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Assessment of Salmonella enterica Biofilms and Expression Differences Among Serovars

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Assessment of *Salmonella enterica* Biofilms and Expression Differences Among Serovars

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Abstract

Biofilms are communities of microorganisms associated by a matrix of extracellular polymers. In this state, microorganisms occupy an ecological niche distinct from their free-floating, planktonic counterparts. Also, biofilm bacteria become biologically unique as they form communities and lose motility. The acquisition of these physiological attributes enables the biofilm to persist through harsh environmental conditions, including antimicrobial induced stress and to resist sanitization efforts. Because of these features, biofilms can rapidly disseminate across numerous surfaces and as they establish, become challenging to remove. This is a particular issue for the food industry as processing plants offer favorable conditions for biofilm formation by providing complex surfaces composed of diverse materials that are frequently inoculated with pathogens and provide an abundance of nutrients and water. This thesis initiates investigations into the mechanisms behind biofilm formation in processing plants, and with such knowledge potentially result in novel treatments in the future. In particular, *Salmonella enterica*, one of the most prevalent foodborne pathogens worldwide, can produce biofilms that are difficult to remove. The thesis starts with a literature review detailing the mechanisms behind biofilm formation, evaluating the state of biofilms in food processing, and finishing with current and future mitigation strategies (**Chapter 1**). Next, this thesis includes four research chapters, with the first evaluating the ability of disinfectants to reduce *Salmonella* biofilms **(Chapter 2)**; the second with a genome announcement about our genomic elucidation of four *Salmonella* strains isolated from poultry sources that are known to produce biofilms **(Chapter 3)**; the third detailing our exploration of the pellicle forming properties of *Salmonella* with a focus on the lesser studied Kentucky serovar (**Chapter 4)**; before ending with an evaluation of transcriptional dynamics of poultry isolates of different *Salmonella* serovars during biofilm formation (**Chapter 5**). Data

presented herein will provide novel insight into *Salmonella* biofilm dynamics, mitigation, and genetics.

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To everyone: thanks for all the help, I really appreciate it.

Dedication

Dedicated to my ever supportive family and friends.

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Chapter 3: Shi, Z., Kaldhone, P.R., Khajanchi, B.K., Foley, S.L. and Ricke, S.C., 2018. Draft Genome Sequences of *Salmonella enterica* Serovar Enteritidis and Kentucky Isolates from Retail Poultry Sources. Genome Announcements, *6*(14): e00193-18.

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Chapter 4: Characterization of Pellicle Formation by Poultry *Salmonella* Kentucky Strains and other Poultry-Associated *Salmonella* Serovars in Luria Bertani Broth
Submitted as: Characterization of Pellicle Formation by Poultry *Salmonella* Kentucky Strains and other Poultry-Associated *Salmonella* Serovars in Luria Bertani Broth.
In Journal: Frontiers of Veterinary Science

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Submitted as: Gene Expression differences between *csgD* and *bcsA* of three important poultry serovars of *Salmonella enterica*.
In Journal: Frontiers of Veterinary Science

I. Introduction

The perseverance of foodborne *Salmonella* in the processing chain has remained an issue, causing high incidences of foodborne illnesses each year despite the efforts of government agencies and food industries to control it. Since the recognition of *Salmonella* as a cause of disease decades ago, various efforts, including the development of regulations, Hazard Analysis Critical Control Point (HAACP) programs, and post-harvest intervention strategies, have been developed to reduce the number of people afflicted each year, but for the most part achieved less than ideal success. Consequently, *Salmonella* remains a significant challenge to the food industry as it perpetually adapts and overcomes mitigation strategies. This is due to its ability to maintain numerous pathogenicity islands, as well as its ability to acquire multi-modal virulence and antimicrobial resistance mechanisms through horizontal gene transfer, enabling it to invade and persist not only in multiple cell types but in varied hostile environments as well.

Perhaps one of its most crucial contributors to virulence is the ability of *Salmonella* to form biofilms on a variety of surfaces. *Salmonella* as a biofilm is capable of resisting sanitation and antimicrobial treatments common in processing plants, creating a significant potential for continued cross-contamination events during food processing production cycles. This presents an ongoing critical issue for the food processing industry as well as from a public health perspective. As a result, it is essential to both elucidate the underlying mechanisms associated with the production of biofilms by *Salmonella* as well as its mitigation.

The central hypotheses of this thesis are (1) that there exist serovar differences in the properties of *Salmonella* biofilms, (2) that they are regulated by transcription factors in which structural regulation genes such as *bcsA* and *csgD* play a central role, and (3) that disinfectants can reduce biofilms. The working hypothesis is that qRT-PCR can be used to monitor

transcription factor activation and that there are differences among common poultry serovars. Furthermore, the reduction of *Salmonella* biofilm formation on coupons derived from materials used in the food industry from disinfectant exposure is possible. A reduction in recoverable and detectable *Salmonella*, as well as a loss in biofilm formation, would demonstrate the disinfectant efficacy.

The thesis organization is as follows:

•**Chapter 1**: A literature review outlining the history of *Salmonella*, the molecular mechanisms of biofilm formation, and the common strategies used by poultry processing to mitigate the problem.

•**Chapter 2** is a peer-reviewed publication demonstrating the various efficacies of common and uncommon disinfectants on commercially derived coupons for the reduction of *Salmonella*.

•**Chapter 3** is a Genome Announcement regarding four biofilm producing *Salmonella* strains isolated from commercial poultry samples.

•**Chapter 4** is an unpublished research paper focused on exploring the pellicle forming properties of *Salmonella enterica* serovar Kentucky under various conditions.

•**Chapter 5** is an unpublished research paper elucidating the transcriptional factor activation and biofilm formation of *Salmonella* poultry isolates representing three critical serovars.

Ultimately, this thesis delivers not only a new understanding of the molecular diversity associated with biofilm formation, but it also evaluates mechanisms to reduce biofilm formation in poultry processing. By accomplishing both tasks, it is recognized that *Salmonella* biofilms are not homogenously synthesized across all *Salmonella* serovars. Therefore, it is unlikely one disinfectant will be useful in the reduction of biofilm formation. This thesis serves as the

stepping stone to understanding the deeper underlying mechanisms associated with *Salmonella* survival in processing plants. That knowledge may be used to develop new sanitation methods in the future.

II. Chapter 1. Literature Review - *Salmonella* biofilms: mechanisms and treatments

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Introduction

Foodborne illness resulting from the ingestion of pathogenic microorganisms and their byproducts is estimated to cause approximately 9.4 million cases of illnesses annually, leading to 56,000 hospitalizations and 1,400 deaths (Scallan et al., 2011). Of these, nontyphoidal *Salmonella* species are estimated to cause approximately a tenth of the cases, a third of the hospitalizations, and a third of the deaths, resulting in up to an estimated 10.9 billion dollars in losses due to medical care, productivity loss, and loss of life (Scallan et al., 2011; Scharff, 2012). Because of the immense impact of *Salmonella* on public health, there have been ongoing efforts by both the government and private industry to reduce the prevalence of *Salmonella* during processing with policies such as the Food Safety Modernization Act and *Salmonella* Action Plan being passed with new performance standards and intervention strategies being developed (USDA-FSIS, 2013). This issue is particularly concerning for the poultry industry as poultry products have been identified as reservoirs of various *Salmonella* serovars, with *S.* Enteritidis associated with eggs and *S.* Typhimurium and *S.* Heidelberg found in meat products (Foley et al., 2008, 2011, 2013; Finstad et al., 2012; Howard et al., 2012; Ricke, 2017). Furthermore, *Salmonella* cells have the ability to congregate together to form into complex communities of biofilms, attaching themselves to processing environments causing a food safety issue for the food industry (Steenackers et al., 2012).

Biofilms consist of microbial cells associated together within a matrix of extracellular polymeric substances (EPS) that confer stability and antimicrobial resistance to the cells (Donlan, 2002). Due to these benefits, microbial cells in nature tend to prefer existing in this state, undergoing a cyclical lifestyle of initial attachment of planktonic cells into microcolonies and priming a favorable microenvironment, growth and cooperation as a functional community,

and finally production of planktonic cells which may migrate and colonize new locations (Costerton et al., 1995). Within biofilms, microbial cells exhibit increased resistance to efforts to remove them, due in large part to the role played by the EPS matrix. This structure provides a firm anchor for their attachment to materials, increased antimicrobial resistance with a concurring increased production of resistance compounds such as catalases, as well as the EPS serving as a barrier to antimicrobial penetration (Fux et al., 2005). The EPS matrix is composed of mainly polysaccharides such as cellulose, but also contains other biopolymers including proteins, nucleic acids, lipids, as well as bacterial structures including flagella, pili, and fimbriae (Flemming and Wingender, 2010). The regulation of EPS compound production allows biofilms to adapt to pressures ranging from heat stress to antimicrobial interventions, and allows attachment of pathogens such as *Salmonella* to a variety of food environments including abiotic surfaces consisting of plastic, concrete, steel, and even to food products themselves, such as produce (Steenackers et al., 2012). The overall objective of this review is to outline the history of biofilms and its impact on the food industry, discuss the molecular basis for biofilm formation and regulation, and explore the strategies used by the food industry to combat this perennial issue with an emphasis on the foodborne pathogen, *Salmonella*.

Salmonella **Background**

Nontyphoidal *Salmonella* infections continue to be a major ongoing public health issue in the United States despite the best efforts of the U.S. government and industry to solve it. Divided based on differences in the expression of antigenic lipopolysaccharides (O), flagella (H), as well as differences in biochemical characters, pathogenicity, and habitat, the White-Kauffmann-Le Minor scheme of classification currently identifies over 2,500 serovars of *Salmonella* (Grimont

and Weill, 2007). Of the two main *Salmonella* species, *S. enterica* and *S. bongori*; and five subspecies within *S. enterica*, only *S. enterica* subspecies *enterica* is usually associated with warm-blooded animals, with 99% of human infections attributed to this group (Brenner et al., 2000). This subspecies, with over 1,500 serovars demonstrates how *Salmonella* can adapt to a myriad of ecological niches, infect different hosts, and occupy diverse environments to be a threat to public health. Even so, government surveillance has shown that exposure to contaminated poultry remains the public's most common infection path, highlighting the need for both the U.S. government and the poultry industry to reduce *Salmonella* in poultry products (USDA-FSIS, 2016). In addition, the recent rise in popularity in raising backyard chickens, by avid but relatively unknowledgeable amateurs, has presented another problem with outbreaks occurring nearly every year since 2010 and has demonstrated the need to better educate the public on food safety practices (Beam et al., 2013; CDC, 2018b).

Within the poultry industry, there are several serovars of *Salmonella* that have been historically associated with poultry products. Data from the USDA-FSIS PR/HACCP testing program from 1998-2014 has shown that Kentucky, Enteritidis, Typhimurium, and Heidelberg to consistently rank in the top five isolated serovars from broilers and eggs (USDA-FSIS, 2016). Although *Salmonella* Enteritidis was rarely encountered in the early twentieth century, outbreaks involving it steadily increased in the 80s and 90s until it became the serovar most associated with undercooked eggs and is now the most commonly reported outbreak strain in the U.S. (Rabsch et al., 2000; Ricke, 2017; CDC, 2018a). This increase in incidence is thought to have been a result of the successful eradication campaigns of *Salmonella* Gallinarum and Pullorum from poultry, as *S.* Enteritidis exhibits similar surface antigens and occupies the same ecological niche (Bäumler et al., 2000). *Salmonella* Typhimurium exhibits a wide range of possible infection transfer

methods and has been implicated in foodborne outbreaks ranging from produce to peanut butter to chicken, leading it to be the third most frequently isolated serovar in 2016 and second in causing illness overall (USDA-FSIS, 2016; CDC, 2018a, b). *Salmonella* Heidelberg is mainly associated with poultry meat, having been implicated as the causative agent in the 2013 Foster Farms outbreak, but has also shown to colonize the poultry reproductive system and undergo vertical transmission to infect eggs (Gast et al., 2004, 2007; Kaldhone et al., 2017; CDC, 2018b). *Salmonella* Kentucky has been the serovar most commonly identified from poultry isolates, but is not usually associated with human illness and has been implicated in few outbreaks in the United States (Foley et al., 2011, 2013; CDC, 2018b).

Infection with nontyphoidal *Salmonella* in healthy individuals generally leads to acute gastroenteritis, consisting primarily of diarrhea, fever, and abdominal cramps and naturally passes within a week (CDC, 2015). Individuals with compromised immune systems, as well as infants and the elderly, have greater susceptibility and may experience more severe symptoms (Shimoni et al., 1999). Among these at-risk populations, *Salmonella* infection has also been linked to cases of bacteremia, pneumonia, and meningitis, causing additional complications or even fatalities (Trevejo, 2003). One reason for the increased infection rates among these populations may be because both the very young and the very old exhibit greater incidences of lowered gastric acid production, allowing more pathogens to pass and invade the intestinal epithelium (Blaser and Newman, 1982). Infants have been found to be the group most commonly hospitalized for *Salmonella* infections, sometimes with additional secondary infections, but usually recover and survive (Shimoni et al., 1999; Trevejo, 2003). However, elderly patients have exhibited the greatest rate of comorbidity with other illnesses and account for over half of

the deaths resulting from *Salmonella* infections (Mandal and Brennand, 1988; Shimoni et al., 1999; Chen et al., 2012).

An issue gaining prominence in more recent decades has been the rise of antibiotic resistance among pathogens, including *Salmonella*. In the 1990s, *Salmonella* Typhimurium strain DT104, resistant against multiple antibiotics and exhibiting greater invasiveness than other common strains, was implicated in several outbreaks, highlighting the danger posed by multidrug-resistant pathogens (Poppe et al., 1998; Threlfall et al., 2000). More recently other antibiotic-resistant *Salmonella* from multiple serovars have been isolated, commonly exhibiting resistance to multiple antibiotics (Cui et al., 2005; Foley and Lynne, 2008; Lestari et al., 2009). Historically, antibiotics have been added to poultry feed in subtherapeutic levels as antibiotic growth promoters due to their beneficial effects in increasing feed conversion and production efficiency (Thomke and Elwinger, 1998). However, due to concerns that this usage could lead to the transmission of antibiotic resistance to human pathogens, a recommendation for the elimination of antibiotic growth promoters was made (Dibner and Richards, 2005). This has led to an increased interest into alternative antimicrobial compounds such as essential oils, botanicals, and bacteriophages, among others (Joerger, 2003; Calo et al., 2015; O'Bryan et al., 2015).

In the poultry industry, there has been considerable interest in prebiotics, food additives that promote the growth of beneficial gut microbiota, and in the direct introduction of favorable live microorganisms in feed in the form of probiotics (Patterson and Burkholder, 2003). Although this shift in pre-harvest food safety intervention measures may assist in the reduction of antibiotic resistance in *Salmonella* from poultry, *Salmonella* exposed to food processing environments may still exhibit some natural antibiotic resistance as a result of biofilm

formations. The ability of *Salmonella* isolated from food processing environments and retail meat samples to form biofilms is well documented and presents an issue for the food industry as well as public health due to its tenacity and widespread distribution (Joseph et al., 2001; Stepanović et al., 2004; Solomon et al., 2005; Kim and Wei, 2007). In fact, Vestby et al. (2009) observed a correlation between the capacity of different *Salmonella* serovars and strains to form biofilms with their ability to persist in processing settings. Further testing demonstrated that persistent strains exhibited increased biofilm forming ability compared to other isolated strains, suggesting a sort of selection for better biofilm formers in these settings (Vestby et al., 2009).

Biofilm Formation

There are several stages in the life cycle of a biofilm whereupon the component bacterial cells undergo several changes in phenotypes, gene expression, and protein production to adapt to changes in their lifestyles as they transition from motile planktonic microorganisms into sessile communities of cells exhibiting multicellular behavior. This process accounts for the life cycle of the majority of bacteria found in nature, where planktonic bacteria can essentially be thought of as displaced individuals searching for new areas to colonize (Costerton et al., 1995; Watnick and Kolter, 2000). In general, the process proceeds with the initial attachment of planktonic bacteria to a favorable environmental surface, the clustering of bacteria and formation of cell cluster microcolonies accompanied by a loss of motility, the growth and development of additional layering and clusters, and finally the dispersion of new motile planktonic bacteria away from the clusters to form new colonies (Costerton et al., 1995; Sauer et al., 2002). Changes in the expression of genes regulating attributes from motility appendages to the production of EPS compounds enable the biofilm community to initiate the processes of attachment and proceed

through the stages of biofilm development before finally becoming fully integrated in their environments (O'Toole et al., 2000). Although the control of biofilm development is governed by different genetic elements in different organisms, the general process of development is similar for bacteria ranging from *Pseudomonas aeruginosa* to *Salmonella enterica* as they proceed through the stages of biofilm formation and can be used to generalize the types of genes involved (Davey and O'Toole, 2000).

Initial Attachment

The initial step in the life cycle of a biofilm is characterized by free-floating planktonic bacteria traveling through their surroundings. These planktonic cells will subsequently attach to surfaces in response to favorable environmental cues such as optimal nutrient availability, or as a reaction to adverse stressors such as exposure to antimicrobials (O'Toole et al., 2000; Jefferson, 2004). Hoffman et al. (2005) demonstrated this defensive aspect of biofilm formation by exposing *Pseudomonas aeruginosa* cultures to subinhibitory levels of antibiotics, resulting in increased production of biofilm pathways and induction of biofilm formation. Reduced nutrient availability also appears to promote the faster formation of biofilms for some organisms when compared to cultures grown in more rich media (Dewanti and Wong, 1995; Ryu et al., 2004).

Due to the importance of motility in this stage, the expression of motility appendages and surface proteins appear to be necessary among many bacterial species (Davey and O'Toole, 2000). These structures, such as flagella controlled by *flg*, *flh*, and *fli* genes, comprise a prominent structure in many microorganisms, allowing them to swim in liquids and play a crucial role in biofilm formation (Iino et al., 1988; Guttenplan and Kearns, 2013). In *P. aeruginosa*, *s*urface attachment defective (*sad*) transposon mutants were deficient in either

flagella synthesis, corresponding with mutations in the *flgK* flagellum locus, or had issues related to *pil* gene loci which govern type IV pili biogenesis; either deficiency would prevent biofilm formation (O'Toole and Kolter, 1998a, b).

Although the presence of surface motility appendages are important during the attachment and early growth stages of biofilm formation, the exact role and interactions for flagella, pili, and others can also vary depending on environmental conditions and the species of bacteria involved. Under flow chamber conditions using citrate as a carbon source, Klausen et al. (2003b) observed that a flat *P. aeruginosa* biofilm was formed without the use of flagella for attachment, but also noted that it played a role afterwards. Likewise, Pratt and Kolter (1998) found that type I pili, also known as fimbriae, rather than flagella were critical in the attachment and interactions of *E. coli* cells, with *fim* mutants often wholly unable to attach to abiotic surfaces. Curli, a type of surface fimbriae governed by *csg* (curli-specific gene) operons, has also been found to play a role in the adhesion of *E. coli* to surfaces in place of flagella (Prigent-Combaret et al., 2000).

Transition to Irreversible Attachment and Biofilm Growth

After the initial contact with a suitable surface, planktonic cells undergo a process of attachment in two stages: 1) reversible attachment where the bacteria may be easily removed by shear forces and 2) irreversible attachment where the cells complex with the surface and must be forcefully removed (Palmer et al., 2007). During the reversible attachment stage, the bacteria are kept in place through a combination of van der Waals, steric, electrostatic, and hydrophobic interactions, during which cells frequently attach and detach (Garrett et al., 2008). Following this phase, the cells enter an irreversible attachment stage where a monolayer is formed on the

surface and cells aggregate together ultimately becoming microcolonies (O'Toole et al., 2000). This is accomplished through twitching motility where cells utilize type IV pili to crawl across surfaces, the lack of which prevents successful biofilm formation (O'Toole and Kolter, 1998a; Semmler et al., 1999). Transitioning between reversible and irreversible attachment involves the regulation of several genes including the *sadB* and *sadC* factors which downregulate the ability of the cells to swarm and promote biofilm formation in an inverse relationship (Caiazza and O'Toole, 2004; Caiazza et al., 2007; Merritt et al., 2007). Although a swarming state works against biofilm formation, this movement can also allow microorganisms to travel to and colonize favorable niches and later form a biofilm (Verstraeten et al., 2008). The *lap* genes, discovered by Hinsa et al. (2003), are associated with an ATP-binding cassette transporter and are also required in the transition from reversible to irreversible attachment.

With the cells clustered and microcolonies formed, the nascent biofilm continues to expand, undergoing cell growth and the development of complex structures and habitats within the biofilm. As the bacteria grow and divide, they excrete extracellular polymeric substances, stabilizing the biofilm against its substrate and encasing its constituent members in a scaffolding matrix and giving it structure (Czaczyk and Myszka, 2007). This development is mediated by the use of cell-to-cell signaling between organisms within the biofilm as they release extracellular regulatory signals, activating genes for the production of EPS along with other products and aiding the process of differentiation between microcolonies within the biofilm (Davies et al., 1998; Waters et al., 2008). Pores and channels form within the biofilm, transporting oxygen, water, and nutrients while also acting as conduits for the removal of waste products and forming a mushroom-like structure (Tolker-Nielsen and Molin, 2000; Klausen et al., 2003a). Overall, the biofilm becomes an efficient and stable community of bacteria living together in mutual benefit.

After the establishment of a permanent biofilm formation, eventually a number of motile cells leave through openings in the biofilm, revert to planktonic cells, and begin the process over again (Sauer et al., 2002).

Salmonella Biofilm Formation

As motile organisms, *Salmonella* species use its cell surface protein structures to aid in biofilm formation. Planktonic *Salmonella* cells primarily use their flagella for the purposes of movement and for bacterial swarming, situations generally opposed to biofilm formation (Wang et al., 2004). However, during the invasion of a host, flagella have been found to be necessary for *Salmonella* to attach to host cells. For the colonization of the gallbladder and of gallstones, flagella, with the *fliC* gene in particular, have been found to mediate the initial binding of cholesterol-coated surfaces (Prouty et al., 2002; Crawford et al., 2010). Mutant *Salmonella* strains defective for the major flagellar subunit, motility, and chemotaxis have also been also been shown to result in a decrease in attachment to poultry gut cells (Allen-Vercoe and Woodward, 1999).

In addition to flagella, various types of fimbriae play a crucial role in *Salmonella* attachment with type I and thin aggregative fimbriae playing an important role in attachment and biofilm formation. Type I fimbriae are characterized by their ability to cause hemagglutination in a mannose-dependent manner and are associated with possessing adhesive properties (Clegg and Gerlach, 1987). Austin et al. (1998) observed type I fimbriae working in conjunction with thin aggregative fimbriae in attachment to both hydrophobic (Teflon) and hydrophilic (stainless steel) food contact surfaces. These aggregated cells could easily be sloughed off and serve as sources of cross contamination in food processing environments (Austin et al., 1998). Type I fimbriae are

also required in the formation of biofilms on animal cells as demonstrated by Ledeboer et al. (2006) with their study applying biofilm mutants to HEp-2 and murine intestinal tissue.

The other major type of fimbriae associated with biofilm development in *Salmonella* is curli. Curli are thin coiled cell surface amyloids which can interact with a wide variety of bacterial cell matrix proteins and has roles in surface adhesion as well as in infection through interactions with major histocompatibility complex molecules (Olsén et al., 1998). Found in a highly conserved fashion in both *Salmonella* and *E. coli*, curli fibers interchangeably termed as thin aggregative fimbriae (Tafi) with production controlled by the *csg* gene cluster (*agf*) (Römling et al., 1998). Curli, along with cellulose, predominate as the major part of the extracellular matrix of *Salmonella* biofilms (Zogaj et al., 2001). Both biopolymers are required for optimum biofilm and pellicle formation, synergistically providing resistance against antimicrobials as well as granting long term survival capabilities, allowing 10% *Salmonella* to survive nine months in storage conditions (Solano et al., 2002; White et al., 2006).

Overall, multiple processes of biofilm formation are directed by the transcriptional regulator *csgD* which acts on multiple downstream targets and regulates curli production, cellulose production, and other products. Belonging to the FixJ/UhpA/LuxR family of regulators, *csgD* activates the production of curli fibers by directly interacting with and positively regulating the *csgBA* operon which then encodes the CsgA and CsgB protein subunits that join to produce curli (Barnhart and Chapman, 2006; Zakikhany et al., 2010). Concurrently, *csgD* also directly interacts with the *adrA* promoter region, leading to the coding of a GGDEF domain protein. and subsequent production of the signal molecule cyclic diguanylic acid (c-di-GMP) (Verstraeten et al., 2008; Zakikhany et al., 2010). C-di-GMP then binds to the regulatory BcsB region of the cellulose synthase and acts as an allosteric activator of cellulose synthase, promoting cellulose

production (García et al., 2004; Römling et al., 2005). C-di-GMP also inhibits the production and rotation of flagellum, establishing *csgD*'s role in promoting biofilm formation (Ogasawara et al., 2011).

Advantages of Biofilm Formation

The aggregation of bacteria into biofilms confers numerous beneficial advantages to the organisms residing within during the change from an originally singular planktonic life to a multicellular symbiotic community. In terms of raw materials, aqueous environments tend to concentrate nutrients near solid surfaces. Therefore the formation of an attached biofilm allows better access to food (Dunne, 2002). Organisms within biofilms also experience higher levels of gene exchange, increasing genetic diversity and increasing overall fitness (Hausner and Wuertz, 1999; Molin and Tolker-Nielsen, 2003). Finally, the most important aspect from a public health perspective is the ability of biofilms to resist antimicrobial action. The first significant barrier to effective antibiotic treatment of biofilms is the EPS matrix surrounding the cells. This dense medium of various organic biopolymers hinders the diffusion of antimicrobials, as they are exposed to deactivating compounds or bind to the matrix (Mah and O'Toole, 2001; Fux et al., 2005). Cells in the biofilm can also excrete protective compounds such as catalases which subsequently diffuse throughout the EPS, neutralizing damage from hydrogen peroxide and other agents, as well as protecting other bacterial cells and creating a synergistic effect between different organisms excreting different compounds (Elkins et al., 1999; Stewart et al., 2000). Furthermore, exposure to antibiotics can result in the upregulation of proteins such as β-lactamases and efflux pumps within the cells, reducing potential damage (Bagge et al., 2004; Matsumura et al., 2011). In fact, Baugh et al. (2012) demonstrate that chemical disruption of

multidrug efflux pumps in *Salmonella* resulted in a halt in curli production and resulted in a significant reduction in biofilm formation.

The Biofilm Matrix

One of the most unique aspects of biofilms and perhaps the most crucial characteristic of bacterial biofilms is the matrix of extracellular polymeric substances spread throughout the biofilm and surrounding every microorganism. The EPS matrix acts as the main setting with which each microorganism within the biofilm can directly and indirectly interact with other cells, its local microenvironment, and the outer environment as a whole (Flemming et al., 2007). In fact, when studying hydrated *Pseudomonas* and *Vibrio* biofilms using scanning confocal laser microscopy, Lawrence et al. (1991) concluded that only a small proportion of the biofilm area was actually comprised of the cells themselves, with between 73 and 98% of the biofilm area being comprised of EPS and pore spaces. The EPS matrix is composed of multiple biopolymers including polysaccharides, proteins, nucleic acids, and lipids as well as metabolic products such as enzymes from its member cells and materials picked up from the surrounding environment (Flemming and Wingender, 2010). Each of these different components works together to form a sophisticated synergistic network and provides the biofilm microbial consortia a successful existence.

The EPS matrix provides many roles for its biofilm community including benefits in durability, protection, nutrient transport, hydration, and housing, giving architectural and structural support. Many different types of morphologies can be exhibited by biofilms depending on the types of EPS products including smooth and flat, rough, fluffy, and filamentous formations, allowing for different habitats for the microorganisms internally (Flemming and

Wingender, 2010). The shear rate of fluid passing over the biofilm as well as the nutrient availability can also cause changes in biofilm morphology as the cells adapt to shifting environmental conditions (Stoodley et al., 1998). Pores and water channels are present throughout the EPS matrix, allowing for the management of nutrient and water flow with channels flowing over clusters of cells, sometimes with liquid flowing against bulk flow (Stoodley and Lewandowski, 1994). Oxygen can also be efficiently distributed through the voids between the cell clusters, providing a much needed resource for cells located deeper within the biofilm structure (De Beer et al., 1994).

Different species of bacteria may cluster together as islets of microcolonies, each contributing to the EPS with different products and creating compartmentalized microenvironments favorable for their survival (Xiao et al., 2012). Møller et al. (1998) tagged different species of *Pseudomonas* and *Acinetobacter* with green fluorescent protein and found the microcolonies to be spatially distributed with different organisms dominating at different stratum levels, sometimes even creating enclaves with clusters of one species surrounding another. When exposed to antimicrobials, microorganisms can adapt by changing their spatial layout in relation to others to increase resistance, working synergistically to increase overall survivability (Leriche et al., 2003; Burmølle et al., 2006). Lawrence et al. (1991) also found that *Pseudomonas* biofilms exhibited tighter clustering at their attachment sites with less density towards their outer surfaces, while *Vibro* biofilms displayed the opposite, demonstrating differences in structural growth between species. Within the biofilm, membrane vesicles are excreted in these zones to alter their environments as well as aiding in communication via quorum sensing and protection through the binding and inactivation of antimicrobials (Schooling and Beveridge, 2006; Kulp and Kuehn, 2010).

Biofilm Polysaccharides

As one of the most versatile types of biopolymers, polysaccharides make up a major element of the EPS matrix and play a variety of roles within it. This includes adhesion of cells to the EPS matrix as well as of the biofilm to its attaching substrate; protection against environmental effects, predators, and antimicrobials; and structurally in providing the framework for microbial microcolonies and subpopulations within the biofilm as well as the network of nutrient and water flow channels (Limoli et al., 2015). Production of the polysaccharide cellulose has been shown to be a major contributor in the formation of pellicle type biofilms as well as in providing roles in providing resistance to antimicrobials and in cell adhesion (Spiers et al., 2003; Limoli et al., 2015). Cellulose, produced by the *bcs* (bacterial cellulose synthesis) operon, is the major polysaccharide component of *Salmonella* biofilms, interacting with curli in the extracellular matrix to provide structure and supporting cell adhesion, especially in *Salmonella* pellicles (Zogaj et al., 2001). Solano et al. (2002) observed cellulose production to be common among *Salmonella* serovars, that cellulose conferred strong resistance against chlorine treatment, and that the inability to produce cellulose severely hindered the ability for *Salmonella* to produce biofilms. *Salmonella* also produces an extracellular O-antigen polysaccharide, controlled by *yih* genes and regulated by *csgD*, which aids the biofilm in persisting through desiccation stress (Gibson et al., 2006). Both cellulose and the O-antigen polysaccharide have been linked to *Salmonella*'s ability to attach and colonize plants and plant food products (Barak et al., 2007).

In *P. aeruginosa* biofilms, the Psl, Pel, and alginate polysaccharides have been implicated in having a critical role in attachment and structure (Friedman and Kolter, 2004; Limoli et al., 2015). Colvin et al. (2012) found that in non-mucoid *Pseudomonas* biofilms, Psl and Pel are used as the main components in the structural matrix, but that the lack of Psl severely affected the

ability of cells to attach. Furthermore, they concluded that different strains utilized Psl and Pel at varying levels, hypothesizing that the differences served as adaptations to environmental niches (Colvin et al., 2012). Meanwhile in mucoid biofilms, alginate predominates as the main polysaccharide and has been shown to provide significantly increased resistance to antimicrobials and host immune responses, a significant issue in medical settings as mucoid *P. aeruginosa* is a major cause of cystic fibrosis (Hentzer et al., 2001; Leid et al., 2005). Alginate also plays a structural role, greatly increasing the volume of the biofilm as well as giving it additional architectural complexity and enhanced microcolony formation (Hentzer et al., 2001; Nivens et al., 2001).

Biofilm Issues in Food Processing

The ability of bacteria to form biofilms throughout natural and manmade environments presents an issue to humans across many disciplines. In the sphere of public health, pathogens may enter into public water supplies and become incorporated into biofilms, potentially causing illnesses especially in developing countries and areas where the water supply is unreliable (Wingender and Flemming, 2011; Kumpel and Nelson, 2013). In medicine, pathogens can form biofilms and cause chronic diseases such as cystic fibrosis and pneumonia as well as form on medical devices including catheters and other implants, often causing cases of sepsis as a result (Donlan, 2002; Wolcott and Ehrlich, 2008; Francolini and Donelli, 2010). For the food industry, the formation of biofilms by pathogens is a major concern as food processing environments can be seen as ideal environments for biofilm formation if proper cleaning and hygienic standards are not followed (Holah and Kearney, 1992). Food processing environments can provide a consistent source of nutrients and water from the food products being processed, a variety of surfaces and

material types to colonize including hard to clean areas such as drains and pipes, and even the initial inoculation event with bacteria initiating the process of colonizing equipment between scheduled disinfection times (Carpentier and Cerf, 1993). Inadequate sanitation processes can be seen as one of the chief causes for biofilm formation by allowing for the soiling of equipment and aiding in initial biofilm attachment (Chmielewski and Frank, 2003). Additionally, direct damage can be caused by biofilms containing acid-producing bacteria through corrosion of equipment and pipes (Flint and Wolfaardt, 2012).

Contamination caused by pathogenic bacteria from sources such as biofilms can also cause a significant economic impact if an outbreak occurs. Once an outbreak has occurred and the source determined, often the company involved issues a recall of the product. This has become a regular occurrence with 18 food-related recalls happening in just July of 2018, with 6 linked to foodborne pathogens, 5 of which were because of *Salmonella* (FDA, 2018). A 2011 report by the Grocery Manufacturer's Association, a food industry trade association representing some of the largest companies in the food industry, found that 48% of the recalls made for health and safety reasons cost up to $9 million, 29% between $10 and $29 million, and the remainder above $30 million (GMA, 2011). Additional economic damage can be caused through the decrease in consumer trust if the public perception decreases due to outbreaks and requires tremendous effort by management to resolve (Doeg, 1995; Kaptan et al., 2017). In 2013-2014, a massive multistate outbreak of *Salmonella* Heidelberg linked to Foster Farms chicken occurred, causing a total number of 634 cases across 29 states and Puerto Rico with over 200 hospitalizations (CDC, 2014). The resulting widespread news coverage, lawsuits, and bad publicity led to Foster Farms launching a new food safety program, improving protocols and

equipment to minimize *Salmonella* and restore public trust in their products, costing $75 million in total (Gabbett, 2015).

The formation of biofilms during food processing can serve as a persistent source of pathogens, allowing for the cross-contamination of food products between initial processing and further processed goods (Lillard, 1990; Reij et al., 2004; Brooks and Flint, 2008). In the poultry industry, multiple flocks of birds from diverse production areas and varying levels of *Salmonella* are processed sequentially, creating the possibility of cross contamination of pathogens from one flock to another (Rasschaert et al., 2008). Through the use of serotyping, plasmid profile typing, and phage typing on several control points in the poultry processing line, Olsen et al. (2003) was able to follow the cross contamination of different flocks moving through the processing plant and found that contamination could even be carried back to the farms through poor cleaning of cages. *Salmonella* and other pathogens may be distributed during multiple steps of poultry processing. After exsanguination, the birds enter a scalding tank of hot water to loosen their feathers, after which the feathers are removed using high speed rotating rubber fingers in the plucking stage. This is followed by the evisceration step that removes the gastrointestinal tract of the birds before the birds are cleaned and held in a large shared chiller tank before finally being packaged and shipped out or moved on to be further processed (Owens et al., 2000). These steps may cause cross contamination events through aerosols during the picking stage, tearing of the gastrointestinal tract or transfers from the skin during evisceration, through the sharing of communal tanks in the scalding and chilling stages, or through direct contact with contaminated equipment (Keener et al., 2004; Carrasco et al., 2012). If proper cleaning and sanitization regimens are not followed, bacteria may attach to equipment during any of these steps, forming biofilms and serving as future sources of cross contamination.

Surface Materials

The setting of a processing plant provides numerous opportunities for bacteria to colonize a variety of surface materials. Steel, plastic, and rubber may be found on processing equipment, while the plant itself offers concrete walls, metal pipes and drains, and glass windows, all of which may include difficult to clean crevices and cracks (Corcoran et al., 2013). In attaching to a new surface, several properties must be taken into account including surface roughness, hygienic status and hydrophobicity, as well as the surrounding environmental conditions (Van Houdt and Michiels, 2010). In general, more hydrophobic surfaces with higher surface free energies, as well as surfaces with a rougher texture seem to enhance the initial attachment stage, increasing the likelihood for bacteria to colonize the surface with *Salmonella* found to attach better to plastic followed by rubber followed by stainless steel (Sinde and Carballo, 2000; Donlan, 2002). As food processing equipment undergoes strenuous conditions and repeated abrasive cleaning cycles use over time, their surfaces become rougher and harbor a more favorable environment for trapping bacteria and media from the processing procedure (Chmielewski and Frank, 2003). This media can then coat the exposed surface and create a conditioning film of polymers and other organic materials over time and affect the ability of bacteria to later attach (Donlan, 2002). Brown et al. (2014) reported that chicken juice derived from meat exudates from processing enhanced the ability of bacteria to attach to stainless steel coupons in addition to promoting biofilm development.

The properties of the material in contact with colonizing bacteria can affect initial attachment and subsequent biofilm growth. As stainless steel is the major component of most processing equipment, it also acts as the surface type food products would have the most contact with along the line. The most common types of stainless steel used in the food industry are of

austenitic grades 304 and 316 chosen for their stability at processing temperatures, ease of cleaning, and resistance to corrosion (Van Houdt and Michiels, 2010). In a study comparing biofilm formation on stainless steel against plastics and cement, the biofilm formed on steel exhibited a greater than 1-log decrease in cell density when compared to the plastic and similar densities as the cement (Joseph et al., 2001). Additionally, the finishing of the steel surface can play a role in bacterial colonization and biofilm formation. Schlisselberg and Yaron (2013) studied the influence of four types of stainless steel finishing on biofilm formation by *Salmonella*. Coupons were either mechanically brush polished by hand, cold rolled as Bright annealed stainless steel, or electro-polished via immersion in an electrolyte with a running current. When compared to untreated coupons, they found that the electro-polished coupons were colonized slower and responded better to sanitation (Schlisselberg and Yaron, 2013). Compared to other materials such as plastic compounds and rubber, stainless steel possesses a lower hydrophobicity and therefore provides a less favorable environment for bacterial attachment (Sinde and Carballo, 2000). However, Arnold and Silvers (2000) concluded that the rubber fingers used during the plucking stage of poultry processing resisted attachment of bacteria and inhibited biofilm formation but also stated that if the fingers became worn, cracked, or covered in organic material, they could act as a favorable site for growth.

Biofilm Prevention and Treatment

Due to the tremendous costs and consequences associated with foodborne outbreaks if pathogenic bacteria are allowed to contaminate food, great care is taken by government agencies and the food industry to remove biofilms before additional contamination occurs. If allowed to mature and persist, biofilms may become extremely difficult to remove using common

disinfectants, and therefore efforts must be made to eliminate biofilms before they become established (Corcoran et al., 2013). Government regulations require the implementation of regulations such as Good Manufacturing Practices (GMPs), which in terms of food safety, require companies to enforce good sanitation practices by keeping employees trained, proper maintenance to be performed, and perform regular validated testing methods to decrease the chances of issues (FDA, 2005). In addition, Hazard Analysis Critical Control Point (HACCP) systems must be implemented which attempt to prevent food safety issues by identifying and targeting control points within the processing chain which can be monitored and samples analyzed to eliminate food safety hazards (FDA, 1997). These steps help decrease the chances of biofilms forming by ensuring proper sanitation steps are created and followed regularly with through cleaning recommended before sanitation (FDA, 2004). Good equipment design to minimize contact with products and facilitates cleaning can also help in the prevention of bacterial attachment (Van Houdt and Michiels, 2010).

Contamination within the processing line is generally checked using standard sampling techniques. Swab and sponge sampling of the equipment and of the general processing environment can be done regularly to check for microbial contamination, but due to the tight adhesion of biofilms to their surfaces, may not be enough and require the use of techniques such as ultrasonication (Oulahal-Lagsir et al., 2000; Wirtanen et al., 2000). These samples are then sent to the laboratory where they can be measured using standard plate enumeration or more rapid methods such as qPCR. Another method for detection and checking for sanitation efficacy is the ATP bioluminescence test which can rapidly yield results in 5 to 10 minutes by measuring ATP through a swab test (Chmielewski and Frank, 2003). Fluorescent imaging may also be used to detect biofilms with possible future handheld devices which may be brought in proximity to

the line and used to inspect at risk areas for targeted sanitation (Jun et al., 2010). With the detection of pathogens, additional attention could be given to those areas and pieces of equipment during subsequent sanitation cycles or Cleaning-in-Place (CIP) routines to prevent development. However, care must be taken during CIP to ensure that bacteria detached from up the line don't reattach themselves further down the line (Le Gentil et al., 2010).

Once contamination has been identified, treatments and cleaning must be applied to the area. Traditional antimicrobial sanitizing agents used in the food industry include halogens such as hypochlorite, peroxygens like hydrogen peroxide, acids such as PAA, and quaternary ammonium compounds (Chmielewski and Frank, 2003). Chlorine is a commonly used antimicrobial agent, being administrated at up to 50 ppm in the wash and chiller steps of poultry processing (Keener et al., 2004). However, it has been shown to have decreased efficacy in the presence of particles and dirt such as that which can be found in processing environments where biofilms are likely to form (Van Houdt and Michiels, 2010). Additionally, the production of cellulose in *Salmonella* has been linked to increased chlorine resistance (Solano et al., 2002). Corcoran et al. (2013) found that applying sodium hypochlorite, sodium hydroxide, and benzalkonium chloride to a week old *Salmonella* biofilm were able to reduce viable counts, but none were able to completely destroy the biofilm. Steenackers et al. (2012) suggests that because biofilms often host multiple cell types, combining disinfectant treatments may end up being more effective at eradication. Studies have shown that even if the biofilm resists being killed off by treatments of disinfectants, the biofilm's attachment strength is decreased, allowing for easier removal in repeated cleaning cycles (Eginton et al., 1998). Gibson et al. (1999) recommended intense scrubbing or other mechanical action along with a high-pressure water spray followed by sanitation to effectively remove biofilms along with detergent in the water to reduce aerosols.

In addition to traditional sanitation methods, more novel methods exist and new processes are being developed and tested to remove biofilms. Enzymes such as proteases, glycosidases, and cellulases present an interesting approach towards biofilm removal by attacking the components of the EPS matrix that house the biofilm (Johansen et al., 1997; Chaignon et al., 2007). The use of bacteriophages may also be useful as they can infiltrate through the EPS matrix and disrupt the biofilm as well as aid in preventing initial colonization (Endersen et al., 2014). They may even be biologically engineered to enzymatically attack biofilms (Lu and Collins, 2007). Nanoparticles present another interesting path as they may be modified by researchers to create composite with additional properties (Rai et al., 2016). Finally, there is an increasing popularity in using compounds such as essential oils as an alternative to chemical treatments due to increasing consumer demand for natural products (Valeriano et al., 2012).

Conclusions

The natural state of microorganisms is to tend towards existing as stable bacterial communities in biofilm formations rather than motile planktonic individuals. As biofilm communities, microorganisms experience the benefits of increased protection against harsh environments and antimicrobial compounds, better and more efficient nutrient management, and increased fitness through the exchange of genetic information with its neighbors within cells clusters. When the microorganism is a foodborne pathogen like *Salmonella enterica*, this can pose an issue from a public health as well as an economic perspective if any illnesses occur due to contamination of food processing environments or cross contamination across production groups. Because of this, there is a need both among food industry safety specialists as well as

public health officials to address the issue by better understanding the underlying mechanisms behind them and think up new ways to prevent and treat biofilm formations to prevent illnesses from occurring.

The formation of a biofilm structure occurs through several steps, each governed by different underlying genetic mechanisms and stages of development. Starting from planktonic organisms, cells proceed from reversible attachment through irreversible attachment followed by biofilm growth and finally dispersion of new cells. Throughout these stages, different components predominate, with motility proteins such as flagella and pili initially positioning the cells for attachment to favorable surfaces. This is followed by the downregulation of these flagellar genes as cells transition from reversible to irreversible attachment. Finally, the biofilm grows as extracellular matrix components such as curli and cellulose for *Salmonella* are synthesized and released. Each of these stages could act as a potential target for prevention and treatment of a biofilm by either targeting the biofilm itself or creating unfavorable environmental conditions to discourage attachment or growth.

Additionally, research on *Salmonella* biofilms usually only look at a few strains within a serovar during biofilm formation and apply their findings in a broader sense. As different *Salmonella* serovars have been shown to be associated with different ecological and hosts, future research should investigate whether there exist serovar differences in biofilm formation and survival. This could be used in the development of more targeted treatment approaches to prevent the initiation of attachment or to create more effective treatment or prevention plans in the future. Therefore, the objectives for this thesis include investigating the differences between *Salmonella* serovars in biofilm formation as well as their treatment and control.

References

Allen-Vercoe, E. and Woodward, M.J., 1999. The role of flagella, but not fimbriae, in the adherence of *Salmonella enterica* serotype Enteritidis to chick gut explant. *Journal of Medical Microbiology, 48* (8), 771-780.

Arnold, J. and Silvers, S., 2000. Comparison of poultry processing equipment surfaces for susceptibility to bacterial attachment and biofilm formation. *Poultry Science, 79* (8), 1215-1221.

Austin, J.W., Sanders, G., Kay, W.W., and Collinson, S.K., 1998. Thin aggregative fimbriae enhance *Salmonella enteritidis* biofilm formation. *FEMS Microbiology Letters, 162* (2), 295-301.

Bagge, N., Schuster, M., Hentzer, M., Ciofu, O., Givskov, M., Greenberg, E.P., and Høiby, N., 2004. *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and β-lactamase and alginate production. *Antimicrobial Agents and Chemotherapy, 48* (4), 1175-1187.

Barak, J.D., Jahn, C.E., Gibson, D.L., and Charkowski, A.O., 2007. The role of cellulose and O-antigen capsule in the colonization of plants by *Salmonella enterica*. *Molecular Plant-Microbe Interactions, 20* (9), 1083-1091.

Barnhart, M.M. and Chapman, M.R., 2006. Curli biogenesis and function. *Annual Reviews in Microbiology, 60*, 131-147.

Baugh, S., Ekanayaka, A.S., Piddock, L.J., and Webber, M.A., 2012. Loss of or inhibition of all multidrug resistance efflux pumps of *Salmonella enterica* serovar Typhimurium results in impaired ability to form a biofilm. *Journal of Antimicrobial Chemotherapy, 67* (10), 2409-2417.

Bäumler, A.J., Hargis, B.M., and Tsolis, R.M., 2000. Tracing the origins of *Salmonella o*utbreaks. *Science, 287* (5450), 50-52.

Beam, A., Garber, L., Sakugawa, J., and Kopral, C., 2013. *Salmonella* awareness and related management practices in US urban backyard chicken flocks. *Preventive Veterinary Medicine, 110* (3-4), 481-488.

Blaser, M.J. and Newman, L.S., 1982. A review of human salmonellosis: I. Infective dose. *Reviews of Infectious Diseases, 4* (6), 1096-1106.

Brenner, F.W., Villar, R.G., Angulo, F.J., Tauxe, R.V., and Swaminathan, B., 2000. *Salmonella* Nomenclature. *Journal of Clinical Microbiology, 38* (7), 2465-2467.

Brooks, J.D. and Flint, S.H., 2008. Biofilms in the food industry: problems and potential solutions. *International Journal of Food Science & Technology, 43* (12), 2163-2176.

Brown, H.L., Reuter, M., Salt, L.J., Cross, K.L., Betts, R.P., and van Vliet, A.H., 2014. Chicken juice enhances surface attachment and biofilm formation of Campylobacter jejuni. *Applied and Environmental Microbiology*, AEM. 02614-14.

Burmølle, M., Webb, J.S., Rao, D., Hansen, L.H., Sørensen, S.J., and Kjelleberg, S., 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. *Applied and Environmental Microbiology, 72* (6), 3916-3923.

Caiazza, N.C., Merritt, J.H., Brothers, K.M., and O'Toole, G.A., 2007. Inverse regulation of biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. *Journal of Bacteriology, 189* (9), 3603-3612.

Caiazza, N.C. and O'Toole, G.A., 2004. SadB is required for the transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas aeruginosa* PA14. *Journal of Bacteriology, 186* (14), 4476-4485.

Calo, J.R., Crandall, P.G., O'Bryan, C.A., and Ricke, S.C., 2015. Essential oils as antimicrobials in food systems–A review. *Food Control, 54*, 111-119.

Carpentier, B. and Cerf, O., 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. *Journal of Applied Bacteriology, 75* (6), 499-511.

Carrasco, E., Morales-Rueda, A., and García-Gimeno, R.M., 2012. Cross-contamination and recontamination by *Salmonella* in foods: a review. *Food Research International, 45* (2), 545-556.

CDC. 2014. *Multistate Outbreak of Multidrug-Resistant Salmonella Heidelberg Infections Linked to Foster Farms Brand Chicken (Final Update)* [Online]. CDC. Available: https://www.cdc.gov/salmonella/heidelberg-10-13/index.html [Accessed 7/28/18].

CDC. 2015. *Salmonella Homepage - Questions and Answers* [Online]. CDC. Available: https://www.cdc.gov/salmonella/general/index.html [Accessed 6/16/18].

CDC. 2018a. *National Enteric Disease Surveillance: Salmonella Annual Report 2016* [Online]. CDC. Available: https://www.cdc.gov/nationalsurveillance/pdfs/2016-Salmonella-report-508.pdf [Accessed 6/15/18].

CDC. 2018b. *Reports of Selected Salmonella Outbreak Investigations* [Online]. Centers for Disease Control and Prevention. Available: https://www.cdc.gov/salmonella/outbreaks.html [Accessed 6/15/18].

Chaignon, P., Sadovskaya, I., Ragunah, C., Ramasubbu, N., Kaplan, J., and Jabbouri, S., 2007. Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. *Applied Microbiology and Biotechnology, 75* (1), 125-132.

Chen, P., Lee, H., Lee, N., Wu, C., Lin, S., Shih, H., Lee, C., Ko, W., and Chang, C., 2012. Non-typhoidal *Salmonella* bacteraemia in elderly patients: an increased risk for endovascular infections, osteomyelitis and mortality. *Epidemiology & Infection, 140* (11), 2037-2044.

Chmielewski, R. and Frank, J., 2003. Biofilm formation and control in food processing facilities. *Comprehensive Reviews in Food Science and Food Safety, 2* (1), 22-32.

Clegg, S. and Gerlach, G., 1987. Enterobacterial fimbriae. *Journal of Bacteriology, 169* (3), 934.

Colvin, K.M., Irie, Y., Tart, C.S., Urbano, R., Whitney, J.C., Ryder, C., Howell, P.L., Wozniak, D.J., and Parsek, M.R., 2012. The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. *Environmental Microbiology, 14* (8), 1913-1928.

Corcoran, M., Morris, D., De Lappe, N., O'connor, J., Lalor, P., Dockery, P., and Cormican, M., 2013. Commonly used disinfectants fail to eradicate *Salmonella enterica* biofilm from food contact surface materials. *Applied and Environmental Microbiology,* AEM. 03109-13.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappin-Scott, H.M., 1995. Microbial biofilms. *Annual Reviews in Microbiology, 49* (1), 711-745.

Crawford, R.W., Reeve, K.E., and Gunn, J.S., 2010. Flagellated but not hyperfimbriated *Salmonella enterica* serovar Typhimurium attaches to and forms biofilms on cholesterol-coated surfaces. *Journal of Bacteriology, 192* (12), 2981-2990.

Cui, S., Ge, B., Zheng, J., and Meng, J., 2005. Prevalence and antimicrobial resistance of *Campylobacter* spp. and *Salmonella* serovars in organic chickens from Maryland retail stores. *Applied and Environmental Microbiology, 71* (7), 4108-4111.

Czaczyk, K. and Myszka, K., 2007. Biosynthesis of extracellular polymeric substances (EPS) and its role in microbial biofilm formation. *Polish Journal of Environmental Studies, 16* (6).

Davey, M.E. and O'Toole, G.A., 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews, 64* (4), 847-867.

Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W., and Greenberg, E.P., 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science, 280* (5361), 295-298.

De Beer, D., Stoodley, P., Roe, F., and Lewandowski, Z., 1994. Effects of biofilm structures on oxygen distribution and mass transport. *Biotechnology and Bioengineering, 43* (11), 1131-1138.

Dewanti, R. and Wong, A.C., 1995. Influence of culture conditions on biofilm formation by Escherichia coli O157: H7. *International Journal of Food Microbiology, 26* (2), 147-164.

Dibner, J. and Richards, J., 2005. Antibiotic growth promoters in agriculture: history and mode of action. *Poultry Science, 84* (4), 634-643.

Doeg, C., 1995. *Crisis management in the food and drinks industry,* Boston, MA, Springer.

Donlan, R.M., 2002. Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases, 8* (9), 881-890.

Dunne, W.M., 2002. Bacterial adhesion: seen any good biofilms lately? *Clinical Microbiology Reviews, 15* (2), 155-166.

Eginton, P., Holah, J., Allison, D., Handley, P., and Gilbert, P., 1998. Changes in the strength of attachment of micro-organisms to surfaces following treatment with disinfectants and cleansing agents. *Letters in Applied Microbiology, 27* (2), 101-105.

Elkins, J.G., Hassett, D.J., Stewart, P.S., Schweizer, H.P., and McDermott, T.R., 1999. Protective role of catalase in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide. *Applied and environmental microbiology, 65* (10), 4594-4600.

Endersen, L., O'Mahony, J., Hill, C., Ross, R.P., McAuliffe, O., and Coffey, A., 2014. Phage therapy in the food industry. *Annual Review of Food Science and Technology, 5*, 327-349.

FDA. 1997. *HACCP Principles & Application Guidelines* [Online]. U.S. Food & Drug Administration. Available: https://www.fda.gov/Food/GuidanceRegulation/HACCP/ucm2006801.htm#defs [Accessed 7/31/18].

FDA. 2004. *GMPs Section Two: Literature Review of Common Food Safety Problems and Applicable Controls* [Online]. U.S. Food & Drug Administration. [Accessed 7/31/18].

FDA. 2005. *Food CGMP Modernization Report* [Online]. U.S. Food & Drug Administration. Available: https://www.fda.gov/Food/GuidanceRegulation/CGMP/ucm207458.htm [Accessed 7/31/18].

FDA. 2018. *Recalls, Market Withdrawals, & Safety Alerts* [Online]. U.S. Food and Drug Administration. Available: https://www.fda.gov/Safety/Recalls/default.htm [Accessed 7/31/18].

Finstad, S., O'Bryan, C.A., Marcy, J.A., Crandall, P.G., and Ricke, S.C., 2012. *Salmonella* and broiler processing in the United States: relationship to foodborne salmonellosis. *Food Research International, 45* (2), 789-794.

Flemming, H.-C., Neu, T.R., and Wozniak, D.J., 2007. The EPS matrix: the "house of biofilm cells". *Journal of Bacteriology, 189* (22), 7945-7947.

Flemming, H.-C. and Wingender, J., 2010. The biofilm matrix. *Nature Reviews Microbiology, 8* (9), 623-33.

Flint, S. and Wolfaardt, G., 2012. Corrosion and Fouling. *In:* Lear, G. and Lewis, G. D. (eds.) *Microbial Biofilms: Current Research and Applications.* Norfolk, UK: Caister Academic Press.

Foley, S.L., Johnson, T.J., Ricke, S.C., Nayak, R., and Danzeisen, J., 2013. *Salmonella* pathogenicity and host adaptation in chicken-associated serovars. *Microbiology and Molecular Biology Reviews, 77* (4), 582-607.

Foley, S.L. and Lynne, A.M., 2008. Food animal-associated *Salmonella* challenges: Pathogenicity and antimicrobial resistance 1. *Journal of Animal Science, 86* (14_suppl), E173-E187.

Foley, S.L., Lynne, A.M., and Nayak, R., 2008. *Salmonella* challenges: prevalence in swine and poultry and potential pathogenicity of such isolates. *Journal of Animal Science, 86* (14 Suppl), E149-62.

Foley, S.L., Nayak, R., Hanning, I.B., Johnson, T.J., Han, J., and Ricke, S.C., 2011. Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. *Applied and Environmental Microbiology, 77*, 4273-4279.

Francolini, I. and Donelli, G., 2010. Prevention and control of biofilm-based medical-device-related infections. *FEMS Immunology & Medical Microbiology, 59* (3), 227-238.

Friedman, L. and Kolter, R., 2004. Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. *Molecular Microbiology, 51* (3), 675-690.

Fux, C.A., Costerton, J.W., Stewart, P.S., and Stoodley, P., 2005. Survival strategies of infectious biofilms. *Trends in Microbiology, 13* (1), 34-40.

Gabbett, R.J., 2015. Fostering Food Safety. *Meatingplace.*

García, B., Latasa, C., Solano, C., Portillo, F.G.-d., Gamazo, C., and Lasa, I., 2004. Role of the GGDEF protein family in *Salmonella* cellulose biosynthesis and biofilm formation. *Molecular Microbiology, 54* (1), 264-277.

Garrett, T.R., Bhakoo, M., and Zhang, Z., 2008. Bacterial adhesion and biofilms on surfaces. *Progress in Natural Science, 18* (9), 1049-1056.

Gast, R.K., Guard-Bouldin, J., and Holt, P.S., 2004. Colonization of reproductive organs and internal contamination of eggs after experimental infection of laying hens with *Salmonella heidelberg* and *Salmonella enteritidis*. *Avian Diseases, 48* (4), 863-869.

Gast, R.K., Guraya, R., Guard-Bouldin, J., Holt, P.S., and Moore, R.W., 2007. Colonization of specific regions of the reproductive tract and deposition at different locations inside eggs laid by hens infected with *Salmonella* Enteritidis or *Salmonella* Heidelberg. *Avian diseases, 51* (1), 40-44.

Gibson, D., White, A., Snyder, S., Martin, S., Heiss, C., Azadi, P., Surette, M., and Kay, W., 2006. *Salmonella* produces an O-antigen capsule regulated by AgfD and important for environmental persistence. *Journal of Bacteriology, 188* (22), 7722-7730.

Gibson, H., Taylor, J., Hall, K., and Holah, J., 1999. Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. *Journal of Applied Microbiology, 87* (1), 41-48.

GMA, 2011. Capturing recall costs: measuring and recovering the losses. *The Association of Food, Beverage and Consumer Products Companies*.

Grimont, P.A. and Weill, F.X., 2007. Antigenic formulae of the *Salmonella* serovars. *WHO Collaborating Centre for Reference and Research on Salmonella, 9*, 1-166.

Guttenplan, S.B. and Kearns, D.B., 2013. Regulation of flagellar motility during biofilm formation. *FEMS Microbiology Reviews, 37* (6), 849-871.

Hausner, M. and Wuertz, S., 1999. High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. *Applied and Environmental Microbiology, 65* (8), 3710-3713.

Hentzer, M., Teitzel, G.M., Balzer, G.J., Heydorn, A., Molin, S., Givskov, M., and Parsek, M.R., 2001. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *Journal of Bacteriology, 183* (18), 5395-5401.

Hinsa, S.M., Espinosa-Urgel, M., Ramos, J.L., and O'toole, G.A., 2003. Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. *Molecular Microbiology, 49* (4), 905-918.

Hoffman, L.R., D'argenio, D.A., MacCoss, M.J., Zhang, Z., Jones, R.A., and Miller, S.I., 2005. Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature, 436* (7054), 1171.

Holah, J. and Kearney, L., 1992. Introduction to biofilms in the food industry. *Biofilms—Science and Technology*. Springer.

Howard, Z.R., O'Bryan, C.A., Crandall, P.G., and Ricke, S.C., 2012. *Salmonella* Enteritidis in shell eggs: current issues and prospects for control. *Food Research International, 45* (2), 755-764.

Iino, T., Komeda, Y., Kutsukake, K., Macnab, R.M., Matsumura, P., Parkinson, J.S., Simon, M.I., and Yamaguchi, S., 1988. New unified nomenclature for the flagellar genes of *Escherichia coli* and *Salmonella typhimurium*. *Microbiological Reviews, 52* (4), 533.

Jefferson, K.K., 2004. What drives bacteria to produce a biofilm? *FEMS Microbiology Letters, 236* (2), 163-173.

Joerger, R.D., 2003. Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. *Poultry Science, 82* (4), 640-647.

Johansen, C., Falholt, P., and Gram, L., 1997. Enzymatic removal and disinfection of bacterial biofilms. *Applied and Environmental Microbiology, 63* (9), 3724-3728.

Joseph, B., Otta, S., Karunasagar, I., and Karunasagar, I., 2001. Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. *International Journal of Food Microbiology, 64* (3), 367-372.

Jun, W., Kim, M.S., Cho, B.-K., Millner, P.D., Chao, K., and Chan, D.E., 2010. Microbial biofilm detection on food contact surfaces by macro-scale fluorescence imaging. *Journal of Food Engineering, 99* (3), 314-322.

Kaldhone, P.R., Foley, S.L., and Ricke, S.C., 2017. Salmonella Heidelberg in layer hens and egg production: incidence and potential issues. *In:* Ricke, S. C. and Gast, R. K. (eds.) *Producing Safe Eggs.* San Diego, CA: Elsevier, Inc.

Kaptan, G., Fischer, A.R.H., and Frewer, L.J., 2017. Extrapolating understanding of food risk perceptions to emerging food safety cases. *Journal of Risk Research*, 10.1080/13669877.2017.1281330, 1-23.

Keener, K., Bashor, M., Curtis, P., Sheldon, B., and Kathariou, S., 2004. Comprehensive review of Campylobacter and poultry processing. *Comprehensive Reviews in Food Science and Food Safety, 3* (2), 105-116.

Kim, S.-H. and Wei, C.-i., 2007. Biofilm formation by multidrug-resistant Salmonella enterica serotype Typhimurium phage type DT104 and other pathogens. *Journal of Food Protection, 70* (1), 22-29.

Klausen, M., Aaes-Jørgensen, A., Molin, S., and Tolker-Nielsen, T., 2003a. Involvement of bacterial migration in the development of complex multicellular structures in Pseudomonas aeruginosa biofilms. *Molecular Microbiology, 50* (1), 61-68.

Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aaes-Jørgensen, A., Molin, S., and Tolker-Nielsen, T., 2003b. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Molecular Microbiology, 48* (6), 1511-1524.

Kulp, A. and Kuehn, M.J., 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annual Review of Microbiology, 64*, 163-184.

Kumpel, E. and Nelson, K.L., 2013. Comparing microbial water quality in an intermittent and continuous piped water supply. *Water Research, 47* (14), 5176-5188.

Lawrence, J., Korber, D., Hoyle, B., Costerton, J.W., and Caldwell, D., 1991. Optical sectioning of microbial biofilms. *Journal of Bacteriology, 173* (20), 6558-6567.

Le Gentil, C., Sylla, Y., and Faille, C., 2010. Bacterial re-contamination of surfaces of food processing lines during cleaning in place procedures. *Journal of Food Engineering, 96* (1), 37-42.

Ledeboer, N.A., Frye, J.G., McClelland, M., and Jones, B.D., 2006. *Salmonella enterica* serovar Typhimurium requires the Lpf, Pef, and Tafi fimbriae for biofilm formation on HEp-2 tissue culture cells and chicken intestinal epithelium. *Infection and Immunity, 74* (6), 3156-3169.

Leid, J.G., Willson, C.J., Shirtliff, M.E., Hassett, D.J., Parsek, M.R., and Jeffers, A.K., 2005. The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN-γ-mediated macrophage killing. *The Journal of Immunology, 175* (11), 7512-7518.

Leriche, V., Briandet, R., and Carpentier, B., 2003. Ecology of mixed biofilms subjected daily to a chlorinated alkaline solution: spatial distribution of bacterial species suggests a protective effect of one species to another. *Environmental Microbiology, 5* (1), 64-71.

Lestari, S.I., Han, F., Wang, F., and Ge, B., 2009. Prevalence and antimicrobial resistance of *Salmonella* serovars in conventional and organic chickens from Louisiana retail stores. *Journal of Food Protection, 72* (6), 1165-1172.

Lillard, H., 1990. The impact of commercial processing procedures on the bacterial contamination and cross-contamination of broiler carcasses. *Journal of Food Protection, 53* (3), 202-204.

Limoli, D.H., Jones, C.J., and Wozniak, D.J., 2015. Bacterial Extracellular Polysaccharides in Biofilm Formation and Function. *In:* Ghannoum, M., Parsek, M., Whiteley, M. and Mukherjee, P. (eds.) *Microbial Biofilms, Second Edition.* Washington, DC: American Society of Microbiology.

Lu, T.K. and Collins, J.J., 2007. Dispersing biofilms with engineered enzymatic bacteriophage. *Proceedings of the National Academy of Sciences, 104* (27), 11197-11202.

Mah, T.-F.C. and O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology, 9* (1), 34-39.

Mandal, B. and Brennand, J., 1988. Bacteraemia in salmonellosis: a 15 year retrospective study from a regional infectious diseases unit. *BMJ: British Medical Journal, 297* (6658), 1242.

Matsumura, K., Furukawa, S., Ogihara, H., and Morinaga, Y., 2011. Roles of multidrug efflux pumps on the biofilm formation of *Escherichia coli* K-12. *Biocontrol Science, 16* (2), 69-72.

Merritt, J.H., Brothers, K.M., Kuchma, S.L., and O'Toole, G.A., 2007. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. *Journal of Bacteriology, 189* (22), 8154-8164.

Molin, S. and Tolker-Nielsen, T., 2003. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Current Opinion in Biotechnology, 14* (3), 255-261.

Møller, S., Sternberg, C., Andersen, J.B., Christensen, B.B., Ramos, J.L., Givskov, M., and Molin, S., 1998. In situ gene expression in mixed-culture biofilms: Evidence of metabolic interactions between community members. *Applied and Environmental Microbiology, 64* (2), 721-732.

Nivens, D.E., Ohman, D.E., Williams, J., and Franklin, M.J., 2001. Role of alginate and its O acetylation in formation of Pseudomonas aeruginosa microcolonies and biofilms. *Journal of Bacteriology, 183* (3), 1047-1057.

O'Toole, G.A., Kaplan, H.B., and Kolter, R., 2000. Biofilm formation as microbial development. *Annual Reviews in Microbiology, 54* (1), 49-79.

O'Toole, G.A. and Kolter, R., 1998a. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology, 30* (2), 295-304.

O'Toole, G.A. and Kolter, R., 1998b. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Molecular Microbiology, 28* (3), 449-461.

O'Bryan, C.A., Pendleton, S.J., Crandall, P.G., and Ricke, S.C., 2015. Potential of plant essential oils and their components in animal agriculture–in vitro studies on antibacterial mode of action. *Frontiers in Veterinary Science, 2*, 35.

Ogasawara, H., Yamamoto, K., and Ishihama, A., 2011. Role of the biofilm master regulator CsgD in cross-regulation between biofilm formation and flagellar synthesis. *Journal of Bacteriology, 193* (10), 2587-2597.

Olsén, A., Wick, M.J., Mörgelin, M., and Björck, L., 1998. Curli, fibrous surface proteins of *Escherichia coli*, interact with major histocompatibility complex class I molecules. *Infection and Immunity, 66* (3), 944-949.

Olsen, J., Brown, D., Madsen, M., and Bisgaard, M., 2003. Cross-contamination with Salmonella on a broiler slaughterhouse line demonstrated by use of epidemiological markers. *Journal of Applied Microbiology, 94* (5), 826-835.

Oulahal-Lagsir, N., Martial-Gros, A., Bonneau, M., and Blum, L., 2000. Ultrasonic methodology coupled to ATP bioluminescence for the non-invasive detection of fouling in food processing equipment—validation and application to a dairy factory. *Journal of Applied Microbiology, 89* (3), 433-441.

Owens, C.M., Sams, A.R., and Alvarado, C., 2000. *Poultry Meat Processing*, Boca Raton, FL, CRC Press.

Palmer, J., Flint, S., and Brooks, J., 2007. Bacterial cell attachment, the beginning of a biofilm. *Journal of Industrial Microbiology & Biotechnology, 34* (9), 577-588.

Patterson, J. and Burkholder, K., 2003. Application of prebiotics and probiotics in poultry production. *Poultry Science, 82* (4), 627-631.

Poppe, C., Smart, N., Khakhria, R., Johnson, W., Spika, J., and Prescott, J., 1998. Salmonella typhimurium DT104: a virulent and drug-resistant pathogen. *The Canadian Veterinary Journal, 39* (9), 559.

Pratt, L.A. and Kolter, R., 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Molecular Microbiology, 30* (2), 285-293.

Prigent-Combaret, C., Prensier, G., Le Thi, T.T., Vidal, O., Lejeune, P., and Dorel, C., 2000. Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains: role of flagella, curli and colanic acid. *Environmental Microbiology, 2* (4), 450-464.

Prouty, A., Schwesinger, W., and Gunn, J., 2002. Biofilm Formation and Interaction with the Surfaces of Gallstones by *Salmonella* spp. *Infection and Immunity, 70* (5), 2640-2649.

Rabsch, W., Hargis, B.M., Tsolis, R.M., Kingsley, R.A., Hinz, K.-H., Tschäpe, H., and Bäumler, A.J., 2000. Competitive exclusion of *Salmonella* Enteritidis by *Salmonella* Gallinarum in Poultry. *Emerging Infectious Diseases, 6* (5), 443-448.

Rai, M., Ingle, A., Gaikwad, S., Gupta, I., Gade, A., and Silvério da Silva, S., 2016. Nanotechnology based anti-infectives to fight microbial intrusions. *Journal of Applied Microbiology, 120* (3), 527-542.

Rasschaert, G., Houf, K., Godard, C., Wildemauwe, C., Pastuszczak-Frak, M., and De Zutter, L., 2008. Contamination of carcasses with *Salmonella* during poultry slaughter. *Journal of Food Protection, 71* (1), 146-152.

Reij, M., Den Aantrekker, E., and Force, I.E.R.A.i.M.T., 2004. Recontamination as a source of pathogens in processed foods. *International Journal of Food Microbiology, 91* (1), 1-11.

Ricke, S.C., 2017. Insights and challenges of *Salmonella* infection of laying hens. *Current Opinion in Food Science, 18*, 43-49.

Römling, U., Bian, Z., Hammar, M., Sierralta, W.D., and Normark, S., 1998. Curli fibers are highly conserved between *Salmonella typhimurium* and *Escherichia coli* with respect to operon structure and regulation. *Journal of Bacteriology, 180* (3), 722-731.

Römling, U., Gomelsky, M., and Galperin, M.Y., 2005. C-di-GMP: the dawning of a novel bacterial signalling system. *Molecular Microbiology, 57* (3), 629-639.

Ryu, J.-H., Kim, H., and Beuchat, L.R., 2004. Attachment and biofilm formation by *Escherichia coli* O157: H7 on stainless steel as influenced by exopolysaccharide production, nutrient availability, and temperature. *Journal of Food Protection, 67* (10), 2123-2131.

Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W., and Davies, D.G., 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *Journal of Bacteriology, 184* (4), 1140-1154.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., and Griffin, P.M., 2011. Foodborne illness acquired in the United States--major pathogens. *Emerging Infectious Diseases, 17* (1), 7-15.

Scharff, R.L., 2012. Economic burden from health losses due to foodborne illness in the United States. *Journal of Food Protection, 75* (1), 123-31.

Schlisselberg, D.B. and Yaron, S., 2013. The effects of stainless steel finish on *Salmonella* Typhimurium attachment, biofilm formation and sensitivity to chlorine. *Food Microbiology, 35* (1), 65-72.

Schooling, S.R. and Beveridge, T.J., 2006. Membrane vesicles: an overlooked component of the matrices of biofilms. *Journal of Bacteriology, 188* (16), 5945-5957.

Semmler, A.B., Whitchurch, C.B., and Mattick, J.S., 1999. A re-examination of twitching motility in *Pseudomonas aeruginosa*. *Microbiology, 145* (10), 2863-2873.

Shimoni, Z., Pitlik, S., Leibovici, L., Samra, Z., Konigsberger, H., Drucker, M., Agmon, V., Ashkenazi, S., and Weinberger, M., 1999. Nontyphoid *Salmonella* bacteremia: age-related differences in clinical presentation, bacteriology, and outcome. *Clinical Infectious Diseases, 28* (4), 822-827.

Sinde, E. and Carballo, J., 2000. Attachment of *Salmonella* spp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. *Food Microbiology, 17* (4), 439-447.

Solano, C., García, B., Valle, J., Berasain, C., Ghigo, J.M., Gamazo, C., and Lasa, I., 2002. Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. *Molecular Microbiology, 43* (3), 793-808.

Solomon, E.B., Niemira, B.A., Sapers, G.M., and Annous, B.A., 2005. Biofilm formation, cellulose production, and curli biosynthesis by *Salmonella* originating from produce, animal, and clinical sources. *Journal of Food Protection, 68* (5), 906-912.

Spiers, A.J., Bohannon, J., Gehrig, S.M., and Rainey, P.B., 2003. Biofilm formation at the air–liquid interface by the Pseudomonas fluorescens SBW25 wrinkly spreader requires an acetylated form of cellulose. *Molecular Microbiology, 50* (1), 15-27.

Steenackers, H., Hermans, K., Vanderleyden, J., and De Keersmaecker, S.C.J., 2012. *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. *Food Research International, 45* (2), 502-531.

Stepanović, S., Ćirković, I., Ranin, L., and Svabić-Vlahović, M., 2004. Biofilm formation by Salmonella spp. and Listeria monocytogenes on plastic surface. *Letters in Applied Microbiology, 38* (5), 428-432.

Stewart, P.S., Roe, F., Rayner, J., Elkins, J.G., Lewandowski, Z., Ochsner, U.A., and Hassett, D.J., 2000. Effect of catalase on hydrogen peroxide penetration into Pseudomonas aeruginosa biofilms. *Applied and Environmental Microbiology, 66* (2), 836-838.

Stoodley, P., Dodds, I., Boyle, J.D., and Lappin-Scott, H., 1998. Influence of hydrodynamics and nutrients on biofilm structure. *Journal of Applied Microbiology, 85* (S1), 19S-28S.

Stoodley, P. and Lewandowski, Z., 1994. Liquid flow in biofilm systems. *Applied and Environmental Microbiology, 60* (8), 2711-2716.

Thomke, S. and Elwinger, K. Growth promotants in feeding pigs and poultry. I. Growth and feed efficiency responses to antibiotic growth promotants. Annales de Zootechnie, 1998. 85-97.

Threlfall, E.J., Ward, L.R., Frost, J.A., and Willshaw, G.A., 2000. The emergence and spread of antibiotic resistance in food-borne bacteria. *International Journal of Food Microbiology, 62* (1-2), 1-5.

Tolker-Nielsen, T. and Molin, S., 2000. Spatial organization of microbial biofilm communities. *Microbial Ecology, 40* (2), 75-84.

Trevejo, R.T., 2003. Epidemiology of *Salmonellosis* in California, 1990-1999: Morbidity, Mortality, and Hospitalization Costs. *American Journal of Epidemiology, 157* (1), 48-57.

USDA-FSIS. 2013. *Salmonella Action Plan* [Online]. United States Department of Agriculture. Available: https://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/get-answers/food-safety-fact-sheets/foodborne-illness-and-disease/salmonella/sap [Accessed 6/11/18].

USDA-FSIS. 2016. *Serotypes Profile of Salmonella Isolates from Meat and Poultry Products, January 1998 through December 2014* [Online]. United States Department of Agriculture. Available: https://www.fsis.usda.gov/wps/wcm/connect/3866026a-582d-4f0e-a8ce-851b39c7390f/Salmonella-Serotype-Annual-2014.pdf?MOD=AJPERES [Accessed 6/14/18].

Valeriano, C., De Oliveira, T.L.C., De Carvalho, S.M., das Graças Cardoso, M., Alves, E., and Piccoli, R.H., 2012. The sanitizing action of essential oil-based solutions against *Salmonella enterica* serotype Enteritidis S64 biofilm formation on AISI 304 stainless steel. *Food Control, 25* (2), 673-677.

Van Houdt, R. and Michiels, C.W., 2010. Biofilm formation and the food industry, a focus on the bacterial outer surface. *Journal of Applied Microbiology, 109* (4), 1117-1131.

Verstraeten, N., Braeken, K., Debkumari, B., Fauvart, M., Fransaer, J., Vermant, J., and Michiels, J., 2008. Living on a surface: swarming and biofilm formation. *Trends in Microbiology, 16* (10), 496-506.

Vestby, L.K., Møretrø, T., Langsrud, S., Heir, E., and Nesse, L.L., 2009. Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal-and feed factories. *BMC Veterinary Research, 5*, 20.

Wang, Q., Frye, J.G., McClelland, M., and Harshey, R.M., 2004. Gene expression patterns during swarming in *Salmonella typhimurium*: genes specific to surface growth and putative new motility and pathogenicity genes. *Molecular Microbiology, 52* (1), 169-187.

Waters, C.M., Lu, W., Rabinowitz, J.D., and Bassler, B.L., 2008. Quorum sensing controls biofilm formation in Vibrio cholerae through modulation of cyclic di-GMP levels and repression of vpsT. *Journal of Bacteriology, 190* (7), 2527-2536.

Watnick, P. and Kolter, R., 2000. Biofilm, city of microbes. *Journal of Bacteriology, 182* (10), 2675-2679.

White, A., Gibson, D., Kim, W., Kay, W., and Surette, M., 2006. Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of *Salmonella*. *Journal of Bacteriology, 188* (9), 3219-3227.

Wingender, J. and Flemming, H.-C., 2011. Biofilms in drinking water and their role as reservoir for pathogens. *International Journal of Hygiene and Environmental Health, 214* (6), 417-423.

Wirtanen, G., Storgards, E., Saarela, M., Salo, S., and Mattila-Sandholm, T., 2000. Detection of Biofilms in the Food and Beverage Industry. *In:* Walker, J., Surman, S. and Jass, J. (eds.) *Industrial Biofouling: Detection, Prevention and Control.* John Wiley & Sons Ltd.

Wolcott, R.D. and Ehrlich, G.D., 2008. Biofilms and chronic infections. *JAMA, 299* (22), 2682-2684.

Xiao, J., Klein, M.I., Falsetta, M.L., Lu, B., Delahunty, C.M., Yates III, J.R., Heydorn, A., and Koo, H., 2012. The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. *PLoS Pathogens, 8* (4), e1002623.

Zakikhany, K., Harrington, C.R., Nimtz, M., Hinton, J.C., and Römling, U., 2010. Unphosphorylated CsgD controls biofilm formation in *Salmonella enterica* serovar Typhimurium. *Molecular Microbiology, 77* (3), 771-786.

Zogaj, X., Nimtz, M., Rohde, M., Bokranz, W., and Römling, U., 2001. The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. *Molecular Microbiology, 39* (6), 1452-1463.

III. Chapter 2. Comparison of methods for quantitating *Salmonella enterica* Typhimurium and Heidelberg strain attachment to reusable plastic shipping container coupons and preliminary assessment of sanitizer efficacy

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Abstract

Salmonella serovars, one of the leading contributors to foodborne illness and are especially problematic for foods that are not cooked before consumption, such as fresh produce. The shipping containers that are used to transport and store fresh produce may play a role in cross contamination and subsequent illnesses. However, methods for quantitatively measuring attached cells are somewhat variable. The overall goal of this study was to compare conventional plating with molecular methods for quantitating attached representative strains of *Salmonella* Typhimurium and Heidelberg on reusable plastic container (RPC) coupons, respectively. We attached *Salmonella enterica* serovar Typhimurium ATCC 14028 and serovar Heidelberg SL486 (parent and an antibiotic resistant marker strain) to plastic coupons (2.54 cm^2) derived from previously used shipping containers by growing for 72 h in tryptic soy broth. The impact of the concentration of sanitizer on log reductions between unsanitized and sanitized coupons was evaluated by exposing attached *S.* Typhimurium cells to 200 ppm and 200,000 ppm sodium hypochlorite (NaClO). Differences in sanitizer effectiveness between serovars were also evaluated with attached *S.* Typhimurium compared to attached *S.* Heidelberg populations after being exposed to 200 ppm peracetic acid (PAA). Treatment with NaClO caused an average of 2.73 ± 0.23 log CFU of *S.* Typhimurium per coupon removed with treatment at 200 ppm while 3.36 ± 0.54 log CFU was removed at 200,000 ppm. Treatment with PAA caused an average of 2.62 ± 0.15 log CFU removed for *S.* Typhimurium and 1.41 ± 0.17 log CFU for *S.* Heidelberg (parent) and 1.61 ± 0.08 log CFU (marker). Lastly, scanning electron microscopy (SEM) was used to visualize cell attachment and coupon surface topography. SEM images showed that remaining attached cell populations were visible even after sanitizer application. Conventional plating and qPCR yielded similar levels of enumerated bacterial populations indicating a high

concordance between the two methods. Therefore, qPCR could be used for the rapid quantification of *Salmonella* attached on RPC.

Keywords: *Salmonella* Typhimurium; *Salmonella* Heidelberg; shipping containers; sanitization; attachment; fresh produce

Introduction

Salmonella is a major cause of foodborne illness in the United States (US), resulting in an estimated 20,000 hospitalizations and 400 deaths per year, the most of any foodborne bacterium (Scallan et al., 2011). *Salmonella* is a Gram-negative enteropathogenic bacterium that can cause a range of illnesses from gastroenteritis to potentially life threatening conditions such as bacteremia if an infection becomes invasive in at risk population (CDC, 2015b). In 2014, various *Salmonella* species caused 10 multistate outbreaks in the US with approximately 1,000 outbreak cases (CDC, 2015a). One characteristic associated with *Salmonella* is the ability to form biofilms as a protective adaptation against environmental challenges, with this being the preferred state in their natural habitats (Hall-Stoodley et al., 2004; Giaouris et al., 2012).

Biofilms are composed of communities of bacterial cells within an extracellular matrix that can adhere to biotic as well as abiotic surfaces (Jahid et al., 2015). Aggregation into biofilms allows these communities to tolerate greater stresses and persist in hostile environments, which presents a problem to the food industry as potential reservoirs of contamination (Steenackers et al., 2012; Sadekuzzaman et al., 2015). Numerous studies have been conducted on major foodborne bacteria known to form biofilms such as *Salmonella* Typhimurium (Stepanović et al., 2004; Ban et al., 2012; Veluz et al., 2012; Park and Kang, 2014), *Listeria monocytogenes* (Rodriguez and McLandsborough, 2007; Belessi et al., 2011; Hingston et al., 2013), and *Escherichia coli* O157:H7 (Wang et al., 2012) to evaluate attachment to abiotic surfaces including stainless steel and plastics, which are often encountered in food processing and transportation systems. It can be assumed that these surfaces could pass pathogenic cells to food products with the sloughing off of cells from biofilm matrices (Jensen et al., 2013).

It has been suggested that determining the efficacy of sanitizers to remove attached microorganisms from food storage and transportation equipment will be important to determine the extent to which attached microorganisms can persist in food processing and shipping environments (Corcoran et al., 2013; Clayborn et al., 2015). Recent reports have resulted in a heightened recognition and debate on the risk that may be associated with reusable plastic containers (RPCs) due to cell attachment, biofilm formation, and fresh produce contamination (Clayborn et al., 2015; Suslow, 2015). The RPCs can retain considerable levels of bacteria, for example in survey studies 37.5% and 8.3% of the RPCs from the field contained > log 5 CFU/swab and > log 6 CFU/swab, respectively (Suslow 2015). RPCs are most often used in the harvesting, processing, packing and shipping of fresh produce, which may be problematic as any microbial contamination could eventually be transferred to fresh produce (Jensen et al., 2013; Carrasco et al., 2012). These RPCs are designed for several cycles of use, are often placed directly on soil, and because of that, there is a potential risk of cross contamination, especially if not thoroughly sanitized (Sholberg, 2004). A previous report based in Italy indicated that their RPCs could hypothetically by reused on average, 200 times over a lifetime of 10 years before being removed from circulation (Levi et al., 2011).

This research is unique in that the RPCs evaluated in this study were previously in the distribution stream. Upon receipt, some of these containers had visually discernible surface wear, which may provide a more realistic model for evaluating the efficacy of sanitizer treatments on containers that have been through several cycles of use, sanitization, and reuse. The challenge was to adopt and develop methods for direct quantitation of laboratory attached *Salmonella* to these complex materials that already contained a background of unidentified bacteria. The

primary objective of study was to compare standard plating methodology with qPCR for recovery and quantitation of *Salmonella* that were attached to these surfaces in the laboratory. While this was not an attempt to assess the broader aspects of RPC contamination, general industry sanitizer conditions were simulated as a part of this initial methodology development study. We chose to look at the best characterized representative strains of two serovars (*S.* Typhimurium and *S.* Enteritidis) that have been identified as commonly observed in *Salmonella* outbreaks in produce (Jackson et al., 2013). The quantitative methods represented a comparison of independent experimental approaches ranging from standard selective plate enumeration of both serovars, generation of a specific *S.* Heidelberg marker strain that allowed direct recovery, and finally a quantitative PCR assay based on primers specific for *S.* Heidelberg.

Materials and methods

Bacterial growth conditions and marker strain preparation

Isolated colonies of *S.* Typhimurium ATCC 14028, *S.* Heidelberg SL486 (parent strain), or a *S.* Heidelberg nalidixic acid (NA) resistant marker strain derived from SL486 were added to 5 mL of tryptic soy broth (TSB) (Neogen, Lansing, MI) and incubated for 18 h at 37°C, 110 rpm. The marker strain was generated by daily subculture of SL486 into growth media containing increasing amounts of NA over the course of seven days until a final resistance concentration of 20 µg/mL NA was achieved.

Sanitizer preparation

Both sodium hypochlorite (NaClO, Sigma-Aldrich, St. Louis, MO) and peracetic acid (PAA, Sigma-Aldrich, St. Louis, MO) were prepared by diluting stock sanitizer in sterile

deionized water to a final concentration of 200 ppm, the maximum residue allowed on food contact surfaces without further removal (FDA, 2015). Additionally, NaClO was prepared at 200,000 ppm for comparison of effectiveness with 200 ppm.

Coupon preparation and attachment of cells

Coupons of 1 x 1 inch (2.54 x 2.54 cm) size were cut from RPCs provided by a commercial company using a band saw with uniform coupons without holes selected for use. Attachment of cells on coupons was based on procedures described previously (Clayborn et al., 2015) with some modifications. Initially, coupons were scrubbed in distilled water and soaked in 70% ethanol (5 min exposure with agitation) to remove surface contamination. These were subsequently dried for 2 min and placed in sterile 90 mL specimen cups (Clarity Diagnostics, Boca Raton, FL) with 40 mL TSB and 0.5 mL of overnight culture for an initial inoculum level of approximately 10^7 colony forming units (CFU). Two coupons were placed in each cup with one designated for sanitization and one remaining unsanitized for comparison. Cups were incubated at 37°C at 110 rpm for 24 h to initiate RPC surface attachment. Following 24 h incubation, the coupons were rinsed thoroughly with deionized water to remove planktonic cells, dried for 2 min, and placed in new sterile specimen cups. Forty mL of new TSB was added to each cup and the coupons were incubated at 37°C at 110 rpm for 72 h to generate the final population level of attached bacterial cells.

Bacterial enumeration

Coupons were rinsed with deionized water, dried for 2 min, and transferred to sterile 50 mL centrifuge tubes (VWR, Radnor, PA). Twenty mL of phosphate buffered saline (PBS, pH 7.4)

and 3 g of glass beads (3 mm, EMD Millipore, Billerica, MA) were added to the tubes to facilitate removal of attached bacterial cells. Tubes were vigorously shaken for 1 min to remove attached cells as previously described by Park and Kang (Park and Kang, 2014). Rinsates were serially diluted with PBS to produce 10-fold diluted samples and spread-plated on tryptic soy agar plates (Neogen) in duplicate. Non-selective TSA was used to reduce stress caused by selective agents present in other media. Plates were incubated at 37°C for 24 h to determine the CFU per coupon.

Sanitizer treatment

Out of the two coupons from each specimen cup, one was designated to be sanitized and one to remain unsanitized. The unsanitized coupons were removed from their cups and rinsed with 40 mL of distilled water to remove residual TSB and planktonic cells before proceeding to bacterial enumeration. The sanitized coupons were first sprayed five times on each side with 43°C tap water to simulate how the RPC would be sprayed with water in a commercial environment before treatment with sanitizer. Afterwards, they were transferred to cups containing 150 mL of NaClO at 200 ppm or 200,000 ppm, or to cups containing PAA at 200 ppm and were subjected to vigorous agitation for 30 s at room temperature. Coupons were subsequently removed from the sanitizer, dried for 2 min, and enumerated using the previously described procedure in subsection “bacterial enumeration”. Antibacterial treatments were performed on five biological replicates for NaClO and on five replicates for PAA.

DNA extraction and *Salmonella* confirmation by conventional PCR

Conventional PCR was performed to confirm *Salmonella* presence in the rinsate. Fifteen mL of PBS containing detached cells was centrifuged for 10 min at 11,000 rpm and 14 mL of the supernatant removed. The remaining 1 mL was centrifuged with 950 μL of supernatant removed to obtain a concentrated DNA sample. Samples were subsequently boiled and placed in ice to extract DNA from the cells. The PCR reaction volume consisted 1 μL of sample DNA, 500 nM of each primer (F: TTT GGC GGC GCA GGC GAT TC; R: GCC TCC GCC TCA TCA ATC CG) (Kim et al., 2006), which amplifies the 423 bp fragment within the genomic DNA of *Salmonella*, 10 μL of 2X premix ExTaq (Takara, Mountain View, CA), and 7 μL of distilled water. The PCR steps included initial denaturation of 94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s followed by a 5 min elongation step at 72°C. The amplified product was electrophoretically separated on 1% agarose gel in 1X TAE buffer.

DNA extraction and enumeration of *S*. Heidelberg in rinsate by quantitative PCR

Quantitative PCR with a Mastercycler® ep realplex (Eppendorf, Hauppauge, NY) was used to quantify detached cells and confirm plate enumerations of the *S.* Heidelberg samples. Extraction of DNA from the PBS rinsate containing detached cells was performed with a Qiagen Blood and Tissue Kit (Qiagen, Valencia, CA). Each duplicate PCR aliquot consisted of 5 μL of DNA, 500 nM of each primer (F: TGT TTG GAG CAT CAT CAG AA; R: GCT CAA CAT AAG GGA AGC AA) (Park and Ricke, 2015), 10 μL of SYBR® Premix Ex Taq™ II (Takara, Shiga, Japan), and nuclease free water to bring to a final reaction volume of 20 μL. Aliquots were subsequently denatured at 95°C for 2 min and followed by 40 cycles of 95°C for 15 s,

annealing at 60°C for 15 s, and extension at 68°C. Melting curve analysis consisted of an increasing temperature of 0.5°C per min for 20 min from 60 to 95°C.

Scanning electron microscopy (SEM) imaging

The evaluation of coupons via scanning electron microscopy (SEM) was performed based on Clayborn et al. (2015). Briefly, coupons were attached to an aluminum specimen mount with a double-coated carbon conductive tab (Ted Pella Inc., Redding, CA) and viewed with a Philips SL 30 ESEM (FEI Company, Hillsboro, OR) in a low vacuum mode.

Statistical analysis

Plate counts were performed in duplicate, and the average and standard error log CFU per coupon were determined by averaging all biological replicates subjected to the same experimental conditions. A student's t-test ($P \leq 0.05$) was performed to compare differences with JMP® Genomics 7.0 (SAS Institute Inc., Cary, NC).

Results

Effect of concentration on NaClO sanitizer efficacy on *S*. Typhimurium

Salmonella Typhimurium ATCC 14028 attached cells were exposed to 200 and 200,000 ppm NaClO treatments and the difference in lethality between concentrations in decreasing cell numbers was compared (Fig. 1). The *S.* Typhimurium exposed to 30 s of 200 ppm NaClO sanitizer exhibited an average baseline cell population of 7.32 ± 0.14 log CFU recovered per coupon from unsanitized coupons and an average cell population of 4.59 ± 0.14 log CFU recovered from sanitized coupons for a log reduction of 2.73 ± 0.23 due to sanitizer activity.

Application of 200,000 ppm sanitizer resulted in baseline cell populations of 6.92 ± 0.31 log CFU and sanitized treatment counts of 3.57 ± 0.31 log CFU for a reduction of 3.36 ± 0.54 log CFU per coupon due to sanitizer activity. The log CFU reduction values were significantly different between the two sanitizer concentrations ($P < 0.05$).

Response of serovar representative strains to PAA sanitizer efficacy

Salmonella Typhimurium ATCC 14028 and *S.* Heidelberg SL486 (parent and derivative marker strain) attached cells were exposed to 200 ppm PAA treatments and the efficacy of sanitizer was compared between the two serovars (Fig. 2). *S.* Typhimurium attached cells treated with 200 ppm PAA sanitizer for 30 s exhibited baseline plate populations of 7.56 ± 0.10 log CFU per coupon and sanitized coupon cell populations of 4.93 ± 0.13 log CFU for a log reduction of 2.62 ± 0.15. The parent strain of *S.* Heidelberg attached cells exhibited unsanitized treatment population levels of 7.19 ± 0.27 log CFU per coupon and sanitized treatment population levels of 5.78 ± 0.13 log CFU for a log reduction of 1.41 ± 0.17. The marker strain yielded an unsanitized treatment population level of 7.52 ± 0.12 log CFU per coupon, a sanitized treatment population level of 5.91 ± 0.14 log CFU per coupon, and a log reduction of 1.61 ± 0.08. There was no significant difference in the reduction rate between parent and the marker strain ($P > 0.05$)

***Salmonella* confirmation by PCR**

As a non-selective media was used, conventional PCR was used to confirm the presence of *Salmonella* on sanitized coupons as well as unsanitized coupons. Samples (PBS rinsates from bead exposed coupons) were confirmed to consist of *Salmonella*, regardless of sanitization

treatment. Each aliquot successfully amplified a 423 bp region of the targeted gene from *Salmonella*.

Salmonella Heidelberg enumeration by quantitative PCR

The log CFU per coupon for samples before and after treatment with PAA (200 ppm) for 30 s was also evaluated using qPCR to confirm the cell number on each coupon. The efficiency and correlation coefficient (R^2) obtained from the standard curves were 96% and 0.998, respectively. Based on the qPCR analysis, the average log population of the parent strain was 6.12 ± 0.26 log CFU/coupon (unsanitized coupon) and 5.52 ± 0.22 log CFU/coupon (sanitized coupon) for PAA treatment (200 ppm for 30 s). In case of the marker strain, before and after log populations with PAA treatment were 7.07 ± 0.21 and 6.26 ± 0.17 log CFU/coupon, respectively (Table 1).

Scanning electron microscopy (SEM) images

Scanning electron microscopy images were generated from selected coupons throughout the experimental process. An inoculated *S.* Typhimurium coupon was examined by SEM to evaluate the topography of the coupons (Fig. 3A). Cells were shown to be attached following 72 h of growth (Fig. 3B) with the attached cell matrix revealed upon additional magnification (Fig. 3C). Some residues appeared to remain following sanitization with 200 ppm PAA (Fig. 3D).

Discussion

Both NaClO and PAA are representative sanitizers commonly used to disinfect equipment/utensil including plastic containers and other food contact surfaces in the food

industry (Park and Ricke, 2015; Fukuzaki, 2006; Pflug, 2000; Rossoni and Gaylarde, 2000). Previous studies have investigated the efficacy of these sanitizers for removal of attached cells from a variety of possible food contact surfaces from stainless steel to concrete to the produce itself (Corcoran et al., 2014; Srey et al., 2014; Kostaki et al., 2012; Patel and Sharma, 2010). These studies are difficult to compare due to the different materials tested as well as the contrasting experimental approaches that have been used among laboratories (Corcoran et al., 2014). However, based on the current study, usage of these sanitizers with protocols derived from industry standards (IFCO, 2014) appears to be insufficient for removal of all laboratory attached *Salmonella* cells from the RPCs (Fig. 1 and 2). Even when NaClO was applied at a level a thousand times greater (200,000 ppm) than the recommended concentration (200 ppm), the reduction in *S.* Typhimurium population between treated and untreated samples was only increased by 0.63 log CFU. The ability of the attached *Salmonella* cells to persist in even when exposed to such a high concentration highlights the necessity of maintaining clean containers and preventing attachment from occurring.

Some potential differences in sanitizer effectiveness between the strain of *S.* Typhimurium and the strain of *S.* Heidelberg were also observed. *S.* Typhimurium attached cells exhibited a tenfold greater reduction due to sanitizer than the *S.* Heidelberg cells (Fig. 2). This result suggests that there may be differences in sanitizer effectiveness against *S.* Typhimurium and *S.* Heidelberg. However, more strains of each serovar would need to be tested to confirm this as being a serovar difference. Different serovars of *Salmonella* are known to associate with different poultry products (Foley et al., 2011) as well as exhibit differences in attachment to produce (Patel and Sharma, 2010; Shi et al., 2007). This indicates that there may be differences

between serovars due to adapting to different environments as González-Gil et al. (2012) found with different serovars showing differences in virulence gene responses while under acid stress. It is possible that this specific strain was particularly resistant to the activity of PAA which highlights the issue of different bacterial contaminants on RPCs possibly requiring different treatments. Thus, introduction of effective interventions or multiple hurdles in the sanitization stages with the use of several sanitizers with different modes of action may be necessary to eliminate attached cells from plastic shipping containers. These methods along with different bacterial strains and serovars combined as a mixture or cocktail of serovars would probably need to be employed for routine testing to ensure maximum efficacy of a corresponding sanitizer against a range of possible *Salmonella* responses.

In general, recovered populations were not significantly different between the two enumeration methods (conventional plating method and qPCR) indicating a high concordance between two methods. Only one case (parent strain for unsanitized coupon) resulted in a minimal difference ($P = 0.02$). Some variations of means were observed; however, most of the data exhibited similar bacterial cell populations. Some variance of means was to be expected because the two methods are based on different targets, namely recovery of viable cells versus DNA.

The SEM images demonstrated that residues and bacterial cells remained after sanitizer treatment and mechanical agitation (Fig. 3). The SEM images revealed the extent to which these plastic materials are a potential reservoir for microbial contamination. Coupons appeared to be rough and worn after many cycles of use thus providing a potentially better environment for *Salmonella* to attach. The coupons evaluated in this study consisted of a diverse topography with

considerable variation in the surface characteristics. Any microbial populations that became embedded into these cracks could potentially escape the action of sanitizers and provide a reservoir, which after repeated contact with food products, may lead to consumer illness and a lower overall product yield. This is consistent with the previous surveys of RPC used in the field, where RPCs contained > log 5 CFU/swab (9 out of 24 or 37.5% of the RPCs) and > log 6 CFU/swab (2 out of 24 or 8.3%) (Suslow, 2015). These results suggest that RPC surfaces could play an important role in cross-contamination of bacteria to the corresponding food products transported in RPCs. RPCs which escape full cleaning where dirt and organic matter remain may protect any organisms which are attached. Nyeleti et al. (2004) observed that *Salmonella enterica* appeared to survive better against ultraviolet radiation treatment on stainless steel surfaces after coating with bovine serum albumin. In the current study, treatment with 200 ppm NaClO and PAA caused an average of 2.73 and 2.62 log CFU of *S.* Typhimurium, respectively. For *S.* Heidelberg, only a 1.41 and 1.61 log reduction was obtained with 200 ppm PAA. If an RPC contains more than 3 log CFU/coupon of the respective pathogenic bacteria, they may not be entirely eliminated with current industry methods for sanitizing containers. Indeed, the attached *Salmonella* cells were recovered with standard plating methods and confirmed visually upon SEM examination.

In summary, it appears that standard food contact surface sanitizers may be insufficient for eradicating microorganisms from certain food equipment surfaces (Corcoran et al., 2014), and that these surfaces should be evaluated to develop proper risk assessments and subsequently reduce foodborne illness. Due to the nature of fresh produce, contamination may enter a supply system and increase the likelihood of cross contamination while in storage and during

transportation if microorganisms are allowed to attach to containers (Suslow, 2015; Galiş et al., 2013; Lundén et al., 2000). The attachment of bacterial cells on food contact surfaces such as RPCs is especially concerning from a food safety aspect for food items such as fresh produce as these foods do not undergo a heat treatment step during preparation that occurs in other food products such as meat (Lynch et al., 2009). However, predictive modeling and risk assessment may be difficult for these shipping containers due to the variability of the RPC surfaces among a set of containers which may affect attachment and cleaning efficacy. Future studies should compare the level of bacterial cell attachment and sanitizer efficacy against completely new RPCs versus RPCs after different cycles of reuse. Future research must also focus on a variety of conditions that mimic fluctuating environmental conditions such as those brought about by temperature or humidity due to seasonal changes (Ward et al., 2015). The environments in which the containers are exposed should also be assessed to determine factors (high-risk areas, environmental contamination, among others) that may impact container handling equipment as well as contamination occurring during transportation and microbial interactions since many factors can contribute to cell attachment (Corcoran et al., 2014; Veluz et al., 2012). Additionally, research should be performed to examine various aspects of transfer rates from attached cells on shipping containers to fresh produce. Finally, field studies to determine the prevalence of foodborne pathogens before and after sanitation are needed to assess the frequency and potential risk.

In conclusion, we demonstrated that qPCR could offer a reasonable estimate to quantify *Salmonella* populations attached on RPC when compared with standard plating methodology. The distinct attribute of qPCR is that it represents a much more rapid quantification method

(requiring time for whole process: < 4 h) since no further confirmation step is needed. This may be important for the produce and food industry to routinely screen the contamination levels of bacteria to assure limited exposure to cross contamination of their products during manufacturing, transportation and distribution. Thus, it appears that molecular quantification can be utilized for the rapid quantification of *Salmonella* and other foodborne pathogens attached on RPC and may also be helpful for maintaining hygienic quality of RPC as well as food safety of products which are in contact with RPCs.

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Author Contributions

ZS, CAB, and SIL performed experiments, drafted the manuscript, collected test data, and analyzed the data. SAK revised the manuscript. ZS, SHP, and SCR designed the study and revised the manuscript.

References

Ban, G.-H., Park, S.-H., Kim, S.-O., Ryu, S., and Kang, D.-H., 2012. Synergistic effect of steam and lactic acid against *Escherichia coli* O157: H7, *Salmonella Typhimurium*, and *Listeria monocytogenes* biofilms on polyvinyl chloride and stainless steel. International Journal of Food Microbiology, *157* (2), 218-223.

Belessi, C.-E.A., Gounadaki, A.S., Psomas, A.N., and Skandamis, P.N., 2011. Efficiency of different sanitation methods on *Listeria monocytogenes* biofilms formed under various environmental conditions. International Journal of Food Microbiology, *145*, S46-S52.

CDC. 2015a. *Reports of selected Salmonella outbreak investigations.* [Online]. CDC. Available: https://www.cdc.gov/salmonella/outbreaks.html [Accessed 12/1/15].

CDC. 2015b. *Salmonella, Technical Information.* [Online]. CDC. Available: https://www.cdc.gov/salmonella/general/technical.html [Accessed 12/1/15].

Carrasco, E., Morales-Rueda, A., García-Gimeno, R.M., 2012. Cross-contamination and recontamination by *Salmonella* in foods: a review. Food Res. Int., *45* (2), 545-556.

Clayborn, J., Adams, J., Baker, C.A., and Ricke, S.C., 2015. Assessment of Salmonella spp. Attachment to Reusable Plastic Containers Based on Scanning Electron Microscopy and BAX® PCR. Journal of Food Research, *4* (2), 166.

Corcoran, M., Morris, D., De Lappe, N., O'connor, J., Lalor, P., Dockery, P., and Cormican, M., 2013. Commonly used disinfectants fail to eradicate *Salmonella enterica* biofilm from food contact surface materials. Applied and Environmental Microbiology, *80* (4), 1507-1514.

FDA, 2015. Code of Federal Regulations Title 21- Chapter I-Subchapter B-Part 178-Subpart B-Substances Utilized to Control the Growth of Microorganisms. Sec. 178.1010 Sanitizing Solutions. Food and Drug Administration. Accessed: Dec. 1, 2015. Available at: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=178.1010

Fukuzaki, S., 2006. Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. Biocontrol Sci., *11* (4), 147-157.

Foley, S.L., Nayak, R., Hanning, I.B., Johnson, T.J., Han, J., Ricke, S.C., 2011. Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. Appl. Environ. Microbiol., *77* (13), 4273-4279.

Galiş, A.M., Marcq, C., Marlier, D., Portetelle, D., Van, I., Beckers, Y., Théwis, A., 2013. Control of *Salmonella* contamination of shell eggs - Preharvest and postharvest methods: A review. Compr. Rev. Food Sci. Food. Saf., *12* (2), 155-182.

Giaouris, E., Chorianopoulos, N., Skandamis, P., and Nychas, G.-J., 2012. Attachment and biofilm formation by Salmonella in food processing environments. *Salmonella-a dangerous foodborne pathogen.* InTech.

González-Gil, F., Le Bolloch, A., Pendleton, S., Zhang, N., Wallis, A., Hanning, I., 2012. Expression of *hilA* in response to mild acid stress in *Salmonella enterica* is serovar and strain dependent. J. Food Sci., *77* (5), M292-M297.

Hall-Stoodley, L., Costerton, J.W., and Stoodley, P., 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nature Reviews Microbiology, *2* (2), 95.

Hingston, P.A., Stea, E.C., Knøchel, S., and Hansen, T., 2013. Role of initial contamination levels, biofilm maturity and presence of salt and fat on desiccation survival of *Listeria monocytogenes* on stainless steel surfaces. Food Microbiology, *36* (1), 46-56.

IFCO. IFCO RPCs and food safety: 5 things you need to know. IFCO Systems **2014**. Accessed: Aug. 20, 2015. Available online: http://rpcresource.hs-sites.com/foodsafety.

Jahid, I.K., Mizan, M.F.R., Ha, A.J., and Ha, S.-D., 2015. Effect of salinity and incubation time of planktonic cells on biofilm formation, motility, exoprotease production, and quorum sensing of *Aeromonas hydrophila*. Food Microbiology, *49*, 142-151.

Jackson, B.R., Griffin, P.M., Cole, D., Walsh, K.A., Chai, S.J., 2013. Outbreak-associated *Salmonella enterica* serotypes and food commodities, United States, 1998-20 Emerg. Infect. Dis., 19 (*8*), 1239-1244.

Jensen, D.A., Friedrich, L.M., Harris, L.J., Danyluk, M.D., and Schaffner, D.W., 2013. Quantifying transfer rates of *Salmonella* and *Escherichia coli* O157: H7 between fresh-cut produce and common kitchen surfaces. Journal of Food Protection, *76* (9), 1530-1538.

Kim, H.J., Park, S.H., Kim, H.Y., 2006. Comparison of *Salmonella enterica* serovar Typhimurium LT2 and non-LT2 *Salmonella* genomic sequences, and genotyping of *Salmonella* by using PCR. Appl. Environ. Microbiol., *72* (9), 6142-6151.

Kostaki, M., Chorianopoulos, N., Braxou, E., Nychas, G.J., Giaouris, E., 2012. Differential biofilm formation and chemical disinfection resistance of sessile cells of *Listeria monocytogenes* strains under monospecies and dual-species (with *Salmonella enterica*) conditions. Appl. Environ. Microbiol., *78* (8), 2586-2595.

Levi, M., Cortesi, S., Vezzoli, C., Salvia, G., 2011. A comparative life cycle assessment of disposable and reusable packaging for the distribution of Italian fruit and vegetables. Packaging Technol. Sci., *24* (7), 387-400.

Lundén, J.M., Miettinen, M.K., Autio, T.J., Korkeala, H.J., 2000. Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. J. Food Prot., *63* (9), 1204-1207.

Lynch, M.F., Tauxe, R.V., Hedberg, C.W., 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. Epidemiol. Infect., *137* (3), 307-315

Nyeleti, C., Cogan, T.A., Humphrey, T.J. 2004. Effect of sunlight on the survival of *Salmonella* on surfaces. J. Appl. Microbiol., *97* (3), 617-620.

Park, S.H., Ricke, S.C., 2015. Development of multiplex PCR assay for simultaneous detection of *Salmonella* genus, *Salmonella* subspecies I, *Salm.* Enteritidis, *Salm.* Heidelberg and *Salm.* Typhimurium. J. Appl. Microbiol., *118* (1), 152-160.

Park, S. H. and Kang, D.H., 2014. Inactivation of biofilm cells of foodborne pathogens by steam pasteurization. European Food Research and Technology, *238* (3), 471-476.

Patel, J., Sharma, M., 2010. Differences in attachment of *Salmonella enterica* serovars to cabbage and lettuce leaves. Int. J. Food Microbiol., *139* (1), 41-7.

Pflug, I.J., Evans, K.D., 2000. Carrying out biological qualification, the control operation of moist-heat (steam sterilization) processes for producing sterile pharmaceuticals and medical devices. PDA J. Pharm. Sci. Tech., *54*, 117-135.

Rodriguez, A. and McLandsborough, L.A., 2007. Evaluation of the transfer of *Listeria monocytogenes* from stainless steel and high-density polyethylene to Bologna and American cheese. Journal of Food Protection, *70* (3), 600-606.

Rossoni, E.M.M., Gaylarde, C.C., 2000. Comparison of sodium hypochlorite and peracetic acid as sanitising agents for stainless steel food processing surfaces using epifluorescence microscopy. Int. J. Food Microbiol., *61* (1), 81-85.

Sadekuzzaman, M., Yang, S., Mizan, M., and Ha, S., 2015. Current and recent advanced strategies for combating biofilms. Comprehensive Reviews in Food Science and Food Safety, *14* (4), 491-509.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., and Griffin, P.M., 2011. Foodborne illness acquired in the United States--major pathogens. Emerging Infectious Diseases, *17* (1), 7-15.

Shi, X., Namvar, A., Kostrzynska, M., Hora, R., Warriner, K., 2007. Persistence and growth of different *Salmonella* serovars on pre-and postharvest tomatoes. J. Food Prot., *70* (12), 2725-2731.

Sholberg, P.L., 2004. Bin and storage room sanitation. In *Washington Tree Fruit Postharvest Conference*, Yakima, WA, Dec 8, 2004, WSU – TFREC Postharvest Information Network: Wenatchee, WA,. Accessed: Dec. 1, 2015. Available online: http://postharvest.tfrec.wsu.edu/PC2004F.pdf.

Srey, S., Park, S.Y., Jahid, I.K., Oh, S.R., Han, N., Zhang, C.Y., Kim, S.H., Cho, J.I., Ha, S.D., 2014. Evaluation of the removal and destruction effect of a chlorine and thiamine dilaurylsulfate combined treatment of *L. monocytogenes* biofilm. Foodb. Pathog. Dis., *11* (8), 658-663.

Steenackers, H., Hermans, K., Vanderleyden, J., and De Keersmaecker, S.C.J., 2012. *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. Food Research International, *45* (2), 502-531.

Stepanović, S., Ćirković, I., Ranin, L., and Svabić-Vlahović, M., 2004. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Letters in Applied Microbiology, *38* (5), 428-432.

Suslow, T.V., 2015. Minimizing microbial risks in multiple use containers. Food Quality & Safety. Accessed: Dec. 1, 2015. Available online: http://www.foodqualityandsafety.com/article/minimizing-microbiological-risks-in-multiple-use-containers/

Veluz, G., Pitchiah, S., and Alvarado, C., 2012. Attachment of *Salmonella* serovars and *Listeria monocytogenes* to stainless steel and plastic conveyor belts. Poultry Science, *91* (8), 2004-2010.

Wang, R., Bono, J.L., Kalchayanand, N., Shackelford, S., and Harhay, D.M., 2012. Biofilm formation by Shiga toxin–producing *Escherichia coli* O157: H7 and Non-O157 strains and their tolerance to sanitizers commonly used in the food processing environment. Journal of Food Protection, *75* (8), 1418-1428.

Ward, M., Dhingra, R., Remais, J.V., Chang, H.H., Johnston, L.M., Jaykus, L.A., Leon, J., 2015. Associations between weather and microbial load on fresh produce prior to harvest. J. Food Prot., *78* (4), 849-854.

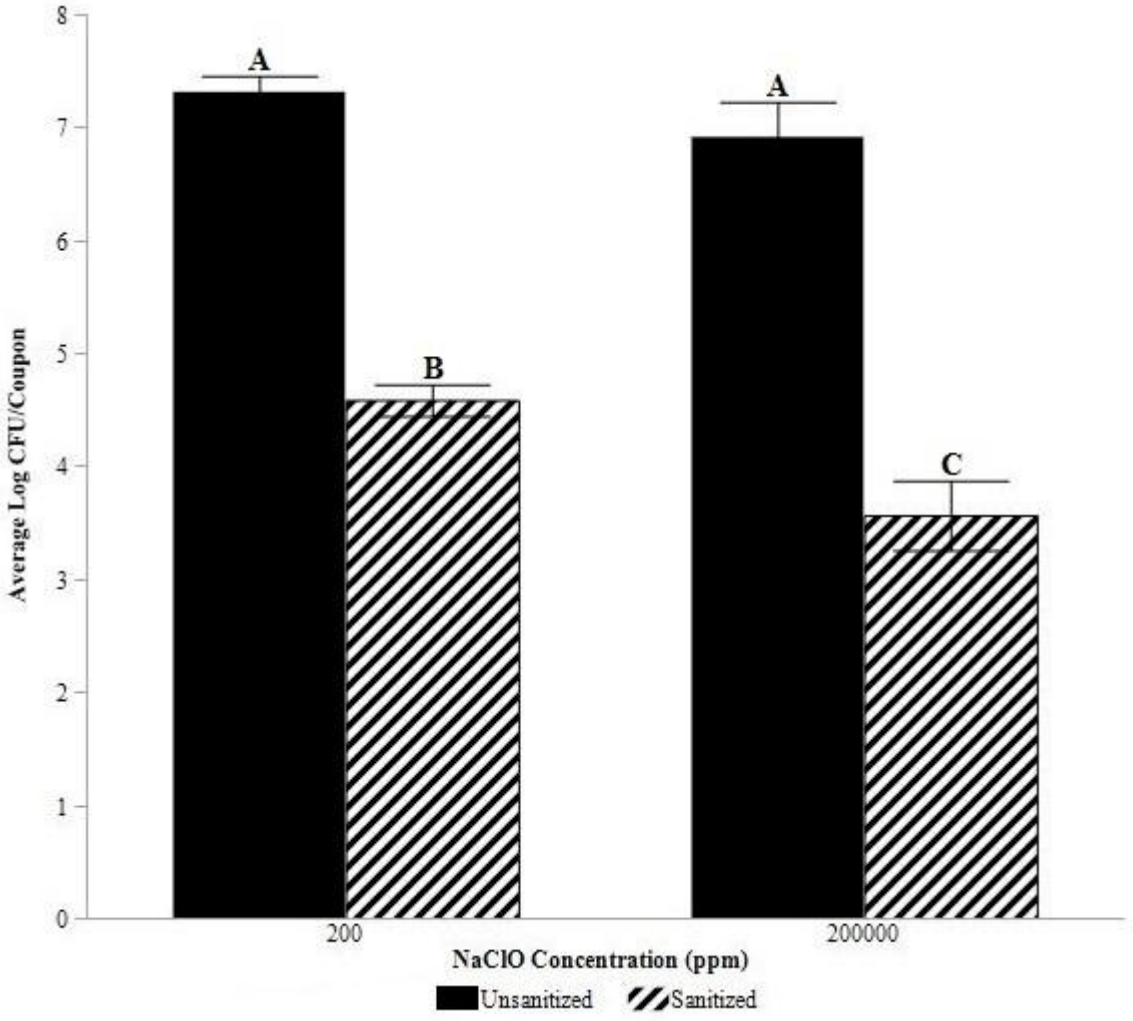

Figure 2.1. Average log CFU *Salmonella* Typhimurium ATCC 14028 cell populations recovered from unsanitized and sanitized coupons treated with 200 ppm and 200,000 ppm sodium hypochlorite (NaClO) (n =5). Different letters above each bar indicate statistically significant differences between values ($P < 0.05$).

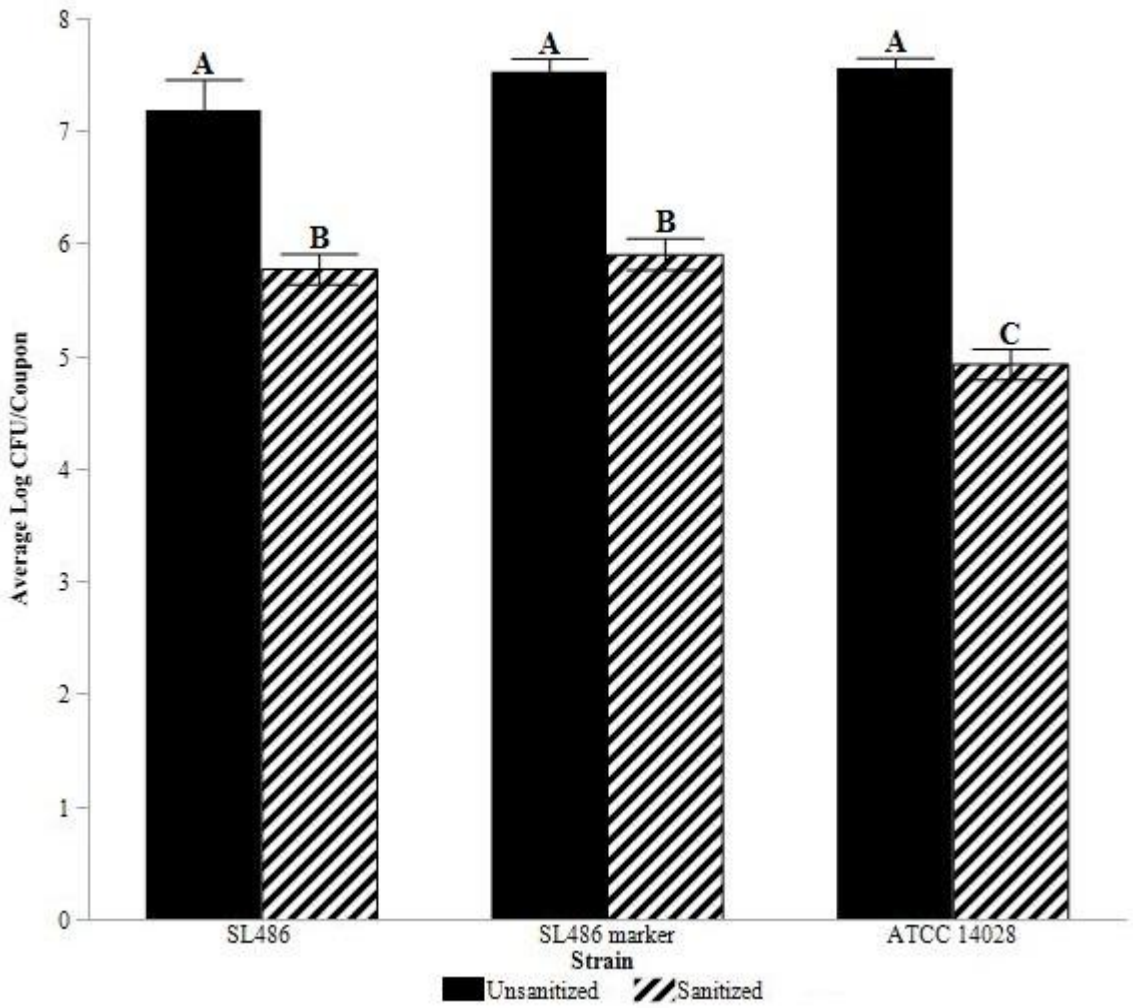

Figure 2.2. Average log CFU *Salmonella* Heidelberg SL486 (parent and marker) and *Salmonella* Typhimurium ATCC 14028 cell populations recovered from unsanitized and sanitized coupons treated with 200 ppm peracetic acid (PAA) (n =5). Different letters above each bar indicate statistically significant differences between values ($P < 0.05$).

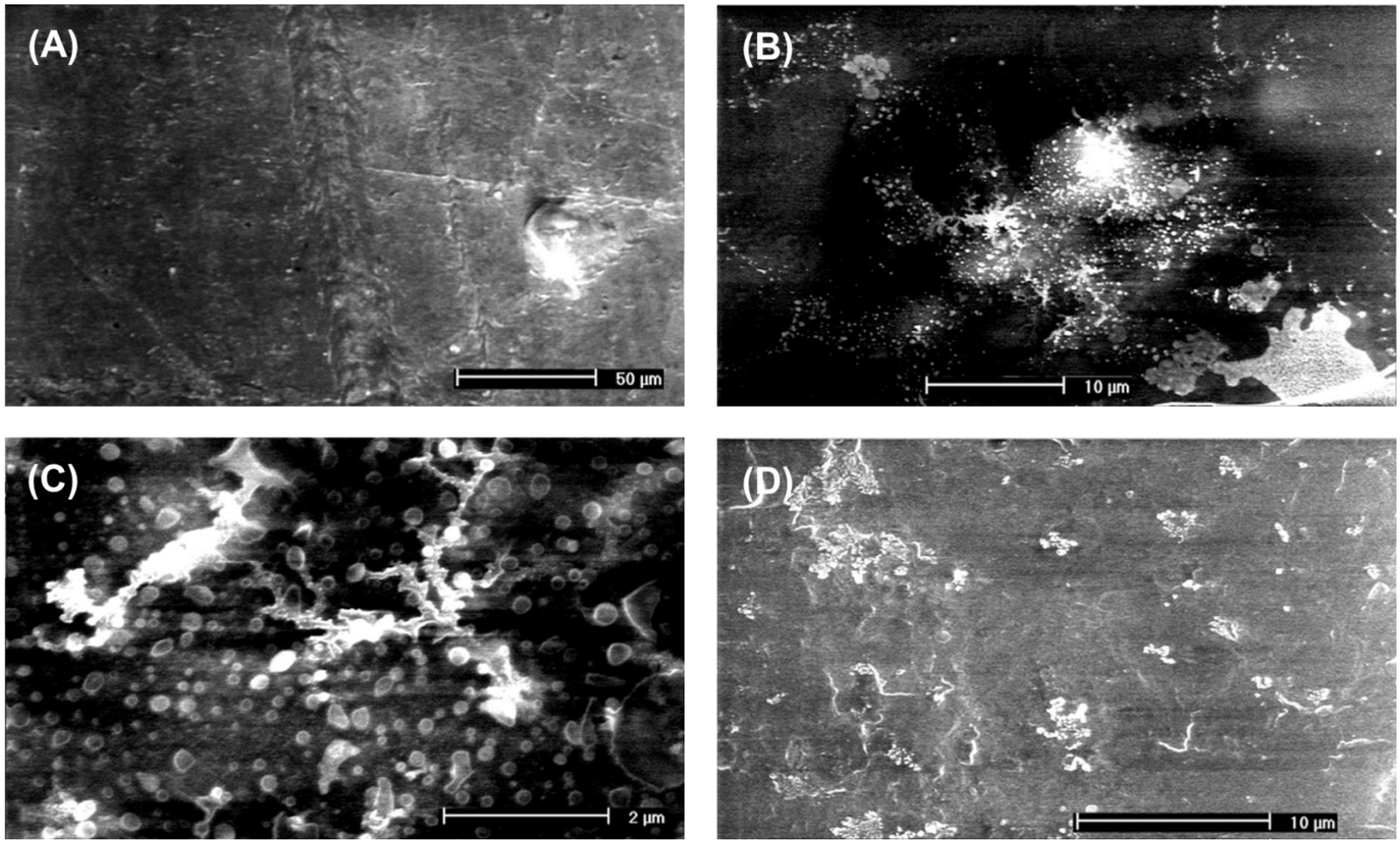

Figure 2.3. Scanning electron microscopy images of (A) an uninoculated reusable plastic containers (RPC) coupon, (B) attached cells prior to sanitization, (C) attached cells at a higher magnification, and (D) a RPC coupon post sanitization with 200 ppm peracetic acid (PAA) and attached cell removal.

Table 2.1. Quantitative PCR and plate log population comparisons of *S.* Heidelberg parent and marker strains on reusable plastic container (RPC) coupons

Strain	Unsanitized coupon		Sanitized coupon*	
	Plating	qPCR	Plating	qPCR
Parent strain	7.19 ± 0.27[a]	6.12 ± 0.26[b]	5.78 ± 0.13	5.52 ± 0.22
Marker strain	7.52 ± 0.12	7.07 ± 0.21	5.91 ± 0.14	6.26 ± 0.17

*Treated with 200 ppm of peracetic acid (PAA) for 30 s
Results are expressed as the mean ± standard error (n = 5)
[a-b] Different letters indicates significant difference in recovered population between two enumeration methods within each strain ($P < 0.05$)

IV. Chapter 3. Draft Genome Sequences of *Salmonella enterica* Serovar Enteritidis and Kentucky Isolates from Retail Poultry Sources

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Abstract

The draft genome sequences of four *Salmonella enterica* serovar Enteritidis and Kentucky isolates were evaluated for biofilm formation and antibiotic resistance. The *Salmonella* serovar Kentucky strains CFS84 and CFS85 and *Salmonella* serovar Enteritidis strains CFS86 and CFS87 were isolated from retail poultry sources in Arkansas.

Genome Announcement

Salmonella enterica remains one of the most common foodborne pathogens causing illnesses leading to numerous hospitalizations and causing millions of dollars in health care costs and productivity losses (Scallan et al., 2011; Minor et al., 2015). Within the food industry, *Salmonella* spp. have been shown to possess the ability to form biofilms on processing equipment (Arnold and Silvers, 2000; Chia et al., 2009). This ability can confer resistance to disinfection and allow bacteria to persist over time and serve as a reservoir for future contamination (Vestby et al., 2009). *Salmonella enterica* serovar Enteritidis is one of the primary serovars associated with human illnesses in the United States and is often associated with the consumption of contaminated poultry products (Foley et al., 2011). *S. enterica* serovar Kentucky has been identified as one of the more commonly isolated serovars from poultry production and often possesses a multidrug resistance phenotype (Foley et al., 2011). Although *S.* Kentucky has been affiliated with fewer hospitalizations than other *Salmonella* serovars, it has demonstrated the ability to obtain and spread plasmids that contribute to increased virulence and colonization in poultry (Johnson et al., 2010). These abilities could become problematic if the strains are allowed to persist in processing and storage environments.

Four strains of *S. enterica* isolated from retail poultry carcasses from Arkansas were sequenced (Melendez et al., 2010) (Table 1). Of these, two (CFS84 and CFS85) belonged to serovar Kentucky and two to serovar Enteritidis (CFS86 and CFS87). Phenotypic testing of the *S.* Enteritidis strains showed wild-type morphologies and biofilm growth, while the *S.* Kentucky strains exhibited morphologies and growth associated with increased extracellular matrix component production (our unpublished data). All strains were previously found to exhibit

resistance to multiple antimicrobial agents, with each strain showing resistance to sulfisoxazole and novobiocin. Strain CFS84 demonstrated additional resistance to neomycin, and CFS86 encoded resistance to ampicillin and nalidixic acid as well. Both *S.* Kentucky strains were detected to carry plasmids identified as incompatibility type I1 (IncI1), while both *S.* Enteritidis strains carried IncFIIA plasmids (Melendez et al., 2010). Analysis of the genome sequences may be useful in identifying mitigation strategies to control *Salmonella* spp. found in retail environments.

To carry out whole-genome sequencing, total bacterial DNA was extracted using a DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA). DNA libraries were constructed using the Nextera XT DNA sample kits (Illumina, San Diego, CA, USA). Sequencing reactions were carried out on an Illumina MiSeq instrument to generate 2 × 300 paired-end reads (Khajanchi et al., 2016). Trimming and *de novo*assembly were performed using CLC Genomics Workbench version 9 (Qiagen, Germantown, MD, USA). Annotation of the draft genomes was done using Rapid Annotations using Subsystems Technology (RAST) (Aziz et al., 2008), Pathosystems Resource Integration Center (PATRIC) (Wattam et al., 2013), and the NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) (Angiuoli et al., 2008) (Table 1). Table 1 lists the numbers of contigs, predicted coding sequences, and functional proteins, as well as the G+C content for each of the sequenced strains.

Accession number(s). This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession numbers listed in Table 1.

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References

Angiuoli, S.V., Gussman, A., Klimke, W., Cochrane, G., Field, D., Garrity, G.M., Kodira, C.D., Kyrpides, N., Madupu, R., and Markowitz, V., 2008. Toward an online repository of Standard Operating Procedures (SOPs) for (meta) genomic annotation. *OMICS A Journal of Integrative Biology, 12* (2), 137-141.

Arnold, J. and Silvers, S., 2000. Comparison of poultry processing equipment surfaces for susceptibility to bacterial attachment and biofilm formation. *Poultry Science, 79* (8), 1215-1221.

Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., and Kubal, M., 2008. The RAST Server: rapid annotations using subsystems technology. *BMC Genomics, 9* (1), 75.

Chia, T., Goulter, R., McMeekin, T., Dykes, G., and Fegan, N., 2009. Attachment of different *Salmonella* serovars to materials commonly used in a poultry processing plant. *Food Microbiology, 26* (8), 853-859.

Foley, S.L., Nayak, R., Hanning, I.B., Johnson, T.J., Han, J., and Ricke, S.C., 2011. Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. *Applied and Environmental Microbiology, 77,* 4273-4279.

Johnson, T.J., Thorsness, J.L., Anderson, C.P., Lynne, A.M., Foley, S.L., Han, J., Fricke, W.F., McDermott, P.F., White, D.G., and Khatri, M., 2010. Horizontal gene transfer of a ColV plasmid has resulted in a dominant avian clonal type of *Salmonella enterica* serovar Kentucky. *PLoS One, 5* (12), e15524.

Khajanchi, B.K., Han, J., Gokulan, K., Zhao, S., Gies, A., and Foley, S.L., 2016. Draft genome sequences of four *Salmonella enterica* strains isolated from turkey-associated sources. *Genome Announcements, 4* (5), e01122-16.

Melendez, S.N., Hanning, I., Han, J., Nayak, R., Clement, A., Wooming, A., Hererra, P., Jones, F., Foley, S., and Ricke, S., 2010. *Salmonella enterica* isolates from pasture-raised poultry exhibit antimicrobial resistance and class I integrons. *Journal of Applied Microbiology, 109* (6), 1957-1966.

Minor, T., Lasher, A., Klontz, K., Brown, B., Nardinelli, C., and Zorn, D., 2015. The per case and total annual costs of foodborne illness in the United States. *Risk Analysis, 35* (6), 1125-1139.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., and Griffin, P.M., 2011. Foodborne illness acquired in the United States--major pathogens. *Emerging Infectious Diseases, 17* (1), 7-15.

Vestby, L.K., Møretrø, T., Langsrud, S., Heir, E., and Nesse, L.L., 2009. Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal-and feed factories. *BMC Veterinary Research, 5*, 20.

Wattam, A.R., Abraham, D., Dalay, O., Disz, T.L., Driscoll, T., Gabbard, J.L., Gillespie, J.J., Gough, R., Hix, D., and Kenyon, R., 2013. PATRIC, the bacterial bioinformatics database and analysis resource. *Nucleic Acids Research, 42* (D1), D581-D591.

Table 3.1. Summary of the genome sequence analysis of *Salmonella enterica* strains from poultry in Arkansas.

Strain	Serovar	No. of Contigs	Assembly size (bp)	G+C content (%)	No. of CDS[a]	No. of functional proteins	GenBank Accession no.
CFS84	Kentucky	232	4,935,761	51.99	5,081	4,293	PHUN00000000
CFS85	Kentucky	151	4,908,583	51.98	4,987	4,230	PHUO00000000
CFS86	Enteritidis	128	4,665,166	52.13	4,724	4,159	PHUP00000000
CFS87	Enteritidis	95	4,656,278	52.14	4,705	4,136	PIJU00000000

[a]CDS- coding sequences.

V. Chapter 4. Characterization of Pellicle Formation by Poultry *Salmonella* Kentucky Strains and other Poultry-Associated *Salmonella* Serovars in Luria Bertani Broth

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Abstract

Salmonella spp., a leading group of foodborne pathogens related to the consumption of poultry, have the ability to form biofilms, making it arduous to eliminate their presence in processing facilities. Furthermore, there is limited literature concerning the biofilm forming capabilities of *S.* Kentucky, a common poultry-associated serovar. Thus, the objective of the current study was to elucidate the difference in pellicle formation of poultry-originating strains of *S.* Kentucky compared to other better-characterized *Salmonella* strains that are also associated with poultry. The strains utilized in the current study included *S.* Kentucky (UA CFS# 38-0055 through 38-0085, excluding 38-0068), *S.* Enteritidis (UA CFS# 38-0086, through 38-0089, 38-0091), and *S.* Heidelberg (UA CFS# 38-00126, 38-00127, 38-00128, 38-00152). In three separate experiments, *Salmonella* strains and serovars were tested for (1) their ability to form biofilms in different Luria Bertani (LB) broth compositions; (2) pellicle formation in 5 mL and 50 mL of LB broth with no salt; and (3) their subsequent pellicle formation and potential priming effects after pellicles were transferred three consecutive times. Data were analyzed using One-Way ANOVA in JMP 14.0 with means being separated using Tukey's protected HSD and a significance level of $P \leq 0.05$. Results of the first experiment demonstrated that there was not a significant effect between strain and serovars ($P > 0.05$), but media type affected pellicle formation significantly with LB Miller and LB broth minus NaCl plus 2% glucose resulting in no pellicle formation ($P < 0.001$). Although there were no detected differences between serovars and strains when grown in 5 mL of LB broth ($P > 0.05$), when grown in 50 mL, a strain of Kentucky, 38-0085, produced larger pellicles than Kentucky 38-0055, and a strain of Heidelberg 38-0127 ($P < 0.0001$). The serial transfer of pellicles did not significantly affect pellicle formation ($P > 0.05$); however, *S.* Kentucky 38-0084, 38-0085, and 38-0086 produced larger pellicles than *S.* Kentucky strains 38-

0055 and 38-0056 and *S.* Heidelberg strains 38-0126, 38-0127, and 38-0152. The current study demonstrates the strong biofilm forming capabilities of *S.* Kentucky strains and may explain why *S.* Kentucky is frequently isolated in poultry processing facilities.

Keywords: pellicle, *Salmonella* Kentucky, poultry, *Salmonella* Enteritidis, *Salmonella* Heidelberg

Introduction

Combating foodborne illnesses caused by non-typhoidal *Salmonella enterica* strains continues to be an ongoing concern for public health officials and food industry specialists due to the high number of cases and economic damage caused annually (Scallan et al., 2011). Despite the passage of regulatory control measures such as the Food Safety Modernization Act in 2011, which included updates to performance standards and the implementation of a *Salmonella* Action Plan, the incidence of cases has remained relatively constant (USDA-FSIS, 2013; CDC, 2018a). Outbreaks of salmonellosis have been attributed to exposure to various contaminated food items (CDC, 2018b). However, according to the USDA FSIS, human exposure to *Salmonella* is highly linked to the consumption of poultry products, with the greatest route of exposure being through the ingestion of broiler chicken carcasses (USDA-FSIS, 2016).

One possible route of *Salmonella* contamination of poultry products is through the direct contact with biofilms present in the environment of commercial processing facilities. As a defense mechanism, biofilms allow *Salmonella* to resist the action of antimicrobials and increase overall fitness (Steenackers et al., 2012). In addition, poultry processing environments can provide ample opportunities for *Salmonella* to form biofilms on a variety of possible surface types, ranging from plastic to glass to stainless steel. These surfaces allow for the attachment of *Salmonella* biofilms and supply the biofilms with a continual source of nutrients through the residual organic matter left on these surfaces (Srey et al., 2013). Furthermore, as both rough surfaces and pipe structures are arduous to sanitize and clean, these surfaces provide environmental matrices that favor the development of biofilms and bacterial attachment (Van Houdt and Michiels, 2010; Kumpel and Nelson, 2013). If biofilms are not fully removed, they may re-grow and act as persistent sources of re-contamination.

Out of the over 2,500 known serovars of *Salmonella,* Enteritidis, Newport, and Typhimurium are the most commonly reported culture confirmed isolate strains (CDC, 2018a). Due to their clinical significance, these serovars, along with other high incident serovars within the food industry, such as *Salmonella* Heidelberg, have been the typical focus of research. For instance, *Salmonella* Typhimurium has been the subject of numerous studies characterizing the biofilm formation in standard surface-air and pellicle-type liquid-air biofilms (Zogaj et al., 2001; Scher et al., 2005). However, other serovars such as *S.* Kentucky can also play an important role in the control of foodborne illness. *S.* Kentucky is the most frequently isolated serovar from poultry samples, comprising nearly 61% of the all isolated broiler samples tested under the Pathogen Reduction/Hazard Analysis Critical Control Point program in 2014 (Foley et al., 2011; Finstad et al., 2012; USDA-FSIS, 2016). Although *S.* Kentucky does not directly cause human illness, numerous *S.* Kentucky isolates have been found to contain transferrable plasmids conferring antimicrobial resistance and virulence factors to other bacteria (Fricke et al., 2009; Johnson et al., 2010; USDA-FSIS, 2016). Within a biofilm, these plasmids and genes may be more easily transferred, increasing the necessity to control for *S.* Kentucky in a processing facility (Molin and Tolker-Nielsen, 2003). Given the prevalence of *S.* Kentucky in poultry environments such as processing plants, it is of interest to characterize the serovar's capability for biofilm formation.

Therefore, the objective of the current study was to elucidate the differences in pellicle type biofilm formations of several *Salmonella* serovars when grown in different environments with a particular focus on *S.* Kentucky strains. Thus, pellicle formation was evaluated in different variations of Luria Bertani broth media, quantity of media (5 or 50 mL), and in serial transfers to investigate the effect nutrient composition, size of containment, and the potential priming effects

of older pellicles on new pellicle formation. It was hypothesized that pellicles would grow differently in different environments and that the priming effects would enhance pellicle formation.

Materials and Methods

Bacterial strain preparation and pellicle formation

Thirty-eight strains of *Salmonella* from the University of Arkansas Center for Food Safety Culture Collection were used in this study. This included thirty strains of *S.* Kentucky (UA CFS# 38-0055 through 38-0085, excluding 38-0068 which could not be cultured), four strains of *S.* Enteritidis (UA CFS# 38-0086, 38-0087, 38-0088, 38-0089, 38-0091), and four strains of *S.* Heidelberg (UA CFS# 38-00126, 38-00127, 38-00128, 38-00152). Quadrant streaks from frozen stocks were prepared on Luria-Bertani (LB) (BD Biosciences, Franklin Lakes, NJ) agar plates and incubated for 24 h at 37 °C. After incubation, overnight cultures were prepared by selecting single colonies and growing in 5 mL of LB broth overnight in a 37 °C shaking incubator for 18 hours. Standard pellicles were formed by taking overnight cultures of each of the *Salmonella* strains, diluting 1:10, inoculating into media, and placing at room temperature for 96 h (Solano et al., 2002). Pellicle growth was evaluated at the end of this time period. Pellicle weights were obtained by removing pellicles using sterile loops and air drying in an oven at 150 °C for 24 h.

Pellicle formation in various LB media compositions

Two strains of *S.* Kentucky (UA CFS# 38-0070, 38-0085) and two strains of *S.* Enteritidis (UA CFS# 38-0086, 38-0091) were grown in test tubes containing 5 mL of five

variations of Luria Broth. This included LB Miller (BD Biosciences, Franklin Lakes, NJ), LB broth without salt (LB - NaCl), LB broth with KCl and without NaCl (LB – NaCl + KCl), LB broth with 2% glucose and without salt (LB-NaCl + 2% glucose), and LB broth with 2% sucrose and without salt (LB – NaCl + 2% sucrose). Standard pellicles were formed and pellicle weights measured.

Pellicle formation in test tubes and flasks

S. Kentucky strains (UA CFS# 38-0055 through 38-0085) were grown at room temperature for 96 h in 16 x 100 mm test tubes (11 mL capacity) containing 5 mL of LB broth without salt. After visible pellicle formation occurred, the corresponding pellicles were dried and weighed (mg) accordingly. In addition, four strains of *S.* Kentucky which produced average sized pellicles (UA CFS# 38-0055, 38-0056, 38-0084, 38-0085) and two strains of *S.* Heidelberg (UA CFS# 38-00126, 38-00127) were grown in separate 125 mL flasks containing 50 mL of LB broth without salt. Standard pellicles were observed and pellicle weights measured (mg).

Pellicle serial transfers

Four strains of *S.* Kentucky (UA CFS# 38-0055, 38-0056, 38-0084, 38-0085), *S.* Enteritidis (UA CFS# 38-0086, 38-0087, 38-0088, 38-0089), and *S.* Heidelberg (UA CFS# 38-00126 , 38-00127, 38-00128, 38-00152) were grown in 125 mL flasks containing 50 mL of LB broth without salt with the pellicles transferred to inoculate several consecutive sets (3). Standard pellicles were formed. After 96 h, pellicles were removed using sterile loops and used to inoculate new 125 mL flasks. Flasks were placed in a 37 °C shaking incubator for 24 h before pellicles were removed and weighed. Flasks were allowed to incubate at room temperature for an

additional 72 h. After new pellicles were formed, the process was repeated. If no stable pellicle formed after 96 h, a new flask was inoculated with either a loop of liquid from the previous flask or any floating more visibly fragile pellicle fragments.

Statistical analysis

For this study, data were analyzed using one-way ANOVA in JMP 14.0 (SAS Institute, Cary, NC). Means were separated using Tukey's protected HSD with a significance level of $P \leq 0.05$.

Results

Effect of media composition on pellicle formation

In the current study, *Salmonella* serovars were grown in several variations of LB broth to determine their effects on pellicle formation (**Figure 4.1**). LB Miller was chosen due to its role as one of the standard LB formulations, LB broth without salt was chosen as the standard pellicle inducing media, LB broth without NaCl, but with KCl added in as an alternative salt component, and LB broth without NaCl with the addition of either 2% glucose or 2% sucrose as additional sugar carbon sources. In the current study, LB Miller and the LB broth without NaCl, with 2% glucose failed to produce pellicles in any of the serovars or strains of *Salmonella* examined. There was a significant effect of media type on pellicle growth ($P<0.0001$). Differences were noted between the media that inhibited pellicle growth, LB Miller and LB without NaCl plus 2% glucose (0.0 mg), and the media that allowed for the development of viable pellicles, LB without NaCl, LB without NaCl plus 2% sucrose, and LB without NaCl plus KCl (1.65, 2.20 and 1.28 mg, respectively). The average pellicle weights of the pellicle inducing media ranged from approximately 1.3 mg (without NaCl + KCl) to 2.2 mg (without NaCl + 2% sucrose) but were

not statistically different from one another. Additionally, there was no difference in *Salmonella* strains (P = 0.5182). As there was no statistical difference among the different pellicle inducing LB media, the remaining experiments were conducted using only the LB broth without salt.

Comparison of pellicle formation among *Salmonella* isolates

Salmonella Kentucky strains were grown in LB broth without NaCl in 16 x 100 mm test tubes (**Figure 4.2**). The thirty strains had weights ranging from a minimum of 0.5 mg to a maximum of 3.6 mg with an average weight of 1.6 mg. Although there were differences in the variation between each strain, there were no overall statistical differences (P > 0.05). In addition, two strains of *S.* Heidelberg and four strains of *S.* Kentucky were grown in 125 mL flasks to observe if the larger volume would affect pellicle formation (**Figure 4.3**, P < 0.0001). The overall average weight of the pellicles was 28.4 mg and ranged from 25.3 mg to 31.3 mg. *Salmonella* Heidelberg UA CFS# 38-00127 was unable to form a biofilm. There were no differences between the *S.* Kentucky strains and the *S.* Heidelberg strain that was capable of forming a pellicle; however, there was a statistical difference between the largest *S.* Kentucky pellicle former (38-0085), and the smallest (38-0055).

Pellicle serial transfers

Transferring formed pellicles to new flasks containing fresh media was performed to determine if transferring the pellicles elicited a priming effect on subsequent pellicle formation and size (mg). There was a significant effect on pellicle size (mg) when *Salmonella* strains and serovars were evaluated (P < 0.0001; **Figure 4.4**). Once again, *S.* Heidelberg strain UA CFS# 38-00127 was unable to form a pellicle. In addition, *S.* Heidelberg strains UA CFS# 38-00126, 38-

00152, and *S.* Kentucky strains 38-0055, 38-0056 formed visibly fragile pellicles that broke apart when attempting to transfer over. Overall, *S.* Kentucky strains 38-0084 and 38-0085, and *S.* Enteritidis strain 38-0086 (41.2, 41.0, and 38.9 mg) had larger pellicles after 4 days than *S.* Kentucky strains 38-0055 and 38-0056 (10.7 and 9.2 mg), *S.* Heidelberg strains 38-0127, 38-0128, and 38-0152 40 (9.4, 0.0, and 0.0 mg). No statistical differences were detected between *S.* Enteritidis 38-0087, 38-0088, 38-0089, *S.* Kentucky 38-0055, 38-0056, and *S.* Heidelberg 38-0126 and 38-0127 (27.5, 36.0, 34.1, 10.7, 9.2, 36.3, and 9.4 mg, respectively). There were also no significant differences between pellicle size and transfer number ($P > 0.05$; **Figure 4.5**).

Discussion

The purpose of this study was to investigate the growth and re-growth of *S.* Kentucky pellicles in varying environmental conditions to further characterize the pellicle formation properties of this serovar. This was done by growing pellicles in several types of LB broth combinations, in test tubes and flasks, and through the serial transfer of pellicles. An additional pilot study was conducted where *Salmonella* strains were grown under anaerobic conditions. However, no pellicles formed after several weeks of incubation (data not shown). These scenarios reflect some of the possibilities for biofilm growth in processing settings with the goal of achieving a better understanding of factors that may influence *S.* Kentucky biofilm formation under environmental conditions associated with poultry processing plants.

In the first experiment, pellicles were grown in various LB media including with the removal of sodium chloride salt and the addition of potassium chloride, the monosaccharide glucose, or the disaccharide sucrose. Stepanović et al. (2004) studied the growth of *Salmonella* spp. in plastic wells containing media ranging from rich brain heart infusion broth (BHI) to

tryptic soy broth diluted to a 1/20 concentration, and found that biofilms formed best in less nutrient rich media. A meat broth was used similar in composition to the LB with glucose solution used in our study and produced biofilm formations on plastic surfaces much smaller than those in their most effective media. In addition, high osmolarity has been shown by to inhibit both the biosynthesis of flagella and the activity of *csgD*, both of which are necessary during the initiation stages of biofilm formation in *Salmonella* (Prigent-Combaret et al., 2001; Goller and Romeo, 2008). This would account for why pellicles were unable to form in the LB Miller (1% NaCl w/v) and the LB without NaCl with 2% glucose. However, pellicles were still visibly formed in the LB without NaCl, but with KCl and LB without salt with 2% sucrose. This suggests that potassium ions and sucrose may play some role in pellicle formation despite the high osmolarity.

Among the pellicle producing compositions in the current study, there were no statistical differences among the different media, but pellicle formation in 2% sucrose was numerically higher than the pellicle formation in other media amendments. The presence of sugars such as glucose and sucrose have been tied to increased cellulose production, a vital component of pellicles (Mikkelsen et al., 2009). However, the LB supplemented with 2% glucose was unable to support visible pellicle formation. This is consistent with the observation by Korhonen et al. (1980) that glucose was slightly inhibitory towards agglutination by type I pili in *Salmonella* Typhimurium, another crucial stage in biofilm formation, compared to sucrose, which exhibited no inhibitory effect.

In the current study, *Salmonella* pellicles were formed in test tubes and flasks, as well as serially transferred to observe any differences between strains or serovars, and to observe if there were any priming effects from pellicle transfers. Turki et al. (2012) studying *S.* Kentucky isolates

from various clinical, food, environmental, and waste samples found that only 42% of their isolates formed rigid pellicles in LB and that only 3.5% of the clinical isolates formed pellicles. All of the *S.* Kentucky strains used in this study were isolated from poultry samples or environmental samples related to poultry production, and all formed rigid pellicles in test tubes. This suggests that there may be differences among strains, possibly due to environmental differences from where the strain was isolated. It would be of interest in future studies to compare poultry isolates with non-poultry isolates to determine if environmental origin does play some sort of selective impact on the capability to form biofilms.

The ability of biofilms to re-form and re-contaminate surfaces is an ongoing issue for the food industry. Commonly used sanitizers have been shown to be unable to completely remove *Salmonella* biofilms from food processing surfaces (Corcoran et al., 2013). With incomplete removal, biofilms are given the opportunity to re-grow as well as spread if the improper cleaning led to the transference of biofilm components and the establishment of new colonies. In the current experiment, serial transfers of pellicles sometimes led to the initiation of pellicle formation being observed up to a day early (results not shown). However, no significant differences were observed by the 96 h time point. This suggests that in a processing environment, any biofilms not completely removed would be able to return to their optimal state perhaps more rapidly after an incomplete removal attempt, highlighting the importance of complete removal of not only the biofilms but the biofilm forming *Salmonella* populations as well.

Conclusions

While *Salmonella* Kentucky is not well-characterized in terms of biofilm formation, this serovar is a relatively frequent isolate associated with poultry environments. The ability to form biofilms may contribute to the frequent occurrence of this serovar. The current study supports the concept that *S.* Kentucky is capable of forming visible pellicles that are consistent with previous observations for other *Salmonella* serovars. However, there may be strain differences which need to be further investigated to determine if this is a contributor to variations in persistence in the environment. In addition, the environmental origin may need to be considered as a factor and thus warrant a broader comparison among *S.* Kentucky stains from both poultry and non-poultry environments. Likewise, the residence time in a particular environment may have an impact as well. Finally, genomic studies need to be conducted to determine if *S.* Kentucky has biofilm genes and regulatory components similar to the more extensively characterized serovars such as *S.* Typhimurium and *S.* Enteritidis.

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Author Contributions Statement

All authors significantly contributed to the work of the current study. ZS and SCR designed the experiments and ZS conducted the laboratory experiments. DKD and ZS analyzed the data and ZS wrote the manuscript with assistance from DKD and SCR.

References

CDC. 2018a. *National Enteric Disease Surveillance: Salmonella Annual Report 2016* [Online]. CDC. Available: https://www.cdc.gov/nationalsurveillance/pdfs/2016-Salmonella-report-508.pdf [Accessed 6/15/18].

CDC. 2018b. *Reports of Selected Salmonella Outbreak Investigations* [Online]. Centers for Disease Control and Prevention. Available: https://www.cdc.gov/salmonella/outbreaks.html [Accessed 6/15/18].

Corcoran, M., Morris, D., De Lappe, N., O'connor, J., Lalor, P., Dockery, P., and Cormican, M., 2013. Commonly used disinfectants fail to eradicate *Salmonella enterica* biofilm from food contact surface materials. *Applied and Environmental Microbiology, 80* (4), 1507-1514.

Finstad, S., O'Bryan, C.A., Marcy, J.A., Crandall, P.G., and Ricke, S.C., 2012. *Salmonella* and broiler processing in the United States: relationship to foodborne salmonellosis. *Food Research International, 45* (2), 789-794.

Foley, S.L., Nayak, R., Hanning, I.B., Johnson, T.J., Han, J., and Ricke, S.C., 2011. Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. *Applied and Environmental Microbiology, 77,* 4273-4279.

Fricke, W.F., McDermott, P.F., Mammel, M.K., Zhao, S., Johnson, T.J., Rasko, D.A., Fedorka-Cray, P.J., Pedroso, A., Whichard, J.M., and LeClerc, J.E., 2009. Antimicrobial resistance-conferring plasmids with similarity to virulence plasmids from avian pathogenic *Escherichia coli* strains in *Salmonella enterica* serovar Kentucky isolates from poultry. *Applied and Environmental Microbiology, 75* (18), 5963-5971.

Goller, C. and Romeo, T., 2008. Environmental influences on biofilm development. *In:* Romeo, T. (ed.) *Bacterial Biofilms.* Springer.

Johnson, T.J., Thorsness, J.L., Anderson, C.P., Lynne, A.M., Foley, S.L., Han, J., Fricke, W.F., McDermott, P.F., White, D.G., and Khatri, M., 2010. Horizontal gene transfer of a ColV plasmid has resulted in a dominant avian clonal type of *Salmonella enterica* serovar Kentucky. *PLoS One, 5* (12), e15524.

Korhonen, T., Lounatmaa, K., Ranta, H., and Kuusi, N., 1980. Characterization of type 1 pili of *Salmonella typhimurium* LT2. *Journal of Bacteriology, 144* (2), 800-805.

Kumpel, E. and Nelson, K.L., 2013. Comparing microbial water quality in an intermittent and continuous piped water supply. *Water Research, 47* (14), 5176-5188.

Mikkelsen, D., Flanagan, B.M., Dykes, G., and Gidley, M., 2009. Influence of different carbon sources on bacterial cellulose production by *Gluconacetobacter xylinus* strain ATCC 53524. *Journal of Applied Microbiology, 107* (2), 576-583.

Molin, S. and Tolker-Nielsen, T., 2003. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Current Opinion in Biotechnology, 14* (3), 255-261.

Prigent-Combaret, C., Brombacher, E., Vidal, O., Ambert, A., Lejeune, P., Landini, P., and Dorel, C., 2001. Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the *csgD* gene. *Journal of Bacteriology, 183* (24), 7213-7223.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., and Griffin, P.M., 2011. Foodborne illness acquired in the United States--major pathogens. *Emerging Infectious Diseases, 17* (1), 7-15.

Scher, K., Romling, U., and Yaron, S., 2005. Effect of heat, acidification, and chlorination on *Salmonella enterica* serovar Typhimurium cells in a biofilm formed at the air-liquid interface. *Applied and Environmental Microbiology, 71* (3), 1163-1168.

Solano, C., García, B., Valle, J., Berasain, C., Ghigo, J.M., Gamazo, C., and Lasa, I., 2002. Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. *Molecular Microbiology, 43* (3), 793-808.

Srey, S., Jahid, I.K., and Ha, S.-D., 2013. Biofilm formation in food industries: a food safety concern. *Food Control, 31* (2), 572-585.

Steenackers, H., Hermans, K., Vanderleyden, J., and De Keersmaecker, S.C.J., 2012. *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. *Food Research International, 45* (2), 502-531.

Stepanović, S., Ćirković, I., Ranin, L., and Svabić-Vlahović, M., 2004. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Letters in Applied Microbiology, 38* (5), 428-432.

Turki, Y., Ouzari, H., Mehri, I., Aissa, R.B., and Hassen, A., 2012. Biofilm formation, virulence gene and multi-drug resistance in *Salmonella* Kentucky isolated in Tunisia. *Food Research International, 45* (2), 940-946.

USDA-FSIS. 2013. *Salmonella Action Plan* [Online]. United States Department of Agriculture. Available: https://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/get-answers/food-safety-fact-sheets/foodborne-illness-and-disease/salmonella/sap [Accessed 6/11/18].

USDA-FSIS. 2016. *Serotypes Profile of Salmonella Isolates from Meat and Poultry Products, January 1998 through December 2014* [Online]. United States Department of Agriculture. Available: https://www.fsis.usda.gov/wps/wcm/connect/3866026a-582d-4f0e-a8ce-851b39c7390f/Salmonella-Serotype-Annual-2014.pdf?MOD=AJPERES [Accessed 6/14/18].

Van Houdt, R. and Michiels, C.W., 2010. Biofilm formation and the food industry, a focus on the bacterial outer surface. *Journal of Applied Microbiology, 109* (4), 1117-1131.

Zogaj, X., Nimtz, M., Rohde, M., Bokranz, W., and Römling, U., 2001. The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. *Molecular Microbiology, 39* (6), 1452-1463.

Figures

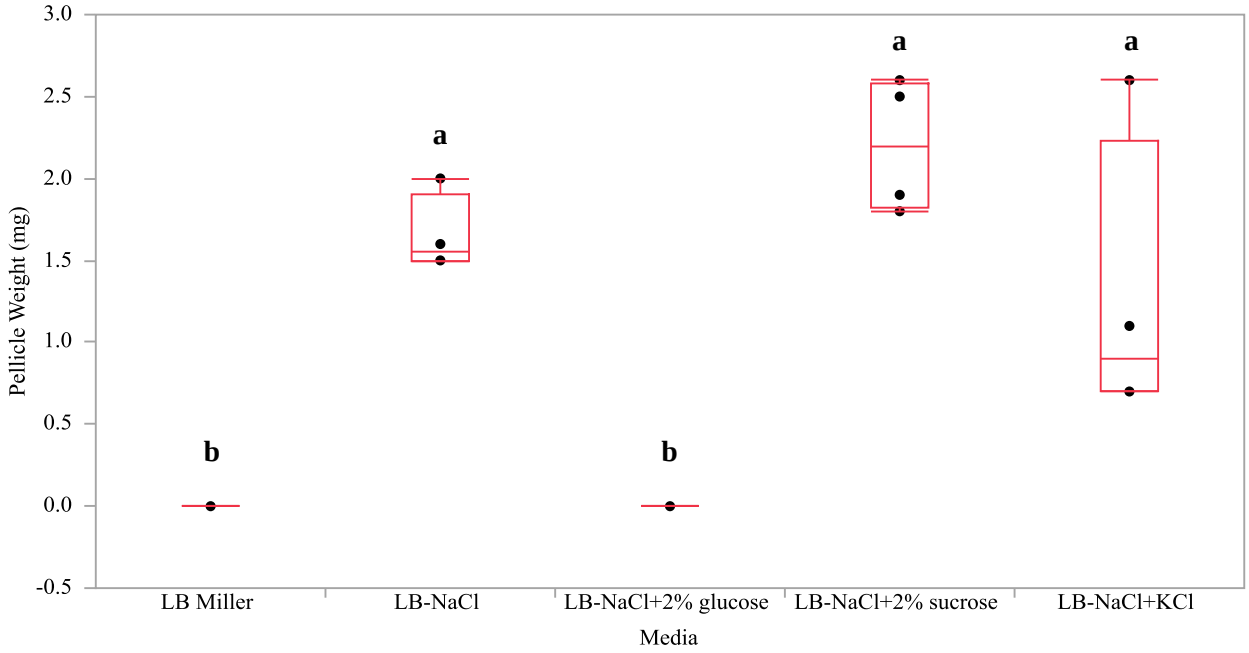

Figure 4.1. The pellicle growth of poultry-related strains of *Salmonella* serovar pellicles in various Luria Bertani (LB) compositions.[1,2] *Salmonella* Kentucky and Enteritidis strains were grown in test tubes containing 5 mL of LB Miller, LB no salt (LB - NaCl), LB - NaCl with 2% glucose, LB - NaCl with 2% sucrose, or LB - NaCl with KCl. Pellicles were formed for 96 h in stationary conditions at room temperature, oven dried, and weighed.
[1]N = 20, n = 4, P < 0.0001
[2]Means with different superscripts are considered significantly different (a-b)

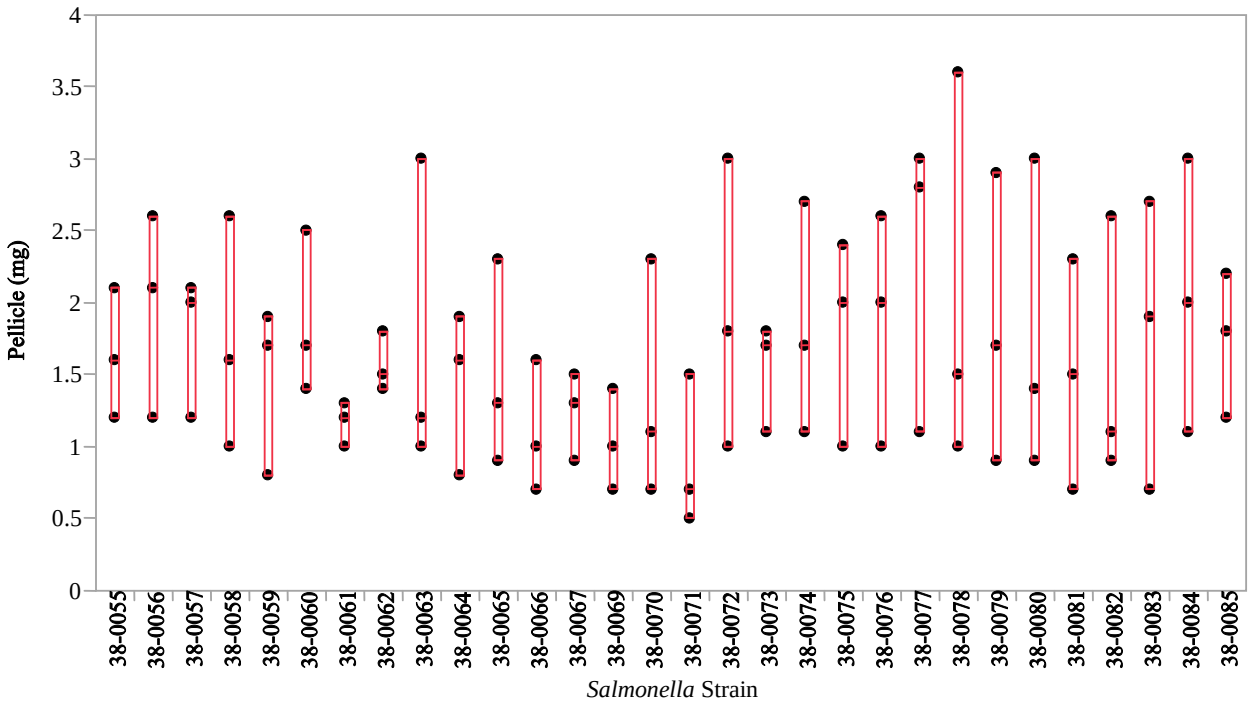

Figure 4.2. The pellicle formation of poultry-related strains of *Salmonella* Kentucky in test tubes of Luria Bertani no salt broth.[1] *Salmonella* Kentucky strains were grown in 16 mm x 100 mm test tubes containing 5 mL of LB - NaCl broth. Pellicles were formed for 96 h in stationary conditions at room temperature, oven dried, and weighed (mg).
[1]N = 90, n = 30, P = 0.9675

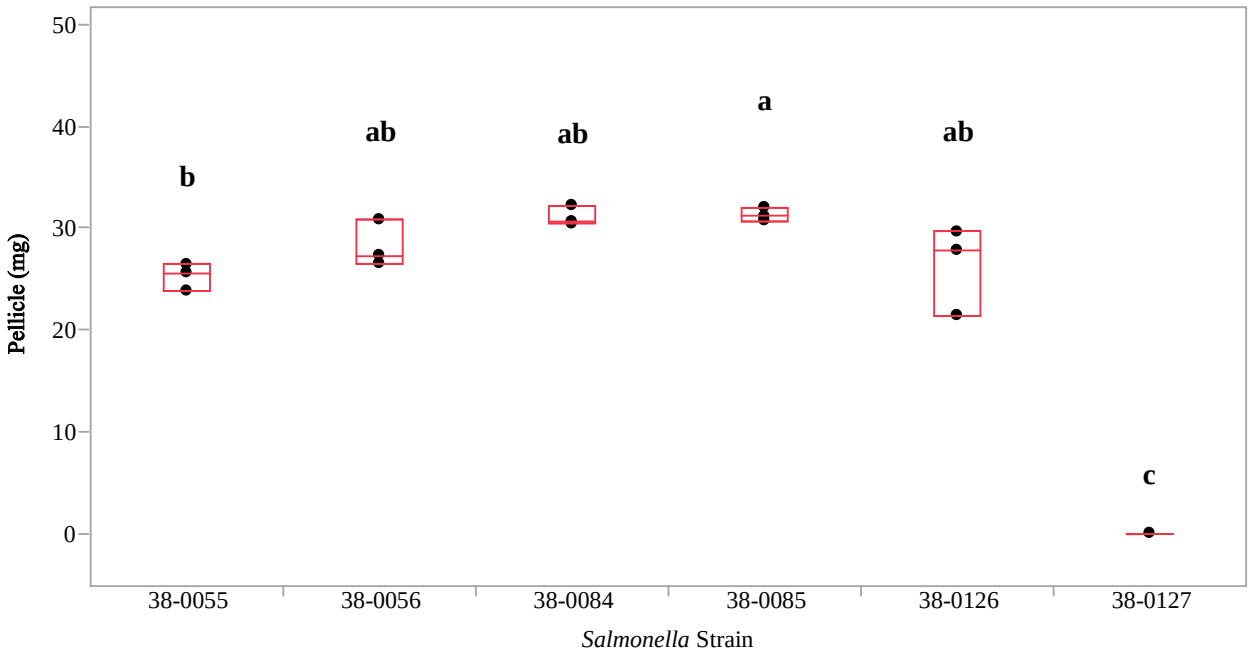

Figure 4.3. The pellicle growth of poultry-related strains of *Salmonella* Kentucky and Heidelberg in flasks of Luria Bertani no salt broth.[1,2] *Salmonella* Kentucky and Heidelberg strains were grown in 125 mL flasks containing 50 mL of LB - NaCl broth. Pellicles were formed for 96 h in stationary conditions at room temperature, oven dried, and weighed (mg).
[1]N = 18, n = 6, P < 0.0001
[2]Means with different superscripts are considered significantly different (a-c)

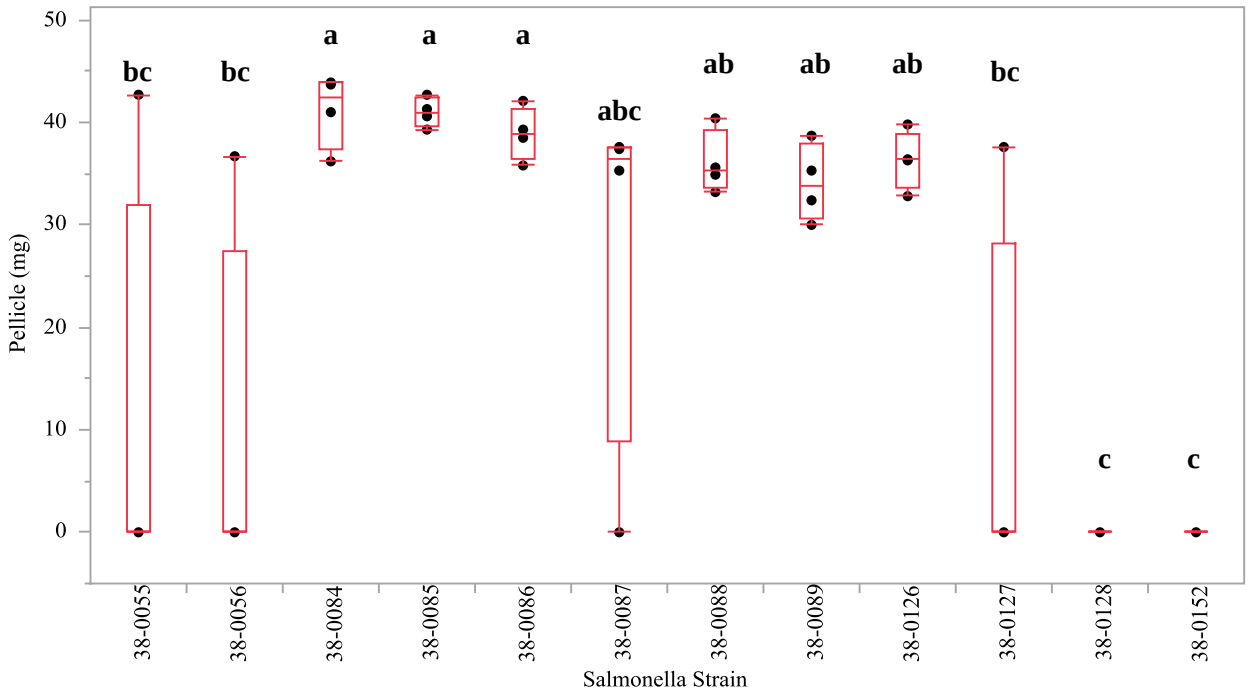

Figure 4.4. The growth of poultry-related strains of *Salmonella* Heidelberg, Kentucky, and Enteritidis pellicles in flasks of Luria Bertani no salt broth (LB - NaCl) after consecutive transfers.[1,2] *Salmonella* Heidelberg, Kentucky, and Enteritidis strains were grown in 125 mL flasks containing 50 mL of LB - NaCl broth. Pellicles were formed for 96 h in stationary conditions at room temperature, transferred to new flasks, and incubated overnight. After 24 h, pellicles were removed, oven dried, and weighed (mg). Pellicles were formed in the new flasks over the course of an additional 72 h and the process repeated three consecutive times.
[1]N = 48, n = 4, $P < 0.0001$
[2]Means with different superscripts are considered significantly different (a-c)

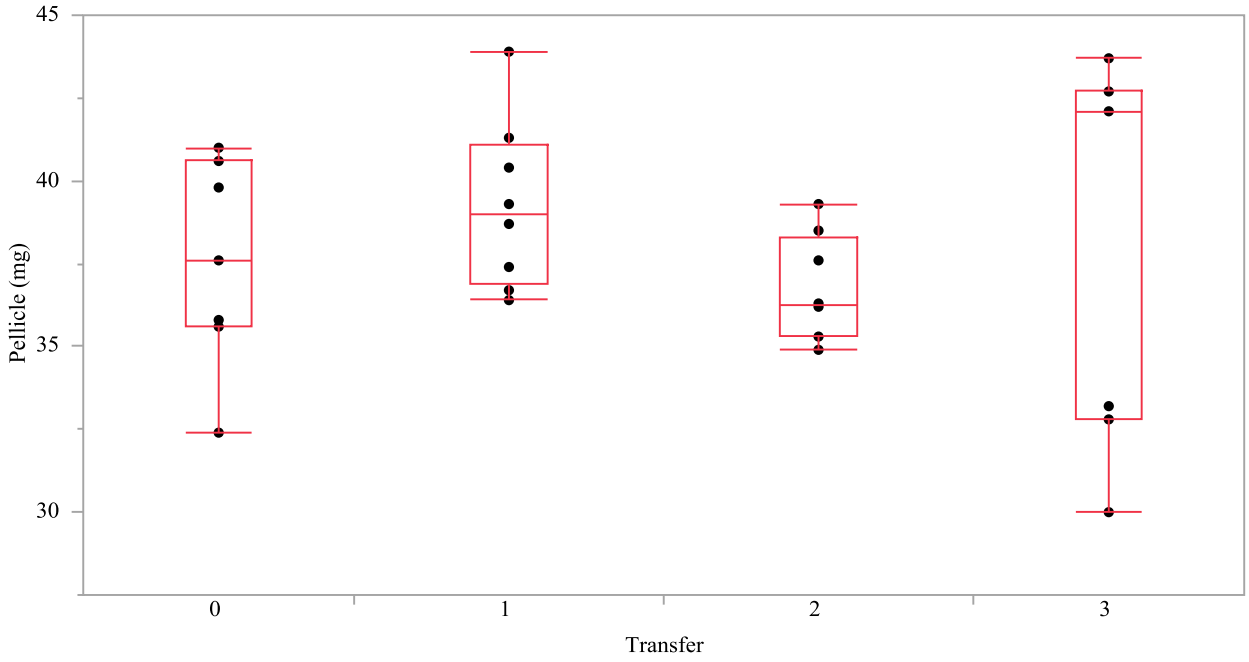

Figure 4.5. The effect of serial transfers of pellicles of poultry-related strains of *Salmonella* Heidelberg, Kentucky, and Enteritidis on subsequent pellicle formation.[1] *Salmonella* Heidelberg, Kentucky, and Enteritidis strains were grown in 125 mL flasks containing 50 mL of Luria Bertani no salt broth. Pellicles were formed for 96 h in stationary conditions at room temperature, transferred to new flasks, and incubated overnight. After 24 h, pellicles were removed, oven dried, and weighed (mg). Pellicles were formed in the new flasks over the course of an additional 72 h, and the subsequent process was repeated three consecutive times.
[1]N = 30, n = 8, P = 0.5378

VI. Chapter 5. Expression differences between *csgD* and *bcsA* in *Salmonella enterica* serovars

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Abstract

Salmonella enterica is one of the most prevalent and varied foodborne pathogens. The large number of serovar types results in the colonization of many types of hosts, with different environmental conditions and hazards. This range of possible settings can lead to the development of differences in phenotype and gene expression during their adaptation to their surroundings and become ingrained as a serovar trait. The aim of this study was to evaluate the differences in gene expression (*bcsA* and *csgD*) of *Salmonella enterica* serovars Heidelberg, Kentucky, and Enteritidis during biofilm formation. Quantitative reverse-transcriptase polymerase chain reaction assays were used to determine gene expression. Throughout the four-day period, *S.* Kentucky had a 2.95-fold lower *csgD* expression than the average of the other serovars ($P<0.0001$), while *S.* Enteritidis had the lowest expression of *bcsA* with a 3.10 lower-fold change ($P=0.0019$). Individual strains also exhibited variability in expression over time ranging from up to a 39.05-fold increase in expression of *csgD* in *S.* Kentucky 38-0085 on Day 4 to a 7.05-fold decrease in expression on Day 1 for *S.* Heidelberg 38-0128. Overall, there appeared to be differences in expression between the different serovars with high variation between strains.

Keywords: biofilm, pellicle, *Salmonella* Enteritidis, *Salmonella* Kentucky, *Salmonella* Heidelberg, qRT-PCR

Introduction

Non-typhoidal *Salmonella enterica* is one of the most commonly encountered bacterial foodborne pathogens (Scallan et al., 2011). There are over *Salmonella* 2,500 serovars. Out of these serovars that have been identified, several have been associated with produce, animal, and poultry products (Foley et al., 2008; Hanning et al., 2009; Foley et al., 2011; Foley et al., 2013; USDA-FSIS, 2016). *Salmonella* serovars Enteritidis, Heidelberg, and Kentucky are among the five most identified serovars from poultry and poultry products (Foley et al., 2008; Foley et al., 2011; Finstad et al., 2012; Howard et al., 2012; Foley et al., 2013). *Salmonella* Enteritidis and Typhimurium are considered the most frequently associated with the consumption of undercooked poultry and egg products (Rabsch et al., 2000; Ricke, 2017). *Salmonella* Heidelberg is also associated with eggs; however, it is more typically identified with outbreaks of contaminated poultry meat (Gast et al., 2004; Gast et al., 2007; Kaldhone et al., 2017; CDC, 2018). While *Salmonella* Kentucky is noted to be the most frequently isolated from poultry samples, this serovar does not generally cause illnesses among humans (Foley et al., 2011; Foley et al., 2013). Therefore it is important to investigate the differences between host-adapted serovars that have a broad host range but have altered disease potential in different hosts such as *Salmonella* Typhimurium, and host-restricted serovars which are adapted and only cause disease to certain hosts like *Salmonella* Gallinarum in poultry (Kingsley and Bäumler, 2000; Uzzau et al., 2000; McClelland et al., 2004; Feasey et al., 2012).

Outside of the host, *Salmonella* expresses a variety of survival mechanisms to the environment, one of which is the ability to form biofilms. Biofilms enable *Salmonella* to resist antimicrobials and thrive in a variety of habitats (Donlan, 2002). Biofilms may form on biotic surfaces such as on organic plant structures, on abiotic surfaces such as stainless steel and

plastic, as well as form pellicle type biofilms at the air-liquid interface (Steenackers et al., 2012). Because of this ability, *Salmonella* has immense potential to magnify its ability as health concern if it colonizes a food processing area and forms a biofilm. Any *Salmonella* strains allowed to persist may form stronger biofilms in the future and become more resistant to removal than freshly introduced strains (Vestby et al., 2009). This may be an issue for example, in pipes with stagnant water as well as in other areas where sanitation is challenging where pellicles are able to form (Carpentier and Cerf, 1993). This process is enhanced with the development of the biopolymer matrix of extracellular polymeric substances (EPS) within the pellicle.

Comprising up to 90% of the total matter of a biofilm, EPS plays a pivotal role in functions related to structural support, nutrient transport, and protection (Flemming and Wingender, 2010). In *Salmonella*, the principle polysaccharide structural component of the EPS matrices is cellulose, chiefly regulated by the *bcs* (bacterial cellulose synthase) operon, without which, *Salmonella* is unable to form strong biofilms (Solano et al., 2002). The other major component of the matrix is the amyloid proteinaceous curli fimbriae structures controlled by the *csg* operons (curli specific gene) which interact with cellulose to start the formation of biofilms and enhance the sequential survival (Zogaj et al., 2001; White et al., 2006). These two structural components provide the majority of the EPS for *Salmonella* and are critical in the ability to form a biofilm.

Therefore, the objective of the current study was to examine the differences in the expression of critical *Salmonella* biofilm structural genes across several serovars. Expression levels of the *bcsA* and *csgD* genes were observed in strains of *Salmonella* serovars Enteritidis, Heidelberg, and Kentucky over the course of the development of bacterial pellicles across a 96 h time period. The authors hypothesized that the different serovars would exhibit contrasting

expression levels of the two genes as assessed using quantitative reverse-transcriptase polymerase chain reaction assays (qRT-PCR).

Materials and Methods

Bacterial strains and pellicle formation

Nine strains of *Salmonella* from the University of Arkansas Center for Food Safety Culture Collection were used in this study. This included three strains of *S.* Kentucky (UA CFS# 38-0055, 38-0084, 38-0085), three strains of *S.* Enteritidis (UA CFS# 38-0086, 38-0087, 38-0088), and three strains of *S.* Heidelberg (UA CFS# 38-00126, 38-00127, 38-00128). Quadrant streaks of the isolates from frozen stocks were prepared on Luria-Bertani (LB) (BD Biosciences, Franklin Lakes, NJ, USA) agar plates and incubated for 24 h at 37 °C. After incubation, single colonies were selected and grown in 5 mL of LB broth overnight in a 37 °C shaking incubator for 18 hours. Overnight cultures of each of the *Salmonella* strains were diluted 1:10 and inoculated into 125 mL flasks containing 50 mL of LB broth without salt (10 g Tryptone and 5 g Yeast Extract per L) (BD Biosciences, Franklin Lakes, NJ). Flasks were placed at room temperature for 96 h with the standing cultures developing pellicles by the end of this period. At the 0 h, 24 h, 48 h, 72 h, and 96 h time points, 1 mL of culture was collected directly below the meniscus and total RNA was extracted using a Qiagen RNeasy kit (Qiagen, Valencia, CA, USA). RNA was stored at -80°C until qRT-PCR was performed. Two independent trials were performed for this study.

Quantitative reverse-transcriptase PCR (qRT-PCR) assay

The qRT-PCR assays were performed using the Verso 1-Step RT-qPCR Kit (Thermo Scientific, Waltham, MA, USA) and optimized using an Eppendorf RealPlex^4 Mastercycler epgradient thermocycler (Eppendorf, Hamburg, Germany). To remove any DNA, the RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) before each assay. Primer pairs for the *csgD, bcsA,* and the rRNA housekeeping gene *rsmC* were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA) and confirmed using the National Center for Biotechnology Information (NCBI) BLAST. Primer sets resulted in 156, 136, and 190 bp for the *bcsA*, *csgD*, and *rsmC* products, respectively. A mastermix was prepared to ensure that each 25 µL reaction contained 12.5 µL of 2X 1-Step qPCR SYBR Mix (Thermo Scientific, Waltham, MA, USA), 1.25 µL RT Enhancer, 0.25 µL Verso Enzyme mix, 500 nM of each primer, 100 ng of total RNA template, and nuclease-free water (MBI Growcells, Irving, CA, USA). The qRT-PCR conditions consisted of a 5-min cDNA synthesis step 50°C step followed by a 15-min Hot Start period at 95°C and 40 cycles consisting of 15 s for denaturation at 95°C, 15 s for primer annealing at 55°C, and 20 s for amplicon extension at 68°C with melt curves. The melt curves were produced by cycling from of 95°C for 15s and then by 60°C for 20 min with a 0.5°C increase in temperature per minute until a final temperature of 95°C was reached. Each assay was performed in triplicate.

Statistical Analysis

In order to study the differences in gene expression among the three *Salmonella* serovars, we observed the RNA transcript levels of the pellicle structural genes *csgD* and *bcsA* as determined by qRT-PCR. The difference in C*t* values were compared with values exhibited by

the rRNA housekeeping gene *rsmC* and analyzed using the JMP® 14.0 (SAS Institute, Cary, NC, USA) software suite. Data were analyzed using n-way ANOVA. Means were separated using Tukey's protected HSD with a significance level of $P \leq 0.05$.

Results

CsgD expression

Overall levels of c*sgD* showed a general decrease from Day 0 to Day 3 before increasing on Day 4 (P<0.0001, **Figure 5.1**, **Table 5.1**). Between serovars, *S.* Enteritidis and Heidelberg had higher levels of gene expression than *S.* Kentucky, which experienced a 2.95-fold decrease (P<0.0001, **Figure 5.2**). Within each serovar, any differences in expression tended to appear on Day 4 (P < 0.05, **Table 5.2**); however, there was little difference in the expression of *csgD* between the other days. Within *S.* Kentucky strains, only CFS# 38-0085 expressed a higher level of *csgD* with a 24.64-fold increase on Day 4 when compared to 38-0084 (P=0.0211, **Figure 5.3c**). *S.* Enteritidis 38-0086 had a 13.26-fold lower expression than 38-0087 (P=0.0006, **Figure 5.3a**). For *S.* Heidelberg strains, 38-0127 experienced a 9.42-fold lower *csgD* expression than the average of the other two strains (P=0.003, **Figure 5.3b**). Within each strain, expression levels over time also tended not to be statistically different and differences were only observed only in a few strains (**Table 5.2**). *S.* Kentucky 38-0085 and *S.* Heidelberg 38-0127 increased expression on Day 4 with 39.05-fold and 8.78-fold increases, respectively. *S.* Heidelberg 38-0128 decreased expression on Day 2 with a 7.05-fold decrease.

BcsA expression

Overall expression levels of *bcsA* showed an increase from Day 0 to Day 1, followed by a decrease on Day 2 and Day 3, and ending with an increase again on Day 4 (P<0.0001, **Table 5.3, Figure 5.4**). Between serovars, *S.* Enteritidis exhibited a 3.10-fold lower expression level than the other serovars (P=0.0019, **Figure 5.5**). As with the *csgD* results, there were limited differences in *bcsA* expression between strains within their respective serovars (P < 0.05, **Table 5.2**). *S.* Kentucky strains exhibited no differences with each other (P=0.0152, **Figure 5.6c**). In *S.* Enteritidis strains, CFS# 38-0087 exhibited 16.34-fold higher expression on Day 4 than 38-0086 (P<0.0001, **Figure 5.6a**). For *S.* Heidelberg strains, 38-0128 had higher expression on Day 1 than 38-0126 with a 3.86-fold increase and 38-0127 had higher expression on Day 4 than the others with a 8.21-fold increase (P = 0.0222**, Figure 5.6b**). Looking at strain expression changes over time, differences were observed in four strains, *S.* Kentucky 38-0085, *S.* Enteritidis 38-0087, *S.* Heidelberg 38-0127, and 38-0128, the first three of which exhibited an increase in expression on Day 4 with 20.71-fold, 8.38-fold, and 7.20-fold increases, respectively while 38-0128 experienced an 8.97-fold increase on Day 1 (**Table 5.4**).

Ratio of *csgD* to *bcsA* expression

Next, the ratios between the level of *csgD* and the level of *bcsA* expression was observed to examine any patterns (**Table 5.5**, **Figure 5.7**). Between the serovars, there was large variability on each day for all serovars with up to a 7.78-fold difference in ratios within each group (P = 0.05). Only Day 0 showed a difference in ratios between serovars with *S.* Kentucky showing a 9.63-fold ratio greater ratio than *S.* Enteritidis. Within *S.* Kentucky, there was also a

10.48-fold ratio decrease from Day 0 to Day 1. No other changes occurred in any of the other serovars through time.

Discussion

The purpose of this study was to investigate the potential differences between *Salmonella* serovars in the formation and gene expression of pellicle type biofilms. This was done by performing qRT-PCR to detect RNA expression levels of the two biofilm matrix structural genes, *bcsA* and *csgD*. The study of biofilms offers unique opportunities to study how species of *Salmonella* can adapt to stressful environmental conditions as well as observe the changes in phenotype, genotype, and gene expression during the change from planktonic to settled cells.

With over 2500 serovars of *Salmonella* identified, many exhibit considerable differences in ecological niches and lifestyles such as in the difference between typhoidal and non-typhoidal *Salmonella* serovars (Gal-Mor et al., 2014). This can even be seen as well in within strains occupying similar niches (Porwollik et al., 2005). In our study, the different serovars of *Salmonella* did appear to give off different expression levels of the two studied genes. Differing patterns of expression were observed between serovar types, but also in strains within the same serovar. The evidence of different patterned gene expression further justifies the need to investigate the different strains and the subsequent environments they were isolated from to determine the influence of those factors have on gene expression. In fact, there were large differences in gene expression levels between strains of the same serovar, but isolated from different environments, as well as indication of large levels of variability. This made it difficult to draw conclusions on potential patterns of gene expression for entire serovars.

In biofilms of *Salmonella*, the two major components of the extracellular matrix are the polysaccharide cellulose and curli amyloid fimbrial structures (Zogaj et al., 2001). Together, these biopolymers provide structure to the biofilm and support cell adhesion with the presence of both necessary to produce fully functional and maximally resistant biofilms (Solano et al., 2002). The transcriptional regulator *csgD* controls the production of curli fimbriae by positively regulating the *csgBA* operon which produces the protein components of curli (Barnhart and Chapman, 2006). In addition, *csgD* acts on the *adrA* promoter section, which results in the production of cyclic diguanylic acid (c-di-GMP), an allosteric activator of cellulose synthase encoded by the *bcs* operon (Verstraeten et al., 2008). Because of the critical role these two genes provide in early *Salmonella* biofilm formation, they were chosen to be observed for this study.

As such, it would be expected that increases in *csgD* expression would lead to increases in cellulose production and therefore expression of the *bcsA* gene. In our current experiment, we found that increased or decreased trends in expression of one gene were often similar to the other. However, the ratio of *csgD* to *bcsA* expression varied considerably over the days throughout all strains, showing no trend in most cases towards an increase in the ratio of *bcsA* to *csgD*, which would indicate greater production of cellulose. Da Re and Ghigo (2006) found that neither *csgD* nor *adrA* were involved in the production and regulation of cellulose in *E. coli* 1094 cells, which instead used the YedQ GGDEF domain protein, suggesting that there may be alternative cellulose pathways involved. These alternative pathways may aid *Salmonella* in providing greater adaptability to different environments and situations and should be further investigated.

Conclusions

Addressing the gene expression differences between the serovars of *Salmonella* is an important topic for controlling *Salmonella* contamination. One concern involves any differences in the development of biofilms and the expression of biofilm producing genes. Our study found there to be differences in the expression of biofilm forming genes between serovars and large variations in gene expression between strains within each serovar. This indicates that individual variables accounting for the differences in each strain such as environmental origin, as well as serovar differences must be considered when trying to control for biofilm formation. Future assessments should include a broader variety of serovars, including the most common poultry isolates that are important to human health. A more expansive study of additional biofilm related genes should also be conducted as well as examining gene expression in the pellicle itself.

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References

Barnhart, M.M. and Chapman, M.R., 2006. Curli biogenesis and function. *Annual Reviews in Microbiology, 60,* 131-147.

Carpentier, B. and Cerf, O., 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. *Journal of Applied Bacteriology, 75* (6), 499-511.

CDC. 2018. *Reports of Selected Salmonella Outbreak Investigations* [Online]. Centers for Disease Control and Prevention. Available: https://www.cdc.gov/salmonella/outbreaks.html [Accessed 6/15/18].

Da Re, S. and Ghigo, J.-M., 2006. A CsgD-independent pathway for cellulose production and biofilm formation in *Escherichia coli*. *Journal of Bacteriology, 188* (8), 3073-3087.

Donlan, R.M., 2002. Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases, 8* (9), 881-890.

Feasey, N.A., Dougan, G., Kingsley, R.A., Heyderman, R.S., and Gordon, M.A., 2012. Invasive non-typhoidal *Salmonella* disease: an emerging and neglected tropical disease in Africa. *The Lancet, 379* (9835), 2489-2499.

Finstad, S., O'Bryan, C.A., Marcy, J.A., Crandall, P.G., and Ricke, S.C., 2012. *Salmonella* and broiler processing in the United States: relationship to foodborne salmonellosis. *Food Research International, 45* (2), 789-794.

Flemming, H.-C. and Wingender, J., 2010. The biofilm matrix. *Nature Reviews Microbiology, 8* (9), 623-33.

Foley, S.L., Johnson, T.J., Ricke, S.C., Nayak, R., and Danzeisen, J., 2013. *Salmonella* pathogenicity and host adaptation in chicken-associated serovars. *Microbiology and Molecular Biology Reviews, 77* (4), 582-607.

Foley, S.L., Lynne, A.M., and Nayak, R., 2008. *Salmonella* challenges: prevalence in swine and poultry and potential pathogenicity of such isolates. *Journal of Animal Science, 86* (14 Suppl), E149-62.

Foley, S.L., Nayak, R., Hanning, I.B., Johnson, T.J., Han, J., and Ricke, S.C., 2011. Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. *Applied and Environmental Microbiology, 77,* 4273-4279.

Gal-Mor, O., Boyle, E.C., and Grassl, G.A., 2014. Same species, different diseases: how and why typhoidal and non-typhoidal *Salmonella enterica* serovars differ. *Frontiers in Microbiology, 5,* 391.

Gast, R.K., Guard-Bouldin, J., and Holt, P.S., 2004. Colonization of reproductive organs and internal contamination of eggs after experimental infection of laying hens with *Salmonella heidelberg* and *Salmonella enteritidis*. *Avian Diseases, 48* (4), 863-869.

Gast, R.K., Guraya, R., Guard-Bouldin, J., Holt, P.S., and Moore, R.W., 2007. Colonization of specific regions of the reproductive tract and deposition at different locations inside eggs laid by hens infected with *Salmonella* Enteritidis or *Salmonella* Heidelberg. *Avian diseases, 51* (1), 40-44.

Hanning, I.B., Nutt, J.D., and Ricke, S.C., 2009. Salmonellosis outbreaks in the United States due to fresh produce: sources and potential intervention measures. *Foodborne Pathogens and Disease, 6* (6), 635-648.

Howard, Z.R., O'Bryan, C.A., Crandall, P.G., and Ricke, S.C., 2012. *Salmonella* Enteritidis in shell eggs: current issues and prospects for control. *Food Research International, 45* (2), 755-764.

Kaldhone, P.R., Foley, S.L., and Ricke, S.C., 2017. Salmonella Heidelberg in layer hens and egg production: incidence and potential issues. *In:* Ricke, S. C. and Gast, R. K. (eds.) *Producing Safe Eggs.* San Diego, CA: Elsevier, Inc.

Kingsley, R.A. and Bäumler, A.J., 2000. Host adaptation and the emergence of infectious disease: the *Salmonella* paradigm. *Molecular Microbiology, 36* (5), 1006-1014.

McClelland, M., Sanderson, K.E., Clifton, S.W., Latreille, P., Porwollik, S., Sabo, A., Meyer, R., Bieri, T., Ozersky, P., and McLellan, M., 2004. Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of Salmonella enterica that cause typhoid. *Nature genetics, 36* (12), 1268.

Porwollik, S., Santiviago, C., Cheng, P., Florea, L., and McClelland, M., 2005. Differences in gene content between *Salmonella enterica* serovar Enteritidis isolates and comparison to closely related serovars Gallinarum and Dublin. *Journal of Bacteriology, 187* (18), 6545-6555.

Rabsch, W., Hargis, B.M., Tsolis, R.M., Kingsley, R.A., Hinz, K.-H., Tschäpe, H., and Bäumler, A.J., 2000. Competitive exclusion of *Salmonella* Enteritidis by *Salmonella* Gallinarum in Poultry. *Emerging Infectious Diseases, 6* (5), 443-448.

Ricke, S.C., 2017. Insights and challenges of *Salmonella* infection of laying hens. *Current Opinion in Food Science, 18*, 43-49.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., and Griffin, P.M., 2011. Foodborne illness acquired in the United States--major pathogens. *Emerging Infectious Diseases, 17* (1), 7-15.

Solano, C., García, B., Valle, J., Berasain, C., Ghigo, J.M., Gamazo, C., and Lasa, I., 2002. Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. *Molecular Microbiology, 43* (3), 793-808.

Steenackers, H., Hermans, K., Vanderleyden, J., and De Keersmaecker, S.C.J., 2012. *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. *Food Research International, 45* (2), 502-531.

USDA-FSIS. 2016. *Serotypes Profile of Salmonella Isolates from Meat and Poultry Products, January 1998 through December 2014* [Online]. United States Department of Agriculture. Available: https://www.fsis.usda.gov/wps/wcm/connect/3866026a-582d-4f0e-a8ce-851b39c7390f/Salmonella-Serotype-Annual-2014.pdf?MOD=AJPERES [Accessed 6/14/18].

Uzzau, S., Brown, D.J., Wallis, T., Rubino, S., Leori, G., Bernard, S., Casadesús, J., Platt, D.J., and Olsen, J.E., 2000. Host adapted serotypes of *Salmonella enterica*. *Epidemiology & Infection, 125* (2), 229-255.

Verstraeten, N., Braeken, K., Debkumari, B., Fauvart, M., Fransaer, J., Vermant, J., and Michiels, J., 2008. Living on a surface: swarming and biofilm formation. *Trends in Microbiology, 16* (10), 496-506.

Vestby, L.K., Møretrø, T., Langsrud, S., Heir, E., and Nesse, L.L., 2009. Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal-and feed factories. *BMC Veterinary Research, 5*, 20.

White, A., Gibson, D., Kim, W., Kay, W., and Surette, M., 2006. Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of *Salmonella*. *Journal of Bacteriology, 188* (9), 3219-3227.

Zogaj, X., Nimtz, M., Rohde, M., Bokranz, W., and Römling, U., 2001. The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. *Molecular Microbiology, 39* (6), 1452-1463.

Figures

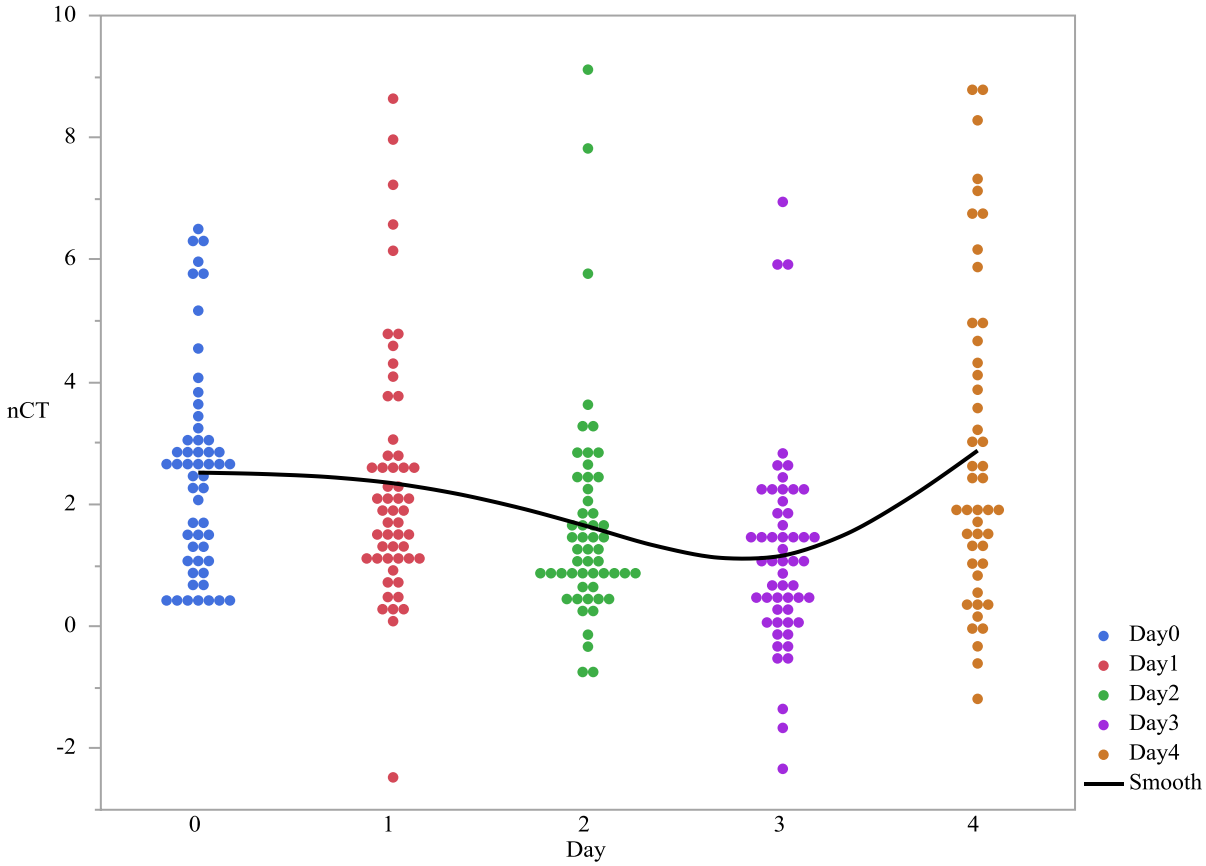

Figure 5.1. Changes in expression of the gene *csgD* from planktonic cells used to form *Salmonella* pellicles over a 4 day time period. [1,2] qRT-PCR was performed on total RNA isolated from bacterial cultures of *Salmonella* as pellicles were being formed over a 4 day time period. [1]N = 270, n = 54, P < 0.0001, Individual SEM for d 0, 1, 2, 3, and 4 was 0.231, 0.285, 0.242, 0.226, and 0.387.

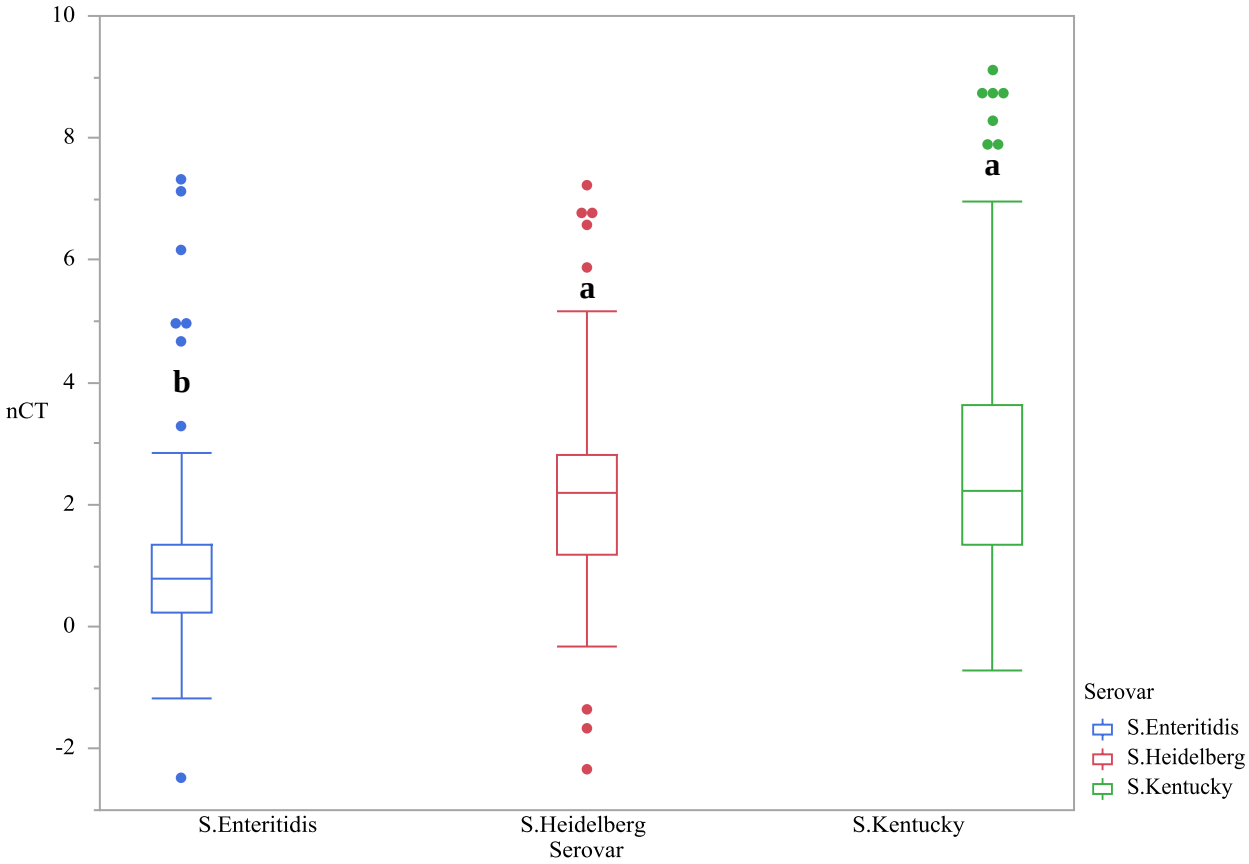

Figure 5.2. Differences in expression of the gene *csgD* between serovars. [1,2] qRT-PCR was performed on total RNA isolated from bacterial cultures of *Salmonella* as pellicles were being formed over a 4 day time period.

[1]N = 270, n = 90, P < 0.000, Individual SEM was 0.170, 0.184 and 0.253 for *S.* Enteritidis, Heidelberg, and Kentucky.

[2]Means with different superscripts are considered significantly different (a-b)

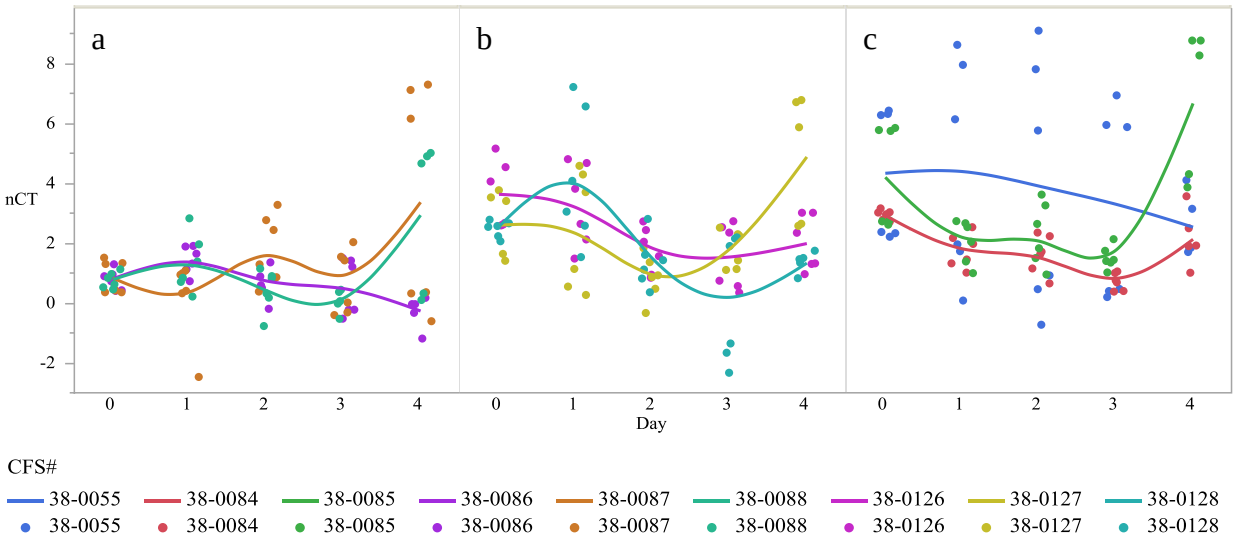

Figure 5.3. Differences in expression of the gene *csgD* over time in strains of *Salmonella* Heidelberg (A), Enteritidis (B), and Kentucky (C). [1] qRT-PCR was performed on total RNA isolated from bacterial cultures of *Salmonella* as pellicles were being formed over a 4-day time period.

[1]N = 90, n = 6, P = 0.0006(A), 0.003(B), 0.0211(C)

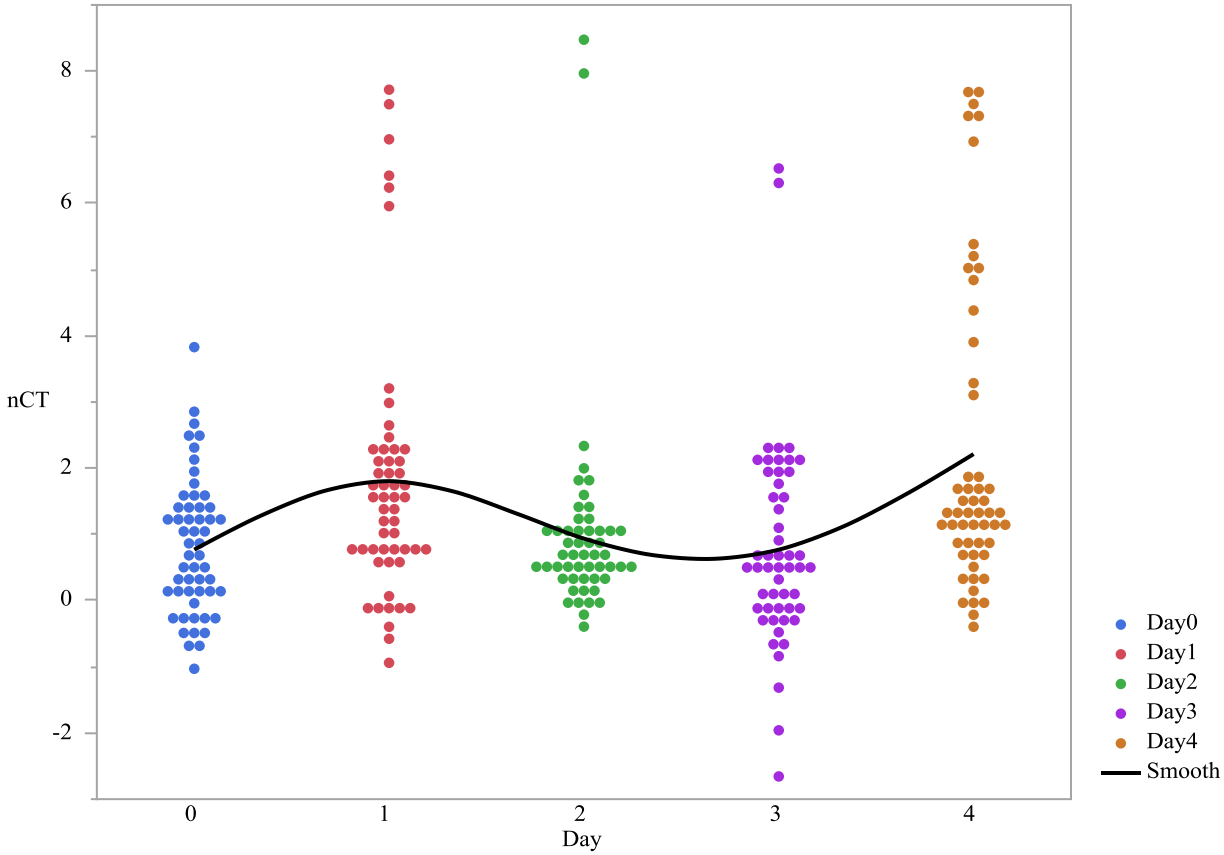

Figure 5.4. Changes in expression of the gene *bcsA* from planktonic cells used to form *Salmonella* pellicles over a 4-day time period. [1] qRT-PCR was performed on total RNA isolated from bacterial cultures of *Salmonella* as pellicles were being formed over a 4-day time period. [1]N = 270, n = 54, P < 0.0001, Individual SEM for d 0, 1, 2, 3, and 4 was 0.138, 0.273, 0.219, 0.220, and 0.319.

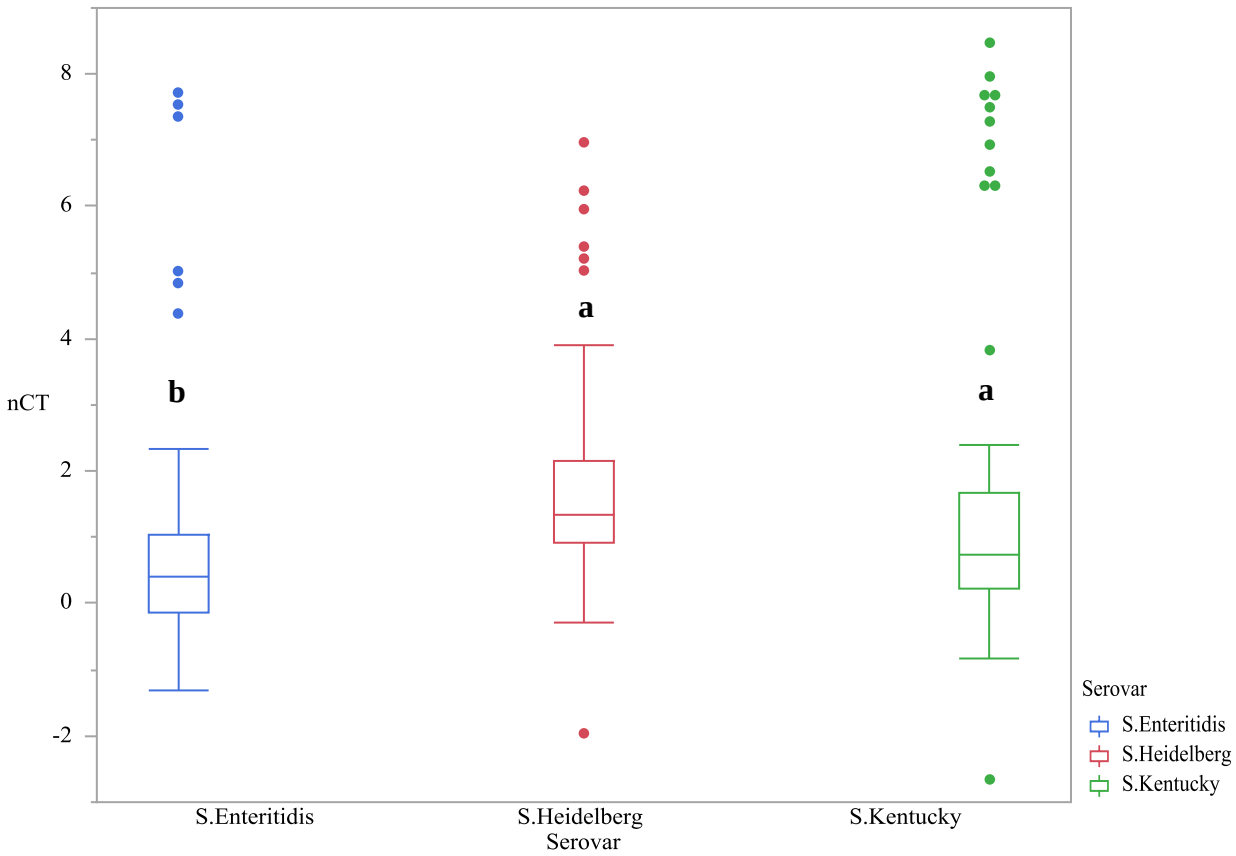

Figure 5.5. Differences in expression of the gene *bcsA* between serovars. [1,2] qRT-PCR was performed on total RNA isolated from bacterial cultures of *Salmonella* as pellicles were being formed over a 4-day time period.
[1]N = 270, n = 90, P < 0.0019, Individual SEM was 0.175, 0.152 and 0.242 for *S.* Enteritidis, Heidelberg, and Kentucky.
[2]Means with different superscripts are considered significantly different (a-b).

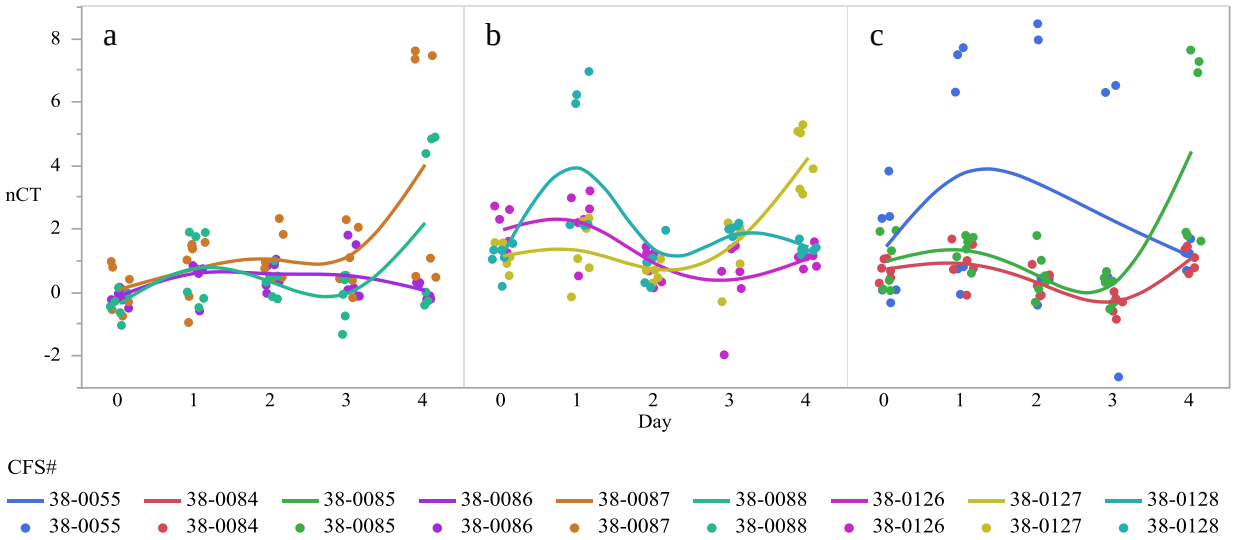

Figure 5.6. Differences in expression of the gene *bcsA* over time in strains of *Salmonella* Heidelberg (A), Enteritidis (B), and Kentucky (C).[1] qRT-PCR was performed on total RNA isolated from bacterial cultures of *Salmonella* as pellicles were being formed over a 4-day time period.

[1]N = 90, n = 6, P < 0.0001(A), 0.0222(B), 0.0152(C).

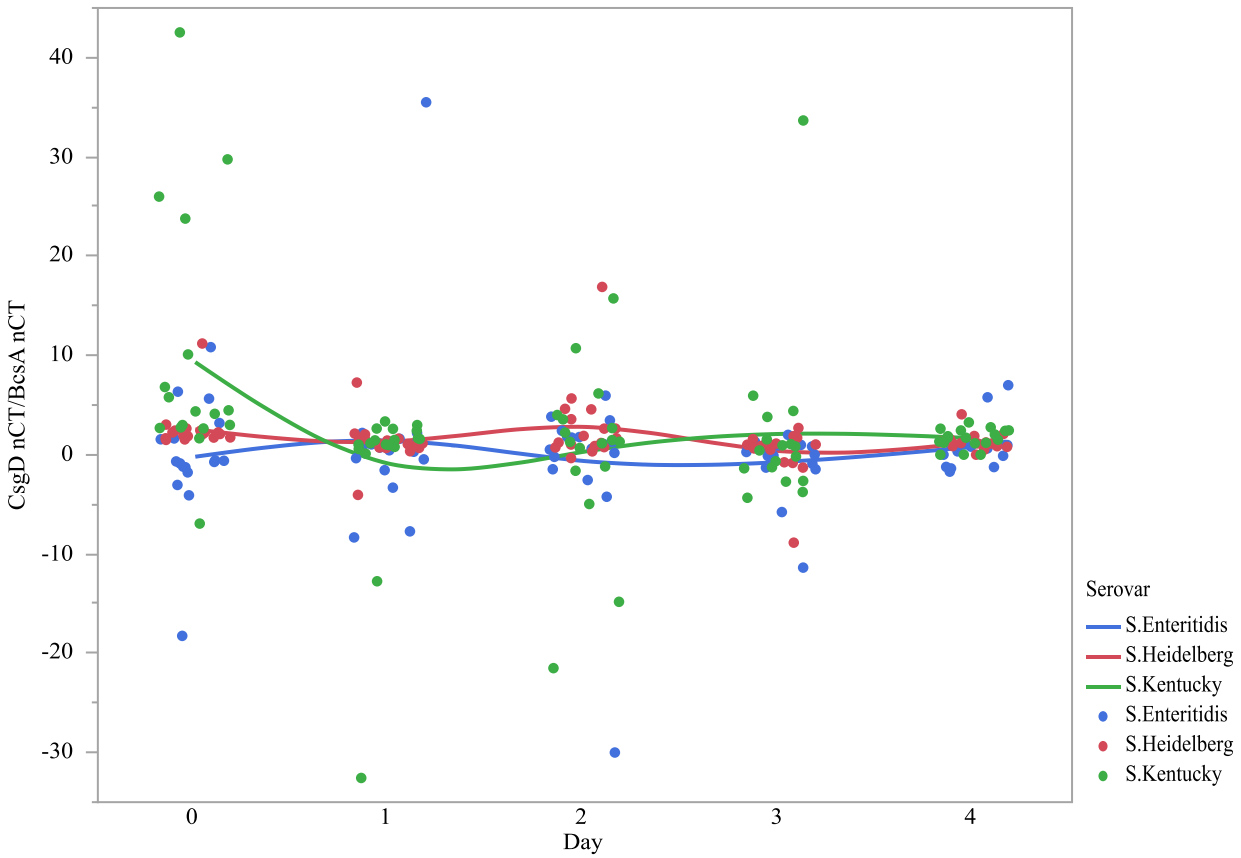

Figure 5.7. Comparison of changes in expression of the ratio of the genes *csgD* and *bcsA* in *Salmonella* serovars over time.[1] qRT-PCR was performed on total RNA isolated from bacterial cultures of *Salmonella* as pellicles were being formed over a 96 h time period. Individual SEM was 1.450, 2.126, 1.808, 0.741, and 0.560, for *S*. Enteritidis, 0.516, 0.472, 0.963, 0.620, and 0.209 for *S*. Heidelberg, and 0.2.960, 2.026, 2.062, 2.067, and 0.227 for *S*. Kentucky on d 0, 1, 2, 3, and 4, respectively.
[1]N = 270, n = 18, P = 0.0065,

Tables

Table 5.1. Changes in expression of the gene *CsgD* from planktonic cells used to form *Salmonella* pellicles over a 4-day time period. [1, 2]

	Day				
	0	1	2	3	4
CsgD nCT	2.517 ± 0.231 [ab]	2.346 ± 0.285 [ab]	1.641 ± 0.242 [bc]	1.151 ± 0.226 [c]	2.884 ± 0.387 [a]

[1]N = 270, n = 54, Means of nCT values and individual SEMs are given for each strain at each time point.
[2]Means with different superscripts are considered significantly different (a-b)

Table 5.2. Changes in expression of the gene *csgD* from planktonic cells used to form pellicles in *Salmonella* Kentucky, Enteritidis, and Heidelberg over a 4-day time period.[1, 2, 3]

	Day				
	0	1	2	3	4
S. Kentucky					
38-0055	4.333 ± 0.904 ^{ab}	4.425 ± 1.476 ^{ab}	3.898 ± 1.714 ^{ab}	3.313 ± 1.330 ^{ab}	2.538 ± 0.474 ^{ab}
38-0084	2.955 ± 0.068 ^{ab}	1.752 ± 0.235 ^{b}	1.628 ± 0.263 ^{b}	0.719 ± 0.117 ^{b}	2.186 ± 0.421 ^{b}
38-0085	4.256 ± 0.692 ^{ab}	2.068 ± 0.296 ^{b}	2.308 ± 0.427 ^{b}	1.522 ± 0.155 ^{b}	6.809 ± 1.114 ^{a}
S. Enteritidis					
38-0086	0.788 ± 0.139 ^{abc}	1.442 ± 0.190 ^{abc}	0.667 ± 0.210 ^{bc}	0.530 ± 0.385 ^{bc}	-0.280 ± 0.240 ^{c}
38-0087	0.887 ± 0.227 ^{abc}	0.218 ± 0.553 ^{bc}	1.843 ± 0.472 ^{abc}	0.722 ± 0.437 ^{abc}	3.449 ± 1.545 ^{a}
38-0088	0.762 ± 0.108 ^{abc}	1.328 ± 0.389 ^{abc}	0.438 ± 0.284 ^{bc}	0.057 ± 0.141 ^{c}	3.008 ± 1.142 ^{ab}
S. Heidelberg					
38-0126	3.617 ± 0.461 ^{abc}	3.267 ± 0.565 ^{abcd}	1.767 ± 0.317 ^{bcde}	1.552 ± 0.451 ^{bcde}	2.002 ± 0.374 ^{bcde}
38-0127	2.575 ± 0.452 ^{abcde}	2.430 ± 0.810 ^{abcde}	0.860 ± 0.305 ^{de}	1.794 ± 0.261 ^{bcde}	4.928 ± 0.956 ^{a}
38-0128	2.483 ± 0.111 ^{abcde}	4.182 ± 0.928 ^{ab}	1.363 ± 0.343 ^{cde}	0.155 ± 0.876 ^{e}	1.383 ± 0.152 ^{bcde}

[1]Each serovar was analyzed separately from others to determine the interaction between specific strains within strains and day. Means of nCT values and individual SEMs are given for each strain at each time point.

[2]N = 90, n = 6

[3]Means with different superscripts in the same serovar are considered significantly different (a-e).

Table 5.3. Changes in expression of the gene *bcsA* from planktonic cells used to form *Salmonella* pellicles over a 4-day time period. [1, 2]

	Day				
	0	1	2	3	4
BcsA nCT	0.766 ± 0.138 [c]	1.815 ± 0.273 [ab]	0.932 ± 0.219 [bc]	0.766 ± 0.220 [c]	2.217 ± 0.319 [a]

[1]N = 270, n = 54, Means of nCT values and individual SEMs are given for each strain at each time point.
[2]Means with different superscripts are considered significantly different (a-c).

Table 5.4. Changes in expression of the gene *bcsA* from planktonic cells used to form pellicles in *Salmonella* Kentucky, Enteritidis, and Heidelberg over a 4-day time period.[1, 2,3]

	Day				
	0	1	2	3	4
S. Kentucky					
38-0055	1.403 ± 0.688 [abc]	3.842 ± 1.511 [ab]	3.382 ± 1.982 [abc]	2.228 ± 1.805 [abc]	1.134 ± 0.159 [abc]
38-0084	0.727 ± 0.128 [abc]	0.938 ± 0.260 [abc]	0.332 ± 0.160 [bc]	-0.359 ± 0.126 [c]	1.126 ± 0.149 [abc]
38-0085	0.963 ± 0.361 [abc]	1.378 ± 0.183 [abc]	0.587 ± 0.302 [abc]	0.160 ± 0.192 [bc]	4.532 ± 1.237 [a]
S. Enteritidis					
38-0086	-0.115 ± 0.101 [b]	0.645 ± 0.275 [b]	0.557 ± 0.175 [b]	0.558 ± 0.354 [b]	0.059 ± 0.105 [b]
38-0087	0.107 ± 0.296 [b]	0.742 ± 0.423 [b]	1.140 ± 0.315 [b]	1.022 ± 0.402 [b]	4.089 ± 1.521 [a]
38-0088	-0.372 ± 0.175 [b]	0.823 ± 0.467 [b]	0.343 ± 0.184 [b]	-0.103 ± 0.314 [b]	2.247 ± 1.105 [ab]
S. Heidelberg					
38-0126	1.948 ± 0.284 [bc]	2.313 ± 0.390 [b]	0.852 ± 0.219 [bc]	0.392 ± 0.513 [c]	1.097 ± 0.124 [bc]
38-0127	1.133 ± 0.162 [bc]	1.390 ± 0.402 [bc]	0.702 ± 0.118 [bc]	1.433 ± 0.390 [bc]	4.281 ± 0.400 [a]
38-0128	1.098 ± 0.194 [bc]	4.263 ± 0.961 [a]	0.899 ± 0.321 [bc]	2.017 ± 0.071 [bc]	1.390 ± 0.068 [bc]

[1]Each serovar was analyzed separately from others to determine the interaction between specific strains within strains and day. Means of nCT values and individual SEMs are given for each strain at each time point.

[2]N = 90, n = 6

[3]Means with different superscripts in the same serovar are considered significantly different (a-e).

Table 5.5. Comparison of changes in the expression of the ratio of the genes *csgD* and *bcsA* in the interaction between *Salmonella* serovars and a 4-day time period.[1,2]

	Day				
	0	1	2	3	4
S. Kentucky	9.400 ± 2.960 [a]	-1.083 ± 2.026 [b]	0.406 ± 2.062 [b]	2.101 ± 2.087 [ab]	1.646 ± 0.227 [b]
S. Enteritidis	-0.230 ± 1.450 [b]	1.485 ± 2.126 [b]	-0.696 ± 1.808 [b]	0.741 ± 0.741 [b]	0.768 ± 0.530 [b]
S. Heidelberg	2.575 ± 0.516 [ab]	1.235 ± 0.472 [b]	2.936 ± 0.963 [ab]	0.264 ± 0.620 [b]	1.384 ± 0.205 [b]

[1]N = 270, n = 18, P = 0.0065, Means of nCT ratios and individual SEMs are given for each strain at each time point.
[2]Means with different superscripts are considered significantly different (a-b).

VII. Conclusion

The ability of foodborne pathogens, especially *Salmonella enterica* to form biofilm communities on a wide variety of surfaces and resist the action of antimicrobials poses an issue to public health. Major economic losses can be accrued if outbreaks of foodborne illnesses occur due to cross contamination or re-contamination of food items due to reservoirs of pathogenic foodborne microorganisms being established in food processing or handling environments. Research continues on understanding all the underlying mechanisms behind biofilm development and on creating improvements in methods of prevention and treatment. One avenue less studied is whether there exist differences between *Salmonella* serovars in the mechanism, development, and resistance capacity to antimicrobials.

This thesis was focused on the growth, treatment, and genetic mechanism of *Salmonella* biofilms. The objective of this research was to observe any differences among serovars in the growth and treatment of various types of biofilms. The results from Chapter Two (the coupon study) provided evidence that *Salmonella* Typhimurium was more susceptible to sanitization from Peracetic Acid treatment when compared to *Salmonella* Heidelberg. Both sanitizers were unable to completely remove attached cells, suggesting the need for multiple hurdles during treatment to fully sanitize materials. Quantitative PCR was also found to be a viable method for rapid quantitation of cell counts, providing CFU values similar to those from plate counts.

In chapter Three (the genome announcement) draft genome sequences of four *Salmonella* Enteritidis and Kentucky isolates from Arkansas retail poultry samples were published to the online GenBank database. Further study of these genomes in the future may be useful in examining the differences in genetics between those two serovars.

Chapter Four observed the formation of *Salmonella* pellicle biofilms in Luria Bertani broth under different conditions. This study focused on the Kentucky serovar due to its association with poultry environments, but also due to its status as a less frequently studied serovar. We examined strains isolated from poultry environments and found that *S.* Kentucky was capable of forming pellicles similar to other serovars as well as the possibility of environmental origin playing a role in the properties of individual strains.

Chapter Five included a look at differences between two *Salmonella* serovars in expression levels over time of two critical biofilm structural genes, *csgD* and *bcsA*, encoding for curli fimbriae and cellulose. Results suggested overall differences between serovars in gene expression, but high variability between strains also suggested the need to address strains uniquely. This research illustrated some of the differences among *Salmonella* but also that further research must be conducted to fully elucidate the mechanisms behind this.

Appendix

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Research Compliance

August 4, 2017

MEMORANDUM

TO:	Dr. Steven Ricke
FROM:	Ines Pinto, Biosafety Committee Chair
RE:	Protocol Renewal
PROTOCOL #:	08027
PROTOCOL TITLE:	Screening of Mechanistically Independent Multiple Antimicrobial Treatments for Effective Decontamination of Salmonella on Poultry
APPROVED PROJECT PERIOD:	Start Date June 30, 2008 Expiration Date June 29, 2020

The Institutional Biosafety Committee (IBC) has approved your request, dated June 23, 2017, to renew IBC # 08027, "Screening of Mechanistically Independent Multiple Antimicrobial Treatments for Effective Decontamination of Salmonella on Poultry".

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

University of Arkansas Institutional Biosafety Committee
Registration for Research Projects

FORM 1 – GENERAL INFORMATION

08027 renewal IBC#:

Please check the boxes for each of the forms that are applicable to the research project you are registering. The General Information Form - FORM 1 (this form) MUST be completed on all submitted project registrations, regardless of the type of research.

- ☐ Recombinant DNA (EVEN IF IT IS EXEMPT from the NIH Guidelines.) (FORM 2)
- ☑ Pathogens (human/animal/plant) (FORM 3)
- ☐ Biotoxins (FORM 4)
- ☐ Human materials/nonhuman primate materials (FORM 5)
- ☐ Animals or animal tissues and any of the above categories; transgenic animals or tissues; wild vertebrates or tissues (FORM 6)
- ☐ Plants, plant tissues, or seed and any of the above categories; transgenic plants, plant tissues, or seeds (FORM 7)
- ☐ CDC regulated select agents (FORM 8)

To initiate the review process, you must attach and send all completed registration forms via email to ibc@uark.edu. All registration forms must be submitted electronically. To complete the registration, print page 1 of this form, PI sign, date, and mail to: Compliance Coordinator-IBC, 120 Ozark Hall, Fayetteville, AR 72701, or FAX it to 479-575-3846.

As Principal Investigator:

- ☑ I attest that the information in the registration is accurate and complete and I will submit changes to the Institutional Biosafety Committee (IBC) in a timely manner.
- ☑ I am familiar with and agree to abide by the current, applicable guidelines and regulations governing my research, including, but not limited to: the NIH Guidelines for Research Involving Recombinant DNA Molecules and the Biosafety in Microbiological and Biomedical Laboratories manual.
- ☑ I agree to accept responsibility for training all laboratory and animal care personnel involved in this research on potential biohazards, relevant biosafety practices, techniques, and emergency procedures.
- ☑ If applicable, I have carefully reviewed the NIH Guidelines and accept the responsibilities described therein for principal investigators (Section IV-B-7).
- ☑ I will submit a written report to the IBC and to the Office of Recombinant DNA Activities at NIH (if applicable) concerning: any research related accident, exposure incident, or release of rDNA materials to the environment; problems implementing biological and physical containment procedures; or violations of NIH Guidelines.
- ☑ I agree that no work will be initiated prior to project approval by the IBC.
- ☑ I will submit my annual progress report to the IBC in a timely fashion.

Principal Investigator Typed/Printed Name: Dr. Steven C. Ricke

Signature (PI): _____ **Date:** _____

FORM 1 – GENERAL INFORMATION (Continued)

CONTACT INFORMATION:

Principal Investigator:

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E-Mail: pmrubine@uark.edu

***Required if research is at Biosafety Level 2 or higher**

PROJECT INFORMATION:

Have you registered ANY project previously with the IBC? Yes

Is this a new project or a renewal?

○ New Project ◉ Renewal

Project Title: Screening of Mechanistically Independent Multiple Antimicrobial Treatments for Effective Decontamination of Salmonella on Poultry
Project Start Date: 7/1/2017
Project End Date: 6/30/2020
Granting Agency: USDA/NIFA

Indicate the containment conditions you propose to use (check all that apply):

☑ Biosafety Level 1 Ref: 1 2	☐ Biosafety Level 1A Ref: 1 2	☐ Biosafety Level 1P Ref: 1 2
☑ Biosafety Level 2 Ref: 1 2	☐ Biosafety Level 2A Ref: 1 2	☐ Biosafety Level 2P Ref: 1 2
☐ Biosafety Level 3 Ref: 2	☐ Biosafety Level 3A Ref: 2	☐ Biosafety Level 3P Ref: 2

References:

1: Biosafety in Microbiological and Biomedical Laboratories (BMBL) 4th Edition
2: NIH Guidelines for Research Involving Recombinant DNA Molecules

FORM 1 – GENERAL INFORMATION (Continued)

3: University of Arkansas Biological Safety Manual

If you are working at Biosafety Level 2 or higher, has your laboratory received an onsite inspection by the Biosafety Officer or a member of the IBC?

(•) Yes ( ) No

If yes, enter date if known: 1/1/2011
If no, schedule an inspection with the Biological Safety Officer.

Please provide the following information on the research project (DO NOT attach or insert entire grant proposals unless it is a Research Support & Sponsored Programs proposal).

Project Abstract:

Because poultry processing has been shown to be a major source of Salmonella cross-contamination during processing, reducing the Salmonella contamination level on poultry has become an important focus. Research efforts have concentrated on determining efficacy of methods to decrease Salmonella contamination in the final processing stages. Overall, we hypothesize that the combination of (1) traditional microbiological methods evaluating the potential of antimicrobial treatment strategies to reduce Salmonella contamination on poultry with (2) comprehensive genetic methods to monitor gene expression in response to these treatments can provide a powerful systematic approach to determine which treatments can be combined to achieve the most effective reduction of Salmonella by isolating treatment strategies without producing overlapping stress response systems. We propose a systematic study to examine the genetic response of the food-borne pathogen Salmonella and its survival following exposure to antimicrobial treatment strategies used in poultry processing.

Specific Aims:

1) Use multiple antimicrobial treatments to evaluate individually and in combination antimicrobial treatments proposed to act in mechanistically distinct ways to reduce Salmonella populations on chicken breast skins. 2) Perform transcriptome analysis of Salmonella recovered after above treatments to investigate the genetic responses of Salmonella to these antimicrobials individually and in combination. Microarrays and Quantitative Real-Time, Reverse-Transcriptase PCR (qRT-PCR) will be utilized to identify and confirm genes affected by the antimicrobial treatments.

Relevant Materials and Methods (this information should be specific to the research project being registered and should highlight any procedures that involve biohazardous or recombinant materials):

a. Preparation of Salmonella cultures: A cryogenic vial containing a bacterial culture in glycerol will be removed from the freezer and placed in a biological safety cabinet. A loop full of the bacterial culture will be inoculated into the appropriate media and allowed to grow in the incubator (37C or 42C). After 24 to 48 hours, the cultures will be washed by centrifugation and resuspended in fresh broth or saline solution and stored at room temperature. Three days before each experiment, the bacteria will be transferred to 5 ml of tryptic soy broth (TSB) and incubated at 37°C for 18 h, followed on the second day by a 1:100 transfer into fresh 5 ml of TSB with incubation again at 37°C for 18 h. The third day a final culture will be inoculated 1:100 into 5 ml of fresh TSB with incubation at 37°C for 12 h. The day before the inoculation of the chicken breast skins, the 12 h culture will be dispensed into sterile centrifuge

FORM 1 – GENERAL INFORMATION (Continued)

tubes (30 ml) and harvested by centrifugation at 5,000 xg for 10 m at room temperature. Each pellet then will be re-suspended in sterile peptone water (Difco) to a final concentration of approximately 10E8 cells/ml. Skin from three chicken breasts purchased from a retail outlet will be used in each of three replicate experiments. One chicken breast skin will be used for all negative control experiments (skins not inoculated with S. Typhimurium and confirmed by qRT-PCR to be Salmonella background negative (Sirsat et al., 2006) and the other two for the experimental treatments (skins inoculated with S. Typhimurium after confirming that they are Salmonella background negative) For surface inoculation, skin will be cut from each chicken breast (~100 cm2) and held with plastic skin holders. The center of each skin will be inoculated with 420 µl of the culture and the culture will be spread evenly across the skin using a sterile glass rod, and then incubated at room temperature for 30 m to allow for bacterial attachment. Skins used for negative controls will be treated the same manner except using 420 µl of sterile peptone water.Application of treatments. Skin sample will be immersed in one experimental treatment (ε-Polylysine, lactic acid, ACS, or 55°C water) or in room temperature phosphate-buffered saline (PBS; process control) for 1 m. At this point 5 cm2 portions of each skin will be removed and enumerated for surviving bacteria using traditional culturing procedures. Skin samples exposed to chemical stresses (ε-Polylysine, lactic acid, or ACS) will be transferred to 55°C water for a second 1 m treatment. In addition, the skin sample treated with room temperature PBS will also be transferred to 55°C water for 1 m (this will serve as a second process control). Skin samples corresponding to each treatment will then be stored at 4oC, the average temperature as determined by an FDA survey of fresh, raw meat in retail environments (FDA, 1999). After five days of storage at 4˚C, the skin samples will again be enumerated for bacterial survival. b. Perform transcriptome analysis of Salmonella recovered after above treatments to investigate the genetic responses of Salmonella to these antimicrobials: A portion of the samples taken from the chicken skins and utilized for the evaluation of Salmonella survival will be used for DNA and RNA extraction. DNA and RNA will be extracted for use in microarrays and Quantitative Real-Time, Reverse-Transcriptase PCR (qRT-PCR) to identify and confirm genes affected by these treatments.

The information requested above can be entered directly or cut & pasted into the space provided, or can be provided as an attached word document. If you provide an attachment, please indicate "See Attached" and list the file name(s) in the space below:

Click here to enter text.

PERSONNEL QUALIFICATIONS & FACILITY INFORMATION:

List all personnel (including PI and Co-PI) to be involved in this project:

Name (First and Last) - Position (Title, academic degrees, certifications, and field of expertise)	**Qualifications/Training/Relevant Experience (Describe previous work or training with biohazardous and/or recombinant DNA; include Biosafety Levels)**
Example: Bob Biohazard - Associate Professor, PhD- Microbiology	14 yrs working with E. coli at BL1, Salmonella enterica at BL2, 8 yrs working with transgenic mice.
Steven C. Ricke (PI), Ph.D., Donald "Buddy" Wray Chair in Food Safety and Director, Center for Food Safety in the Institute of Food Science and Engineering	24 years working with anaerobic bacteria and food-borne pathogens
Peter Rubinelli, Ph.D., cell biology	20 years working with BSL2 organisms
Sun Ae Kim, Ph.D., food science	10 years working with BSL2 organisms
Morgan Wright, M.S., food science	5 years working with BSL2 organisms

FORM 1 – GENERAL INFORMATION (Continued)

Zhaohao Shi, B.S., biology	2 years working with BSL2 organisms
Pravin Khaldone, M.S., biology	3 years working with BSL2 organisms
Laura Meyer, MS, Animal Science Program Technician	1 year working with BSL2 organisms

Additional Personnel Information (if needed):

Click here to enter text.

List all the laboratories/facilities where research is to be conducted:

Building:	Room #:	Category:	*Signage Correct?
Biomass	**132**	**Laboratory**	Yes
Biomass	**102**	Laboratory	Yes
Biomass	**117**	Laboratory	Yes
Biomass	**101**	Autoclave/BioStorage	Yes
		Choose an item.	Choose an item.
		Choose an item.	Choose an item.
		Choose an item.	Choose an item.
		Choose an item.	Choose an item.
		Choose an item.	Choose an item.
		Choose an item.	Choose an item.

*** Biohazard signs are required for entrances to Biosafety Level 2 (including Animal Biosafety Level 2) areas. EH&S will supply these signs. If an updated biohazard sign is required, please indicate the location and what agents/organisms/hazards should be listed on the sign:**

Click here to enter text.

Additional Facility Information (if needed):

For security purposes, the laboratories are in a secure laboratory wing with access limited to authorized personnel. On laboratory 132, warning signs are posted which read – "Biohazard, No Eating, Smoking, or Drinking. This is a restricted area". Emergency contact information with phone numbers are posted on the doors.

SAFETY PROCEDURES:

Please indicate which of the following personal protective equipment (PPE) will be used to minimize the exposure of laboratory personnel during all procedures that require handling or manipulation of registered biological materials.

Gloves:

☐ Latex ☐ Vinyl

FORM 1 – GENERAL INFORMATION (Continued)

☑ Nitrile
☐ Leather
☐ Other
Specify: Click here to enter text.

Face & Eye Protection:

☐ Face Shield
☐ Safety Goggles
☑ Safety Glasses
☐ Other
Specify: Click here to enter text.

Clothing Protection:

☑ Re-usable Lab Coat
☐ Re-usable Coverall
☐ Disposable Clothing Protection
☐ Other
Specify: Click here to enter text.

Dirty or contaminated protective clothing cleaning procedures: (Check all that apply)

☐ Autoclaved prior to laundering or disposal
☑ Laundered on site using bleach
☐ Laundered by qualified commercial service
☐ Other
Specify: Click here to enter text.

Outline procedures for routine decontamination of work surfaces, instruments, equipment, glassware and liquid containing infectious materials. Autoclaving or using fresh 10% bleach as a chemical disinfectant are preferred treatments; please specify and justify any exceptions:

Work surfaces will be decontaminated with a freshly prepared 10% bleach solution before and after working. Exception is biosafety cabinets which will be disinfected before and after use with Lysol® No Rinse Sanitizer in order to avoid the corrosiveness of the bleach on the metal of the biosafety cabinets. Instruments and equipment will be decontaminated by wiping down with 10% bleach. Paper towels used for these purposes will be discarded in biohazard bags. Glassware, waste, and disposable tubes will be autoclaved under standard conditions (15 psi, 121 C, 20 min). Disposable items (pipette tips, pipets, etc) will be discarded into 10% bleach. After 30 minutes it will be permissible to place these items in a biohazard bag for autoclaving before disposal.

Describe waste disposal methods to be employed for all biological and recombinant materials. Include methods for the following types of waste: (ref: UofA BiosafetyManual)

Sharps:

Placed into 10% bleach solution for decontamination followed by discarding into sharps waste container.

Cultures, Stocks and Disposable Labware:
Placed into biohazard bags and autoclaved before disposal. Liquids will be disposed of in drains after autoclaving. Disposable glass will be placed in glass disposal after autoclaving.

Pathological Waste:

FORM 1 – GENERAL INFORMATION (Continued)

Liquid biological waste will always be discarded into freshly made 10% bleach and then autoclaved for decontamination treatment before it is discarded. Other biological waste will be placed carefully into biohazard waste bags, autoclaved at 15 psi, 1210C for 20 min.

Other:

Click here to enter text.

Autoclave(s), to be used in this project, location(s) and validation procedures:

Biomass Res. Ctr. Room 101: Autoclaves are checked monthly using SteriGage test strips (3M) and SporAmpule vials to ensure autoclaves completely sterilize all bacterial life forms including spores.

Will biological safety cabinet(s) be used?

Yes

If yes, please provide the following information:

Make/Model	Serial Number	Certification Expiration	Location (bldg/room)
Biosafety Cabinet Level II FormaScientific Model 1126	12118-128	11-17	BIOR Room 132
Biosafety Cabinet Level II Forma Scientific Model 1284	21160-1813	2-18	BIOR Room 132
Labconco Logic +	170238664B	6-18	BIOR Room 132

Additional Biological Safety Cabinet Information (if needed):

Click here to enter text.

Indicate if any of the following aerosol-producing procedures will occur: (check all that apply)

- ☑ Centrifuging
- ☐ Blending
- ☑ Sonic Disruption
- ☐ Dissection
- ☐ Stomacher
- ☐ Other
- ☐ Grinding
- ☑ Vigorous Shaking or Mixing
- ☑ Pipetting
- ☐ Innoculating Animals Intranasally

Describe: Click here to enter text.

Describe the procedures/equipment that will be used to prevent personnel exposure during aerosol-producing procedures:

All pipetting of infectious material will take place in the biological safety cabinet. Mechanical pipetting devices will be used. Lab coats buttoned over street clothes, gloves and goggles will be worn. All materials needed will be placed in the biological safety cabinet before work begins. Sash of the cabinet

will be lowered and all movements will be slow to avoid disruption of the air currents. Centrifuged cultures will be contained in a closed Eppendorf tube or contained in screw-capped polypropylene or polystyrene tubes with gasket seals to prevent aerosol exposure. Cultures to be vortexed will be contained in screw-capped polypropylene or polystyrene tubes, and vortexing will be done within the biological safety cabinets. Sonicating will be done within the biosafety cabinet or within an enclosure on the bench top.

EMERGENCY PROCEDURES:

In the event of personnel exposure (e.g. mucous membrane exposure or parenteral inoculation), describe what steps will be taken including treatment, notification of proper supervisory and administrative officials, and medical follow up evaluation or treatment:

In the event of accidental exposure of personnel the person exposed should notify the laboratory supervisor immediately. Treatable exposures will be treated by use of the first aid kit containing antimicrobial agents. Mucous membrane exposure or puncture with contaminated material will result in the person being taken to the Health Center for prophylactic antibiotic therapy.

In the event of environmental contamination, describe what steps will be taken including a spill response plan incorporating necessary personal protective equipment (PPE) and decontamination procedures.

For a spill inside the biological safety cabinet, alert nearby people and inform laboratory supervisor. Safety goggles, lab coat buttoned over street clothes and latex gloves should be worn during clean up. If there are any sharps they will be picked up with tongs, and the spill covered with paper towels. Carefully pour disinfectant (freshly made 10% bleach) around the edges of the spill, then into the spill without splashing. Let sit for 20 minutes. Use more paper towels to wipe up the spill working inward from the edge. Clean the area with fresh paper towels soaked in disinfectant. Place all contaminated towels in a biohazard bag for autoclaving. Remove personal protective clothing and wash hands thoroughly.
For a spill in the centrifuge turn off motor, allow the machine to be at rest for 30 minutes before opening. If breakage is discovered after the machine has stopped, re close the lid immediately and allow the unit to be at rest for 30 minutes. Unplug centrifuge before initiating clean up. Wear strong, thick rubber gloves and other personal protective equipment (PPE) before proceeding with clean up. Flood centrifuge bowl with disinfectant. Place paper towels soaked in a disinfectant over the entire spill area. Allow 20 minute contact time. Use forceps to remove broken tubes and fragments. Place them in a sharps container for autoclaving and disposal as infectious waste. Remove buckets, trunnions and rotor and place in disinfectant for 24 hours or autoclave. Unbroken, capped tubes may be placed in disinfectant and recovered after 20 minute contact time or autoclaved. Use mechanical means to remove remaining disinfectant soaked materials from centrifuge bowl and discard as infectious waste. Place paper towels soaked in a disinfectant in the centrifuge bowl and allow it to soak overnight, wipe down again with disinfectant, wash with water and dry. Discard disinfectant soaked materials as infectious waste. Remove protective clothing used during cleanup and place in a biohazard bag for autoclaving. Wash hands whenever gloves are removed. For a spill outside the biological safety cabinet or centrifuge have all laboratory personnel evacuate. Close the doors and use clean up procedures as above.

FORM 1 – GENERAL INFORMATION (Continued)

TRANSPORTATION/SHIPMENT OF BIOLOGICAL MATERIALS:

Transportation of Biological Materials: The Department of Transportation regulates some biological materials as hazardous materials; see 49 CFR Parts 171 - 173. Transporting any of these regulated materials requires special training for all personnel who will be involved in the shipping process (packaging, labeling, loading, transporting or preparing/signing shipping documents).

Will you be involved in transporting or shipping human or animal pathogens off campus?
No

If yes, complete the remaining:

- ☐ Cultures of Human or Animal Pathogens
- ☐ Environmenatl samples known or suspected to contain a human or anumal pathogen
- ☐ Human or animal material (including excreta, secreta, blood and its components, tissue, tissue fluids, or cell lines) containing or suspected of containing a human or animal pathogen.

Transportation/Shipment Training: Have any project personnel who will be involved in packaging, labeling, completing, or signing shipping documents received formal training to ship infectious substances or diagnostic specimens within the past 3 years?
Choose an item.

If yes, please provide the following information:

Name	Date Trained	Certified Shipping Trainer
Click here to enter text.	Click here to enter a date.	Click here to enter text.
Click here to enter text.	Click here to enter a date.	Click here to enter text.
Click here to enter text.	Click here to enter a date.	Click here to enter text.
Click here to enter text.	Click here to enter a date.	Click here to enter text.
Click here to enter text.	Click here to enter a date.	Click here to enter text.

IBC Approval Letter: 08034

UNIVERSITY OF ARKANSAS | Research & Economic Development
Research Compliance

August 4, 2017

MEMORANDUM

TO:	Dr. Steven Ricke
FROM:	Ines Pinto, Biosafety Committee Chair
RE:	Protocol Renewal
PROTOCOL #:	08034
PROTOCOL TITLE:	Real-time PCR detection and quantification of Salmonella virulence in poultry and feed
APPROVED PROJECT PERIOD:	Start Date June 30, 2008 Expiration Date June 29, 2020

The Institutional Biosafety Committee (IBC) has approved your request, dated June 23, 2017, to renew IBC # 08034, "Real-time PCR detection and quantification of *Salmonella* virulence in poultry and feed".

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

University of Arkansas Institutional Biosafety Committee
Registration for Research Projects

FORM 1 – GENERAL INFORMATION

08034 renewal IBC#:

Please check the boxes for each of the forms that are applicable to the research project you are registering. The General Information Form - FORM 1 (this form) MUST be completed on all submitted project registrations, regardless of the type of research.

- ☐ Recombinant DNA (EVEN IF IT IS EXEMPT from the NIH Guidelines.) (FORM 2)
- ☑ Pathogens (human/animal/plant) (FORM 3)
- ☐ Biotoxins (FORM 4)
- ☐ Human materials/nonhuman primate materials (FORM 5)
- ☐ Animals or animal tissues and any of the above categories; transgenic animals or tissues; wild vertebrates or tissues (FORM 6)
- ☐ Plants, plant tissues, or seed and any of the above categories; transgenic plants, plant tissues, or seeds (FORM 7)
- ☐ CDC regulated select agents (FORM 8)

To initiate the review process, you must attach and send all completed registration forms via email to ibc@uark.edu. All registration forms must be submitted electronically. To complete the registration, print page 1 of this form, PI sign, date, and mail to: Compliance Coordinator-IBC, 120 Ozark Hall, Fayetteville, AR 72701, or FAX it to 479-575-3846.

As Principal Investigator:

- ☑ I attest that the information in the registration is accurate and complete and I will submit changes to the Institutional Biosafety Committee (IBC) in a timely manner.
- ☑ I am familiar with and agree to abide by the current, applicable guidelines and regulations governing my research, including, but not limited to: the NIH Guidelines for Research Involving Recombinant DNA Molecules and the Biosafety in Microbiological and Biomedical Laboratories manual.
- ☑ I agree to accept responsibility for training all laboratory and animal care personnel involved in this research on potential biohazards, relevant biosafety practices, techniques, and emergency procedures.
- ☑ If applicable, I have carefully reviewed the NIH Guidelines and accept the responsibilities described therein for principal investigators (Section IV-B-7).
- ☑ I will submit a written report to the IBC and to the Office of Recombinant DNA Activities at NIH (if applicable) concerning: any research related accident, exposure incident, or release of rDNA materials to the environment; problems implementing biological and physical containment procedures; or violations of NIH Guidelines.
- ☑ I agree that no work will be initiated prior to project approval by the IBC.
- ☑ I will submit my annual progress report to the IBC in a timely fashion.

Principal Investigator Typed/Printed Name: Dr. Steven C. Ricke

Signature (PI): _____ **Date:** _____

FORM 1 – GENERAL INFORMATION (Continued)

CONTACT INFORMATION:

Principal Investigator:

Name: Steven C. Ricke
Department: Food Science
Title: Professor
Campus Address: FDSC E-27
Telephone: 479-575-4678
*After Hours Phone: 479-387-4433
Fax: 479-575-6936
E-Mail: sricke@uark.edu

Co-Principal Investigator:

Name: Peter Rubinelli
Department: Food Science
Title: Program Associate
Campus Address: BIOR 128
Telephone: 479-236-0562
*After Hours Phone: 479-236-0562
Fax: 479-575-6936
E-Mail: pmrubine@uark.edu

***Required if research is at Biosafety Level 2 or higher**

PROJECT INFORMATION:

Have you registered ANY project previously with the IBC? Choose an item.

Is this a new project or a renewal?

○ New Project ◉ Renewal

Project Title: Real-time PCR detection and quantification of Salmonella virulence in poultry and feed
Project Start Date: 7/1/2008
Project End Date: 6/30/2014
Granting Agency: Cobb-Vantress

Indicate the containment conditions you propose to use (check all that apply):

☑ Biosafety Level 1 Ref: 1 2	☐ Biosafety Level 1A Ref: 1 2	☐ Biosafety Level 1P Ref: 1 2
☑ Biosafety Level 2 Ref: 1 2	☐ Biosafety Level 2A Ref: 1 2	☐ Biosafety Level 2P Ref: 1 2
☐ Biosafety Level 3 Ref: 2	☐ Biosafety Level 3A Ref: 2	☐ Biosafety Level 3P Ref: 2

References:

1: Biosafety in Microbiological and Biomedical Laboratories (BMBL) 4th Edition
2: NIH Guidelines for Research Involving Recombinant DNA Molecules

FORM 1 – GENERAL INFORMATION (Continued)

3: University of Arkansas Biological Safety Manual

If you are working at Biosafety Level 2 or higher, has your laboratory received an onsite inspection by the Biosafety Officer or a member of the IBC?

◉ Yes ○ No

If yes, enter date if known: Click here to enter a date.
If no, schedule an inspection with the Biological Safety Officer.

Please provide the following information on the research project (DO NOT attach or insert entire grant proposals unless it is a Research Support & Sponsored Programs proposal).

Project Abstract:

The proposed research aims at reducing and preventing Salmonella colonization in poultry. Salmonella is a leading cause of foodborne bacterial diarrhea in the U.S. Poultry and poultry products are considered to be a major source of Salmonella infections in humans. Salmonella can colonize the gut of the chicken without causing any symptoms of disease. Infection of poultry breeder flocks with Salmonella is not tolerated and infected flocks are destroyed causing a large loss of profits. Poultry feed is considered to be a major source of Salmonella and therefore control of this initial contamination is crucial to preventing flock colonization. Processing of feed aims at eliminating Salmonella, but may not always be effective. Detection of Salmonella in feed may be hindered by inadequate sampling procedures, levels of Salmonella being too low to detect and / or inhibitors of PCR present in the feed.

Specific Aims:

1) To develop a nucleic-acid based PCR assay for the detection of Salmonella in feed to be used to determine if the feed process reduces or eliminates Salmonella. 2) Determine if processing of feed contaminated with Salmonella enhances virulence of Salmonella using a reverse transcriptase PCR assay and a bird model.

Relevant Materials and Methods (this information should be specific to the research project being registered and should highlight any procedures that involve biohazardous or recombinant materials):

Handling:a. Feed processing simulation experiments. A cryogenic vial containing a bacterial culture in glycerol will be removed from the freezer and placed in a biological safety cabinet. A loop full of the bacterial culture will be inoculated into the appropriate media and allowed to grow in the incubator (37C or 42C). After 24 to 48 hours, the cultures will be washed by centrifugation and resuspended in fresh broth or saline solution. Samples will be taken and enumerated on plates to determine the exact starting concentration. Feed components, such as corn and soy bean, will be soaked in Salmonella cultures to allow bacteria to attach and penetrate the foods. After incubation, feed components will be rinsed to remove any unattached cells with sterile PBS. Feed components then will be dried, by freezing or vacuuming. Feed components will be treated by heating to 70C for 2 minutes to simulate heat treatment in a feed mill processing. The feed components then will be suspended in an enrichment broth of Rappaport medium for 24 h at 37C. Serial diluted portions of the enriched samples will be inoculated onto Brilliant Green agar to determine the viability of Salmonella.b. Real-time and reverse transcriptase PCR assays. A cryogenic vial containing a bacterial culture in glycerol will be removed from the freezer and placed in a biological safety cabinet. A loop full of the bacterial culture will be inoculated into the appropriate media and allowed to grow in the incubator (37C or 42C). After 24 to 48 hours, the cultures will be washed by centrifugation and resuspended in fresh broth or saline solution. Salmonella

FORM 1 – GENERAL INFORMATION (Continued)

will be inoculated into feed and dried as described above. The feed will be processed under simulated conditions as described above. Feed will be sampled and samples will be used for DNA preparations utilized in real-time and reverse transcriptase PCR assays. A sample will also be taken and inoculated into pre-enrichment broth as described above to validate the real-time PCR assays. c. Preparation and challenge of Salmonella. A cryogenic vial containing a bacterial culture in glycerol will be removed from the freezer and placed in a biological safety cabinet. A loop full of the bacterial culture will be inoculated into the appropriate media and allowed to grow in the incubator (37C or 42C). After 24 to 48 hours, the cultures are washed by centrifugation and resuspended in fresh broth or saline solution. Samples will be taken and enumerated on plates to determine the exact starting concentration. Poultry will be inoculated with Salmonella by utilizing feed prepared as described above at the Arkansas Veterinary Farm and personnel are required to wear surgical gloves and laboratory coats or overalls during the procedure.d. Isolation of Salmonella from the tissues. Tissue samples will be collected at the University of Arkansas Veterinary farm and personnel are required to wear surgical gloves and laboratory coats or overalls. Tissues will be transported to the lab at POSC (L-311) for determination of pathogen content. Samples will be inoculated into the appropriate media and allowed to grow in an incubator (37 or 42, 48 hours) for enumeration. No bird challenges will be conducted prior to IACUC approval. Because Salmonella is a BSL-2 pathogen, all the same precautions taken in the laboratory will also be taken at the poultry health farm. These procedures will include:For security purposes, the birds will be housed in an isolator access limited to authorized personnel. Only personnel that have been trained and working on this experiment will be permitted to enter the isolator where birds are being housed. On door, warning signs will be posted which reads – "Biohazard, No Eating, Smoking, or Drinking. This is a restricted area". Emergency contact information with phone numbers will be posted on the doors. A list of biohazardous agents in use also will be posted on the door. In the isolator room, a list of emergency phone numbers will be posted which includes phone numbers for medical emergency, poison control center, chemical emergency, chemical/biological spill and the University Health Center.In the event of personnel exposure, depending on the nature of exposure, the lab personnel are trained to take simple measures such as washing using tap water, etc. to decontaminate first and then contact PI and office of Environmental Health and Safety and Pat Walker Health Center for further instruction and treatment. The PI, Pat Walker Health Center, Office of Environmental Health and Safety and Fire Department's contact information will be posted on the front door of the isolator room and by the telephone.

The information requested above can be entered directly or cut & pasted into the space provided, or can be provided as an attached word document. If you provide an attachment, please indicate "See Attached" and list the file name(s) in the space below:

Click here to enter text.

PERSONNEL QUALIFICATIONS & FACILITY INFORMATION:

List all personnel (including PI and Co-PI) to be involved in this project:

Name (First and Last) - Position (Title, academic degrees, certifications, and field of expertise)	Qualifications/Training/Relevant Experience (Describe previous work or training with biohazardous and/or recombinant DNA; include Biosafety Levels)
Example: Bob Biohazard - Associate Professor, PhD- Microbiology	14 yrs working with E. coli at BL1, Salmonella enterica at BL2, 8 yrs working with transgenic mice.
Dr. Steven Ricke	20+ years of experience working as a PI, running research laboratory, and working with BSL-2 pathogens
Peter Rubinelli, Ph.D., cell biology	20 years working with BSL2 organisms
Sun Ae Kim, Ph.D., food science	10 years working with BSL2 organisms

FORM 1 – GENERAL INFORMATION (Continued)

Morgan Wright, M.S., food science	5 years working with BSL2 organisms
Zhaohao Shi, B.S., biology	2 years working with BSL2 organisms
Pravin Khaldone, M.S., biology	3 years working with BSL2 organisms
Laura Meyer, MS, Animal Science Program Technician	1 year experience with BSL2 organisms
Click here to enter text.	Click here to enter text.

Additional Personnel Information (if needed):

Click here to enter text.

List all the laboratories/facilities where research is to be conducted:

Building:	Room #:	Category:	*Signage Correct?
Biomass	132	**Laboratory**	Yes
Biomass	101	Autoclave/BioStorage	Yes
Biomass	102	**Laboratory**	**Yes**
Poultry Vet Farm	Determined by Vet. Farm	Animal Care	Yes
		Choose an item.	Choose an item.
		Choose an item.	Choose an item.
		Choose an item.	Choose an item.
		Choose an item.	Choose an item.

*** Biohazard signs are required for entrances to Biosafety Level 2 (including Animal Biosafety Level 2) areas. EH&S will supply these signs. If an updated biohazard sign is required, please indicate the location and what agents/organisms/hazards should be listed on the sign:**

Click here to enter text.

Additional Facility Information (if needed):

Click here to enter text.

SAFETY PROCEDURES:

Please indicate which of the following personal protective equipment (PPE) will be used to minimize the exposure of laboratory personnel during all procedures that require handling or manipulation of registered biological materials.

Gloves:

☐ Latex
☑ Nitrile
☐ Other
☐ Vinyl
☐ Leather
Specify: Click here to enter text.

Face & Eye Protection:

FORM 1 – GENERAL INFORMATION (Continued)

☐ Face Shield ☐ Safety Goggles

☑ Safety Glasses

☐ Other Specify: Click here to enter text.

Clothing Protection:

☑ Re-usable Lab Coat ☐ Re-usable Coverall

☐ Disposable Clothing Protection

☐ Other Specify: Click here to enter text.

Dirty or contaminated protective clothing cleaning procedures: (Check all that apply)

☐ Autoclaved prior to laundering or disposal ☑ Laundered on site using bleach

☐ Laundered by qualified commercial service

☐ Other Specify: Click here to enter text.

Outline procedures for routine decontamination of work surfaces, instruments, equipment, glassware and liquid containing infectious materials. Autoclaving or using fresh 10% bleach as a chemical disinfectant are preferred treatments; please specify and justify any exceptions:

Work surfaces will be decontaminated with a freshly prepared 10% bleach solution before and after working. Exception is biosafety cabinets which will be disinfected before and after use with Lysol® No Rinse Sanitizer in order to avoid the corrosiveness of the bleach on the metal of the biosafety cabinets. Instruments and equipment will be decontaminated by wiping down with 10% bleach. Paper towels used for these purposes will be discarded in biohazard bags. Glassware, waste, and disposable tubes will be autoclaved under standard conditions (15 psi, 121 C, 20 min). Disposable items (pipette tips, pipets, etc) will be discarded into 10% bleach. After 30 minutes it will be permissible to place these items in a biohazard bag for autoclaving before disposal.

Describe waste disposal methods to be employed for all biological and recombinant materials. Include methods for the following types of waste: (ref: UofA BiosafetyManual)

Sharps:

Placed into 10% bleach solution for decontamination followed by discarding into sharps waste container.

Cultures, Stocks and Disposable Labware:
Placed into biohazard bags and autoclaved before disposal. Liquids will be disposed of in drains after autoclaving. Disposable glass will be placed in glass disposal after autoclaving.

Pathological Waste:

Liquid biological waste will always be discarded into freshly made 10% bleach and then autoclaved for decontamination treatment before it is discarded. Other biological waste will be placed carefully into biohazard waste bags, autoclaved at 15 psi, 1210C for 20 min.

Other:

Click here to enter text.

FORM 1 – GENERAL INFORMATION (Continued)

Autoclave(s), to be used in this project, location(s) and validation procedures:

Autoclaves are located in L-344 and BIOR 101. All the materials and disposables contaminated with the pathogens will be either 1) disposed into biohazard bags (procedures conducted in L311) or 2) burned in the farm incinerator (performed at the farm). The glassware and containers as well as the biohazard bags will be autoclaved at 121C and 15psi for 15 to 45 min in the autoclave in POSC L-344. Autoclaved dishware is washed with detergent for future use. For biohazard bags, the autoclave tapes will be checked after autoclave to ensure sterilization. The bags then will be placed in an ordinary trash bag for disposal.Autoclaves conditions are also validated using a sterilization integrator (VWR catalog # 34010-019).

Will biological safety cabinet(s) be used?

Yes

If yes, please provide the following information:

Make/Model	Serial Number	Certification Expiration	Location (bldg/room)
Biosafety Cabinet Level II FormaScientific Model 1126	12118-128	11-17	BIOR Room 132
Biosafety Cabinet Level II Forma Scientific Model 1284	21160-1813	2-18	BIOR Room 132
Labconco Logic +	170238664B	6-18	BIOR Room 132

Additional Biological Safety Cabinet Information (if needed):

Click here to enter text.

Indicate if any of the following aerosol-producing procedures will occur: (check all that apply)

- ☑ Centrifuging
- ☐ Blending
- ☐ Sonic Disruption
- ☐ Dissection
- ☐ Stomacher
- ☐ Other
- ☐ Grinding
- ☐ Vigorous Shaking or Mixing
- ☑ Pipetting
- ☐ Innoculating Animals Intranasally

Describe: Click here to enter text.

Describe the procedures/equipment that will be used to prevent personnel exposure during aerosol-producing procedures:

FORM 1 – GENERAL INFORMATION (Continued)

All personnel are required to wear surgical gloves and laboratory coats or overalls during procedures involving infectious the agent. Any procedure involving pipetting will be done under a biosafety cabinet to prevent personnel exposure to aerosols. All centrifuges are contained units to prevent exposure to aerosols.

EMERGENCY PROCEDURES:

In the event of personnel exposure (e.g. mucous membrane exposure or parenteral inoculation), describe what steps will be taken including treatment, notification of proper supervisory and administrative officials, and medical follow up evaluation or treatment:

In the event of exposure, the affected area will be rinsed or washed thoroughly (eyes, nose, mouth or skin abrasion). The PI will immediately be informed. The individual will be encouraged to consult with the physicians in the University of Arkansas Center for any symptoms related to the diseases that could be caused by the agent. In the event of personnel exposure, depending on the nature of exposure, the lab personnel are trained to take simple measures such as washing using tap water, etc. to decontaminate first and then contact PI and office of Environmental Health and Safety and Pat Walker Health Center for further instruction and treatment. The PI, Pat Walker Health Center, Office of Environmental Health and Safety and Fire Department's contact information is posted on the front door of L-311 and by the telephone.A list of Emergency phone numbers is posted in the laboratory (L-311, BIOR 132). This includes the phone numbers for medical emergency, poison control center, chemical emergency, chemical/biological spill and the University of Arkansas Health Center.

In the event of environmental contamination, describe what steps will be taken including a spill response plan incorporating necessary personal protective equipment (PPE) and decontamination procedures.

In case of spills, the responsible researcher will wear gloves and cover the spill area with paper towels (small spills) or chemical sorbent pads and soak in disinfectant for 5 minutes. The materials will be discarded into the biohazard bag. Material Safety Data Sheets are located in L-311 and BIOR 132 for reference. In addition, a first aid kit, biohazardous spill kit, and chemical spill kit are located in L-311 and BIOR 132. A spill kit for large chemical spills is located at the end of the hall inside the lab wing and in BIOR 132. A list of Emergency phone numbers is posted in the laboratory (L-311, BIOR 132). This includes the phone numbers for medical emergency, poison control center, chemical emergency, chemical/biological spill and the University of Arkansas Health Center. All researchers handling the infectious agent are encouraged to consult a physician in the University Health Center for any symptoms related to the disease that could be caused by the agent.

TRANSPORTATION/SHIPMENT OF BIOLOGICAL MATERIALS:

Transportation of Biological Materials: The Department of Transportation regulates some biological materials as hazardous materials; see 49 CFR Parts 171 - 173. Transporting any of these regulated materials requires special training for all personnel who will be involved in the

FORM 1 – GENERAL INFORMATION (Continued)

shipping process (packaging, labeling, loading, transporting or preparing/signing shipping documents).

Will you be involved in transporting or shipping human or animal pathogens off campus?

No

If yes, complete the remaining:

- ☐ Cultures of Human or Animal Pathogens
- ☐ Environmenatl samples known or suspected to contain a human or anumal pathogen
- ☐ Human or animal material (including excreta, secreta, blood and its components, tissue, tissue fluids, or cell lines) containing or suspected of containing a human or animal pathogen.

Transportation/Shipment Training: Have any project personnel who will be involved in packaging, labeling, completing, or signing shipping documents received formal training to ship infectious substances or diagnostic specimens within the past 3 years?

Choose an item.

If yes, please provide the following information:

Name	Date Trained	Certified Shipping Trainer
Click here to enter text.	Click here to enter a date.	Click here to enter text.
Click here to enter text.	Click here to enter a date.	Click here to enter text.
Click here to enter text.	Click here to enter a date.	Click here to enter text.
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