The aim of the present study was to evaluate the microbiological properties of three probiotic candidate strains of lactic acid bacteria (LAB) (128; 131; CE11_2), their effect on intestinal epithelial permeability as well as their ability to reduce intestinal colonization of *Salmonella* Typhimurium (ST) individually or as a batch culture in neonatal turkey poults. Isolates were characterized morphologically, and identified by 16S rRNA sequence analyses. Additionally, each of the isolates was evaluated for: Tolerance and resistance to acidic pH, high osmotic concentration of NaCl, and bile salts resistance in broth medium. *In vitro* assessment of antimicrobial activity against different enteropathogenic bacteria was determined with an overlay technique. Moreover, *in vitro* intestinal permeability was evaluated using a stressed Caco-2 cell culture assay treated with/without the probiotic candidates. However, the *in vivo* effect of the selected LAB strains on ST cecal colonization was determined in two independent trials with neonatal turkey poults. The results obtained in this study showed the tolerance of LAB candidates to a pH of 3, NaCl concentration of 6.5 %, and high bile salts resistance (0.6 %). All strains evaluated showed *in vitro* antibacterial activity against *Salmonella* Enteritidis, ST, and *Campylobacter jejuni*. Candidates 128 and 131 showed coccus morphology and were identified as *Enterococcus faecium*, whereas bacterial strain CE11_2 showed clusters of cocci-shaped morphology and was identified as *Pediococcus parvulus.* All three candidate probiotics significantly ($P < 0.05$) increased transepithelial electrical resistance (TEER) in Caco-2 cells following a 3 h incubation period with hydrogen peroxide when compared with control and blank groups. The combination of all three candidates as a batch culture showed significant efficacy in controlling intestinal colonization of ST in neonatal turkey poults. Evaluation of the combination

of these selected LAB candidate strains in performance and intestinal health parameters of chickens and turkeys are currently in process.

5.2 Introduction

Prokaryotes are widespread in all environments, establishing diverse interactions with many eukaryotic taxa (Bronstein et al., 2006; Gnad et al., 2010). The cooperative interactions between species (mutualism) have a central role in the generation and maintenance of all forms of life (Kikuchi et al., 2009; Jones et al., 2010). One example of a beneficial group of microorganisms is the astonishing abundant ensemble of microbes that harbors in the gastrointestinal tract (GIT) of metazoans (Neish, 2009). The GIT is more heavily populated with microorganisms than any other organ and is an interface where the microbiota may have a pronounced impact on animal biology (Yegani and Korver, 2008; Maslowski and Mackay, 2010; Musso et al., 2010). Clearly, the association of gut microbiota is regulated by elaborate and combinatorial host–microbial and microbial–microbial interactions developed over the course of evolution (Xu and Gordon, 2003); (Xu et al., 2007; Fraune and Bosch, 2010). Comparison of gnotobiotic rodents with control rodents colonized with a normal microbiota have revealed a wide range of host functions affected by indigenous microbial communities such as assembly of the gut-associated lymphoid tissue; integrity of the intestinal mucosal barrier; proliferation and differentiation of its epithelial lineages; angiogenesis; and activation of the enteric nervous system (Bergman, 1990; Moran, 2007; Duerkop et al., 2009; Martin et al., 2010; Sekirov et al., 2010; Tlaskalová-Hogenová et al., 2011; Walter et al., 2011). The microbiota can metabolize a wide range of by product converting them to end products including short chain fatty acids, a process which has direct benefits on digestive physiology (Tellez et al., 2006; Dass et al., 2007; Tellez et al., 2012). As with most complex ecosystems, it appears that the majority of these microbial species cannot be cultured when

removed from the niches in their host animals (Moran, 2007). The fragile composition of the gut microbiota can be affected by various factors such as age, diet, environment, stress and medication (Choct, 2009; Maslowski and Mackay, 2010; Bäckhed, 2011). Dietary ingredients have a profound effect on the composition of the gut microflora, which in turn, regulates the physiology of all animals (Fraune and Bosch, 2010). Hence, nutritional components of the diet are of critical importance for both the host and its intestinal microbiota, which in turn, will determine the balance between health and disease. Colonization of different microflora in the mucosal surface of metazoans initiates at birth, and is tracked by progressive assembly of a complex and dynamic microbial society maintaining a perfect homeostasis (Martin et al., 2010; Di Mauro et al., 2013).

Perhaps, one of the most intriguing aspects of a balance microbiome is its impact on the innate and adaptive immune system (Hammes and Hertel, 2002; Parvez et al., 2006; Parracho et al., 2007). In this context, a short window of time during birth exists that enables the colonization of symbiotic bacteria to all mucosal surfaces, which may modify the future immune phenotype of the host (Martin, 2012; Almqvist et al., 2012; Hansen et al., 2013). Delayed microbial colonization of the gut mucosa, the largest immune organ of the body, could cause significant changes in the immune system possibly having long term impacts on systemic immunity (Thavagnanam et al., 2008; Martin et al., 2010; Guibas et al., 2013). Animal and human studies have provided evidence that specific bacterial strains are capable of stimulating multiple aspects of innate immunity (Alvarez-Olmos and Oberhelman, 2001; Reveneau et al., 2002; Farnell et al., 2006; Feng and Elson, 2010; Maslowski and Mackay, 2010; Salzman, 2011; Jounai et al., 2012) and humoral immunity (Arvola et al., 1999; Joint, 2001; Kalliomaki et al., 2001; Ouwehand et al., 2002; Parvez et al., 2006). Fascinatingly, through a process of “cross talk” with the mucosal immune system, the microbiota negotiates mutual growth, survival, and inflammatory control of the intestinal

ecosystem (Salzman, 2011), having remarkable effects on gut permeability (Lyte, 2011; Yu et al., 2012b; Howarth and Wang, 2013). In this regard, recent studies published in our laboratory suggest that early infection with *Salmonella* Enteritidis (SE) induce severe inflammation, increase intestinal permeability and mucosal barrier dysfunction that were associated with increase organ invasion and intestinal colonization of SE. However these adverse effects were prevented by the administration of a commercial lactic acid base probiotic in chickens (Prado-Rebolledo et al., 2017). Hence, the objectives of the present study were to characterize different lactic acid bacteria (LAB) probiotic candidates based on multiple microbiological properties, and evaluate the effect of these selected LAB isolates on epithelial permeability *in vitro*, and *in vivo Salmonella* Typhimurium (ST) cecal colonization in neonatal turkey poults.

5.3 Material and Methods

5.3.1 Isolation and Selection of Bacterial Candidates

A total of 90 probiotic candidates were isolated from 34 week-old broiler chickens. Briefly, cecal epithelium, cecal content, and ileal epithelium were separated, homogenized, serially diluted with 0.9 % sterile saline solution and plated on de Man Rogosa Sharpe (MRS) agar plates (Catalog no. 288110, Becton Dickinson and Co., Sparks, MD 21152 USA). Single colonies were obtained, identified with a number and evaluated for *in vitro* antimicrobial activity against enteropathogenic bacteria. Three candidates were identified as 128, 131 and CE11_2 and selected because all showed the highest zone of inhibition against different enteropathogens. These isolates were identified by 16S rRNA sequence analyses (Microbial ID Inc., Newark, DE 19713, USA) and were further evaluated for multiple microbiological properties as probiotic candidates, as well as their *in vitro* effect on intestinal epithelial permeability and *in vivo* ability to reduce ST intestinal colonization in turkey poults as described below.

5.3.2 Candidate Probiotic Culture Conditions, Morphology Characterization, and Biochemical Tests

Bacterial strains were maintained as frozen stocks at -80°C in 50% glycerol aliquots. Selected candidates were routinely cultured at 37°C under microaerophilic conditions in MRS broth. Each isolate was passed three times every 8 h in MRS broth. Then, bacteria were washed three times, resuspended in sterile 1 x PBS and adjusted an optical density (OD_{600}) of 0.8-0.9. Each isolate was tested for Gram stain affinity, catalase and oxidase production (Lanyi, 1988).

5.3.3 Evaluation of Probiotic Candidates to Different Biochemical Conditions: pH, Temperature, and Sodium Chloride Concentration

A basal MRS medium was used in these series of *in vitro* studies. An overnight culture of each isolate was used as the inoculum whereby the cells were centrifuged and re-suspended in 0.9 % sterile saline. The suspension (100 µl) was inoculated into 10 mL of MRS broth. Two incubation time points (2 and 4 h) were evaluated for each of the variables (pH, temperature, and sodium chloride concentration). The rationale for these two points was mainly based on digesta feed passage time through the gastrointestinal tract of poultry. The temperatures tested were 15 and 45°C, and the concentrations of sodium chloride (**NaCl**) tested were 3.5 and 6.5 % (w/v). Additionally, the LAB candidates were tested for survivability under two different pH conditions (2.0 and 3.0). The tubes were incubated with reciprocal shaking, at the specific test temperatures or at 37°C for the tests of pH and concentrations of NaCl. At each time point evaluated, each sample was struck onto MRS agar for presence or absence of growth, to confirm livability of the strains. The turbidity of each tube was also noted as an indication of growth or no-growth. Each treatment was tested by triplicate.

5.3.4 Bile Salts Tolerance

The method of Gilliland et al. (1984), with some modifications, was used to determine bile salt tolerance. MRS broth containing 0 %, 0.4 %, 0.5 %, or 0.6 % of bile salts No. 3 (Catalog no. 213010, Becton Dickinson and Co., Sparks, MD 21152 USA) was inoculated with 107 cfu/mL of each probiotic strain, after being centrifuged at 3000 g for 15 minutes and washed three times from their overnight growth cultures. Samples were incubated for 24 h at 37°C with shaking at 100 rpm. Growth in control (no bile salts) and test cultures was evaluated at 2, 4, and 24 h by streaking samples onto MRS agar for presence or absence of growth.

5.3.5 *In vitro* Assessment of Antimicrobial Activity against Enteropathogenic Bacteria

The LAB isolates were screened for *in vitro* antimicrobial activity against *Salmonella enterica* serovar Enteritidis (SE), *Salmonella enterica* serovar Typhimurium (ST), and *Campylobacter jejuni* (CJ). Ten microliters of each isolate were placed in the center of MRS plates. After 24 h of incubation at 37°C, the plated samples were overlaid with tryptic soy agar (TSA, catalog no. 211822, Becton Dickinson, Sparks, MD) containing 10^6 cfu/mL of SE or ST. After 24 h of incubation at 37°C, plates were evaluated and those colonies that produced zones of inhibition were selected. A similar overlay method as described above was used for CJ, where 10^6 cfu/mL of CJ was inoculated in TSA containing 0.2 g of sodium thioglycolate as a reducing agent, and overlaid over the solid agar. Plates were incubated in a microaerophilic environment for 48 h at 42°C. Colonies that produced zones of inhibition were selected by triplicate.

5.3.6 Cell Culture of Epithelial Caco-2 cells

Epithelial Caco-2 cells, obtained from the American Type Culture Collection, were grown in high-glucose Dulbecco's modified Eagle's media (DMEM). Medium was supplemented with

10% Fetal Bovine Serum, 2% L-glutamine and 1% penicillin/streptomycin. Caco-2 cells cultivated in permeable filter inserts were washed twice with Hank's Balanced Salt Solution and maintained under a humidified atmosphere of 5% CO_2 at 37°C.

5.3.7 Evaluation of Lactic Acid Bacteria Probiotic Candidates on Transepithelial Electrical Resistance of Stressed Caco-2 Cells

A Transepithelial Electrical Resistance (TEER) assay was performed in epithelial Caco-2 cell cultures stressed by exposure to 5mM of hydrogen peroxide (H_2O_2) during different time points to induce an experimental inflammatory process. Briefly, cells were seeded on fibronectin coated polycarbonate membrane inserts in transwell plates (0.4μm pore size, Corning #3413) at a density of 2×10^5 cells/ml. Culture media were replaced every two days until a confluent monolayer was obtained. A Millicell-ERS voltohmmeter (Millipore, Bedford, MA) was used to measure the TEER. After two weeks of cell culture when TEER measurements reached above 1,000 ohm, Caco-2 cell monolayers were used to evaluate the effect of the probiotic on cell permeability. Briefly, Caco-2 cells cultivated in permeable filter inserts were washed twice with Hank's Balanced Salt Solution. The monolayer on the apical side of inserts were treated with probiotic candidates mixed in DMEM at an incubation ratio [Multiplicity of Infection] of 10:1 bacteria : epithelial cell ratios. TEER was measured at different time points: before probiotic treatment of monolayers, and 3 or 5 h after treatment with H_2O_2. TEER was expressed as percentage of resistance, normalized to initial value. Each probiotic candidate strain was replicated in 4 transwells. In the non-treated group, the transwells were not incubated with any probiotic strains, but treated with 5mM H_2O_2. A blank group was also included. In this group, Caco-2 cell cultures were not incubated with any probiotics and not treated with H_2O_2.

5.3.8 *Salmonella* Culture for *In Vivo* Trials

The enteropathogen ST which served as a challenge pathogen was obtained from the USDA National Veterinary Services Laboratory (Ames, Iowa). This isolate was selected for resistance to novobiocin (NO) and naladixic acid (NA). For the present studies, 100µL of ST from a frozen aliquot were added to 10 mL of Tryptic Soy broth (TSB, Catalog no. 211822, Becton Dickinson, Sparks, MD) and incubated at 37°C for 8 h. This was followed by two passages every 8 h into fresh TSB for a total of 24 h, to ensure log phase growth. Post incubation, bacterial cells were washed 3 times in sterile 0.9% saline by centrifugation (1,864 × *g*, 4°C, 15 min), quantified with a spectrophotometer (Spectronic 20D+, Spectronic Instruments Thermo Scientific) at 625nm using an established concentration curve, and diluted in sterile 0.9% saline as per required concentrations (cfu/mL) for the trials. Concentrations of ST were also determined retrospectively by serial dilution and further plating on BGA with NO (25µg/mL, catalog no. N-1628, Sigma) and NA (20µg/mL, catalog no. N-4382, Sigma) for enumeration of actual cfu/mL used for challenge, as reported below.

5.3.9 Experimental Animals

Day-of-hatch, turkey poults were obtained from a local hatchery (Gentry, AR, USA) and placed in isolators with controlled age-appropriate environment. Poults were provided ad libitum access to water and a balanced unmedicated corn-soybean diet meeting the nutrition requirements of poultry recommended by the NRC (1994). All animal handling procedures were in compliance with Institutional Animal Care and Use Committee at the University of Arkansas. Twelve poults for each trial were humanely killed and sampled upon arrival at the laboratory. Whole ceca-cecal tonsils (CCT), liver/spleen, and trachea were aseptically removed from these neonate poults,

incised and cultured in 10 ml tetrathionate enrichment broth (Catalog no. 210420, Becton Dickinson, Sparks, MD) and incubated overnight at 37°C. The samples were confirmed negative for *Salmonella* spp. by plating onto selective Brilliant Green Agar (BGA, Catalog no. 70134, Sigma) with NO (25µg/mL) and NA (20µg/mL).

5.3.10 Experimental Design

Two independent trials were conducted. In each trial, sixty neonatal turkey poults were randomly assigned to one of five groups (n=12/group); 1) Control ST challenged; 2) ST challenged plus probiotic candidate 128; 3) ST challenged plus probiotic candidate 131; 4) ST challenged plus probiotic candidate CE11_2; 5) ST challenged plus batch probiotic culture. The challenge with ST was administered to all groups of poults on arrival at the laboratory by oral gavage (4×10^4 cfu/0.25 mL/bird) using an animal feeding needle. Each candidate probiotic strain was grown individually or combined as a batch culture (1:1:1) on MRS broth. At 1 h post challenge, probiotic candidates were administered to poults by oral gavage (4×10^6 cfu/0.25 mL/bird). In each trial, at 24 h post ST challenge, all poults in all groups were euthanized by CO_2 asphyxiation. In both trials, CCT were aseptically collected for ST recovery as explained below.

5.3.11 Recovery of *Salmonella*

Briefly, whole CCT were aseptically removed, collected in sterile bags, weighed, homogenized, and serially diluted with sterile 0.9% saline (1:4 wt/vol). Tenfold dilutions of each sample, according to groups, were subsequently made in a sterile 96 well flat bottom plate and the diluted samples were plated on BGA with NO and NA, incubated at 37°C for 24 h to enumerate total *Salmonella* cfu.

5.3.12 Data and Statistical Analysis

Epithelial permeability TEER values as well as Log_{10} cfu/g of ST in cecal contents were subjected to ANOVA as a completely randomized design, using the GLM procedure of SAS (SAS Institute, 2002). Significant differences among the means were determined by Duncan's multiple-range test at $P < 0.05$.

5.4 Results

Table 1 shows the results of the morphological characteristics and identification of LAB probiotic candidates evaluated. In the present study, all three selected LAB candidate strains were classified as Gram-positive with a negative catalase and oxidase reaction. Candidates 128 and 131 showed coccus morphology and were identified by 16S RNA sequencing as *Enterococcus faecium.* Candidate CE11_2 showed a cluster of cocci-shaped morphology and was identified as *Pediococcus parvulus* (Table 1). All candidates evaluated were able to grow when cultured at 0.4 %, 0.5 %, and 0.6 % bile salts concentration at 2, 4, and 24 h of incubation (data not shown). The results of the tolerance of LAB bacteria probiotic candidates to different pH, temperature and NaCl concentrations are summarized in Table 2. Candidate CE11_2 did not survive an incubation period of 2 or 4 h at pH 2.0. Candidates 128 and 131 did survive at pH 2.0 for only 2 h. However, all strains were tolerant after 2 and 4 h of incubation at a pH of 3.0, temperatures of 15 and 45°C, or high concentration (6.5 %) of NaCl (Table 2). Table 3 shows the results of the *in vitro* assessment of antimicrobial activity of LAB probiotic candidates against enteropathogenic bacteria. All three lactic acid isolates exhibited *in vitro* antimicrobial activity against SE, ST, and CJ (Table 3). The results of the *in vitro* effect of LAB probiotic candidates on TEER of Caco-2 cells stressed at 3 and 5 hours with H_2O_2 are summarized in Table 4. All three-candidate probiotics significantly increased TEER following 3 h of H_2O_2 administration when compared with negative

control or blank cells. However, following 5 h of H_2O_2 stress, only those Caco-2 cells treated with the CE11_2 strain, showed a significant increase of TEER when compared with Caco-2 cells without probiotic (Table 4). Table 5 shows the results of the evaluation of LAB probiotic candidates on ST cecal colonization in turkey poults. The combination of all three bacteria candidates as a batch culture (1:1:1), significantly reduced cecal colonization of ST when compared with non-treated control poults or the groups that received a single candidate strain (Table 5).

5.5 Discussion

Salmonellosis remains one of the most comprehensive foodborne diseases that can be transmitted to humans through animal and plant products (Hernández-Reyes and Schikora, 2013) (Zheng et al., 2013), however, in recent years, several studies have demonstrated the use of probiotics as alternative tool to antibiotic growth promoters in poultry, preventing or controlling the presentation of *Salmonella* outbreaks (Hammes and Hertel, 2002; Tellez et al., 2012). Furthermore, probiotic bacteria have been shown to regulate production of pro-inflammatory and anti-inflammatory cytokines (Lyte, 2011), exert anti-oxidant properties (Howarth and Wang, 2013), enhance barrier integrity (Yu et al., 2012a,b), as well as innate (Vanderpool et al., 2008; Molinaro et al., 2012) and humoral immunity (Howarth and Wang, 2013). In the present study, the 3 probiotic candidates showed good tolerance to conditions present in the intestinal tract and also exhibited *in vitro* antimicrobial activity against three important poultry enteropathogens (SE, ST and CJ). Moreover, the combination of all three bacteria candidates as a batch culture (1:1:1), significantly reduced the cecal colonization of ST when compared with control poults with no probiotic in two independent trials. On the other hand, intestinal epithelial cells are not only responsible for digestion, secretion and absorption of nutrients, but act as a physical barrier

separating external environmental agents from the internal host environment (Salzman, 2011). In these epithelial cells, tight junctions (TJ) act as intercellular cement regulating the permeability and dissemination of microorganisms and antigens from the intestinal lumen to the systemic circulation (Ulluwishewa et al., 2011). Endotoxins from *Salmonella* spp. have been shown to activate aldose reductase (AR) and the nuclear factor kappa B (NF-κB), iducing the expression of several inflammatory cytokines that are responsible disruption of TJ complex proteins and loss of barrier function (Ozinsky et al., 2000; Liao et al., 2008; Steed et al., 2010; Overman et al., 2012; Pastel et al., 2012). Interestingly, microarray analysis with probiotic cultures in broiler chickens challenged with SE showed a significant reduction in intestinal gene expression associated with the NF-κB complex and AR (Higgins et al., 2011). In the present study, the increase TEER following 3 hours of H_2O_2 administration by the probiotic candidates on Caco-2 cells are in agreement with previous published studies suggesting that these candidates suppressed the oxidant-induced intestinal permeability, and improve intestinal barrier function (Johansson *et al*., 2010; Yu et al., 2012a; Nava and Vidal, 2016). TEER measures the flux of all ions across the epithelial lining, and recently it has been demonstrated that probiotics increase gene expression of TJ proteins (Alvarez et al., 2016). Increased TEER can be used as an indicator of the integrity of TJ proteins, hence reducing paracellular permeability. Metchnikoff (1907) founded the research field of beneficial microorganisms for animals and humans (probiotics), aimed at modulating the intestinal microflora. Currently, new molecular techniques are helping us to understand how the anti-inflammatory, cell integrity and anti-oxidant properties of probiotics can improve gut and barrier integrity. Given the recent international legislation and domestic consumer pressures to withdraw growth-promoting antibiotics and limit antibiotics available for treatment of bacterial infections, probiotics may offer alternative options. Together, the results of the present

investigation show critical and important characteristics of LAB to be evaluated when selecting strains to be used as probiotics for poultry. Furthermore, the selected LAB probiotic candidates enhanced *in vitro* intestinal epithelial permeability and reduced ST intestinal colonization in neonatal turkey poults. The supplementation of these strains on growth performance of turkeys is currently being evaluated.

5.6 Acknowledgements

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5.7 References

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5.8 Tables

Table 1. Morphological characteristics and identification of lactic acid bacteria probiotic candidates[1]

LAB ID	16S RNA sequencing microbial identification	Isolated region	Gram strain	Morphology	Catalase	Oxidase
128	*Enterococcus faecium*	Ceca	+	Cocci	-	-
131	*Enterococcus faecium*	Ceca	+	Cocci	-	-
CE11_2	*Pediococcus parvulus*	Ceca	+	Cocci (clusters)	-	-

[1]Symbols: (+), positive; (-), negative.

Table 2. Tolerance of lactic acid bacteria probiotic candidates to different pH, temperature and NaCl concentrations[1]

	pH2		pH3		15ºC		45ºC		3.5% NaCl		6.5% NaCl	
LAB ID	2h	4h	2h	4h	2h	4h	2h	4h	2h	4h	2h	4h
128	+	-	+	+	+	+	+	+	+	+	+	+
131	+	-	+	+	+	+	+	+	+	+	+	+
CE11_2	-	-	+	+	+	+	+	+	+	+	+	+

[1]Symbols: (+), tolerant; (-), not tolerant.

Table 3. *In vitro* assessment of antimicrobial activity of lactic acid bacteria probiotic candidates against enteropathogenic bacteria[1]

ID	***Salmonella* Enteritidis**	***Salmonella* Typhimurium**	***Campylobacter jejuni***
128	0.7 cm	1.15 cm	1.1 cm
131	1.2 cm	1.1 cm	1.0 cm
CE11_2	1.0 cm	0.975 cm	0.8 cm

[1]Represents the diameter of the zone of inhibition observed at 24 h of incubation. All lactic acid bacteria candidates were tested by triplicate

Table 4. *In vitro* effect of lactic acid bacteria probiotic candidates on transepithelial electrical resistance (TEER) of Caco-2 cells stressed at 3 and 5 hours with hydrogen peroxide (H_2O_2)[1]

LAB ID	TEER Treated with H_2O_2 for 3 h	TEER Treated with H_2O_2 for 5 h
128	96.27 ± 4.13 [a]	32.48 ± 2.15 [d]
131	98.79 ± 2.01 [a]	32.64 ± 0.84 [d]
CE11_2	103.19 ± 0.78 [a]	49.80 ± 1.46 [b]
Non-treated	81.90 ± 1.43 [b]	41.55 ± 0.90 [c]
Blank	84.64 ± 1.21 [b]	119.66 ± 2.59 [a]

[1]The initial *in vitro* TEER determination of each transwell was normalized as 100% when analyzing these data. Each strain was replicated in 4 transwells. In the negative control, the transwells were not incubated with any probiotic strains, but treated with 5 mM H_2O_2, while blank wells were not incubated with any probiotics nor treated with H_2O_2.

Table 5. Evaluation of lactic acid bacteria probiotic candidates on *Salmonella* Typhimurium cecal colonization in turkey poults[1]

Treatment	Log_{10} *S.* Typhimurium /g Caeca-cecal content Trial 1	Log_{10} *S.* Typhimurium /g Caeca-cecal content Trial 2
Saline	6.43 ± 0.14^{a}	6.51 ± 0.31^{a}
128	6.09 ± 0.20^{ab}	5.77 ± 0.22^{ab}
131	6.89 ± 0.33^{a}	5.47 ± 0.18^{b}
CE11_2	6.27 ± 0.30^{ab}	5.78 ± 0.25^{ab}
Batch[2]	5.20 ± 0.35^{b}	5.54 ± 0.24^{b}

[1]Ceca-cecal tonsils were cultured to enumerate Log_{10} cfu of ST/g of cecal content, and the data are expressed as mean ± standard error of the mean.
[2]Combination on an equal ratio of each of the lactic acid bacteria candidates (1:1:1).
[a–b] Different superscripts within columns indicate significant differences $P < 0.05$; n=12 turkeys/group

CONCLUSION

In this dissertation, the approaches for controlling *Salmonella* infection were determined and estimated as the effective methods. *Salmonella*-based recombinant vaccines have been shown to be effective for controlling the challenges from cross-serotype infection of *Salmonella* Heidelberg. The number of *Salmonella* colonization in the ceca of chicken was decreased by comparing with the control group. The other approach for controlling *Salmonella* infection is isolation of functional probiotics, which showed promising result in decreasing gut permeability and reducing pathogen colonization in the chicken gut. We developed a novel approach for researching *Salmonella* transmission within the chicken flock by inserting 6 random nucleotides into the functional neutral regions in the genome of *Salmonella enteritidis*, which was named as *Salmonella* barcode-tagged isogenic strains. This approach has been estimated as the effective and novel method to analyze *Salmonella* transmission mechanism. The proof of concept has been substantiated by three chicken experiments and providing valuable data to study *Salmonella* transmission within the chicken flock. The project of researching the competitive exclusion theory has also been substantiated by using the constructed *Salmonella* barcode-tagged isogenic strains. It provides a profound insight for the future research in *Salmonella* population transmission.

APPENDIX

IACUC Approval

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO:	Young Min Kwon
FROM:	Craig N. Coon, Chairman
DATE:	Apr 7, 2016
SUBJECT:	IACUC Approval
Expiration Date:	Apr 6, 2019

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # 16049 "Rationale design for stain-specific prebiotics for promotion of gut health and growth performance in chickens".

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond Apr 6, 2019 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

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