Potential Enhanced Stress Tolerance Of Outbreak-Associated Salmonella Enterica From Multiple U.s. Outbreaks

POTENTIAL ENHANCED STRESS TOLERANCE OF OUTBREAK-ASSOCIATED *SALMONELLA ENTERICA* FROM MULTIPLE U.S. OUTBREAKS

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ABSTRACT

Salmonella enterica is a widespread species of bacteria that consists of over 2,600 different serovars, with fewer than 100 serovars accounting for most human salmonellosis cases globally. Serovars are classified by their surface antigens and are commonly grouped into two groups – serovars that cause typhoid fever are known as typhoidal *Salmonella enterica*, and serovars that cause salmonellosis are non-typhoidal *S. enterica*. The CDC estimates that non-typhoidal *S. enterica* (NTS) causes 1.35 million illnesses annually in the United States, with 26,500 hospitalizations and 420 deaths. Of these illnesses, most are outbreak-associated and can be traced back to a common source.

The objective of this work is to determine if outbreak-associated strains of *S. enterica* display enhanced stress tolerance compared to *S. enterica* strains that have not been involved in an outbreak. *S. enterica* serovars Enteritidis, Heidelberg, Typhimurium, Newport, and I 4,5,[12]:i:- (monophasic Typhimurium) outbreak-associated (OA) and non-outbreak-associated (NOA) strains were evaluated for enhanced stress tolerance. To determine the attachment capacity of these strains, 21 OA and 4 NOA isolates were grown statically in 96-well polystyrene plates for 24, 72, and 120 hours in 1X (nutrient-rich) and 1/20X (nutrient-poor) trypticase soy broth (TSB) conditions, at 22°C and 4°C. Sanitizer minimum inhibitory concentration (MIC) was determined for 23 OA and 6 NOA isolates through static incubation of cultures in 1X and 1/20X trypticase soy broth (TSB), with serial dilutions of Chlorox (bleach) and Inspexx-250 (PAA) at 22°C for 24 hours using OD_{600}. Heat tolerance of 9 OA isolates was evaluated; aliquots were taken at the start of stationary phase and serially diluted into phosphate-buffered saline, and then incubated at 56°C and pour-plated at 0-, 3-, 6-, 9-, 15-, 30-, 45-, and 60-minutes post-heat-shock. Plates were incubated at 37°C for 36 hours, after which colony counts were recorded. Statistically significant differences between isolates for all assays were determined via analysis of variance (ANOVA) with Tukey's HSD test, with significance defined at $p_{adj} < 0.05$.

In 1/20X TSB at 4°C, three *Salmonella* Heidelberg OA, two monophasic OA, and one NOA monophasic isolate attached significantly better than ≥3 others. At 22°C, five Heidelberg OA, two monophasic OA, and one NOA monophasic isolate attached significantly better than ≥3 others. In 1X TSB at 4°C and 22°C, only one OA Heidelberg attached significantly better than ≥3 others. MICs for bleach, 1X TSB were >200ppm and >100ppm in 1/20X TSB ($p_{adj} < 0.01$). MICs for PAA, 1X TSB were >175ppm vs 25.9ppm in 1/20X TSB ($p_{adj} < 0.01$). After the initial period of 6 minutes, all isolates showed a significant decrease in growth ($p_{adj} < 0.05$). At 45 minutes post-scald, 3 isolates plated were below detectable limits (less than 1 CFU/mL), followed by 5 isolates at 60 minutes post-scald. One isolate was still detectable at 60 minutes with a count of $3.43*10^3$ CFU/mL. Findings suggest that there is no significant difference in attachment capacity and MICs between the outbreak-associated and non-outbreak-associated isolates. The sanitizer tolerance and heat tolerance observed in several of the strains are high and could pose a threat to the food processing industry.

DEDICATION

This thesis is dedicated to my cat Chickadee, whom I love very much. Thank you for being my moral support during my master’s degree.

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Salmonella enterica

Salmonella enterica is a widespread species of bacteria that consists of over 2,600 different serovars. However, fewer than 100 of these serovars account for most human salmonellosis cases globally, and *Salmonella enterica* is commonly classified into 2 major groups – typhoidal *S. enterica* and non-typhoidal *S. enterica* (NTS) (1, 2). Typhoidal *enterica* is transmitted only human-to-human and can cause an enteric fever known more commonly as typhoid fever. This illness is invasive, leading to systemic blood infections, and is difficult to treat even with antibiotics (3). Non-typhoidal *S. enterica* (NTS) is typically transmitted animal-to-human or food-to-human and accounts for most human salmonellosis cases *(4)*. Non-typhoidal *S. enterica* is most commonly found in food products that have been sourced from animals, such as poultry, pork, or other meat products, food processed in facilities with contaminated products, or in crops that have been exposed to contaminated water or animal feces (5).

Salmonellosis, an infection caused by the presence of non-typhoidal *S. enterica* in the human body, is the second most commonly reported gastrointestinal infection in humans and contributes to many food-borne outbreaks globally (6, 7). The World Health Organization (WHO) notes that NTS is one of the four top contributors to diarrheal illness worldwide. Notably, diarrheal diseases are the leading cause of foodborne disease burden in most regions of the world and the 40^{th} leading cause of child mortality (7, 8). The World Health Organization estimates a median number of 78,707,591 NTS salmonellosis cases occur annually globally, with a median estimate of 59,153 deaths occurring from infection by this pathogen (7). In the United States alone, the CDC estimates that there are 1.35 million infections annually, with 26,500 hospitalizations and 420 deaths resulting from salmonellosis (5). Because of this, non-typhoidal *S. enterica* is also responsible for a massive economic burden annually. Labor loss due to illness, death and funerary costs, and medical bills all factor into the economic burden of this pathogen in the United States, while the WHO also counts food exports lost and contamination in their estimates (5, 9, 10). The WHO notes that for people with salmonellosis, the years lived with disability (YLD), years of life lost (YLL), and disability-adjusted life year (DALY) counts are quite high, at an estimated 78,306 YLD, 3,976,386 YLLs and 4,067,929 DALYs (7). The burden of foodborne pathogens, such as *S. enterica*, in less developed countries, has affected the tourism, agricultural, and food exportation industries, and this, in conjunction with poor record keeping and infrastructure makes it difficult for the WHO to estimate the financial loss in terms of money as a result of this pathogen (7, 8). In the United States, there is an estimated $4.142 billion loss annually as a result of Salmonellosis infections, which as noted above does not include the burden of recalls and the disposal of contaminated food products (10-12). Due to the high economic burden incurred by this pathogen, the United States and many other countries are focused on developing new strategies to reduce the number of foodborne outbreaks annually (5).

S. enterica **Serovars**

Serovars, or serological variants, are groups within a bacterial species which are distinguished by their characteristic surface structures, known as antigens (13). Non-typhoidal *S. enterica* can be classified by serovar using two antigens found on the bacteria; the O antigen, which is on the surface of the outer membrane, and the H antigen, which is a part of the flagella found on *S. enterica* and is a thin, threadlike structure (13). The O (somatic) antigens are determined in serotyping by specific sugar sequences on the outer membrane. H (flagellar) antigens are commonly monophasic (meaning one phase, whether the bacteria display either phase 1 or phase 2), biphasic, where they can change from one phase to the other as desired (phase 1 and phase 2), or non-motile, if they are missing both flagellar phases (14, 15). Once the O and H antigens are determined, the antigenic formula of the *S. enterica* can be used to classify the serovar (14). Some serovars of *S. enterica* are more prevalent than others, with a few in particular standing out in the food industry (16).

In the food industry, *Salmonella* Enteritidis is considered the predominant serovar of non-typhoidal *S. enterica* in the U.S. *Salmonella* Enteritidis also accounts for an estimated 32% of foodborne outbreaks in the United States, and it is most commonly found in poultry and eggs (17-19). *Salmonella* Enteritidis, in conjunction with *Salmonella* Typhimurium, also accounts for 50% of all human NTS serovars reported globally, and 33.1% and 65.2% of all the serovars, respectively, found in serotyped cases of gastroenteritis or salmonellosis (17, 18, 20). *Salmonella* Typhimurium is the second most common serovar of NTS in the U.S. and is often associated with poultry, pork, ground beef, and chicken product outbreaks (17, 19, 21). The third most common NTS serovar noted in the U.S. processing industry is *Salmonella* Newport (21). *Salmonella* Newport, which is more common in produce, is often found colonizing cantaloupe and alfalfa sprouts, as well as poultry and turkey products on occasion (19, 21). Other common serovars include *Salmonella* Javiana, which contaminates and colonizes watermelon, bass, mozzarella, lettuce, and tomatoes; and *Salmonella* Heidelberg, which is often antibiotic resistant, and much like *Salmonella* Enteritidis, is most commonly associated with poultry and egg products (18, 19, 21, 22). *Salmonella* I 4,5,[12]:i:- (also known as monophasic Typhimurium) is an emerging serovar in the U.S. and has been on the rise since 2015, especially in conjunction with the rise of antimicrobial resistance (23).

Foodborne Outbreaks

The Centers for Disease Control and Prevention (CDC) in the United States considers a foodborne outbreak to be an incident in which two or more people experience a similar illness or disease resulting from the ingestion of a common food or drink contaminated with the same type of disease agent, such as viruses, parasites, or bacteria (24). When reporting outbreaks, it is very important to confirm that the outbreak is not an isolated incident and that bacterial strains don't get clumped together under the umbrella of the same outbreak if they are in fact from distinct outbreaks or sporadic cases. Thus, the CDC has posted guidelines for confirmation of outbreaks; to consider an illness as part of an outbreak, the bacteria or other organism causing the illness must be isolated from two or more ill people clinically, and the strains must match (24). The organism must then also be isolated from the epidemiologically-implicated food determined to be the source through interviews with the ill people and microbiological confirmation (24). Every year in the United States there are many foodborne outbreaks from many different bacteria and viruses, including *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter jejuni*, and Norovirus, alongside the outbreaks associated with *S. enterica* (25). These outbreaks can lead to changes in food protection policies and the implementation of new processing strategies.

Relevant Outbreaks of *S. enterica*

There have been many important outbreaks over the last 20 years resulting from the presence of non-typhoidal *S. enterica* as a contaminant in food. From 2003-2004, there was a minor outbreak of antimicrobial-resistant *Salmonella* Enteritidis in raw almonds distributed by Paramount Farms (26). This outbreak resulted in at least 29 infections and 7 hospitalizations, and patients ranged across 12 states and Canada (26). A recall was issued for approximately 18 million pounds of raw almonds, and further investigation into the company revealed that two huller-sheller machines tested positive for the outbreak strain (26). The company lacked an adequate sanitation step at the time of the outbreak to control the presence of potential pathogens on the machinery (26), but following the outbreak in August 2004, the Almond Board of California implemented a voluntary program to ensure that raw almonds are treated with propylene oxide, chlorine dioxide, or heat treatments to reduce microbial contamination by a 5-log pathogen reduction (27). Once treated, these almonds could be labeled as pasteurized for sale, and in 2007 the program was formally adopted and implemented by the USDA as a standard for the processing of raw almonds (28).

In 2008-2009, the Peanut Corporation of America (PCA) was responsible for a massive multistate outbreak of antimicrobial-resistant *Salmonella* Typhimurium in peanut butter products (29). This outbreak caused 714 infections, approximately 173 hospitalizations, and 9 deaths of consumers across 46 states and Canada (29). Traceback investigations found the *Salmonella* Typhimurium strains present in two peanut-processing facilities of the nine owned by the PCA (30). The FDA investigation noted that rodents were present in the facilities, that one of the facilities had a leaky roof which leaked onto the products, and that unprocessed raw peanuts were stored next to roasted peanuts (31). As a result of this outbreak, the PCA declared bankruptcy, while the president of the company and the food broker for the company, brothers Stewart and Mitchell Parnell, were sued and convicted of multiple counts of conspiracy, the sale of misbranded food products, and mail and wire fraud, as they had knowingly sold consumers contaminated products, fabricated certificates of analysis and covered up lab records indicating the presence of *Salmonella enterica* in the majority of their products (30, 32, 33).

Another outbreak of note is the 2011-2012 outbreak of antimicrobial-resistant *Salmonella* Heidelberg in Kosher broiled chicken liver produced by Schreiber Processing Corporation, which caused 190 illnesses and 30 hospitalizations (34). This outbreak was spread across 6 states with the highest percentage of illnesses in New York (109 cases) and New Jersey (62 cases) (34). The wording of "broiled" in the product led consumers and retailers to believe that the product was ready-to-eat, and the product did appear fully cooked (34, 35). However, the product had not been cooked to an internal temperature of 160°F prior to sale, and this was stated in the labeling, which indicated that the product required further thermal processing prior to consumption (35). This misunderstanding contributed to the high case count observed in this outbreak (34, 36). A recall for the contaminated Kosher broiled chicken liver products was promptly issued on November 8, 2011, with the company promptly discontinuing the production of the broiled products, and the outbreak was declared closed on January 11, 2012 (34, 35).

The Foster Farms poultry outbreak in 2013-2014 was an outbreak of several multidrug-resistant strains of *Salmonella* Heidelberg which infected a confirmed 634 people and hospitalized 200 people (37). The USDA Food Safety and Inspection Service found that Foster Farms poultry parts had a positivity rate of 25% during the outbreak, and that the three implicated facilities where the contaminated poultry was being processed were highly unsanitary (37); the carcasses being processed in these facilities were found to have fecal content present on the exterior during processing (37, 38). This outbreak was multi-state, spread across 29 states and Puerto Rico, and led to the implementation of a $75 million food safety program by Foster Farms that reduced naturally occurring *S. enterica* in each stage of the production process, and the eventual recall of over 1 million pounds of contaminated poultry products (37-39).

Finally, in 2015, there was an outbreak of multidrug-resistant *Salmonella* I 4,5,[12]:i:- and *Salmonella* Infantis in roasted pork products produced by Kapowsin Meats that caused 192 infections and 30 hospitalizations (40). This outbreak was spread across 5 states, but centered primarily in Washington, with 184 of the ill consumers residing there (40). Kapowsin Meats issued a recall on August 13, 2015, for 116,262 pounds of whole pigs, and then expanded their recall in mid-August, 2015 to include over 523,380 additional pounds of contaminated pork products (40, 41). The facility where the whole hogs were processed was deemed unsanitary by the USDA-FSIS during traceback investigations, and corrective actions performed by Kapowsin Meats were ultimately deemed insufficient, leading to the shutdown of the processing facility (41).

The outcomes for the consumers involved in each of these outbreaks varied, due to the handling of the outbreaks by the companies, the serovars involved in the outbreaks, the demographics of the consumers, the responsibility of the companies involved, and the rate at which the recalls were issued (30, 38). The Paramount Farms Almond outbreak, the Peanut Corporation of America outbreak and the Foster Farms outbreak were pivotal in the processing industry for the implementation of new regulations and policies to prevent the contamination of food products by *S. enterica* (28, 30, 42). However, outbreaks of *S. enterica* continue to occur; outbreak strains often survive the processing industry to cause illness in consumers, and they have a variety of mechanisms that they employ to colonize and persist in these environments, including the formation of biofilms, tolerance to biocides, antimicrobials, and a variety of other stresses they may encounter in the industry.

Biofilm Formation

Bacteria often form biofilms as a way to colonize the environment. Biofilms are a sessile community of cells that form an extracellular polymeric matrix composed of secreted extracellular polymeric substances (EPS) to cling to a surface (43). The EPS is typically composed of water, extracellular DNA, polysaccharides, and proteins (44). Interactions between these components and the cells in biofilms can prevent the desiccation of cells present in the matrix, prevent cell death by bacteriophages and other predatory microorganisms, aid the adherence of the cells to a surface, and aid in the degradation of chemicals and sanitizers exposed to the matrix (44). Due to the nature of the extracellular polymeric matrix, biofilms may form anywhere there is an appropriate surface to adhere to and sufficient moisture and nutrients.

Biofilms can be found in nature; *Aciditerrimonas* spp., *Bacillus* spp., *Acidithiobacillus* spp., *Lysinibacillus* spp., and *Vibrio* spp. have been found to colonize hydrothermal environments, such as hot springs and deep-sea vents (45). Many species of bacteria are known to form aquatic biofilms on river and stream sediment and rocks, and multiple species of marine bacteria, including *Alcanivorax borkumensis*, have even been known to form biofilms on oil spills in the ocean (46). In agricultural environments, biofilms can be found in soil or colonizing fruits and vegetables, such as *Escherichia coli* establishing itself inside the calyx of an apple, or *S. enterica* attaching to the rind surface of a cantaloupe (47). They can also be found in our bodies; for example, the plaque found on teeth is a biofilm (43).

In the food industry, biofilms can be observed in areas with high concentrations of moisture and nutrients, such as floors or walls, drains, pipes, undersides of processing equipment, and surfaces where food is processed (48, 49). Manufacturing environments have surfaces that are typically composed of stainless steel, rubber, polypropylene, glass, and plastic, or surfaces which are a combination of these components, all of which can make an ideal attachment surface for bacteria to adhere to (43, 47).

Generally, *in vitro* formation of biofilms occurs in five stages. The first stage is the reversible attachment of the cells to an abiotic surface, where the cells have not yet formed layers or begun secreting the extracellular polymeric substances (50). The secretion of the EPS mediates the start of the irreversible attachment of these cells in the second stage of the biofilm formation (50). In the third stage, the biofilm begins to mature; the cells present stop replicating, and microcolonies or clusters within the EPS form (50). The fourth stage is identifiable by the complete formation of the 3-dimensional extracellular polymeric matrix, with the cells present in clusters and channels that run between the clusters transporting water, nutrients, and other waste products (50). At the fifth stage, the biofilm is fully established – cells detach from the matrix and dispose to other surfaces to begin the formation of a new biofilm, and the high cell concentration triggers a series of cell signaling mechanisms known as quorum sensing (47, 50-52).

Contribution of Biofilms to Persistence

Cells in established biofilms are hardier than those in liquid culture, due to the layering of the cells inside the biofilm. The density of the matrix also prevents killing or inhibition by biocides and antimicrobials, chemical stresses such as pH and oxygen changes, and physical stresses, including heat, freezing, desiccation, and pressure (47). The organization of the biofilm matrix into channels helps transport water, nutrients from the consumption of exopolysaccharides, and waste products throughout the biofilm (50). The proximity of the cells also permits the exchange of genetic material between cells, including plasmids and other mobile genetic elements (53). Cells can communicate with each other through a mechanism known as quorum sensing (47, 54). Bacteria in biofilms will produce small diffusible signaling molecules, known as autoinducers, which can be transported through the channels present in the EPS and that other cells in the community can detect and respond to (54, 55). These autoinducers have a wide array of functions; bacteria can use them to communicate across species in biofilm, to respond to environmental stimuli, or to regulate their gene expression (54-56). Gram-negative and Gram-positive bacterial autoinducers vary a little in their composition; gram-negative bacteria produce acylated homoserine lactone autoinducers that passively diffuse through their cell walls, while gram-positive bacteria produce oligopeptides that must be actively transported across their peptidoglycan cell wall using the ATP-binding cassette (ABC) transporter system (56). The extracellular accumulation of these autoinducers eventually reaches a threshold where the expense of autoinducers leaving the cells would be energetically unfavorable to the cells, and as such, this leads to the intracellular increase of autoinducer molecules (56). These autoinducers bind receptors in the cells, triggering a signaling cascade that alters transcription factor activity, leading to a change in the gene expression (56). Quorum sensing via autoinducers plays a very important role in the successful formation and maintenance of biofilms (47).

Certain cells present in biofilms can survive biocidal treatments; these are known as persister cells or tolerant cells (57). Tolerant cells are cells that have genetic elements that encode resistance to antibiotics and sanitizers; they may survive the effects of these treatments and continue their growth due to their genotype (58). Persister cells are cells that are dormant during the treatment, such as cells in the stationary phase of growth, and as such are only phenotypically tolerant to antibiotics and sanitizers; they will remain viable and repopulate the biofilm after exposure to the biocides ceases (58). The presence of tolerant cells in biofilms and their ability to transfer their tolerance genes to other bacteria via plasmids is a huge contributor to the difficulty of eradicating established biofilms (57, 59). Additionally, the matrix laid down by the previous cells may persist on a surface even after the complete eradication of cells within, providing a good structure for other bacteria to colonize (48).

Benefits of Monospecies Biofilm Models

Oftentimes, research studies will look at the effectiveness of sanitizers or other antimicrobials on a single-species biofilm. In a non-research environment, a biofilm will typically be composed of multiple species, since bacteria are ubiquitous, but mono-species biofilms can be beneficial to study *in vivo* (55, 60). Oftentimes, if a researcher wishes to study the biofilm-forming capabilities of a single bacterial species, the presence of multiple other bacterial species may mask the abilities of the species of interest (60). Since the ability of bacteria to form biofilms can vary widely even amongst species (1), it may be hard to detect the effect of certain genes in these bacteria, or the impact that deletions or knockouts in experimental settings may have on the species if other species are present. Even within the species of *S. enterica*, there is a high level of diversity amongst serovars, as some *S. enterica* serovars only colonize plants, while others colonize animals, and some are human pathogens (61). Specific genes involved in biofilm formation, antimicrobial resistance, sanitizer tolerance and stress tolerance may vary amongst different serovars of *S. enterica*, which is why it can be beneficial specifically in *S. enterica* studies to study biofilms from individual serovars (62).

Features of Multispecies Biofilms

In the food processing environment, it is highly unlikely that cells will be in a mono-species biofilm; there are many ways common bacterial species can make it onto the processing floor to establish or join pre-existing biofilms, and most non-pathogenic organisms are not tested for in food processing facilities(63). Additionally, for foodborne pathogens not considered to be an adulterant in some food products, such as

Campylobacter spp., and *Salmonella spp*., the presence of these bacteria in a certain amount of some food products may be permitted (64). These bacteria can enter the processing area and shed from food products onto surfaces where they may attach or join biofilms already established (63). Mixed species biofilms can be beneficial for bacteria, as they have been observed to display higher resistance to disinfectants, such as quaternary ammonium compounds (QACs), and other antimicrobials compared to single-species biofilms (49, 65). Dual-species biofilms containing *Escherichia coli* O45:H2 and O121:H19 and *Salmonella* Typhimurium have been observed to display higher tolerance to chlorine sanitizer than single-species biofilms of these organisms (66). Co-existence of these two pathogens in biofilms has been observed in the processing industry, and these biofilms have also been noted to display enhanced resistance to QACs, broad-spectrum disinfectants, and other sanitizers commonly used in the food industry as a disinfectant (67). The tolerance to sanitizers seen in these dual-species biofilms has also been seen in multi-species biofilms of *S. enterica* and other foodborne pathogens in the processing industry.

Biofilm Genes in *S. enterica*

There are a variety of genes that are important in bacteria for the successful formation of biofilms, including quorum sensing genes that allow communication between cells via the production of autoinducer molecules, motility genes that encode for flagellar genes, biosynthesis genes that encode for EPS components including sugars, and adhesion genes that aid in adherence to a surface and to other cells (54). Quite a few *S.*

enterica genes have been noted to play an important role in the successful formation of biofilms.

Curli proteins play a crucial role in biofilm formation; they are responsible for adhesion to surfaces and cellular aggregation and are encoded by the *csgDEFG* and *csgBAC* operons. The *csgDEFG* operon encodes proteins which are required for curli assembly, as well as *csgD,* which encodes a transcriptional regulator that promotes transcription of the *csgBAC* operon (68-70). Both *csgC* and *csgE* facilitate the synthesis of extracellular thin aggregative fimbriae in *Salmonella* Enteritidis (71). Another important component in *S. enterica* biofilms is cellulose, which, in conjunction with the production of curli, results in the formation of the hydrophobic matrix coating the cells. In order to synthesize cellulose, *bcsABZD* and *bcsEFG* operons are required (69, 72). The biosynthesis of cellulose is positively regulated by CsgD, the transcriptional regulator mentioned prior which stimulates the transcription of AdrA, which then activates cellulose production (69, 72). The expression of another protein, BapA, required by *Salmonella* Enteritidis for successful formation of biofilms and encoded by the *bapA* gene with secretion via the *bapBCD* operon, is also coordinated with the expression of genes encoding curli and cellulose through CsgD (73, 74).

Adhesins and flagella are another major component of successful biofilms. *S. enterica* possesses fimbriae adhesins that aid in the adherence of the cells to a surface, including *fimH*, a gene that encodes the protein subunit responsible for the adhesion of the structural subunit encoded through *fimA* (75, 76). There are a variety of other fimbriae genes in the type 1 fimbriae gene cluster which play more minor roles in the adhesion abilities of the subunit FimA (75), and different serovars of *S. enterica* have also been observed to possess other types of fimbrial genes which vary in their role in the formation of biofilms. SirA, a SPI-1 regulator of the genes *hilA* and *prgH*, regulates virulence but also regulates the *fim* operon that encodes type 1 fimbriae in *S. enterica* (77, 78). The expression of flagella genes in *S. enterica* has been found to be involved in the successful formation and attachment of biofilms on certain surfaces (79). One such flagellar gene which has been noted as important is *fliC*, which is responsible for flagellum formation and motility which aids in cellular movement in *S. enterica* (80). The gene *csrA*, which is a regulator of invasion genes, also affects the expression of motility genes necessary for the early formation of biofilms (78, 81).

Certain serovars of *S. enterica* rely on genes located on the Salmonella Pathogenicity Islands I and II. *RpoS* encodes a sigma factor which mediates the stationary-phase expression of genes involved in resistance to environmental stresses and also regulates the virulence genes of Salmonella; *rpoS* is vital for the transcriptional activation of the *csgD* promoter (82, 83). OmpR is another protein required for the successful transcription of *csgD*, with 6 binding sites for OmpR located in the *csgD* promoter regions. It is encoded in a two-gene operon that regulates virulence and porin production (84). *Salmonella* Enteritidis that lack *ompR* have the inability to create biofilms, and are unable to produce cellulose and curli since those are regulated by *csgD* (85).

Also of importance to proper biofilm formation are genes used in the formation of the EPS, as previously described. *WcaA* is a colonic acid biosynthesis gene observed that produces exopolysaccharide sugars for the biofilm during development; these are a major component of the EPS and provide food to bacteria colonizing it. The gene *rfbA*, encoding a lipopolysaccharide biosynthesis gene, has also been noted to play an important role in biofilm formation, as a *Salmonella* Typhimurium DT104 mutant displays reduced attachment on glass and stainless steel surfaces (79). Quorum sensing genes can also help regulate the formation of biofilms, as seen in the case of SdiA regulating *rck*. SdiA is a receptor that detects the presence of *N*-acylhomoserine lactones (AHLs) secreted by other Gram-negative species of bacteria, which aids the bacteria in quorum sensing; SdiA also regulates the *rck* gene, which mediates adhesion and bacterial biofilm formation (86, 87).

Even in the absence of some genes, *S. enterica* is still capable of forming biofilms if in a multi-species biofilm with another microorganism that produces the necessary proteins or contains copies of the necessary genes (88). A strain of *S. enterica* missing certain genes to form biofilms has been observed in biofilms with *E. Coli* to use the curli proteins secreted by *E. coli* to enhance its' adherence ability in the dual-species biofilm (88).

Biofilms in the Food Industry

Food processing facilities often contain many stainless steel surfaces; untreated surfaces or mechanically sanded steel surfaces make an ideal surface for *S. enterica* attachment and biofilm formation (89). Even when these surfaces are dry and lack a steady source of nutrients, *S. enterica* has been observed to persist on them for over a year, during which time it can shed cells onto food products (51, 90). Detection of this bacteria on processing surfaces may prove difficult because *S. enterica*, like other foodborne pathogens, may exist on these surfaces in a viable but not culturable state; this state prevents most culture-based diagnostics from detecting these cells, as they cannot be cultured by standard methods due to their dormancy (51). It is estimated that approximately 80% of bacterial infections in the U.S. are linked to foodborne pathogens that are residing in biofilms (49, 91).

Common Sanitizers in the Food Industry

A variety of different sanitizers and antimicrobials are commonly utilized in the food processing industry; the most frequent sanitizers used to control levels of *S. enterica* and other common pathogens include sodium hypochlorite (SH or bleach), peroxyacetic acid (PAA), and quaternary ammonium compounds (QACs) (92-95). Sanitizing is the process by which bacteria found on a surface can be reduced to a safe level, considered to be a 99.999% reduction of the bacteria present (a 5-log reduction) within 30 seconds of the sanitizer contacting the surface (95-97). Sanitizers must contain an active ingredient that is toxic to microorganisms, and each active ingredient varies in usage and efficacy (95).

Sodium Hypochlorite

Sodium hypochlorite, otherwise known as bleach, contains chlorine (Cl) as the active ingredient, mixed with sodium hydroxide (NaOH), and it is an effective sanitizer for both direct food application and application to food-contact surfaces (93). It can also be applied directly to food products in a dilute form, and it degrades upon reaction into inactive compounds, making it safe for use on food in certain concentrations (98). Bleach is also a strong oxidizer, which makes it reactive to many cellular components, but also reactive to dirt and other organic materials; since bleach decomposes upon reaction, it can be rather ineffective when used on dirty surfaces, in poultry chillers with lots of organic materials, or on improperly rinsed food products (99, 100). Also, as a strong oxidizer, bleach mixed with acid-based sanitizers will create chlorine gas, and bleach will corrode some metals, which limits some potential uses (98, 101). Due to the reactivity of chlorine, it is commonly used in chilling tanks to reduce the presence of *S. enterica* on chicken carcasses or fruits being processed and functions best when the concentration being used is effectively monitored and replaced upon depletion. However, sodium hypochlorite often leaves a residue that must be rinsed from products after exposure, unlike some of the other sanitizers commonly used in poultry processing (97, 102).

When bleach is used as a disinfectant or biocide against bacteria such as *S. enterica*, the bleach targets the proteins being produced by the bacteria (101). As such a strong oxidizer, the hypochlorite (ClO-) will replace the hydrogens present in the proteins by breaking the hydrogen bonds present, which denatures the proteins, leading to their clustering (98). As many proteins are present in the cell walls of bacteria, this can cause apoptosis or cell death (98). At high concentrations, chlorine can be very effective against bacteria and other contaminants present (101). However, using high concentrations of chlorine as a direct disinfectant on food products will also affect the taste, texture, color, and odor of the products, which is a massive drawback of using chlorine-based sanitizers in the processing industry (101). Additionally, high concentrations of chlorine sanitizers can be hazardous to humans without proper dilution, leading to inhalation of chemical fumes, and direct skin contact burns (101). The use of low-concentration sanitizers can lead to the survival of bacteria, which can then continue to grow and populate the surface (101). Due to the potential dangers of high-concentration sodium hypochlorite sanitizers and their impact on food products, the limit for the amount of free-floating chlorine from sodium hypochlorite allowed in food processing is 20-50 parts per million (ppm) permitted in antimicrobial sprays, washes, rinses, or dips (103), or up to 200 ppm on food-contact surfaces (97).

Peroxyacetic Acid

Peroxyacetic acid (PAA) is a sanitizer that is frequently used in the poultry processing industry, oftentimes in place of chlorine-based sanitizers. Peroxyacetic acid can be used on directly on food-contact surfaces, in poultry chilling tanks or low-temperature immersion baths (less than 40^{o}F), or as a rinse/spray (103). Peroxyacetic acid is an organic acid that is generated from the reaction of hydrogen peroxide and acetic acid; it is a strong oxidizer that decomposes to oxygen and acetic acid after reaction with organic material (97). This sanitizer also denatures proteins in bacteria, much like chlorine, but has been found to be more effective than sodium hypochlorite as an antimicrobial treatment, as the sanitation efficacy is not adversely affected by high organic loads (97, 100, 104). Peroxyacetic acid degrades rapidly, as it is a fairly unstable sanitizer, so monitoring the concentration of this sanitizer is vital; degradation of this sanitizer does not leave a residue (102). Peroxyacetic acid is most commonly permitted for use on food products and food-contact surfaces at concentrations of up to 220 ppm (100, 103), and will cause discoloration and odor changes if used at higher concentrations on poultry products (94, 97, 100, 103). It is most effective when used in cold temperatures, at less than 40^{o}F, works at a wide range of pH levels, and works well when used on biofilms (97, 100).

Quaternary Ammonium Compounds

Quaternary ammonium compounds, or QACs, are a class of sanitizers commonly used in the food processing industry. They can be used for direct food contact or on food-contact surfaces, are active for a longer time than other common sanitizers in the food processing industry, have low toxicity, and work effectively in a wide range of acidity levels (105). QACs are surface-active agents which are cationic, with the central nitrogen binding to a bromine or chlorine molecule to form a salt (105). The most common QACs are benzalkonium chloride, centrimide, and cetylpyridinium chloride, and they differ in their antimicrobial activities and efficacy against certain species due to the difference in the R groups connected to the central nitrogen in each QAC (105). These compounds are large and function via insertion into the cell wall, where they react with the cytoplasmic membrane, causing leakage, degradation of cell materials, and cell lysis (105). QACs can be affected by heavy organic loads on surfaces, the presence of hard water, and anionic surfactants, are generally ineffective in use on biofilms due to their size, and may vary in their efficacy on certain surfaces or food products (105, 106). Quaternary ammonium compounds are permitted for use from 200-400 ppm for various food contact surfaces in an aqueous form (97).

Sanitizer Tolerance and Persistence

Sanitizers are widely and frequently utilized in the food processing industry to kill bacteria and pathogens to prevent contamination of food products or the formation of biofilms. Despite frequent sanitation efforts, however, pathogens are frequently still found in food processing environments. Sanitization does not always eradicate these bacteria completely, as some bacteria, known as persister cells, may tolerate exposure to the biocide and proliferate once the biocide is removed (57). There are several other factors that affect the ability of bacteria to tolerate sanitizers. Incorrect application of the sanitizer, including shortened exposure time to the sanitizer, incorrect concentrations of the active ingredients being used in the sanitizer, or the use of the wrong sanitizer type can lead to the bacteria surviving the sanitization step (97, 107). Inadequate cleaning steps to remove dirt or other organic materials prior to the sanitation step can also contribute to the survival of bacteria on a surface (97, 107), while the presence of niches or crevices in the equipment or surfaces being sanitized can allow bacteria a foothold to form biofilms in (107). Additionally, bacteria already in biofilms are more difficult to sanitize, especially when those bacteria are in stationary phase in a well-established biofilm; the three-dimensional EPS can prevent direct contact with bacterial cells further into the biofilm, leading to just the surface cells being eradicated by sanitizer treatment (107). All of these methods allow bacterial pathogens such as *S. enterica* to persist in the food processing environment.

Tolerance to benzalkonium chloride, a QAC, has been observed in both *Listeria monocytogenes* and *S. enterica*. *L. monocytogenes* has been noted to display increased minimum inhibitory concentrations for benzalkonium chloride due to the presence of the efflux pump genes *qacH* and *bcrABC*. *QacH*, *bcrB* and *bcrC*, all of which are transporters, function to remove the QACs from the cell once they enter (108). In *S. enterica*, the multidrug efflux pump genes *acrAB*, *acrEF*, and *mdsABC* have been found to expel benzalkonium chloride from the cell upon entry (109). These efflux pumps present do not confer full resistance to sanitizers; rather, they act as a part of the sanitizer tolerance mechanism that allows survival in low concentrations of QAC; in higher concentrations, the cells would be unable to expel the QAC molecules quickly enough to prevent them from taking action (108, 109).

Sanitizer Resistance vs Resistance

Oftentimes, it is easy to confuse sanitizer tolerance with sanitizer resistance. Sanitizer tolerance is the ability of a bacteria to survive short-term exposure to high concentrations of biocides or antibiotics, with minimal change in the minimum inhibitory concentration needed to inhibit the growth of this bacterium or kill it (110). Sanitizer resistance is the ability of bacteria to grow at high concentrations of biocides and antibiotics, regardless of the length of time of exposure, and is generally heritable. The difference between tolerance and resistance is observable in minimum inhibitory concentrations, where bacteria that are tolerant to the biocide can be seen growing at high concentrations while susceptible strains will not (110). When discussing resistance to sanitizers, relatively few bacteria truly possess genes which allow them to resist sanitizers (107). The presence of *polyP*, a chaperone holdase found in *E. coli* and *Pseudomonas aeruginosa*, allows the bacteria to stabilize unfolded proteins and prevents their aggregation, therefore conferring resistance to hypochlorous acid (111, 112). *E. coli* has also been found to contain a locus of heat resistance (LHR), which has three chlorine-sensitive transcription factors encoded on it: *hypT*, *rlcR*, and *nemR.* These genes are activated by oxidation of chlorine ions, and respectively, they function to inhibit unnecessary regulation of target genes, to reduce cellular components which are specifically oxidized by chlorine, and to control genes which encode electrophile detoxification enzymes that increase resistance to bleach (113-115). Several studies suggest that *E. coli* and *Salmonella* Typhimurium, which both possess an *oxyR* gene regulon that enhances the oxidative response of these bacteria, gain resistance to hypochlorous acid through constitutive expression of the regulon (116-118). Resistance to a variety of sanitizers specifically in *S. enterica* has not been found; many studies conclude that while *S. enterica* can tolerate sanitizers, especially when in biofilms, there is no evidence of sanitizer resistance in *S. enterica* (109, 119, 120).

Antimicrobial Resistance

Antimicrobial resistance, also known as antibiotic resistance or AMR, is a rising concern globally. Over the last two decades, there has been a noticeable increase in the ability of bacterial pathogens to resist a variety of antibiotics used in the medical field (121, 122). The rise in antimicrobial resistance in the last 20 years has been attributed to the global overuse and misuse of common and prescribed antibiotics (121, 123). The Centers for Disease Control and Prevention estimated in 2017 that 30% to 50% of all antibiotics that are prescribed in outpatient clinics and 30% of the ones prescribed in hospitals are unnecessary or were prescribed incorrectly (121). In 2019, the CDC estimated that more than 2,868,700 antibiotic-resistant infections occur each year, with the deaths of over 35,900 people annually as a result of these infections (124). Luckily, the number of deaths associated with antimicrobial resistance has decreased by 18% since 2013, likely due to the implementation of new prevention methods in hospitals, including the tracking of AMR pathogens, the increase in antibiotic stewardship, and the improvement of sanitation methods to prevent accidental leaching and of antibiotics into the environment (124). The CDC describes antibiotic stewardship as the effort to improve the use of antibiotics by patients through improving the prescription of antibiotics by clinicians, to reduce the accidental prescription or misuse of antibiotics (124).

Misusage of Antibiotics in the Livestock and Food Industry

The use of antibiotics in food and animal industries has contributed greatly to the rise of antimicrobial resistance in foodborne pathogens. Antibiotics have commonly been used previously as growth promoters, and metals such as copper, zinc, and silver are frequently used as additives in animal feed to prevent illness in livestock (121, 125). In 2006, the European Union banned antibiotic usage for livestock growth promotion, including the imports of animal feed containing these products and imported livestock or other animals that were treated with antibiotics as a regular course of action (126, 127). As of 2012, the United States Food and Drug Administration has banned the use of antibiotics on animals without a valid prescription; however, antibiotics are still widely used in livestock (127, 128). Approximately 80% of the antibiotics sold in the U.S. annually are for use in animal agriculture, and of that 80%, 70% are from classes of antibiotics commonly used in human medicine (129). Improper dosing of livestock can provide differential selective pressure on bacteria that retain antimicrobial resistance genes (130, 131). When the livestock go to slaughter, they may bring bacteria with these genes into the processing facility; once inside the processing facility, these bacteria can exchange plasmids with other bacteria already present in the facility, facilitating the transfer of antibiotic resistance genes (131, 132). These bacteria can also be shed into the environment through the feces of dosed livestock, where they can exchange resistance genes with other bacteria present; approximately 40-90% of administered antibiotic doses to livestock are excreted in the feces and urine as well (132), which can provide the selective pressure to soil pathogens, or leech into nearby water, soil, or plants (133, 134). While water can be treated for contamination, the complete removal of antibiotics in wastewater is impossible, and the effluent and sludge can still contain antibiotics when they exit the water treatment facilities (135); the sludge may be used as manure fertilizer, and the excess water may runoff into rivers and streams, creating an issue for crops grown in agricultural fields that can potentially enter the food processing industry later (132).

The Acquisition of Antimicrobial Resistance Genes

Bacteria most often acquire antimicrobial resistance through the horizontal gene transfer of mobile genetic elements (MGEs), such as plasmids or transposons; these can be transferred from cell to cell through direct contact by a pilus in a process called conjugation (136). The process of conjugation happens ubiquitously in bacterial communities in the environment, occurring in soil, water, sewage, biofilms, plants, crops, and any surface where bacteria are present (136). Bacteria in these environments will oftentimes exchange genes that aid in virulence, genes that confer resistance to antimicrobials and heavy metals, genes that aid in the tolerance of stress, cold, heat, bile, salt, and a host of other tolerances, and genes that aid in the proper functioning of metabolic processes necessary inside the bacterial cell (137, 138). This transfer allows bacteria previously not exposed to an antibiotic to gain resistance to it; the circulation of bacterial hosts – livestock to humans – makes it very easy for antibiotic resistance genes to enter the food industry, where they can pass to other microbes (130, 138). Over the years, the incidence of drug resistance in bacteria has risen steeply, and many bacteria have obtained resistance to more than one common class of drug. Bacteria that have resistance to three or more classes of antibiotics are known as multi-drug resistant bacteria, or MDR bacteria (138, 139).

The Rise of Antimicrobial Resistance in *S. enterica*

Several serovars of *S. enterica* exist which have an unusually high carriage of antibiotic resistance genes. These include *Salmonella* serovars Heidelberg, Newport, and Typhimurium, as well as *Salmonella* I 4,5,[12]:i:-, or monophasic Typhimurium (140). *Salmonella* I 4,5,[12]:i:- was first identified in poultry products during the late 1980's in Portugal, and since has risen to become one of the most common serotypes of *S. enterica*; since 2011, it has consistently been among the three most common serotypes isolated from humans in the EU (6, 141). *Salmonella* I 4,5,[12]:i:- is characterized by the presence of the *Salmonella* Genomic Island 4, and an insertion into the *flj*B region of the chromosome, where the genetic code of the *flj*AB operon was replaced by the presence of a 28-kb region encoding $bla_{\mathrm{TEM\text{-}1}}$, *strA*, *strB*, *sul2*, and *tet*(B) (125, 142, 143). The deletion of the *flj*B gene results in the monophasic form of this isolate, as *flj*B encodes for the phase 2 flagellin in *S. enterica* (15). Three distinct clonal lineages of *Salmonella* I 4,5,[12]:i:- emerged in the 2000s, and the Spanish and European clones both display high amounts of antimicrobial resistance, as well as tolerances to copper, silver, and mercury, which as mentioned above, have been commonly used in animal husbandry to promote growth, maintain health, and control infections (130, 141). The European clone has since become one of the most common serovars in the United States, and is characterized by the tetra-resistance ASSuT profile and the presence of the SGI-4 (Salmonella Genomic Island 4) (142). The ASSuT resistance profile confers resistance to the common antibiotics ampicillin, streptomycin, sulfonamides and tetracycline via the gene cassette inserted into the *flj*AB operon (125, 142).

Antimicrobial Resistance Mechanisms in *S. enterica*

There are a variety of antimicrobial resistance genes in *S. enterica* which inactivate antibiotics and biocides in a few different ways. Efflux genes are one of the better-known ways *S. enterica* resists antibiotics and other biocides, and are often the most effective mode of resistance to multiple antimicrobials (144). Efflux pumps in the cell membrane function to pump out antibiotics and other biocides upon entry to the cell, preventing the concentration of a lethal dose of certain antibiotics (145). There are nine important efflux genes found in *S. enterica* antibiotic resistant serovars which have been known to affect antibiotics and bile salts, and also aid in the removal of certain sanitizers from the cell (145, 146). The genes *tolC* and *mdsC* are outer membrane proteins (OMPs) that aid in the proper function of many of these efflux pumps (145). The *acrAB* and *acrEF* operons are found in both *S. enterica* and *E. coli* and rely on the presence of the OMP TolC to function properly (146, 147). These operons, in conjunction with the efflux proteins AcrD, MdsABC, and MdtABC , account for five of the nine important efflux systems in *S. enterica*, and are all part of the resistance nodulation cell division (RND) transporter family (145, 148). The RND transporter family is a category of efflux pumps identified commonly in Gram-negative organisms which is located in the cytoplasmic membrane and function as a tripartite complex which is generally composed of an inner membrane component, a membrane-fusion protein (also known as a periplasmic adaptor protein), and the outer membrane protein (148). The remaining four efflux pump systems present in *S. enterica* include the *emrAB* operon and the *mdfA* gene, from the major facilitator superfamily (MFS), which are tripartite complexes (EmrAB) or single-component active transporters (MdfA) (149); the efflux protein MdtK from the multidrug and toxic compound extrusion (MATE) transporter family, transporters which use electrochemical gradients to drive the movement of substrates and molecules (144, 145); and the *macAB* operon from the ATP-binding cassette (ABC) transporter superfamily, which uses free energy released by the hydrolysis of ATP to ADP to drive the transport of substrates both directions across the lipid membrane (144, 145). These are all active efflux pumps, meaning they function through the use of ATP and electrochemical gradients to pump substrates or antibiotic molecules against the gradient and remove them from the cells (145).

Other mechanisms that *S. enterica* employs to resist antimicrobials and biocides include modifying the target site to change the affinity of certain antimicrobials or modifying target molecules to prevent their inhibition via antibiotic action; changing the permeability of the cell by modifying OMPs to restrict the access of the antibiotic to the target site; and the use of enzymes to modify or degrade antibiotics (144, 145). The limitations of these mechanisms are that most of these genes target single antibiotic classes, while efflux pumps can recognize and target multiple antibiotic classes (144, 150, 151). Most of these genes are encoded on the Salmonella Genomic Islands previously mentioned; some of the more common genes in this category are listed here (152). *bla*$_{\text{TEM-1}}$ is a gene that encodes a β-lactamase which hydrolyzes the beta-lactam ring present in β-lactam antimicrobial agents; this inactivates the antibiotic (153). Other genes which encode enzymes that inactive antibiotics include *strA* and *strB*, linked genes which target streptomycin's streptidine ring for phosphorylation (154, 155). Common β-lactam agents include penicillins, cephalosporins, carbapenums, monobactams and related derivatives of these classes (153). Sulfonamide-resistance genes such as *sul1*, *sul2*, and *sul3* are also commonly found in *S. enterica*, and encode forms of dihydropteroate synthase protein which will not be inhibited by the presence of sulfonamides, therefore preventing the correct function of the antimicrobial (156).

Many antimicrobial resistance genes are encoded on special gene cassettes that are present in *S. enterica*, known as Salmonella Genomic Islands (SGIs), along with genes associated with virulence and invasiveness, cellular metabolism, stress tolerance, attachment, and heat shock (152). A subset of the SGIs are the Salmonella Pathogenicity Islands (SPIs), recognized as one of the main contributors to the virulence of *S. enterica*, as many virulence genes, including a type III secretion system and effector proteins, are encoded on SPI-1 and SPI-2 (152). These genes are able to be copied to plasmids and transferred to other bacteria or other serovars of *S. enterica*.

Control of Pathogens in Industry: Temperature

In the food processing industry, there are a variety of ways that *S. enterica* can be controlled. One such way is through the use of temperature combined with antimicrobials. *S. enterica*, alongside many other foodborne pathogens, can grow rapidly on food when the food is held between 4°C and 60°C (40-140°F); this is known as the temperature danger zone (157). Therefore, control of the temperature in food processing facilities is vital to ensuring minimal growth of bacteria present.

There are several methods of temperature control frequently used in the poultry processing industry; the control of room temperature and chilling room temperatures is one utilized for the processing of food products, specifically poultry and other meat products (158). During active processing, room temperatures should be below 4°C (40°F) to prevent the outgrowth of foodborne pathogens (158). Scalding is another method commonly used to control pathogens; both steam-spraying and immersion scalds are considered a good method for reduction of *S. enterica* in the poultry processing facility (159). The length and temperature of the scalds may vary; a hard scald is considered to be 30-75 seconds of exposure at 59-64°C, whereas a soft scald is 90-120 seconds of exposure at 51-54°C, and turkey is commonly scalded at 59-63°C for 50-125 seconds, according to the FDA guidelines for controlling *S. enterica* in raw poultry (159). Scalding above 47°C controls the growth of *Salmonella*, while scalding at 60°C for higher results in the reduction of *Salmonella* by an additional 0.3-0.5 log more than scalding at 52°C or 56°C does (160). Hard scalds are more common in the poultry industry than soft scalds due to shorter time at a higher temperature, as well as the removal of the epidermis being easier due to the higher temperatures (159).

Chilled immersion tanks are also commonly used in the poultry processing industry to reduce levels of *S. enterica* on food products; these come in the form of pre-chillers, main-chill, and post-chill dips, which function mainly to reduce the temperature of the carcasses and inhibit the growth of microbes (158). Oftentimes, antimicrobials are incorporated into the chiller water to reduce the presence of potential microbes on the carcasses, with the most frequent antimicrobial in use being PAA (103, 158). These immersion tanks are held at or below 4°C, and studies report the efficacy of these tanks as an antimicrobial intervention step in processing, with an approximate 71.8% *Salmonella* incidence prior to immersion, reduced to 20.2% incidence post-chill (158, 161).

Several temperature controls are also utilized in tree nut processing, another food that has been noted to contain *S. enterica* (162). Oftentimes, nuts are roasted or broiled as a pathogen reduction step – almonds are commonly dry roasted at 130-154°C, or until the nut attains an internal temperature of 130°C and the moisture has evaporated (162-164). Different exposure times are used depending on the desired roast of the nut; peanuts being processed are dry roasted at 140-160°C for 40-60 minutes, or oil roasted at 138-143°C for 3-10 minutes (162). Steam can also be used to destroy *S. enterica* that may be present on the nut; temperatures for the steam processing vary as with the application of vacuum pressure, the target temperature for the steam doesn't need to be as high as it does in ambient pressure (162, 165). Using steam for too long can compromise the quality of the product (166); one study found that 25 seconds of steam pasteurization at 95-102°C served as an adequate control step to reduce the presence of *Salmonella* Enteritidis on almonds (167).

Heat Tolerance

Several studies on heat tolerance in bacteria have shown that *S. enterica* and *E. coli* that have previously been exposed to sub-lethal levels of stress develop resistance to heat (168-170). The pre-exposure to stress and conditions for growth prior to thermal treatments in processing facilities has also been noted to increase the survival capability of *S. enterica* during processing (171), and prior heat exposure or starvation stresses can lead to the exhibition of higher thermal tolerance in *S. enterica* (172). Another factor that contributes to heat tolerance in *S. enterica* includes the growth stage of the cells being exposed to heat; when in stationary phase, cells display more resistance to heat than cells in exponential phase, or cells that are still replicating (172). Additionally, *S. enterica* has been known to have a hysteretic response; when exposed to a stress, cells may display an increase in the upregulation of heat shock genes, even after the environmental stress has ceased; this may aid in the survival of *S. enterica* in the food industry (172).

S. enterica is known to possess genes that encode chaperones, sigma factors, and heat shock proteins, as well as the thermosensors which detect the presence of heat in the cell and upregulate these genes (172). Thermosensors are the cellular thermometer, and are found in the form of proteins, lipids, temperature-responsive RNA's, and the topology and structure of DNA (172). Expression of heat shock proteins are regulated by the regulator heat shock sigma factor (σ^H/σ^{32}) encoded by *rpoH*, or the extreme heat stress sigma factor (σ^E/σ^{24}) encoded by *rpoE*, depending on the level of heat stress detected by the thermosensors (172, 173). RpoS is a sigma factor which functions in general stress response and the repair of DNA and is also involved in the virulence capabilities of *S. enterica* (82, 172). These sigma factors function to upregulate over 30 chaperones and proteases to help repair cellular components and control the expression of membrane heat shock proteins during heat exposure as well as over 100 other heat shock genes (172). Inadequate heat treatments during food processing may actually increase the thermal resistance and survival of *S. enterica* and other foodborne pathogens, as these treatments increase virulence capabilities which have been linked to increased thermal tolerance through the upregulation of related genes (174).

Summary

Salmonella enterica has a variety of ways that it persists in the food processing environment, including the formation of biofilms to resist disinfectants, the resistance of sanitizers and antimicrobials through the use of efflux pumps and other antimicrobial resistance genes, and tolerance to heat and other stresses (151, 175). As such, removal of *S. enterica* from the food processing industry requires a strong understanding of how this foodborne pathogen persists in the processing industry and the potential of the strains that pass through the food processing industry to display enhanced tolerance to common stresses encountered previously by these pathogens. While there is quite a bit of research on common foodborne outbreak strains, there is still a knowledge gap on the potential of those strains to have enhanced abilities to resist stresses, including stresses these strains may have previously encountered and survived in the processing industry. If outbreak-associated strains do have enhanced stress tolerance abilities, the knowledge of these abilities could help to develop new strategies in the processing industry to prevent the persistence of *S. enterica* and potential future illness of consumers.

CHAPTER 2: MANUSCRIPT

Introduction

Non-typhoidal *S. enterica* is a foodborne pathogen that is commonly found in poultry, eggs, pork, nuts, and some types of produce (176). This pathogen is responsible for a high number of foodborne outbreaks annually in the United States, and is a common contaminant in processing facilities (177). *S. enterica* also incurs quite a high economic burden, with the loss of over $4.142 billion annually in the U.S. (10). As such, *S. enterica* is a major foodborne pathogen of interest, especially in conjunction with the rise of antimicrobial resistance (122). Previous research done by Etter et al., 2019, indicated that 6 *Salmonella* Heidelberg isolates from the Foster Farms poultry outbreak in 2013-2014 displayed enhanced tolerance to stress (178). Of these 6 isolates, one demonstrated an enhanced ability to form biofilms under stressful conditions, and two of the strains displayed significantly higher heat tolerance than a non-outbreak reference strain used in the study (178). The stress-tolerant strains also displayed a higher expression of stress proteins, chaperone proteins and heat shock proteins in stationary phase than the reference strain (178). Ultimately, Etter et al., 2019, concluded that there was a need for a broader research study to test the tolerance of outbreak and non-outbreak associated strains of *S. enterica* to common stresses found during food processing, and to conclude if outbreak-associated strains truly do display enhanced stress tolerance capabilities. This work has focused primarily on determining whether outbreak-associated strains from multiple U.S. outbreaks display enhanced stress tolerance to common processing stresses when compared to non-outbreak strains. To achieve this goal, the attachment capacity, sanitizer tolerances and heat tolerances of a variety of *S. enterica* strains was observed.

Transcriptomes of stress-tolerant and stress-sensitive *S. enterica* were also going to be observed, but unexpected delays prevented the processing of these transcriptomes in time. In place of the transcriptomes, antimicrobial genes, metal tolerance genes and stress tolerance genes have been noted for most of the isolates in this study.

Methods

Acquisition of Culture Collection

There were 35 strains of *S. enterica* collected for this study from a variety of sources. All strains are described in greater detail in **Table 1**. 25 of the microbial strains used in this work were provided by the USDA-ARS Culture Collection (NRRL). Six strains were retrieved from the Food and Drug Administration (FDA), and 3 more were obtained from the New York State Food Laboratory. One strain was ordered from the American Type Culture Collection (ATCC).

Upon acquisition, cultures and dehydrated isolates were transferred into sterile trypticase soy broth (TSB, BD, Franklin Lakes, NJ) for growth, and incubated at 37^{o}C, 200 RPM (rotations per minute), for 24 hours. Cultures were then streaked out for isolation onto trypticase soy agar (TSA, BD, Franklin Lakes, NJ) and the TSA was incubated at 37^{o}C for 24 hours of growth. A single colony from each plate was collected and inoculated into TSB broth for incubation at 37^{o}C overnight. A 1:1 ratio of culture to 50% glycerol was transferred aseptically to a sterile cryovial for storage at -80^{o}C as a working stock for future use.

Crystal Violet Attachment Assay

Attachment to the polystyrene plates was evaluated for the isolates as follows, using a procedure adapted for the *S. enterica* cultures in this thesis from methods previously described by Wang et al., 2015, and Etter et al., 2019 (178, 179).

Preparation of Cultures:

Isolates were recovered from the working stock solutions by streaking 10 µl onto fresh TSA plates in a 4-zone streak and incubated at 37^{o}C for 24 hours. A single colony from each plate was aseptically transferred to 10 mL of fresh TSB (1x or 1/20x) and incubated at 37^{o}C for 24 hours with shaking at 200 RPM.

Microtiter Plate Preparation:

Twelve 96-well polystyrene microtiter plates (Corning, NY) were prepared as follows. For the nutrient-high and the nutrient-low plates, the procedure was identical except for the corresponding concentrations of the media used. 100 microliters of culture broth were pipetted in the wells in triplicate, followed by 100 microliters of sterile TSB, for a total of six 1x TSB microtiter culture plates and six 1/20x TSB plates. Sterile TSB broth (200 microliters) was added to three wells on each plate to act as a negative control. Prior to incubation, a spectrophotometer was used to measure the optical density of cells present at 600 nanometers (OD_{600nm}). Half of the 1x and 1/20x microtiter plates were incubated at 22^{o}C, and the other half were incubated at 4^{o}C for 24, 72, and 120 hours.

Microplate Rinsing and Staining:

Four microplates, one from each nutrient condition and each temperature, were rinsed and stained at 24 hours post-incubation, 72 hours post-incubation, and 120 hours post-incubation. All media in the microplates at these time points were removed and disposed of, and microtiter wells were rinsed thoroughly with sterile deionized water three times to remove any excess cells. Once the water was removed, 200 microliters of 0.4% aqueous crystal violet were added to each cell; the crystal violet solution was incubated in the wells for 30 minutes to stain the cells. Afterward, the crystal violet solution was removed from the microplates, and the wells were rinsed with water until the water ran clear. Plates were inverted in an undisturbed cabinet to dry for later quantification.

Cell Quantification:

Dried microtiter plates were retrieved, and 200 microliters of 95% ethanol was added to each well and agitated to elute the crystal violet from the cells. The ethanol was then transferred from each well to a corresponding well in a sterile 96-well microtiter plate, and the OD_{600nm} was read and recorded for each plate. An Epoch microplate spectrophotometer was used to determine the retention of crystal violet as an approximation of the cells attached to the polystyrene plate. All crystal violet attachment assays were repeated in their entirety in triplicate for technical replicates.

Minimum Inhibitory Concentration Assay

Minimum Inhibitory Concentrations (MICs) for sodium hypochlorite and peroxyacetic acid were determined as follows, using a procedure adapted for the *S. enterica* cultures in this thesis from methods previously described by Wang et al. and Etter et al. (178, 179).

Preparation of Cultures:

Isolates were recovered from the working stock solutions by streaking 10 microliters onto fresh TSA plates in a 4-zone streak and incubated at 37°C for 24 hours. Approximately 15-25 isolated colonies from each strain were selected with a sterile swab and suspended in 3 mL of TSB. Optical density (OD_{600}) of these cultures was read with an Epoch microplate spectrophotometer (BioTek, Winooski, VT) and adjusted to a range of 0.600 – 0.800. Adjusted culture was then diluted 1:100 into 1 mL of 2x concentration TSB or 1/10x concentration TSB; media was further diluted later by the addition to sanitizer, resulting in a final concentration of 1x (nutrient-rich) or 1/20x (nutrient-poor) TSB.

Sanitizer Preparation (Bleach):

A solution of sodium hypochlorite (Clorox, Oakland, CA) was prepared to achieve a final concentration of 400 ppm. Aliquots of 200 microliters of the sanitizer was added to 7 wells of the first column on a 96-well polystyrene microtiter plates (9017 Corning, Corning, NY). Into the next 5 columns, 100 microliters of sterile deionized water was added to 7 wells, and the sanitizer in the first column was serially diluted 1:2 in the wells containing the sterile deionized water. After serial dilution, 100 microliters of the sanitizer mixture were disposed of from the 6^{th} column wells to achieve the same amount of sanitizer as the previous 5 columns. 50 microliters of the sanitizer from each well was then transferred to the remaining 6 columns of the plate, creating a duplicate of the first half of the plate.

Sanitizer Preparation (PAA):

A solution of peroxyacetic acid (PAA, Inspexx-250, Saint Paul, MN) was prepared to achieve a final concentration of 400 ppm. Aliquots of 100 microliters of the sanitizer were added to 7 wells of the first column, fifth column, and ninth column on a 96-well polystyrene microtiter plate (9017 Corning, Corning, NY). Into the next 3 columns following each sanitizer column, 50 microliters of sterile deionized water were added to 7 wells, and the sanitizer in the 1st, 5th, and 9th columns was serially diluted 1:2 in the wells containing the sterile deionized water. After serial dilution, 50 microliters of the sanitizer mixture were disposed of from the 4th, 8^{th,} and 12th column wells to achieve the same amount of sanitizer as the previous columns.

Plate Inoculation and Incubation (Bleach):

Each 96-well plate with sodium hypochlorite contained enough wells to test two isolates in triplicate at nutrient-rich (1x TSB) and nutrient-poor (1/20x TSB) conditions. 50 microliters of adjusted culture in 2x TSB was added to the first set of 3 rows of sanitizer concentrations, and 50 microliters of adjusted culture in 1/10x TSB was added to the second half of the rows A-F for each isolate in triplicate. This resulted in 100 microliters each of 200, 100, 50, 25, 12.5, and 6.5 ppm sodium hypochlorite per nutrient condition (1x or 1/20x) for each isolate in triplicate. For the preparation of negative controls, 50 microliters of sterile TSB media was added to the 7th row (G) of the plate; 2x TSB was added to the first 6 wells (columns 1-6) and 1/10x TSB was added to the second set of wells (columns 7-12). For the preparation of the positive controls, 50 microliters of 2x and 1/10x sterile TSB media was added to the final row (H) of the plate and inoculated with 50 microliters of adjusted cultures for each isolate at both nutrient conditions in triplicate. Plates were read at hour zero, immediately following the inoculations to determine a baseline for the OD_{600}, and plates were read again at 24 hours post-inoculation to measure the growth of the cultures at 22^{o}C. The minimum inhibitory concentration was determined from the comparison of the 24-hour wells to the hour-zero wells, where the MIC was the concentration of sanitizer at which no growth occurred in the wells.

Plate Inoculation and Incubation (PAA):

Each 96-well plate with peroxyacetic acid contained enough wells to test three isolates in triplicate at nutrient-rich (1x TSB) and nutrient-poor (1/20x TSB) conditions. 50 microliters of adjusted culture in 2x TSB were added to the first set (A-C) of 3 rows of sanitizer concentrations, and 50 microliters of adjusted culture in 1/10x TSB was added to the second half of the rows (D-F) for each isolate in duplicate. This resulted in 100 microliters each of 200, 100, 50, and 25 ppm peroxyacetic acid per nutrient condition (1x or 1/20x) for each isolate in triplicate across the plate. For the preparation of the negative controls, 50 microliters of sterile TSB media was added to the 7th row (G) of the plate; 2x TSB was added to the first 4 wells (columns 1-4) and 1/10x TSB was added to the second set of wells (columns 5-8). Positive controls were prepared as follows: 50 microliters of 2x and 1/10x sterile TSB media were added to the last 3 columns of row G and the final row (H) of the plate (2x in 10G-12G, 1H-6H, and 1/10x in 7H-12H) and the wells were inoculated with 50 microliters of adjusted cultures for each isolate at nutrient-rich conditions (2x) in triplicate and nutrient-poor conditions (1/10x) in duplicate. Plates were read at hour zero, immediately following the inoculations to determine a baseline for the

OD_{600}, and plates were read again at 24 hours post-inoculation to measure the growth of the cultures at 22°C. The minimum inhibitory concentration was determined from the comparison of the 24-hour wells to the hour-zero wells, where the MIC was the concentration of sanitizer at which no growth occurred in the wells.

Growth Curves & Heat Shock Assay

Growth Curves and Heat Shock Assays were performed using procedures adapted from methods previously described by Etter et al. (178).

Preparation of Cultures:

Isolates were recovered from the working stock solutions by streaking 10 microliters onto fresh TSA plates in a 4-zone streak and incubated at 37°C for 24 hours. A single colony from each plate was aseptically transferred to 10 mL of fresh TSB and incubated at 37°C for 16 hours with shaking at 200 RPM. After incubation, each culture tube was serially diluted with 5 1:10 dilutions, and dilute culture tubes were re-incubated at 37°C for 8 hours with shaking (200 rpm) in duplicate.

Generation of Growth Curves:

From timepoint zero (the initial dilution and incubation) to timepoint 8, every hour aliquots of 1 mL of culture were collected, serially diluted in fresh phosphate-buffered saline (PBS) to the desired concentrations and spread-plated onto 3-day dehydrated TSA plates in duplicate. Growth curves were used to determine how many hours post-inoculation each isolate reached stationary phase, to act as a starting point in the heat shock assays.

Heat Shock and Incubation:

Culture from each isolate tube was aliquoted in 1 mL amounts into sterile 2.0 mL Eppendorf tubes (epi-tubes) labeled in single for 0, 3, 6, and 9 minutes, in duplicate for 15 minutes, and in triplicate for 30, 45, and 60 minutes. Strains were transferred to plastic racks in preheated 56^{o}C water baths for heat shock and incubation was staggered by 3 minutes for each strain. At the times labeled on the tubes, aliquots were removed from the hot water baths and serially diluted in fresh PBS to the desired concentrations. Dilutions were handed to assistants for immediate pour-plating using liquid TSA into pre-labeled sterile round polystyrene plates (15x100mm, Corning, Corning, NY), and plates were set aside for incubation. Non-diluted cultures fresh from the hot water baths were collected for a single tube at 15 minutes and two tubes at 30, 45, and 60 minutes for direct plating of 100 microliters or 250 microliters. At the end of the experiment, all plates were permitted to solidify prior to transfer to a 37^{o}C incubator for 36 hours of growth.

Plate Counting:

After 36 hours of growth, plates were removed from the incubator, and colonies on the plates were counted using a counter-pen and verified by individual counts. Counts were labeled on the plates and recorded after counting was complete. Plates with more than 300 visible colonies, or lawn-like coverage, were labeled as too numerous to count (TNTC), and plates with fewer than 25 colonies were recorded but not used for eventual analysis. Plates that displayed contamination were labeled as such and not used in the analysis.

Antimicrobial Resistance and Heavy Metal Tolerance Genes

Antibiotic resistance genes, stress tolerance genes, and heavy metal resistance genes for each isolate were determined by Andrea Etter, using AMRfinder (v. 3.8.4) from data shown on NCBI Isolates Browser.

Statistical Methods

All significant differences for the crystal violet attachment assays, the minimum inhibitory concentration assays, and the heat shock assays were assessed using Analysis of Variance (ANOVA), followed by Tukey's honest significance test in R (v. 4.2.3) (180).

Results

Growth of Biofilm on Polystyrene Plates

When isolates were grown in the nutrient-rich condition at 22^{o}C, there was significantly more biomass present on day 5 compared to day 3 and 1, and day 3 compared to day 1 ($p_{adj} < 0.05$). In the nutrient-poor condition at 22^{o}C, significantly more biomass was also present on day 5 compared to day 3 and 1, and day 3 compared to day 1 ($p_{adj} < 0.05$). In the nutrient-rich condition at 4^{o}C, significantly more biomass was observed on day 5 compared to day 1, and day 3 also had significantly more biomass compared to day 1 ($p_{adj} < 0.05$). In the nutrient-poor condition at 4°C, we observed significantly more biomass at day 5 compared to both day 3 and 1 ($p_{adj} < 0.05$).

Our criteria for labelling an isolate as a high-biofilm former was significantly higher growth than ≥ 3 other isolates. For the isolates grown at nutrient-rich conditions at 22°C (**Figure 1**), one isolate from the Roast Pork outbreak (isolate 12) displayed significantly better attachment than 5 other isolates, and one from the Foster Farms outbreak (isolate 3) attached significantly better than one other isolate ($p_{adj} < 0.05$). In nutrient-poor conditions at 22°C (**Figure 2**), only a few of the Roast Pork outbreak isolates attached significantly better than others, including isolate 17, which displayed better attachment than 19 of the 25 total isolates observed; isolate 20, which attached significantly better than 2 other isolates; and isolate 12, which attached better than one other isolate in a significant way ($p_{adj} < 0.05$). For the Foster Farms outbreak isolates, isolate 3 attached significantly better than 15 others, isolate 10 attached significantly better than 9 other isolates, isolates 2 and 6 attached significantly better than 8 other isolates, isolate 5 attached significantly better than 7 others, and isolate 7 attached significantly better than 2 other isolates ($p_{adj} < 0.05$). One of the non-outbreak food isolates, isolate 25, attached significantly better than 8 others to the polystyrene plates ($p_{adj} < 0.05$).

For the isolates grown in nutrient-rich conditions at 4°C (**Figure 3**), only one isolate from the Roast Pork outbreak displayed significantly better attachment than the other isolates, and that was isolate 12, with better attachment than 4 others ($p_{adj} < 0.05$). In nutrient-poor conditions at 4°C (**Figure 4**), for the Roast Pork outbreak isolates, only two displayed significantly better attachment. This includes isolate 17, which attached significantly better than 13 other isolates, and isolate 20, which attached better than 3 others in a significant way ($p_{adj} < 0.05$). For the Foster Farms outbreak isolates, isolate 3 attached significantly better than 13 other isolates, followed by isolate 2 which attached significantly better than 11 others, and isolate 1 which attached significantly better than 5 others ($p_{adj} < 0.05$). Isolates 4 also attached significantly better than 2 other isolates in nutrient-poor conditions at 4°C ($p_{adj} < 0.05$). One of the non-outbreak food isolates, isolate 24, attached significantly better than one other isolate in these conditions as well ($p_{adj} < 0.05$).

Our criteria for labelling an isolate as a low-biofilm former was significantly worse growth than ≥ 3 other isolates. For the isolates grown at nutrient-rich conditions at 22°C (**Figure 1**), there were no isolates observed that performed significantly poorly. For nutrient-poor conditions in 22°C, attachment varied widely amongst isolates. For the Roast Pork outbreak isolates, isolates 14 and 23 attached significantly worse than 7 others, and isolates 22 attached significantly worse than 10 others ($p_{adj} < 0.05$). For the Foster Farms outbreak isolates, isolate 6 attached significantly worse than 10 other isolates, and isolates 1, 8, and 9 attached significantly worse than 7 others ($p_{adj} < 0.05$). One of the non-outbreak food isolates, isolate 24, attached significantly worse than 7 other isolates in nutrient-poor conditions at 22°C ($p_{adj} < 0.05$).

For the isolates grown in nutrient-rich conditions at 4°C, there were no isolates observed that performed significantly poorly. In nutrient-rich conditions at 4°C, multiple isolates from the Roast Pork outbreak displayed significantly worse attachment, including isolate 15, which attached significantly worse than 7 others, and isolate 14, which attached significantly worse than 6 others ($p_{adj} < 0.05$). Isolate 14 also attached significantly worse than 5 others, followed by isolate 18 which displayed worse attachment than 4 others, and isolates 12 and 23 which attached significantly worse than 3 others each ($p_{adj} < 0.05$). For the Foster Farms outbreak isolates, only 3 isolates displayed significantly worse attachment. Isolate 8 attached significantly worse than 4 other isolates, and both isolates 6 and 9 attached significantly worse than 3 other isolates ($p_{adj} < 0.05$).

Isolates displayed better attachment in 22^{o}C than 4^{o}C, as observed in **Figures 1-4**. Log_{10} cell counts in 22^{o}C in nutrient-rich and nutrient-poor conditions displayed higher attachment, under 2.5 Log_{10}, than those incubated at 4^{o}C in nutrient-rich and nutrient-poor conditions. The entirety of the attachment abilities of each isolate are summarized and can be viewed in **Table 4**.

Minimum Inhibitory Concentrations

At room temperature (22^{o}C), the minimum inhibitory concentrations of PAA in nutrient-poor conditions ranged from 25-50 ppm, while those in nutrient-rich conditions ranged from 100 ppm to 200 ppm (**Table 2**). For the Roast Pork outbreak isolates, almost all isolates displayed low tolerance (25 ppm) to PAA in nutrient-poor conditions; isolate 11 displayed an intermediate tolerance (50 ppm) to PAA. For the Foster Farms outbreak isolates, all 10 isolates displayed low tolerance to PAA, and all 6 non-outbreak food isolates displayed low tolerance to the PAA as well in nutrient-poor conditions. In the nutrient-rich conditions, the Roast Pork outbreak isolates 11, 13 and 17 displayed intermediate tolerance (100 ppm) to the PAA, while the other 10 all had high tolerance (200 ppm). In nutrient-rich conditions, two of the Foster Farms isolates – 2 and 10 –

displayed intermediate tolerance (100 ppm), while the other 8 had high tolerance to the PAA. Of the non-outbreak food isolates, all displayed low tolerance to the PAA in nutrient-poor conditions. One isolate displayed intermediate tolerance (100 ppm) to the PAA in nutrient-rich conditions, and the other 5 displayed high tolerance to the PAA. The nutrient-poor tolerance range observed in all the isolates is well below the recommended 220 ppm usage on food and food-contact surfaces in food processing (103), but the nutrient-rich concentrations come close to reaching that recommendation level.

For sodium hypochlorite (bleach) at room temperature (22ºC), the minimum inhibitory concentrations for the nutrient-poor conditions ranged from 50 ppm to over 200 ppm, while nutrient-rich concentrations were all ≥200 ppm (**Table 2**). For the Roast Pork outbreak isolates, in nutrient-poor conditions all isolates displayed intermediate tolerance (50-100 ppm) to the bleach, except for isolate 23 which had high tolerance (200 ppm). For the Foster Farms isolates in nutrient-poor conditions, all isolates displayed intermediate tolerance to the bleach. Three of the non-outbreak food isolates (isolates 29, 25, and 26) displayed intermediate tolerance to the bleach in the nutrient-poor conditions, and high tolerance was observed for the remaining three isolates. While the nutrient-poor tolerance range for some isolates was on the lower end, the overall tolerance displayed by these isolates was close to or at the 200 ppm recommendation guidelines for sodium hypochlorite on food contact surfaces (94, 103), and the nutrient-rich tolerance met or surpassed the recommended amount.

There were no significant differences observed among isolates for either peroxyacetic acid tolerance or sodium hypochlorite tolerance. For both sanitizers, all isolates grown under nutrient-rich conditions exhibited significantly greater tolerance to the sanitizers than the isolates grown under nutrient-poor conditions. Overall, the tolerance displayed by the isolates grown in nutrient-rich conditions in peroxyacetic acid and sodium hypochlorite was quite high; for sodium hypochlorite, the tolerance displayed matched the 200 ppm guideline for use in food processing facilities (103), indicating that label-strength sanitizer would be ineffective, while the peroxyacetic acid was close to the 220 ppm limit for food processing (103). The tolerance in nutrient-poor conditions for sodium hypochlorite was also quite high in some of the isolates, matching the tolerance observed in nutrient-rich conditions for some of the isolates. Tolerances for all isolates can be observed in both **Table 2** and **Table 4**; the latter provides a summary of all results obtained in the study for each isolate by outbreak.

Growth Curves and Heat Shock

Growth curves were generated for 23 of the 35 isolates to early stationary phase, as seen in **Figure 5**. There were no noticeable differences in the growth of the isolates, and all isolates reached early stationary phase by hour seven. We further extended the growth curves for several isolates to eight hours and confirmed that isolates were in stationary phase at hour seven. We conducted heat shock and generated survival curves for nine outbreak-associated *S. enterica* isolates (**Figure 6**). After the initial incubation period of 6 minutes, all isolates displayed a significant decrease in growth ($p_{adj} < 0.05$). No significant differences in survival were observed for the isolates at the same timepoints ($p_{adj} < 0.05$). All isolates survived until at least 45 minutes of heat-stress. At 45 minutes scald, two of the Roast Pork outbreak isolates – isolates 11 and 13 – and one of the Chicken Liver outbreak isolates (isolate 31) when plated were below detectable levels, at less than 1 colony forming unit (CFU) per milliliter of liquid. Following 60 minutes scald, the remaining Roast Pork outbreak isolate (isolate 12) and the two Chicken Liver outbreak isolates, 30 and 32, when plated were below detectable levels, along with the two Peanut Butter outbreak isolates, 47 and 48. The only isolate still surviving following 60 minutes scald was the single Almond outbreak isolate (Isolate 33) ($3.43*10^3$ CFU/mL). Isolate 33 had a single experimental replicate that was still alive at 60 minutes following scald. All survival times are noted in **Table 4**.

Antimicrobial Tolerance Genes (NCBI)

Antimicrobial resistance genes, heavy metal tolerance genes and stress tolerance genes varied across the isolates and had wider variance between outbreaks. All genes found in AMRfinder, including the stress tolerance genes and heavy metal genes, can be seen in **Table 3**. The Foster Farms outbreak isolates were mostly multidrug resistant. All of the isolates had genes for the efflux pump mdsAB, and eight of the isolates encoded a mixture of antimicrobial genes that conferred resistance to Aminoglycosides, Extended-Spectrum Beta Lactamases, Streptomycin, Spectinomycin, Phleomycin, Fosfomycin, Sulfonamides, Tetracycline, Florfenicol, and Chloramphenicol. These eight isolates also had tolerance genes for tellurium, arsenic, silver, gold, copper and mercury, and a stress tolerance gene encoding antiseptic resistance. Two of the Foster Farms outbreak isolates were pansusceptible and possessed only a single gene for resistance to Fosfomycin in addition to the efflux pump.

While the Roast Pork outbreak isolates were also multidrug resistant, they had fewer antimicrobial resistance genes than the Foster Farms isolates. The Roast Pork outbreak isolates displayed resistance genes for Aminoglycosides, Extended-Spectrum

Beta Lactamases, Sulfonamide, and Tetracycline, and also had genes for the MdsAB efflux pump. Metal tolerance genes across the Roast Pork isolates were pretty consistent, encoding tolerance to arsenic, silver, gold, copper, and mercury, and they did not display any stress-tolerance genes. The Chicken Liver outbreak isolates were antimicrobial resistant and encoded only a few antimicrobial resistance genes; the MdsAB efflux pump, and a Fosfomycin resistance gene. The metal tolerance for these isolates was also less, as they encoded tolerance to arsenic and gold only, and they possessed no stress-tolerance genes. The Peanut Butter outbreak isolates were by far the most susceptible to antibiotics, as they only possessed the genes for the MdsAB efflux pump, and much like the Chicken Liver outbreak isolates, they only had heavy metal tolerance genes to arsenic and gold.

The antimicrobial resistance genes displayed by the non-outbreak food isolates in this study also varied; while all six had the genes for the MdsAB efflux pump, the two *Salmonella* I 4,5,[12]:i:- non-outbreak isolates (isolates 24 and 25) encoded resistances to Aminoglycosides, Extended-Spectrum Beta Lactamases, Quinolones, and Sulfonamides, and tolerances to tellurium, arsenic, gold, copper, and silver. The non-outbreak *Salmonella* Heidelberg and *Salmonella* Enteritidis isolates, 26 and 28, displayed tolerance to Aminoglycosides, Extended-Spectrum Beta Lactamases, Sulfonamides, and Tetracycline, and had heavy metal tolerances to arsenic and gold. Finally, the last two non-outbreak isolates, *Salmonella* Heidelberg isolate 27 and *Salmonella* Typhimurium var 5- isolate 29 encoded only a few resistance genes, which varied amongst them. Isolate 27 encoded resistance to Fosfomycin, and tolerance to arsenic and gold, and isolate 29 encoded resistances to Sulfonamides and Tetracycline, and tolerance to mercury.

Discussion

The *S. enterica* isolates attached very well at room temperature in the nutrient rich conditions, as seen in **Figure 1**. It is likely that the abundance of nutrients that were present in the trypticase soy broth played a role in the growth and attachment capacity of the isolates, which were able to replicate and attach better than those in nutrient-poor conditions. The attachment capacity of the isolates in the nutrient-poor conditions at 22^{o}C was worse than in the nutrient-rich conditions, as observed in **Figure 2**. The lack of nutrients present in the broth could have limited the ability of the bacteria to secrete the extracellular polymeric matrix for the biofilm or prevented the abundant replication of the isolates prior to their attachment to the polystyrene plates, leading to a reduced amount of cellular biomass present in the plates when they were measured. However, the isolates present in the room temperature nutrient-poor and nutrient-rich plates still outperformed those incubated at 4^{o}C.

Previous studies have noted that *S. enterica* can persist at 4^{o}C for up to 28 days on food products and surfaces (181, 182). This accounts for the isolate attachment observed for the nutrient-rich and nutrient-poor plates - which can be seen in **Figure 3** and **Figure 4** –held at that temperature when compared to the 22^{o}C plates. The isolates incubated at 4^{o}C are unlikely to have grown significantly; however, the bacteria present in the plates at inoculation still attached to the polystyrene plates. The attachment of the isolates in the nutrient-rich conditions at 4^{o}C was worse than attachment at 22^{o}C, but there was still a trend of increased attachment over time present in these bacteria. The ability of these bacteria to attach at 4^{o}C and create a biofilm is an excellent persistence mechanism for *S. enterica*.

There were several notable isolates that attached well to the polystyrene plates under stressful conditions. For the Foster Farms outbreak isolates, both isolates 2 and 3 attached significantly well when in a nutrient-poor environment, and they displayed better attachment than other isolates under cold temperatures as well, suggesting that they excel at attaching to the polystyrene surface under stressful conditions. One of the Roast Pork outbreak isolates, isolate 17, also displayed enhanced attachment under stressful conditions as well (nutrient-poor and cold environments). The enhanced attachment of these isolates did not significantly impact their attachment in nutrient-rich conditions, which implies that while these isolates can tolerate nutrient-deprivation and cold better than the other study isolates, they are not significantly better at attachment in a nutrient-abundant environment.

Several isolates notably attached to the polystyrene plates significantly worse than the other isolates in nutrient-poor conditions; three Foster Farms outbreak isolates (6, 8, and 9) and two Roast Pork outbreak isolates (14 and 23) attached significantly worse under nutrient-poor conditions in both temperatures than the other isolates in this study. There were no observed significant differences in attachment for these isolates in nutrient-rich conditions, suggesting that these isolates could just be particularly bad at forming biofilms under stressful conditions compared to the others in this study. These isolates may have reacted slower to the depletion of nutrients than the strains that performed significantly better in the stressful environments, or they could have potentially downregulated biofilm-related genes during cellular starvation, leading to the poorer attachment of these isolates under the stressful conditions. Even with the poor biofilm forming abilities of these particular isolates, they could still pose a threat to the processing industry if they join an already-established biofilm.

The minimum inhibitory concentrations that were observed were concerningly high for sodium hypochlorite (**Table 2**). A possible explanation for why the MICs observed were so high could have to do with the mechanism of sodium hypochlorite. SH is very reactive and will react with any organic material present in the plate, including the bacteria (98); the reaction of the sanitizer with the nutrients as well as the bacteria could have failed to completely eradicate the bacteria present, leading to the re-population of the plates with the bacteria that tolerated the sanitizer. Additionally, bacteria could have been actively replicating using the available nutrients and thus may have outlasted the sodium hypochlorite. Since the sanitizer was not reapplied, it is likely that this could partially account for the high minimum inhibitory concentrations observed for the sodium hypochlorite, especially in the nutrient-rich plates.

The sodium hypochlorite was slightly more effective in the nutrient-poor conditions. In the nutrient-poor conditions, it is likely that the sodium hypochlorite also reacted with the available nutrients, but it also functioned more as a disinfectant with the bacteria present, as is reflected in the MICs of the nutrient-poor condition ranging from 50-200 ppm. However, even in nutrient-poor conditions, a few of the strains still managed to tolerate up to 200 ppm of the sanitizer; this could be due to the high concentration of bacteria present upon inoculation into the plates. Alternatively, the tolerance of the bacteria up to 200 ppm in the nutrient-poor conditions could be an issue of the sensitivity of the concentrations used in the assay. Since there is no intermediate measurement between 100 and 200 ppm, the bacteria could have been inhibited on the lower end of this range (closer to 100 ppm) and recorded as higher.

Forauer, E. C. previously noted that the sanitizer tolerance of *Listeria monocytogenes*, another foodborne pathogen, in nutrient-rich conditions at 22^{o}C ranged from 25-1600 ppm, with an average sanitizer tolerance of 867 ppm observed across all isolates (183). In nutrient-poor conditions, tolerance ranged from 25-400 ppm, with an average sodium hypochlorite tolerance of 151.3 ppm observed by Forauer, E. C. (183). This high tolerance could be attributed to the high nutrient concentration of the media used in her study. However, other studies on *S. enterica* have also found high sodium hypochlorite tolerance levels. Xiao et al. reported a tolerance of greater than 256 ppm for 161 of 172 total *S. enterica* isolates in their study (184), and Obe et al. found that in their study of *S. enterica*, the observed MICs for sodium hypochlorite ranged from 500-1000 ppm (185). Humayoun et al. noted in their study of 88 multidrug-resistant *S. enterica* isolates that these isolates displayed an average MIC of 3,152 ppm for sodium hypochlorite (186). The tolerance observed of *S. enterica* to sodium hypochlorite in this study and others was concerningly high and could account for the continued presence and persistence of *S. enterica* in the food processing industries that commonly use this sanitizer.

The minimum inhibitory concentrations for peroxyacetic acid (PAA) were also high in nutrient-rich conditions, ranging from 100-200 ppm (**Table 2**). Peroxyacetic acid is an unstable sanitizer which degrades fairly rapidly in water, and it also targets organic materials much like sodium hypochlorite (100); the high amount of nutrients present in the MICs may have aided the rapid degradation of the PAA present. This could explain why the isolates displayed higher tolerance to PAA in nutrient-rich conditions. In the nutrient-poor conditions, only one isolate displayed tolerance up to 50 ppm. Since 25 ppm was the lowest measurement used in this minimum inhibitory concentration assay, we can conclude that all but one of the isolates in nutrient-poor conditions displayed inhibition in the presence of the peroxyacetic acid.

In comparison to other studies, the MICs observed for these isolates for peroxyacetic acid in nutrient-rich conditions were intermediate compared to other MICs observed in other studies. Etter et al. found an average MIC of 73.6 ppm in their study of multidrug-resistant *Salmonella* Heidelberg isolates from the Foster Farms outbreak (178). Comparatively, Jolivet-Gougeon et al. found an MIC of 7 ppm was effective for inhibiting *Salmonella* Typhimurium LT2 (187), while Mourao et al. found that the *S. enterica* in their study had an MIC of 60-70 ppm (188). Humayoun et al. found a much higher average MIC of 880 ppm for the 88 multidrug-resistant *S. enterica* isolates in their study (186), and Micciche et al. noted an MIC of 500 ppm for household peroxyacetic acid, and 1000 ppm for industrial grade peroxyacetic acid in their study of *Salmonella* Typhimurium (189). These studies found a wide range of minimum inhibitory concentrations in *S. enterica* for peroxyacetic acid, which can be attributed to the difference in methodologies or media being used in each study, or could possibly be attributed to the fluctuating concentration of the active ingredient in peroxyacetic acid used in each study (188). The comparison between the tolerances in nutrient-rich and nutrient-poor conditions for this sanitizer in this study are stark and indicate that with appropriate monitoring in the processing industry, peroxyacetic acid sanitizers can be used effectively against *S. enterica*.

Eight of the 10 *Salmonella* Heidelberg Foster Farms outbreak-associated strains had a variety of antimicrobial resistance genes which encode resistance to 3 or more common classes of antibiotics, making these strains multidrug resistant, and a variety of heavy metal tolerance genes (**Table 3**). The two strains that were not multidrug resistant still had resistance genes for Fosfomycin and a few heavy metal tolerance genes encoding resistance to arsenic and gold. Of the *Salmonella* I 4,5,[12]:i:- strains, 12 of the strains were multidrug resistant, including 1 non-outbreak strain, and possessed 6 antimicrobial tolerance genes and 6 heavy metal resistance genes which encoded resistance to multiple antibiotic classes including beta lactams, sulfonamides, tetracycline, silver, arsenic, gold, and other antibiotics. One of the *Salmonella* I 4,5,[12]:i:- non-outbreak isolates possess a quinolone resistance gene and is multidrug resistant. Three other non-outbreak associated isolates (serovars Heidelberg, Enteritidis and Newport) are also multidrug resistant. The presence of antimicrobial resistance genes does seem biased towards outbreak-associated strains in this study, which may have acquired these tolerance genes in the food processing facility or in agricultural settings; this is especially likely in the case of the metal tolerance genes present in these strains, especially the Roast Pork outbreak strains (125).

The antimicrobial resistance genes were unsurprisingly present in a higher concentration in the poultry and pork outbreak-associated isolates, which could be attributed to the high amount of exposure to antibiotics these bacteria have in the animal agricultural industry (190), but the liver isolates didn't display a high amount of antimicrobial resistance. Procura et al. had previously noted in their study of *S. enterica* isolated from chicken livers that these isolates possessed few antimicrobial tolerance genes, with the exception of erythromycin resistance in all isolates, and 22% of the isolates had intermediate resistance to streptomycin (191). The non-outbreak associated isolates sourced from meats were a mixed bag in terms of antimicrobial resistance; half were pansusceptible, and half were multidrug resistant. The almond isolate and peanut butter isolates were unsurprisingly pansusceptible; being sourced from nuts cross-contaminated during processing, these strains would presumably be less likely to have the level of exposure to antibiotics that the poultry and pork-associated strains would have. This is corroborated in a study performed by Danyluk et al., which found that 73 of the 83 *S. enterica* isolates they sampled from raw almonds between 2001-2005 were resistant to 2 or less of the 15 antimicrobials screened for, with 52 of these isolates being pansusceptible (192). The remaining 10 isolates in their study displayed a variety of tolerances to 3 or more antimicrobials (192).

The antimicrobial resistance genes found in this study also varied between isolates from the same outbreak, as seen in the Foster Farms isolates, with a few being pansusceptible and most multidrug resistant (**Table 4**) (37). The diversity of these genes in these isolates could have resulted from the sourcing of these isolates, which were isolated from three separate processing facilities - these isolates may have acquired their tolerance genes independently in these facilities (37, 38). Comparatively, the Roast Pork outbreak isolates do not display much antimicrobial tolerance gene variation amongst themselves; they were isolated from a single facility (41). Overall, the variety of antimicrobial resistance genes observed in the isolates for this study could be a result of the direct exposure these isolates may have had to these antibiotics and to other bacteria pre- or post-processing.

All nine of the *S. enterica* strains exposed to heat shock survived past 30 minutes of exposure in a 56°C water bath, as seen in **Figure 1.** Eight of the nine *S. enterica* isolates were below detectable levels at 60 minutes post-scald, and one of the *S. enterica* isolates was detectable at 60 minutes. Several of the isolates used in this study had previously had heat shock performed on them under similar conditions by Etter et al. (178). Three of the Foster Farms outbreak isolates in this study previously displayed enhanced heat tolerance, survival past 60 minutes in 56°C scald, and are noted in **Table 5** (178). The survival of these strains in 56°C has alarming implications for the poultry processing industry, as scalds, one of the control measures commonly used to reduce the presence of *S. enterica* on carcasses, typically last under two minutes in length and involve temperatures from 54-59°C (159). While the heat tolerance observed for the strains isolated from peanut butter and raw almonds is also quite high at 56°C, this temperature often isn't used in the nut processing industry, as steam treatments and roasting treatments commonly use temperatures higher than 95°C (162). Other studies have noted the survival of *S. enterica* strains present in peanut butter at temperatures of up to 90°C, so the survival of these strains at 56°C is unsurprising. Ma et al. studied the survival of three *Salmonella* Tennessee outbreak strains in 90°C peanut butter for 50 minutes and compared the performance of these isolates to a cocktail of three *Salmonella* serovars, *Salmonella* Heidelberg, Enteritidis, and Typhimurium (193). The outbreak strains persisted past 50 minutes, as they were still detectable at 1 log CFU/g at the 50-minute measurement and weren't completely reduced below detectable levels until approximately 120 minutes of exposure to the 90°C treatment (193). Lower temperatures used in this study (71°C, 77°C, and 83°C) showed less efficacy and took longer to reduce the bacteria below detectable levels (193). Shachar and Yaron also observed high heat tolerance in their study of *Salmonella enterica* serovars Agona, Enteritidis and Typhimurium (194). They noted that there was only a 3.2-log reduction in CFU in peanut butter samples after exposure to 90ºC for 50 minutes; efficacy of the treatment was further reduced at the lower temperatures of 70ºC and 80ºC (194). Survival of the strains in this study in the 56ºC scald could be an indicator that these strains of *S. enterica* possess heat-tolerance genes; without the transcriptomics of these isolates, we cannot conclude for sure whether this is the case or which they might possess. Heat tolerance genes use heat shock proteins to help the *S. enterica* survive and mitigate cellular damage in the presence of extreme heat, and is a huge advantage to have in processing industries that use heat as an antimicrobial control (172).

There did not appear to be a correlation between the attachment capabilities of the study isolates and their tolerance to the sodium hypochlorite and peroxyacetic acid sanitizers. There also did not appear to be a link between the antimicrobial resistance of these isolates and their various stress tolerances; the pansusceptible isolates displayed comparable levels of sanitizer tolerance to the multidrug resistant isolates, and there weren't any noticeable trends in the attachment of the isolates based on their antimicrobial resistances. Heat tolerance was not noticeably correlated with increased sanitizer tolerance or attachment capabilities of the isolates and did not appear affected by the presence or absence of the antimicrobial resistance genes, stress tolerance genes, and heavy metal tolerance genes. The attachment to the polystyrene plates did not appear to vary considerably between outbreak and non-outbreak associated *S. enterica*. Both outbreak-associated and non-outbreak associated isolates attached reasonably well overall to the polystyrene plates in the nutrient-rich conditions, and while the isolates in the nutrient-poor conditions varied somewhat in their attachment to the polystyrene plates, there wasn't a noticeable difference in the attachment capabilities of these bacteria between outbreak and non-outbreak strains. Minimum inhibitory concentrations also did not appear to vary significantly between outbreak- and non-outbreak associated *S. enterica*. The non-outbreak associated strains present in the MIC assay displayed similar tolerances to those of the outbreak-associated strains. The lack of observable differences in the performance of the outbreak versus non-outbreak associated *S. enterica* implies that any strain of *S. enterica* could survive the processing industry and become an outbreak-associated strain, given the chance. We did not note any definitive differences in the outbreak- and non-outbreak-associated *S. enterica* in our study; however, this does not mean that there are not any differences in these strains, merely that more study would be needed to conclude definitely whether outbreak-associated strains do have enhanced tolerance to common processing stresses.

Future research on this topic could be beneficial if more non-outbreak associated strains were tested alongside outbreak-associated strains. As biofilms, heat tolerance and antimicrobial tolerance of foodborne pathogens are rising concerns, specific research could look at genes upregulated in stationary-phase cells during the exposure to antimicrobials and heat, and potentially lead to the development of new procedures to control this pathogen more effectively in the food processing industry. Currently, there are several promising methods that are rising in usage to reduce the presence of common foodborne pathogens such as *S. enterica*, *C. jejuni*, *L. monocytogenes*, *E. coli* and others in the processing industry.

The first of these methods is the use of competitive exclusion and involves the addition of probiotics to feed products used in the agricultural industry to prevent the colonization, adhesiveness, and invasiveness of pathogens such as *C. jejuni* and non-typhoidal *S. enterica* (195, 196). Cultures of *Lactobacillus*, *Bacillus*, and *Enterococcus* species are commonly studied for their efficacy in reducing *Campylobacter spp.* in chick and chicken populations (197). These probiotics can be quite effective as feed additives, or as oral pills administered to animals, and can replace antibiotics as an effective method of controlling pathogens in livestock (198). Additionally, the use of probiotics in the processing industry is also rising, as when applied to surfaces following proper cleaning and sanitation, these bacteria can become established in biofilms on surfaces and provide competition for pathogens that are also present (199). This method of using probiotics to outcompete the pathogens present in a biofilm is an excellent addition to potential *S. enterica* control methods for the processing industry and could be effective in reducing the number of outbreaks of this pathogen in the future.

Nanoparticles (NPs) are another useful method – they can reduce microbial growth, both in planktonic form and in biofilms (200). Nanoparticles made up of nanomaterials such as zinc oxide, silver, and titanium dioxide can combat the growth of foodborne pathogens during both processing and storage and inhibit the attachment of these microbes to surfaces (200). Since zinc oxide is a trace element in agricultural feed, this nanoparticle is particularly well-adapted for use in pre-processing; it is reasonably small, and its' antimicrobial action is through the release of reactive oxygen species onto its surface (201). Zinc oxide and titanium oxide nanoparticles have been proven in multiple studies to be effective against *Salmonella* spp., *L. monocytogenes,* and *E. coli* (201-203).

Other nanoparticles are known to interrupt the electron transport in the cellular membrane, penetrate the cellular membrane and affect the normal function of ribosomes, enzymes, and DNA (204-206). The antimicrobial efficacy of different nanoparticles varies, however, depending on the size of the nanoparticles – generally, smaller nanoparticles are better at antimicrobial action against the bacteria (198). Nanoparticles can function as an agricultural feed additive, but are also promising when used as a coating for antimicrobial food-contact surfaces, or used for smart packaging applications to inhibit potential growth on packaging products (200). Nanoparticles can also be used as a sanitizer method, but their efficacy depends on the type of nanoparticle being used to treat the surfaces (200). Additionally, these particles could prove to be less effective in the presence of a pathogen possessing a copy of the corresponding heavy metal tolerance gene, such as silver tolerance (205). In the presence of *S. enterica* which can tolerate sodium hypochlorite, as seen in our studies and other studies in the field, nanoparticles as a sanitation method could prove to be a more effective control method, especially in combination with other antimicrobial control methods.

Antimicrobial coatings, also known as edible films, are a more recent method used in the meat processing industry, specifically, to delay the spoilage of meat products and prevent the growth of common pathogens (198, 207, 208). These coatings prevent the adherence of cells and the formation of biofilms and can limit nutrient availability for any bacteria present on the meat (207). Carnauba wax coatings have been proven to reduce the attachment of *S. enterica* to chicken carcasses, and other wax coatings are commonly used in the fruit processing industry to prevent the growth of molds and to prevent contamination from microbes (209). Antimicrobial coatings are an excellent microbial growth control method and can be used in conjunction with nanoparticles, probiotics, and bacteriophage to control foodborne pathogens in the processing industry.

Bacteriophages are bacterial viruses that target and kill specific bacteria through self-replication, and are considered a promising intervention for the processing industry; however, they are one of the interventions that requires more active oversight than others, as they are inactivated by non-ideal environmental conditions, including low oxygen levels, extreme pH levels, high temperatures in processing, and radiation from UV (210, 211). Bacteriophages can currently be used effectively in several areas of food processing, including for bio-sanitation in the fruit, vegetable, and poultry processing industries, which includes the disinfection of food-contact surfaces and other processing equipment, and bio-control, with usage on a variety of food products to inhibit the colonization of these products by pathogens including *S. enterica* and *Listeria monocytogenes* (198, 211). There are a variety of different phages that work on different foods and target specific pathogens, so choosing the correct bacteriophage can be vital to the efficacy of this treatment.

The use of nanoparticles, food coatings, probiotics and bacteriophages can help to reduce the prevalence of *S. enterica* in the processing industry and may be an effective method to prevent more outbreaks of this pathogen. Currently, interventions such as these are needed in the food processing industry to work in combination with current sanitation control methods – a closer look into the continued use of sanitizers such as sodium hypochlorite, which has shown to be not as effective as other treatments, and the substitution of this sanitizer for another method could be needed to help prevent future outbreaks of non-typhoidal *S. enterica* and other foodborne pathogens.

TABLES

Table 1: All *S. enterica* Strains, Serovars, Sourcing and Outbreaks.

Isolate	**Serovar**	**Outbreak**[a]	**Sourced From**
FSL M2-0001	Heidelberg	Foster Farms, 2013-2014	USDA Culture Collection
FSL M2-0002	Heidelberg	Foster Farms, 2013-2014	USDA Culture Collection
FSL M2-0003	Heidelberg	Foster Farms, 2013-2014	USDA Culture Collection
FSL M2-0004	Heidelberg	Foster Farms, 2013-2014	USDA Culture Collection
FSL M2-0005	Heidelberg	Foster Farms, 2013-2014	USDA Culture Collection
FSL M2-0006	Heidelberg	Foster Farms, 2013-2014	USDA Culture Collection
FSL M2-0007	Heidelberg	Foster Farms, 2013-2014	USDA Culture Collection
FSL M2-0008	Heidelberg	Foster Farms, 2013-2014	USDA Culture Collection
FSL M2-0009	Heidelberg	Foster Farms, 2013-2014	USDA Culture Collection
FSL M2-0010	Heidelberg	Foster Farms, 2013-2014	USDA Culture Collection
FSL M2-0011	I 4,5,[12]:i:-	Roast Pork, 2015	USDA Culture Collection
FSL M2-0012	I 4,5,[12]:i:-	Roast Pork, 2015	USDA Culture Collection
FSL M2-0013	I 4,5,[12]:i:-	Roast Pork, 2015	USDA Culture Collection
FSL M2-0014	I 4,5,[12]:i:-	Roast Pork, 2015	USDA Culture Collection
FSL M2-0015	I 4,5,[12]:i:-	Roast Pork, 2015	USDA Culture Collection
FSL M2-0016	I 4,5,[12]:i:-	Roast Pork, 2015	USDA Culture Collection
FSL M2-0017	I 4,5,[12]:i:-	Roast Pork, 2015	USDA Culture Collection
FSL M2-0018	I 4,5,[12]:i:-	Roast Pork, 2015	USDA Culture Collection
FSL M2-0019	I 4,5,[12]:i:-	Roast Pork, 2015	USDA Culture Collection
FSL M2-0020	I 4,5,[12]:i:-	Roast Pork, 2015	USDA Culture Collection

FSL M2-0021	I 4,5,[12]:i:-	Roast Pork, 2015	USDA Culture Collection
FSL M2-0022	I 4,5,[12]:i:-	OA, Pork Sausage	USDA Culture Collection
FSL M2-0023	I 4,5,[12]:i:-	OA, Ground Pork	USDA Culture Collection
FSL M2-0024	I 4,5,[12]:i:-	NOA, Food (Italian Sausage)	USDA Culture Collection
FSL M2-0025	I 4,5,[12]:i:-	NOA, Food (Pork Feet)	USDA Culture Collection
FSL M2-0026	Heidelberg	NOA, Food (Ground Turkey)	FDA
FSL M2-0027	Heidelberg	NOA, Food (Ground Turkey)	FDA
FSL M2-0028	Enteritidis	NOA, Food (Chicken Breast)	FDA
FSL M2-0029	Typhimurium var. 5-	NOA, Food (Chicken Breast)	FDA
FSL M2-0030	Heidelberg	Kosher Broiled Chicken Livers, 2012	NY State Food Laboratory
FSL M2-0031	Heidelberg	Kosher Broiled Chicken Livers, 2012	NY State Food Laboratory
FSL M2-0032	Heidelberg	Kosher Broiled Chicken Livers, 2012	NY State Food Laboratory
FSL M2-0033	Enteritidis	Paramount Farm Almonds, 2003-2004	ATCC
FSL M2-0047	Typhimurium	Peanut Corp of America, 2008-2009	FDA
FSL M2-0048	Typhimurium	Peanut Corp of America, 2008-2009	FDA

[a]Outbreaks are stated with their corresponding years; NOA, indicating non-outbreak associated, are stated with the isolation source (specific food or environmental).

Table 2: *S. enterica* Minimum Inhibitory Concentrations of Peroxyacetic Acid (Inspexx-250) and Sodium Hypochlorite (Clorox).

Isolate	**Serovar**	**Outbreak/Source**	**PAA MIC (≥) 1/20x,1x (ppm)[a]**	**SH MIC (≥) 1/20x,1x (ppm)[b]**
FSL M2-0001	Heidelberg	Foster Farms, 2013-2014	25, 200	100, 200

FSL M2-0002	Heidelberg	Foster Farms, 2013-2014	25, 100	50, 200
FSL M2-0003	Heidelberg	Foster Farms, 2013-2014	25, 200	100, 200
FSL M2-0004	Heidelberg	Foster Farms, 2013-2014	25, 100	100, 200
FSL M2-0005	Heidelberg	Foster Farms, 2013-2014	25, 200	50, 200
FSL M2-0006	Heidelberg	Foster Farms, 2013-2014	25, 200	100, 200
FSL M2-0007	Heidelberg	Foster Farms, 2013-2014	25, 200	50, 200
FSL M2-0008	Heidelberg	Foster Farms, 2013-2014	25, 200	100, 200
FSL M2-0009	Heidelberg	Foster Farms, 2013-2014	25, 200	100, 200
FSL M2-0010	Heidelberg	Foster Farms, 2013-2014	25, 100	100, 200
FSL M2-0011	I 4,5,[12]:i:-	Roast Pork, 2015	50, 100	200, 200
FSL M2-0012	I 4,5,[12]:i:-	Roast Pork, 2015	25, 200	50, 200
FSL M2-0013	I 4,5,[12]:i:-	Roast Pork, 2015	25, 100	50, 200
FSL M2-0014	I 4,5,[12]:i:-	Roast Pork, 2015	25, 200	100, 200
FSL M2-0015	I 4,5,[12]:i:-	Roast Pork, 2015	25, 200	100, 200
FSL M2-0016	I 4,5,[12]:i:-	Roast Pork, 2015	25, 200	50, 200
FSL M2-0017	I 4,5,[12]:i:-	Roast Pork, 2015	25, 100	50, 200
FSL M2-0018	I 4,5,[12]:i:-	Roast Pork, 2015	25, 200	100, 200
FSL M2-0019	I 4,5,[12]:i:-	Roast Pork, 2015	25, 200	50, 200
FSL M2-0020	I 4,5,[12]:i:-	Roast Pork, 2015	25, 200	50, 200
FSL M2-0021	I 4,5,[12]:i:-	Roast Pork, 2015	25, 200	100, 200
FSL M2-0022	I 4,5,[12]:i:-	Roast Pork, 2015	25, 200	100, 200
FSL M2-0023	I 4,5,[12]:i:-	Roast Pork, 2015	25, 200	200, 200
FSL M2-0024	I 4,5,[12]:i:-	NOA, Food (Italian Sausage)	25, 200	200, 200

FSL M2-0025	I 4,5,[12]:i:-	NOA, Food (Pork Feet)	25, 100	100, 200
FSL M2-0026	Heidelberg	NOA, Food (Ground Turkey)	25, 200	50, 200
FSL M2-0027	Heidelberg	NOA, Food (Ground Turkey)	25, 200	200, 200
FSL M2-0028	Enteritidis	NOA, Food (Chicken Breast)	25, 200	200, 200
FSL M2-0029	Typhimurium var. 5-	NOA, Food (Chicken Breast)	25, 200	100, 200

[a]PAA, peroxyacetic acid sanitizer; MIC, minimum inhibitory concentration required to prevent growth; ppm, parts per million.
[b]SH, sodium hypochlorite sanitizer; MIC, minimum inhibitory concentration required to prevent growth; ppm, parts per million.

Table 3: *S. enterica* Antimicrobial Resistance Genes, Stress Tolerance Genes and Heavy Metal Resistance Genes from AMRfinder (v. 3.8.4).

Isolate	**AMR Genes[a]**	**Heavy Metal Genes**	**Stress Genes**
FSL M2-0001	*aac(3)-VIa, aadA1, aph(3")-Ib, aph(3')-Iia, aph(6)-Ic, aph(6)-Id, ble, fosA7, mdsAB, sul1, tet(B)*	*silABCEFPRS, terDWZ, asr, arsR, golST, pcoABCDERS*	*qacEdelta1*
FSL M2-0002	*ant(2")-1a, aph(3")-1b, aph(6)-1d, blaTEM-1, cmlA5, floR, fosA7, mdsAB, sul2, tet(A)*	*arsR, golST, merABDPRT, asr,*	*qacEdelta1*
FSL M2-0003	*aac(3)-Via, aadA1, aph(3")-1b, aph(3')-11a, aph(6)-Ic, aph(6)-1d, ble, fosA7, mdsAB, sul1, tetB*	*arsR, golST,pcoABCDERS, silABCEFPRS, terDWZ,asr*	*qacEdelta1*
FSL M2-0004	*ant(2")-1a, aph(3")-1b, aph(6)-1d, blaTEM-1, cmlA5, floR, fosA7, mdsAB, sul2, tet(A)*	*arsR, golST, merABDEPRT, asr*	*qacEdelta1*
FSL M2-0005	*fosA7, mdsAB*	*arsR, golST, asr*	
FSL M2-0006	*aac(3)-Via, aadA1, aph(3")-1b, aph(3')-11a, aph(6)-Ic, aph(6)-1d, ble, fosA7, mdsAB, sul1, tetB*	*arsR, golST, pcoABCDERS, SilABCEFPRS, terDWZ, asr*	*qacEdelta1*
FSL M2-0007	*ant(2")-1a, aph(3")-1b, aph(6)-1d, blaTEM-1,*	*arsR, golST, merABDEPRT, asr*	*qacEdelta1*

	cmlA5, floR, fosA7, mdsAB, sul2, tet(A)		
FSL M2-0008	*fosA7, mdsAB*	*arsR, golST, asr*	
FSL M2-0009	*aac(3)-Via, aadA1, aph(3")-1b, aph(3')-11a, aph(6)-Ic, aph(6)-1d, ble, fosA7, mdsAB, sul1, tetB*	*arsR, golST, pcoABCDERS, SilABCEFPRS, terDWZ, asr*	*qacEdelta1*
FSL M2-0010	*aac(3)-Via, aadA1, aph(3")-1b, aph(3')-11a, aph(6)-Ic, aph(6)-1d, ble, fosA7, mdsAB, sul1, tetB*	*arsR, golST, pcoABCDRS, silABCEFPRS, terDWZ, asr*	*qacEdelta1*
FSL M2-0011	*aph(3")-1b, aph(6)-1d, blaTEM-1, mdsAB, sul2, tet(B)*	*arsABCDR, golST, merACDEPRT, pcoACDRS, silABCEFPRS, asr*	
FSL M2-0012	*aph(3")-1b, aph(6)-1d, blaTEM-1, mdsAB, sul2, tet(B)*	*arsABCDR, golST, merACDEPRT, pcoACDRS, silABCEFPRS, asr*	
FSL M2-0013	*aph(3")-1b, aph(6)-1d, blaTEM-1, mdsAB, sul2, tet(B)*	*arsABCDR, golST, merACDEPRT, pcoACDRS, silABCEFPRS, asr*	
FSL M2-0014	*aph(3")-1b, aph(6)-1d, blaTEM-1, mdsAB, sul2, tet(B)*	*arsABCDR, golST, merACDEPRT, pcoACDRS, silABCEFPRS, asr*	
FSL M2-0015	*aph(3")-1b, aph(6)-1d, blaTEM-1, mdsAB, sul2, tet(B)*	*arsABCDR, golST, merACDEPRT, pcoACDRS, silABCEFPRS, asr*	
FSL M2-0016	*aph(3")-1b, aph(6)-1d, blaTEM-1, mdsAB, sul2, tet(B)*	*arsABCDR, golST, merACDEPRT, pcoACDRS, silABCEFPRS, asr*	
FSL M2-0017	*aph(3")-1b, aph(6)-1d, blaTEM-1, mdsAB, sul2, tet(B)*	*arsABCDR, golST, merACDEPRT, pcoACDRS, silABCEFPRS, asr*	
FSL M2-0018	*aph(3")-1b, aph(6)-1d, blaTEM-1, mdsAB, sul2, tet(B)*	*arsABCDR, golST, merACDEPRT, pcoACDRS, silABCEFPRS, asr*	
FSL M2-0019	*aph(3")-1b, aph(6)-1d, blaTEM-1, mdsAB, sul2, tet(B)*	*arsABCDR, golST, merACDEPRT,*	

		pcoACDRS, silABCEFPRS, asr	
FSL M2-0020	*aph(3")-1b, aph(6)-1d, blaTEM-1, mdsAB, sul2*	*arsABCDR, golST, merACDEPRT, pcoACDRS, silABCEFPRS, asr*	
FSL M2-0021	*aph(3")-1b, aph(6)-1d, blaTEM-1, mdsAB, sul2, tet(B)*	*arsABCDR, golST, merACDEPRT, pcoACDRS, silABCEFPRS, asr*	
FSL M2-0022	*aph(3")-1b, aph(3')-1a, aph(6)-1d, blaTEM-1, mdsAB, sul2, tet(B)*	*arsABCDR, golST, merACDEPRT, pcoACDRS, silABCEFPRS, asr*	
FSL M2-0023	*aph(3")-1b, aph(3')-1a, aph(6)-1d, blaTEM-1, mdsAB, sul2, tet(B)*	*arsABCDR, golST, merACDEPRT, pcoACDRS, silABCEFPRS, asr*	
FSL M2-0024	*aph(3")-1b, aph(3')-1a, aph(6)-1d, blaTEM-1, mdsAB, sul2*	*arsABCDR, golST, pcoACDRS, silABCEFPRS, asr*	
FSL M2-0025	*blaSHV-12, mdsAB, qnrB19*	*arsABCDR, golST, pcoACDRS, silABCEFPRS, terDWZ*	
FSL M2-0026	*aac(3)-11d, aadA1, aph(3")-1b, blaTEM-1, fosA7, mdsAB, tet(A)*	*arsR, golST, asr*	
FSL M2-0027	*fosA7, mdsAB*	*arsR, golST, asr*	
FSL M2-0028	*aph(3")-1b, aph(6)-1d, blaTEM-1, mdsAB, sul2, tet(A)*	*golST, asr*	
FSL M2-0029	*mdsAB, sul2, tet(A)*	*golST, merAPR, asr*	
FSL M2-0030	*fosA7, mdsAB*	*arsR, golST, asr*	
FSL M2-0031	*fosA7, mdsAB*	*arsR, golST, asr*	
FSL M2-0032	*fosA7, mdsAB*	*arsR, golST, asr*	
FSL M2-0033	*fosA7, mdsAB*	*arsR, golST, asr*	
FSL M2-0047	*mdsAB*	*golST, asr*	
FSL M2-0048	*mdsAB*	*golST, asr*	

[a]AMR, antimicrobial resistance.

Table 4: Summarized Results of Attachment Assays, MICs, and Heat Shock Experiments Sorted by *S. enterica* Isolates and Outbreaks.

Isolate	Outbreak	Attachment[a] 1/20x in 4ºC, 22ºC	Attachment 1x in 4ºC, 22ºC	MICs[b] (SH) 1/20x, 1x	MICs (PAA) 1/20x, 1x	Heat Tolerance[c]
FSL M2-0001	Foster Farms, 2013-2014	Good (> 5), Poor (< 7)	Intermediate, Intermediate	Intermediate, High	Low, High	No Data
FSL M2-0002	Foster Farms, 2013-2014	Good (> 11), Good (> 8)	Intermediate, Intermediate	Intermediate, High	Low, Intermediate	60+ mins (Etter et al.)
FSL M2-0003	Foster Farms, 2013-2014	Good (> 13), Good (> 15)	Intermediate, Intermediate	Intermediate, High	Low, High	No Data
FSL M2-0004	Foster Farms, 2013-2014	Intermediate, Intermediate	Intermediate, Intermediate	Intermediate, High	Low, Intermediate	No Data
FSL M2-0005	Foster Farms, 2013-2014	Intermediate, Intermediate	Intermediate, Intermediate	Intermediate, High	Low, High	60+ mins (Etter et al.)
FSL M2-0006	Foster Farms, 2013-2014	Poor (> 3), Poor (> 10)	Intermediate, Intermediate	Intermediate, High	Low, High	No Data
FSL M2-0007	Foster Farms, 2013-2014	Intermediate, Intermediate	Intermediate, Intermediate	Intermediate, High	Low, High	No Data
FSL M2-0008	Foster Farms, 2013-2014	Poor (> 4), Poor (> 7)	Intermediate, Intermediate	Intermediate, High	Low, High	60+ mins (Etter et al.)
FSL M2-0009	Foster Farms, 2013-2014	Poor (> 3), Poor (> 7)	Intermediate, Intermediate	Intermediate, High	Low, High	No Data
FSL M2-0010	Foster Farms, 2013-2014	Intermediate, Good (> 8)	Intermediate, Intermediate	Intermediate, High	Low, Intermediate	No Data
FSL M2-0011	Roast Pork, 2015	Intermediate, Intermediate	Intermediate, Intermediate	High, High	Low, Intermediate	45 mins
FSL M2-0012	Roast Pork, 2015	Poor (> 3), Intermediate	Good (> 4), Good (> 5)	Intermediate, High	Low, High	60 mins
FSL M2-0013	Roast Pork, 2015	Intermediate, Intermediate	Intermediate, Intermediate	Intermediate, High	Low, Intermediate	45 mins

FSL M2-0014	Roast Pork, 2015	Poor (> 5), Poor (> 7)	Intermediate, Intermediate	Intermediate, High	Low, High	No Data
FSL M2-0015	Roast Pork, 2015	Poor (> 7), Intermediate	Intermediate, Intermediate	Intermediate, High	Low, High	No Data
FSL M2-0016	Roast Pork, 2015	Intermediate, Good (> 8)	Intermediate, Intermediate	Intermediate, High	Low, High	No Data
FSL M2-0017	Roast Pork, 2015	Good (> 13), Good (> 19)	Intermediate, Intermediate	Intermediate, High	Low, High	No Data
FSL M2-0018	Roast Pork, 2015	Poor (> 4), Intermediate	Intermediate, Intermediate	Intermediate, High	Low, High	No Data
FSL M2-0019	Roast Pork, 2015	Poor (> 6), Intermediate	Intermediate, Intermediate	Intermediate, High	Low, High	No Data
FSL M2-0020	Roast Pork, 2015	Good (> 3), Intermediate	Intermediate, Intermediate	Intermediate, High	Low, High	No Data
FSL M2-0021	Roast Pork, 2015	Intermediate, Intermediate	Intermediate, Intermediate	Intermediate, High	Low, High	No Data
FSL M2-0022	Roast Pork, 2015	Intermediate, Poor (> 10)	Intermediate, Intermediate	Intermediate, High	Low, High	No Data
FSL M2-0023	Roast Pork, 2015	Poor (> 3), Poor (> 7)	Intermediate, Intermediate	High, High	Low, High	No Data
FSL M2-0024	NOA, Food (Italian Sausage)	Intermediate, Poor (> 7)	Intermediate, Intermediate	High, High	Low, High	No Data
FSL M2-0025	NOA, Food (Pork Feet)	Poor (> 3), Good (> 8)	Intermediate, Intermediate	Intermediate, High	Low, Intermediate	No Data
FSL M2-0026	NOA, Food (Ground Turkey)	No Data	No Data	Intermediate, High	Low, High	No Data
FSL M2-0027	NOA, Food (Ground Turkey)	No Data	No Data	High, High	Low, High	No Data

FSL M2-0028	NOA, Food (Chicken Breast)	No Data	No Data	High, High	Low, High	No Data
FSL M2-0029	NOA, Food (Chicken Breast)	No Data	No Data	Intermediate, High	Low, High	No Data
FSL M2-0030	Kosher Broiled Chicken Livers, 2012	No Data	No Data	No Data	No Data	60 mins
FSL M2-0031	Kosher Broiled Chicken Livers, 2012	No Data	No Data	No Data	No Data	45 mins
FSL M2-0032	Kosher Broiled Chicken Livers, 2012	No Data	No Data	No Data	No Data	60 mins
FSL M2-0033	Paramount Farm Almonds, 2003-2004	No Data	No Data	No Data	No Data	60+ mins (A single replicate)
FSL M2-0047	Peanut Corp of America, 2008-2009	No Data	No Data	No Data	No Data	60 mins
FSL M2-0048	Peanut Corp of America, 2008-2009	No Data	No Data	No Data	No Data	60 mins

[a]From the crystal violet attachment assays; good, intermediate, poor attachment (for significance, the isolate performed better (good) or worse (poor) than at least 3 others).
[b]Minimum inhibitory concentrations; high (≥200), intermediate (100, 50), low (25).
[c]Heat tolerance; Time of Survival (60+ indicates beyond 60 minutes).

FIGURES

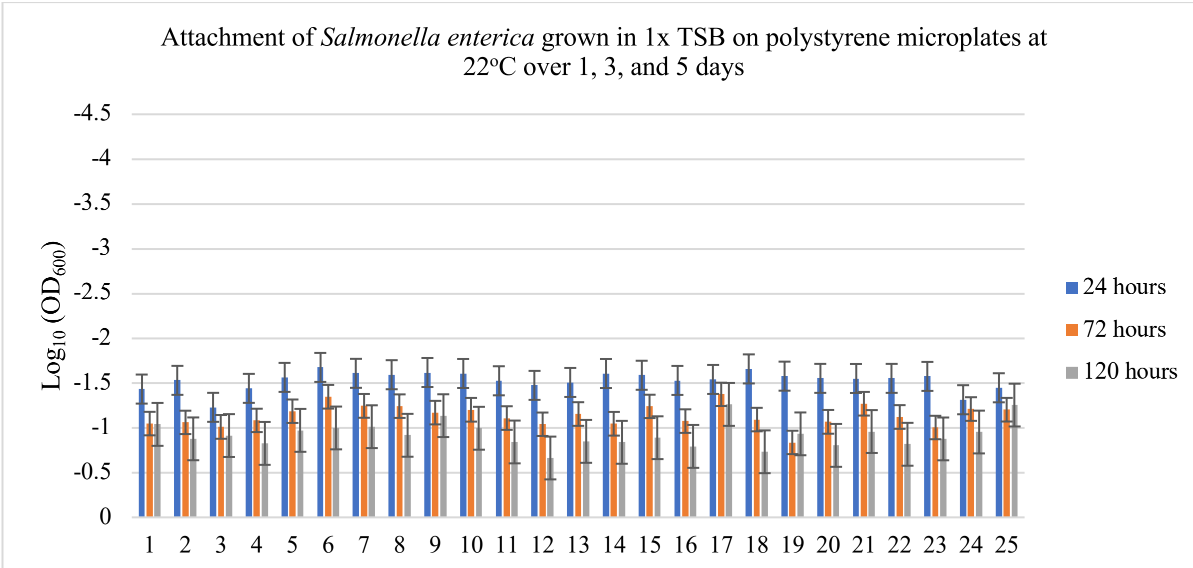

Figure 1: The attachment of Salmonella enterica serovars Heidelberg (Foster Farms, 1-10) and I 4,5,[12]:i:- (Kapowsin Meats, 11-23; NOA 24-25) on polystyrene plates when incubated at 22°C for 24, 72, and 120 hours (1, 3, and 5 days) in nutrient-rich (1x TSB) conditions. Error bars represent standard deviation between experimental replicates.

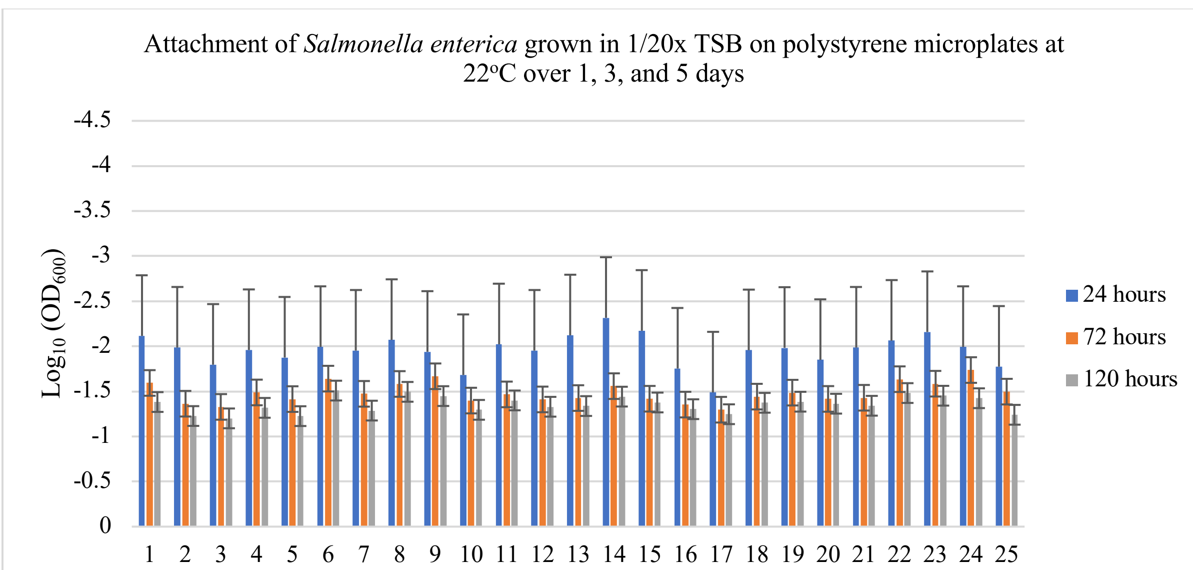

Figure 2: The attachment of *S. enterica* serovars Heidelberg (Foster Farms, 1-10) and I 4,5,[12]:i:- (Kapowsin Meats, 11-23; NOA 24-25) on polystyrene plates when incubated at 22°C for 24, 72, and 120 hours (1, 3, and 5 days) in nutrient-poor (1/20x TSB) conditions. Error bars represent standard deviation between experimental replicates.

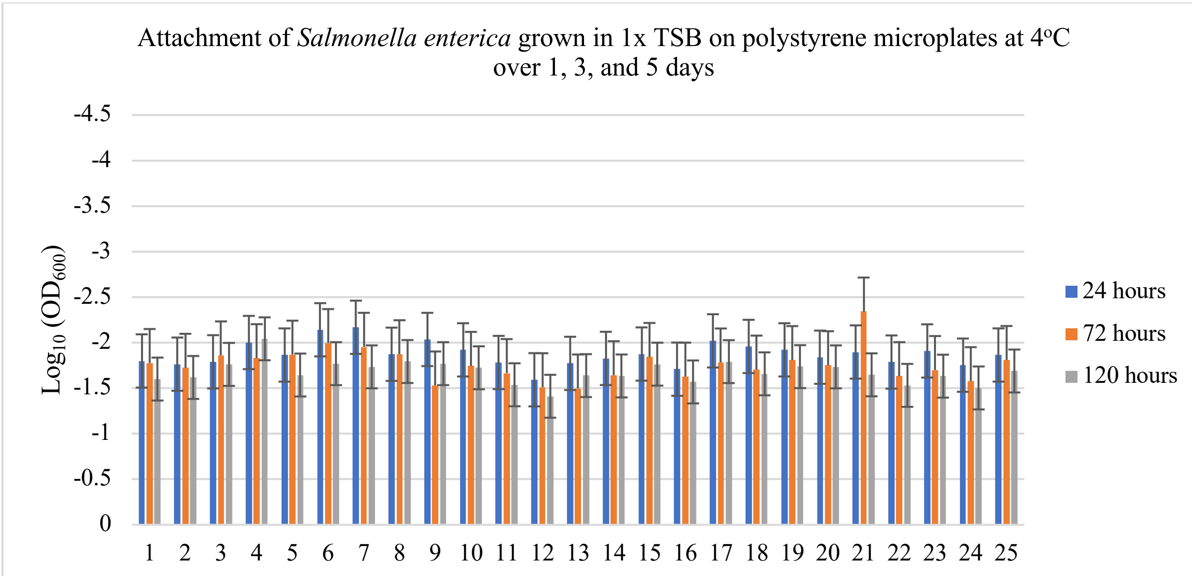

Figure 3: The attachment of Salmonella enterica serovars Heidelberg (Foster Farms, 1-10) and I 4,5,[12]:i:- (Kapowsin Meats, 11-23; NOA 24-25) on polystyrene plates when incubated at 4ºC for 24, 72, and 120 hours (1, 3, and 5 days) in nutrient-rich (1x TSB) conditions. Error bars represent standard deviation between experimental replicates.

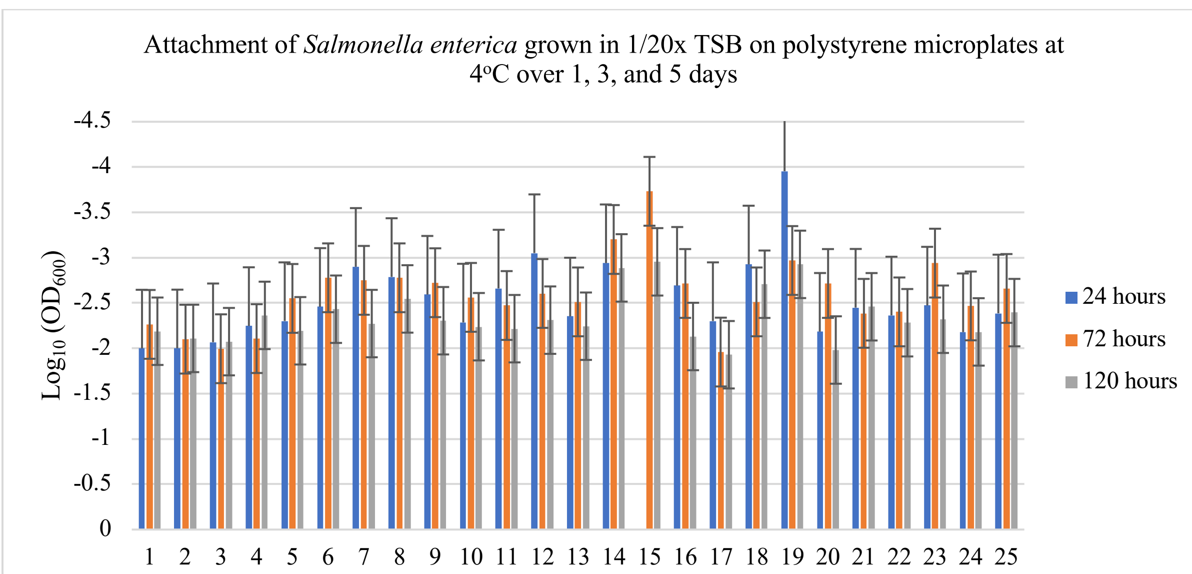

Figure 4: The attachment of *S. enterica* serovars Heidelberg (Foster Farms, 1-10) and I 4,5,[12]:i:- (Kapowsin Meats, 11-23; NOA 24-25) on polystyrene plates when incubated at 4ºC for 24, 72, and 120 hours (1, 3, and 5 days) in nutrient-poor (1/20x TSB) conditions. Error bars represent standard deviation between experimental replicates.

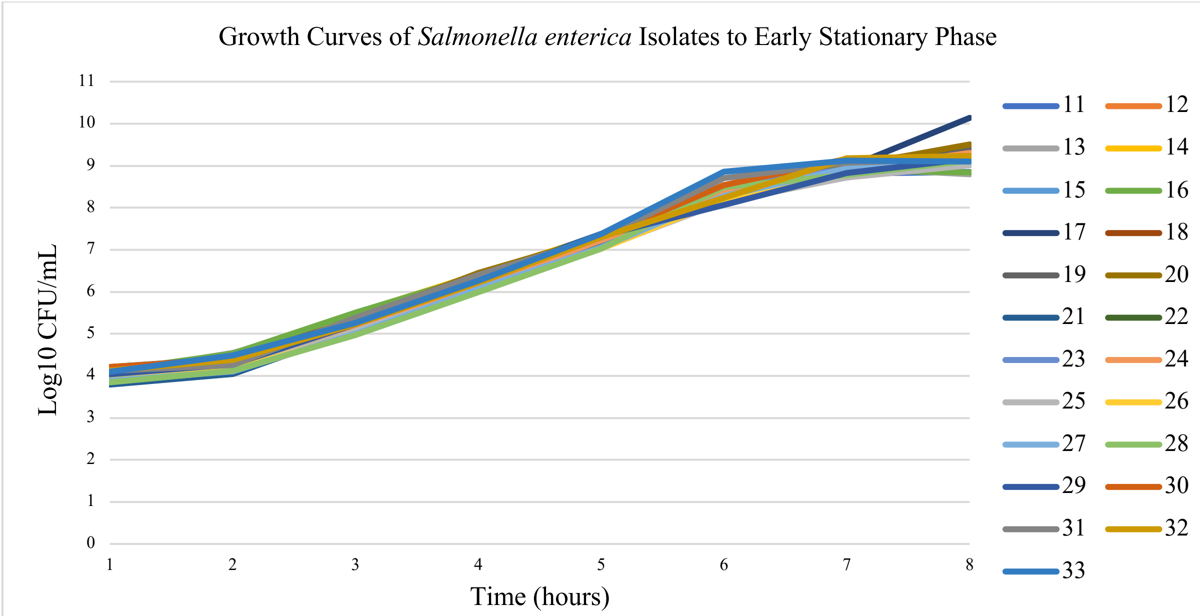

Figure 5: The growth curves of the *S. enterica* isolates over 8 hours from early exponential phase to early stationary phase, where the growth of the bacteria levels off. Isolates 11-23 are from the Roast Pork outbreak, while 24-29 and 34 are NOA isolates, 30-32 are from the Chicken Liver outbreak, and 33 is from the Almond outbreak.

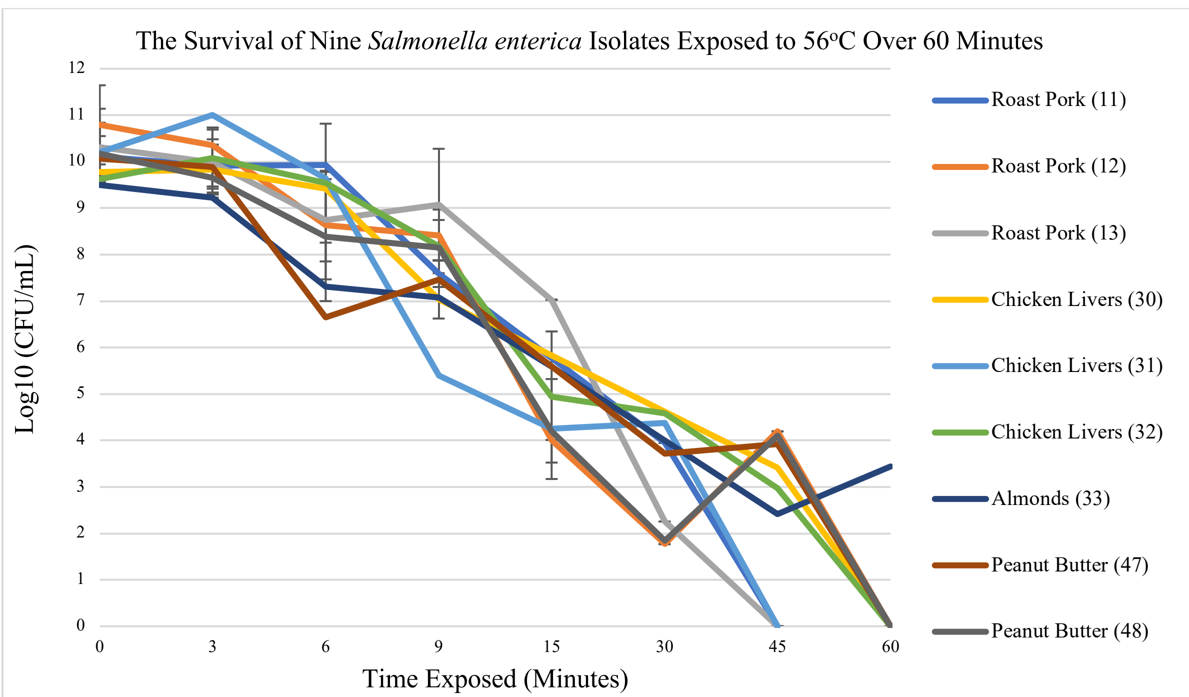

Figure 6: The survival of nine outbreak-associated *S. enterica* isolates exposed to a 56ºC scald from 0-60 minutes.

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