The effects of sub-lethal chlorine induced oxidative stress on biofilm formation and thermal resistance of *Salmonella*

By

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The effect of sub-lethal chlorine stress on various strains/serotypes of *Salmonella* on biofilm formation and thermal resistance was studied. The effect of oxidative stress (induced by 150 ppm of chlorine in TSB) on *Salmonella* biofilm formation on polystyrene and stainless steel surfaces at three temperatures (4°C, 30°C, and room temperature) in nutrient rich (full strength TSB) and nutrient limited conditions (1/10th TSB) was evaluated. On polystyrene surface, chlorine stressed *S.* Heidelberg (strain ID 72), *S.* Newport (strain ID 107) and *S.* Typhimurium (ATCC 14028) formed stronger (P < 0.05) biofilms at 30°C. On stainless steel, the chlorine stressed *S.* Heidelberg (ATCC 8326) and *S.* Enteritidis (ATCC 4931) at room temperature formed stronger (P < 0.05) biofilms as compared to the non-stressed control cells. The thermal resistance of short-term (1h) and long-term (27d) chlorine stressed *Salmonella* Heidelberg and *S.* Typhimurium were compared with the non-stressed controls at three different temperatures (55°C, 58°C and 61°C) and two growth phases (logarithmic and stationary). The short-term stressed log phase cells (both serotypes) were found to be more sensitive
(P< 0.05) to thermal inactivation in TSB. Upon long-term sub-lethal chlorine exposure, *Salmonella* developed a rugose morphotype on tryptic soy agar at 37°C. The rugose morphotype provided significant thermal protection (P< 0.05) against heat stress as compared to smooth morphotype. In chicken broth, at 55°C, short-term chlorine stressed stationary phase *S. Typhimurium* displayed a higher D$_{55}$ value compared to non-stressed cells. The findings from this research reveal that some *Salmonella* strains have the potential to form stronger biofilms and exhibit higher thermal tolerance upon exposure to sub-lethal chlorine concentration.
DEDICATION

To my parents and family
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I would like to express my deep gratitude and sincerity to my advisors Dr. Chander Shekhar Sharma and Dr. Chris McDaniel for their incessant help throughout the study period. Dr. Sharma’s understandings and empathies during my ups and downs in this period helped me to achieve this feat. His constant guidance, expertise and support throughout the study made this research work possible. The research works would have no significance without the open-hearted and tremendous assistance in statistic and data analysis part by Dr. McDaniel.

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CHAPTER I
INTRODUCTION

Non-typhoidal *Salmonella* accounts for 11% of an estimated 9.4 million foodborne illnesses in the U.S. every year (Scallan et al., 2011). In 2014, *Salmonella* was responsible for 27% of foodborne illnesses and 59% of hospitalizations (CDC, 2014). Poultry meat and eggs are among the common sources of non-typhoidal *Salmonella* transmission to the food chain (Howard et al., 2012). Three major outbreaks caused by *Salmonella* linked to handling live poultry were reported in 2015 (CDC, 2015). In 2016, two *Salmonella* related multi-state outbreaks have been reported: one has been linked to live poultry which involves multiple serotypes of *Salmonella* and has claimed three lives and another outbreak has been linked to consumption of eggs involving *S.* Oranienburg (CDC, 2016).

The use of antimicrobials is a common practice for controlling *Salmonella* and other food borne pathogens during poultry processing. Chlorine compounds such as sodium hypochlorite, calcium hypochlorite and chlorine gas are among the most commonly used antimicrobials approved by the USDA (USDA-FSIS, 2016). The *Salmonella* present on poultry carcasses and processing facilities are prone to being exposed to sub-lethal concentrations of chlorine, due to improper sanitation and erroneous dosing of the antimicrobial. *Salmonella* can be exposed to various physiological stressors such as heat, salt, acid, alkali, oxidation, etc. in food and poultry
processing environment. Such environmental stresses can influence bacterial physiology and may affect tolerance to subsequent stresses as well as their pathogenicity. For example, starvation is known to induce cross protection against thermal challenges, acidic pH (Spector et al., 1995), and oxidative stress (Seymour et al., 1996) in *S.* Typhimurium whereas, acid shocked *Salmonella* Typhimurium developed a cross protection against heat, oxidative stress, and osmotic challenges (Leyer and Johnson, 1993).

Biofilm formation by foodborne pathogens such as *Salmonella* in a food processing environment is a major food safety concern. Biofilms act as reservoir for recurrent contamination in the food surfaces. A biofilm is a community of microbes which are embedded in an organic polymeric matrix and are attached to a substrate. Rupture of a mature biofilm can serve as a continuous source of contamination in a food chain. Biofilms are considered an important issue in human health because approximately 80% of bacterial infections are associated with biofilms (Davies, 2003; Steenackers et al., 2012). The nutrient and moisture rich surfaces of equipment and parts in poultry processing facilities are known to facilitate biofilm formation. The process of biofilm formation by *Salmonella* is initiated by the attachment and formation of an extracellular polymeric matrices (EPS). Quorum sensing molecules trigger the release of extracellular DNA which regulates the formation of EPS (Das et al., 2013). The genes involved (such as *bssS* and *ycfR*) in biofilm formation by *Salmonella* and other bacteria were up-regulated after *Salmonella* was exposed to sub-lethal chlorine (Salazar, 2013; Wang et al., 2010; Speranza et al., 2011). The upregulation of these genes have been studied in
terms of measuring the quantity of attached biomass under different environmental conditions.

Inappropriate cooking is known to cause about 67% of the total *Salmonella* related outbreaks (Bean and Griffin, 1990; Juneja *et al.*, 2013; Kimura *et al.*, 2004). *Salmonella* Enteritidis and Typhimurium which were exposed to sub-lethal concentration of tri-sodium phosphate and hydrogen peroxide have been reported to develop resistance against thermal inactivation, showing the possible role of antimicrobials in providing cross-protection against heat treatment. (Sampathkumar *et al.*, 2004; Christman *et al.*, 1985). This study was undertaken to determine the biofilm forming ability and thermal resistance of chlorine stressed *Salmonella*, under different environmental conditions, which simulate the conditions commonly encountered in the processing environment.
References


Kimura, A. C., Reddy, V., Marcus, R., Cieslak, P. R., Mohle-Boetani, J. C., Kassenborg, H. D., ... & Emerging Infections Program FoodNet Working Group. (2004). Chicken consumption is a newly identified risk factor for sporadic *Salmonella* enterica serotype Enteritidis infections in the United States: a case-control study in FoodNet sites. *Clinical Infectious Diseases, 38*(Supplement 3), S244-S252.


CHAPTER II
LITERATURE REVIEW

*Salmonella*

Classification

*Salmonella* spp. are gram negative, non-spore forming, rod shaped, facultative anaerobic bacteria that belong to the Enterobacteriaceae family. The genus *Salmonella* comprises of two species: *S. enterica* and *S. bongori*. *S. enterica* are further divided into six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica* (Popoff and Minor, 1997) (Table 2.1). Using the Kauffman-White serotyping scheme these subspecies are further differentiated based on their biochemical and genomic relatedness into serovars or serotypes (Popoff and Minor, 1997; Brenner et al., 2000). Based on their antigens; lipopolysaccharide (O), flagellar protein (H), and capsular (Vi) antigens, over 2600 serotypes of *Salmonella* have been classified (Gal-Mor et al., 2014).

The majority of *Salmonella* isolates which cause disease in humans and other mammals belong to *S. enterica* subsp. *enterica* (OIE, 2005). Few human pathogens such as *S. Typhi*, *S. Paratyphi*, and *S. Hirschfeldii* are transmitted from person to person and have no significant animal reservoirs. These serotypes are causative agents of typhoidal
salmonellosis whereas the non-typhoidal salmonellosis is caused by *Salmonella* which have animal reservoirs (OIE, 2005).

*Salmonella* spp. are widely distributed in nature. The intestinal tracts of reptiles, birds, mammals, including humans and sometimes insects, are the primary habitat for *Salmonella* spp. (Jay, 2000; Adams and Moss, 2000). Intensive animal husbandry practices provide a conducive environment for *Salmonella* proliferation. Serotypes vary in their distribution, some being specific to a particular region, while others are worldwide in their distribution.

**Table 2.1  *Salmonella* nomenclature**

<table>
<thead>
<tr>
<th>Genus (caps, italic)</th>
<th>Species (italic)</th>
<th>Subspecies (italic)</th>
<th>Serotypes (caps, non-italic)</th>
<th>No. of serotypes (Popoff et al., 2004)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella enterica</em></td>
<td><em>enterica</em> (or subspecies I)</td>
<td></td>
<td>Choleraesuis, Enteritidis, Paratyphi, Typhi, Typhimurium*</td>
<td>1504</td>
</tr>
<tr>
<td>salamae (or subspecies II)</td>
<td></td>
<td>9,46: z: z39</td>
<td></td>
<td>502</td>
</tr>
<tr>
<td>arizonae (or subspecies IIIa)</td>
<td></td>
<td>43: z29: -</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>diarizonae (or subspecies IIIb)</td>
<td></td>
<td>6,7: l, v:1,5,7</td>
<td></td>
<td>333</td>
</tr>
<tr>
<td>houtenae (or subspecies IV)</td>
<td></td>
<td>21:m, t: -</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>indica (or subspecies VI)</td>
<td></td>
<td>59: z36: -</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>bongori</td>
<td>subspecies V</td>
<td>13,22: z39: -</td>
<td></td>
<td>22</td>
</tr>
</tbody>
</table>

*examples of serotypes*

The major route of transmission of *Salmonella* in humans is via the feco-oral route. In many animals, *Salmonella* remains as a resident of their intestines, and create a gall stone-biofilm (Gonzalez-Escobedo et al., 2011), which may continuously or intermittently be shed in their feces. Fomites and insect vector transmission are also reported for *Salmonella* (OIE, 2005). Vertical transmission of *Salmonella* (e.g. *S.*
Enteritidis) is common in birds, whereas in utero transmission in animals has also been reported (Jones, 2011; Ricke et al., 2013). Meat, eggs, and contaminated vegetables and fruits are common sources of Salmonella infection to humans. Salmonella are known to grow at a temperature range of 2-54°C, with an optimum temperature of 37°C, and at a pH range of 3.99-9.5 with an optimum pH being 6.5-7.5 (Doyle and Buchanan, 2012). However, Salmonella can survive under various unfavorable environmental conditions. Salmonella were found to survive over 100 weeks on a plastic surface under refrigeration (Gruzdev et al., 2012).

**Infection and Pathogenesis**

In humans, non-typhoidal salmonellosis displays three types of clinical symptoms: gastroenteritis, bacteremia, and an asymptomatic carrier state (Ryan and Ray, 2004). It is more common in children and elderly patients (Ryan and Ray, 2004). Infectious dose of Salmonella varies with serotypes. For non-typhoidal salmonellosis, a dose of $10^3$ bacilli can make a person ill (Bronze and Greenfield, 2005, Ryan and Ray, 2004). The incubation period varies depending on the serotypes and inoculum size and usually ranges between 5 to 72 hours (Krauss et al., 2003). Salmonella gastroenteritis or food poisoning in humans is manifested by nausea, vomiting, diarrhea, abdominal cramps, headache, and fever (Collins and Kennedy, 1983; Ryan and Ray, 2004; Krauss et al., 2003; Brock et al., 2000). The mild to severe symptoms can last up to a week (Ryan and Ray, 2004). Three to ten percent of the individuals infected with Salmonella enterica develop bacteremia, and the severity of the symptoms depends on the serotype involved (Bronze and Greenfield, 2005, Woods et al., 2008). Immunocompromised individuals are more prone to develop bacteremia.
On the basis of host preference and disease attributes, *Salmonella* are divided into invasive (typhoidal) and non-invasive (non-typhoidal) (Okoro et al., 2012). However, recent reports from many sub-Saharan countries indicate non-typhoidal *Salmonella* can also cause invasive bacterial diseases (Redddy et al., 2010; Graham, 2010), with high fatality rates (22-45%) in both adults and children (Gordon et al., 2002; Gordon et al., 2008; Gordon, 2008; Cheesbrough et al., 1997).

A systemic invasion of *Salmonella* into human host cells involves invasion, attachment or adherence, internalization, and proliferation (Lawhon et al., 2011). *Salmonella* Typhimurium, which escapes the acidic environment of the stomach, possess a highly efficient mechanism of invasion there by invading the distal ileal non-phagocytic M-cells (Gunn, 2011; Bhowmick et al., 2011). *Salmonella*, once attached to the host epithelium, use their type 3 secretion system (T3SS) to inject the effector proteins into eukaryotic host cells (Okoro et al., 2012). The T3SS in *Salmonella* is regulated by two regulatory systems: CpxR/CpxA and PhoP/PhoQ, which inject the effector proteins or virulence factors namely SopE, SopE2, SopB, and SopA into the host cell (Raffatellu et al., 2008; Layton and Galyov, 2007). These effector proteins, once inside the cell, remodel the host cellular and cytoskeletal function (ruffling) there by promoting the pathogen engulfment, and proliferation (Ramos-Morales, 2012). After replication and dissemination of *Salmonella* inside the macrophages, they spread into mesenteric lymph nodes (Misselwitz et al., 2011).

Other than the flagellar system of T3SS, *Salmonella* enterica are also known to encode for two virulence related T3SS: T3SS1 and T3SS2, which are located on *Salmonella* pathogenicity island 1 (SPI1) and *Salmonella* pathogenicity island 2 (SPI2)
respectively (Galan and Curtiss, 1989; Ochman et al, 1996; Shea et al., 1996).

Pathogenicity islands are distinct genetic elements found in the pathogenic bacterial chromosomes. The invasion, which is SPI-1 directed, is dependent on actin rearrangements, and hence bacterial engulfment and release of effector proteins (Clark et al., 2011), whereas systemic infection by *Salmonella* is mediated by SPI-2.

**Salmonella and foodborne illnesses**

*Salmonella* is second only to Noro virus in causing foodborne illnesses in the United States and is the leading cause of hospitalizations and deaths among the known causes of foodborne illnesses in the United States. Non-typhoidal *Salmonella* accounts for 11% of an estimated 9.4 million foodborne illness in the U.S. every year, whereas 28 % of the total hospitalization (55,961) and 35 % of the total deaths (1,351) due to foodborne illnesses occur because of salmonellosis alone (Scallan et al., 2011). In 2014 alone, *Salmonella* were responsible for 30% of total foodborne disease outbreaks, 27% of foodborne illnesses, and 59% of hospitalizations (CDC, 2014). In the U.S., the most common serotypes of *Salmonella* isolated from human cases are: *Salmonella* Enteritidis, *S.* Newport, *S.* Typhimurium, *S.* Javina, *S.* I 4, [5],12: i: -, *S.* Poona, *S.* Muenchen, *S.* Heidelberg, *S.* Saintpaul, and *S.* Infantis (CDC, 2015).

**Poultry as a source of Salmonella**

The United States is the largest broiler producing country and second largest exporter of poultry meat in the world (USDA-ERS, 2014). Poultry are asymptotic carriers of non-typhoidal *Salmonella* and act as a major reservoir of *Salmonella* (Sadeyen et al., 2004). Compared to other livestock, poultry has been found to be most common as a
source of *Salmonella* (OIE, 2008). Undercooked poultry and egg are among the common pathways of *Salmonella* transmission to the human food chain (Howard et al., 2012). Surveillance of *Salmonella* using a pooled cecal sample of spent hens in the United States showed 65.4% of the flocks (Waltman et al., 1992) and 86% of layer houses (Ebel et al., 1992) were positive for *Salmonella*. Siemon et al. (2007) reported a 30% fecal prevalence of *Salmonella* in conventionally housed broilers. Other studies estimated the *Salmonella* prevalence in conventional broiler farms range between 10 and 26% (Bailey et al., 2001; Liljebjelke et al., 2005; Rodriguez et al., 2006). *Salmonella* prevalence in broiler flocks varied considerably among different countries with nearly 0% in Sweden and 68.2% in Hungary (Hald, 2007), 76.9% in Canada (Chambers et al., 1998), 69.8% in France (Rose et al., 1999), 41.3% in Turkey (Carli et al., 2001), and 25 % in Denmark (Chadfield et al., 2001).

*Salmonella* in poultry can be introduced via various sources like feed, water, soil, bedding, litter, rodents or humans. *Salmonella* can survive well in farm environments for months (Petkar et al., 2011). In addition, *Salmonella* after colonizing the gastrointestinal tract of poultry, get continuously shed in the feces. In the poultry production chain, during post-harvest operations, contamination of meat by *Salmonella* can occur at any point. Some steps of the slaughter process are more common for *Salmonella* contamination and cross contamination than others. Cross contamination can occur during transportation (Bolder 1998), scalding (Ono and Yamamoto, 1999; Mead et al., 1994), de-feathering (Ono and Yamamoto, 1994), evisceration, and chilling in the chiller tanks (Lillard, 1986). *Salmonella* positive birds increased from 47% at pre-defeathering to 63% post-defeathering (Nde et al., 2007). Waldroup et al., (1992, 1993) reported a 20
% rise in the incidence of *Salmonella* in post-chill samples as compared to pre-chill samples. Scalding and immersion in the chiller tank were identified as main points of cross contamination during poultry processing (Shackleford 1988).

Poultry meat is one the most common sources of foodborne salmonellosis in the U.S. (Mead et al, 2010). Meat and poultry products accounts for 22% of the total foodborne illnesses, and 29% of deaths related to foodborne illnesses (CDC, 2013). Two S. Enteritidis outbreaks in 2015 were linked to raw, frozen and stuffed chicken entrees and one outbreak linked to live poultry involved different serotypes: S. Enteritidis, S. Hadar, S. Indiana, and S. Muenchen (CDC, 2015). In 2016, two multi-state outbreaks one linked to live poultry involving several serotypes and another linked to eggs linked to S. Oranienburg have been reported and the former has claimed three lives (CDC, 2016). The prevalence of *Salmonella* in retail poultry has fallen to its lowest level since 2002, with only 6% found in ground turkey compared to 19% in 2008 and 9% found in retail chicken as compared to 15% in 2008 (USDA-FDA, 2016).

The USDA-FSIS has recently introduced new performance standards for *Salmonella* in raw poultry products. According to which a maximum of 5 broiler carcasses out of 51 (9.8%) can be *Salmonella* positive to pass the performance standards, whereas in case of ground chicken and chicken parts, a maximum of 13 and 8 (25%, and 15.4% respectively) *Salmonella* positive samples out of 52 samples can pass the performance standard (USDA-FSIS, 2016).
**Salmonella stress response to sub-lethal injury**

**Stress and sub-lethal injury**

Stress is a subtle term, which means not necessarily harm the organism physically, but alter to simply the behavior of the organism. The term stress is used to refer to an exposure of agents or treatments causing injury (Wesche et al., 2009). A bacterial stress in food microbiology can be defined as a deviation in physical, chemical, or nutritional conditions which are insufficient to kill the bacteria thereby resulting in sub-lethal conditions that basically result in injured microbes (Hurst, 1977; Murano and Pierson, 1993). Bacteria including *Salmonella* in food processing environments can encounter a variety of stressors in the form of exposure to acids, alkali, oxidizing agents, osmotic agents, temperature, and starvation (Yousef and Courtney, 2003). Food items when subjected to preservation by heat and pressure, cause denaturation of cellular proteins and enzymes of bacteria, this can lead to oxidative stress (Capozzi et al., 2009). The injured or stressed bacteria can be of food safety concern as these microorganisms can resuscitate to the normal state during favorable conditions (Wu, 2008). Bacterial defenses which are used to survive one kind of stress can then provide cross protection to another form of hostile conditions (Foster and Spector, 1995).

An injury to a bacterium can be described as the effect of one or more sub-lethal treatment(s) against the microbe (Hurst, 1984). Yousef and Courtney (2003) defined sub-lethal injury as damage to bacterial cellular components thus impairing the ability of the microbe to multiply and yielding susceptibility to minor challenges. According to Gilbert (1994) “sub-lethal injury of microorganisms implies damage to structures within the cell,
the expression of which entails some loss of cell function that may be transient or permanent.”

According to Storz and Hengge-Aronis (2000), the level of stress severity in bacteria can vary from minor to severe and eventually become lethal. A low or minor stress causes a transient adaptation in bacteria, which results in transient physiological alteration rendering them more tolerant to increased stress (Yousef and Courtney, 2003). A mild to moderate stress results in a mixed population of healthy and dead cells (Hurst et al., 1976; Hurst 1984; Mackey 2000; Stephens et al., 1997) and leads to increased survival of the surviving cells (Storz and Hengge-Aronis 2000; Archer, 1996).

A 3 h (short-term) versus 5 day (long-term) temperature stress of 4°C or 45°C, NaCl stress of 5.5%, oxidative stress of 15 mm H₂O₂, acidic pH stress of 5.5 and ethanol stress of 5% to S. Enteritidis, E. coli and L. monocytogenes (Hahm and Bhunia 2006) resulted in a decrease expression of stress induced crude cell surface antigens (CCSA). However, a long term stress on S. Enteritidis resulted in higher expression of lipopolysaccharide (LPS) as compared to the short term stress, possibly due to adaptation of cells that survived the harsh conditions.

**Stress response and cross protection in Salmonella**

*Salmonella* may encounter several stresses during food production, processing, preservation, and cooking. In the food processing environment, bacteria face inhibitory chemicals or antimicrobial disinfectants (Marino et al., 2001), thermal (Mackey et al., 1991) and cold treatments (Artes and Allende, 2005). Exposure to heating, chilling and acidity result in bacterial injury and damage to its cellular components including the cell wall, DNA, RNA and proteins (Wesche et al., 2009) depending on the degree of severity.
This means if the antimicrobial or heat treatment of food is insufficient to be lethal, it may enable the bacteria to survive. Stressed bacteria sometimes show an adaptive effect with a better host interaction, invasion and colonization during disease (Dorman, 1994). A homologous cross adaptation is when a pre-exposure to a sub-lethal level of a given stress protects an organism upon exposure to a normally lethal level of the same stress whereas, a protection obtained by exposure to one kind of stress against another kind of stress is referred as non-homologous or heterologous stress adaptation (Yousef and Courtney, 2003).

*Salmonella* displays various stress survival strategies against starvation, a iron depleted growth environment, acid tolerance response (ATR), oxidative stress, heat stress, and cationic peptides. Some of the stressors provide cross protection against subsequent homologous and/or heterologous stressors whereas some do not offer any protection. Starvation, a carbon limitation in the case of *Salmonella* Typhimurium is known to induce cross protection against various environmental stresses such as osmotic, oxidative and heat stressors (McCann et al., 1991). Starvation of *S*. Typhimurium yields cross protection against thermal, oxidative, and osmotic challenges (Foster and Spector, 1995). Acid shocked *Salmonella* are known to develop a significant cross protection against acid, cold, heat, oxidative stress, and osmotic stressors (Xu et al., 2008; Foster and Spector, 1995; Leyer and Johnson, 1993), whereas heat or osmotic stressors are not known to induce acid tolerance (Leyer and Johnson, 1993). Some proteins that are induced by oxidative stress in *E. coli* and *S*. Typhimurium can also be induced by other types of stressors, such as heat shock and starvation (Farr and Kogoma, 1991). Sub-lethal hydrogen peroxide exposure induces 30 proteins in *S*. Typhimurium, out of which five
(dnaK, two katG, and two ahp) are also induced by heat stress (Morgan et al, 1986). So, peroxide adapted cells are technically resistant to heat shock.

**Oxidative stress response in Salmonella**

An excess level of pro-oxidants in a cell is considered oxidative stress. Active oxygen species which are generated as by-products of normal aerobic metabolism, when not neutralized, are known to damage the cellular DNA, RNA, proteins, and lipids (Farr and Kogoma, 1991). As reviewed by Farr and Kogoma, (1991) the detoxification of superoxide in *Salmonella* involves the conversion of superoxide to hydrogen peroxide by the help of superoxide dismutase (SOD) and catalase mediated destruction of hydrogen peroxide. Superoxide radicals (O\(^2^-\)), hydrogen peroxide (H\(_2\)O\(_2\)), and hydroxyl radicals (OH\(^-\)) are major active oxygen species involved in oxidative damage of a cell. Peroxide stress causes at least nine proteins in *S. Typhimurium* to over express which are regulated by the *oxyR* regulon (Farr and Kogoma, 1991). However, Farr and Kogoma reported no cross protection to cells which were stressed by peroxide, when subsequently stressed with superoxide. They suspected this is because the sets of superoxide stimulated proteins are different from those belonging to the peroxide stress proteins.

The *oxyR* regulon is activated after H\(_2\)O\(_2\) treatment in *Salmonella*, which then plays an antioxidant role. Hydroperoxidase I (*katG*) and alkyl hydroperoxide reductase (*ahpCF*) help to eliminate oxidants from the cells and provide another layer of protection to *Salmonella* (Christman et al., 1985). *oxyR* is also known to protect against DNA damage and mutation by inducing the synthesis of nonspecific DNA binding proteins (Altuvia et al., 1994; Martinez and Kolter, 1997). In the fight against OH\(^-\), *oxyR* inducible *fur* acts as an iron uptake repressor thereby lowering the formation of OH\(^-\), which
otherwise is formed by \( \text{H}_2\text{O}_2 \) and intracellular iron (Fenton type reaction) (Zheng et al., 1999). Interestingly, stationary phase cells and starved cells are more resistant to various stresses including high \( \text{H}_2\text{O}_2 \), and \( \sigma^\text{S} \) regulates various antioxidant gene expression (Conter et al., 1997; Groat et al., 1986).

**Chlorine as an oxidative stressor**

The active ingredient of chlorine, hypochlorous acid (HOCl) is a powerful oxidizing agent. Inside a cell, sulphur containing amino acids are most susceptible to HOCl. For example, HOCl mediated oxidation of cysteine thiols forms unstable sulphenyl chloride (R-SCl) as an intermediate, which then reacts with water to form oxidized cysteine sulphenic acid (R-SOH) (Gray et al., 2013). These unstable intermediates either get reduced by thioredoxin or further oxidized to sulfinic (R-SO\(_2\)H) and sulfonic (R-SO\(_3\)H) acids, initiating a cascade of irreversible thiol degradation, which ultimately leads to protein degradation (Gray et al, 2013). Bacteria can be exposed to HOCL stress even inside a host body. An animal’s innate immune systems are known to generate high quantities of oxidants including HOCl, to combat invading pathogens (Hurst, 2012; Klebanoff, 2005; Winterbourn and Kettle, 2013). A reaction catalyzed by myeloperoxidase (MPO) between \( \text{H}_2\text{O}_2 \) and chloride generates HOCl in phagosomes (Klebanoff, 2005). MPO acts as a haloperoxidase, which oxidizes halides to respective hypohalous acids (e.g. \( \text{Cl}^- \) to HOCl). This indicates that the *Salmonella* faces oxidative stress caused by active chlorine species not only in environment but also inside host body.

The expression of certain genes such as *rpoS* (virulence) after exposure to chlorine stress confers protection to the pathogen against other stressors such as osmotic,
heat, and oxidative stress (Foster and Spector 1995). Wang et al., (2010) reported that the oxidative-stress regulator gene in *Salmonella*, *oxyR*, was up-regulated 1.4 times high after exposure to 130 ppm chlorine for 30 min treatment but was not unregulated by exposure to 390 ppm chlorine for 10 min, whereas gene *soxS* (*soxRS*) was under expressed (1.2-fold) by the 130 ppm/30 min chlorine exposure. The differential regulation of the *oxyR* and *soxRS* regulons suggested that they respond differently to chlorine-based oxidation in *S. enterica* thereby proving that oxidative stress is caused by chlorine.

Results at the gene level show that a chlorine stress in *Salmonella* can cause an increase in biofilm formation. Biofilm is a community of microbes which are embedded in an organic polymeric matrix and attach to a substrate. An oxidative stress produced by sub-lethal chlorine concentration up-regulated a number of genes (*bssS, ycfR*), which are involved in biofilm formation in *Salmonella Typhimurium* (Wang et al., 2010). In a separate study, chlorine stress was also found to induce biofilm formation in *E. coli* O157:H7, *S. Typhimurium*, and *S. Enteritidis* via up-regulation of the *ycfR* gene which is involved in the biofilm formation (Salazar et al., 2013). Similarly, Wang et al., (2009) reported that chlorine stressed (390 ppm) *E. coli* in brain heart infusion medium developed resistance against high temperature stress as shown by the over expression of heat-shock proteins such as *htpX, dnaK, dnaJ, htpG*. *S. Typhimurium* which are adapted to 60µM hydrogen peroxide stress displayed a higher thermal resistance at 50°C (Christman et al., 1985) as displayed by their survival curve. The oxidative effect of hydrogen peroxide treatment on *Salmonella Typhimurium* induced 30 proteins, including *Dnak*, a heat shock chaperone out of which five proteins are also induced by heat stress.
(Morgan et al., 1986), providing evidence that hydrogen peroxide treated *Salmonella* might already have some proteins which provide a cross-protection against heat.

**Use of chlorine in food and poultry processing operations**

Due to its easy availability, cost effectiveness, and broad spectrum of activity, chlorine is one of the most frequently used antimicrobials in food and poultry processing plants. In the poultry processing industry, chlorine is used either in the form of chlorine gas (less common), sodium hypochlorite (most common) or calcium hypochlorite. USDA and FDA regulates the level of chlorine that can be used by food and poultry processing operations. Federal regulation (21 CFR Part 178) allows a maximum of 200 ppm of chlorine on food processing equipment and articles. The level of chlorine in poultry chiller or on carcasses and/or in water used in poultry processing should not exceed 50 ppm of free available chorine (USDA- FSIS, 2016).

In a food processing facility, it is always best to apply chlorine after the equipment is cleaned and rinsed because organic load reduces the effectiveness of chlorine (Chang, 1971). The potency of the chlorine depends on the pH and temperature of the sanitizing solution, and it acts best at a pH of 5-6 and at a temperature of 105°F-120°F. The effect of chlorine decreases at lower temperatures, however using it at too high of a temperature may release toxic chlorine gas.

**Mechanism of action of chlorine**

Chlorine once dissolved in water forms hypochlorous acid (HOCl), and hypochlorite ions (OCl-) which are freely available chlorine and are the active forms that can act on microbes (Morris, 1966). The hypochlorite anion is a weak disinfectant as
compared to the neutral HOCl. HOCl is a very strong oxidant which reacts with the cellular biomolecules of the organisms. The mechanism of how it kills bacteria is not completely understood. The inner cellular membrane is known to be the site for lethal damage in vegetative cells (Gray et al, 2013). Another view on the action of reactive oxygen species is through unfolding and aggregation of bacterial essential proteins. Winter et al. (2008) reported that HSP33 chaperone-depleted-bacteria accumulated high amounts of protein aggregation, which were more sensitive to chlorine treatment as compared to the non-chlorine treated bacteria. Sulphur containing amino acids such as cysteine, methionine and glutathione were easily affected by HOCl (Gray et al., 2013). Chloramines, which are formed through the reaction of chlorine with organic compounds, acts as a secondary disinfectant (Miche and Balandreau, 2001). Upon decomposition of chloramines, radicals are produced, which are known to damage other substrates at the cellular level (Hawkins et al, 2003). Some chloramines are also known to react with hydrogen peroxide producing singlet oxygen (Khan and Kasha, 1994). Chloramines also react with iron and copper ions inside cells, generating nitrogen radicals which are very reactive (Hawkins and Davies, 2002). HOCl and chloramines are known to target the primary and secondary amines of nucleotides in DNA and RNA (Gray et al, 2013). Chlorination of double bond fatty acids lead to the formation of chlorohydrins, thus HOCl mediates damage in eukaryotic cells (Carr et al., 1997). The efficacy of chlorine depends on the presence of organic matter in the medium, as free chlorine is easily quenched by organic load forming chloramines (Chang, 1971). Also, pH, concentration of chlorine, temperature, and number and types of microorganisms affect the potency of chlorine.
Effect of chlorine on *Salmonella* growth

It is well documented that microorganisms have different physiological and morphological properties at their different growth stages (Serra and Hengge, 2014). Various studies reported that stationary phase cells are more resistant to a wide range of environmental stresses ranging from pH, osmotic stress, and temperature (Foster and Spector, 1995; McCann et al., 1991). The *Salmonella* virulence system which is encoded by SPI-2 are documented to be induced by Mg\(^{2+}\) and PO\(_4^{2-}\) deprivation, low pH and oxidative stress only during their stationary phase of growth (Pawelek et al., 2002; Monack et al., 2001).

Antimicrobial stressors in food processing facilities such as starvation, osmotic stress, acidic stress, and chlorine stress are known to alter the growth pattern of bacteria on the surface of fresh produce (McMahon et al., 2007; Wesche et al., 2009). Hypochlorous acid, an active ingredient of chlorine, was found to be less effective against stationary phase *S. Typhimurium* as compared to its log phase cells (Oliver, 2005). Alexandrou *et al.*, (1995) discovered that sub-lethal injury caused by antimicrobials, like acetic and lactic acids, in *S. Enteritidis* caused an extension of lag phase growth. Similar findings were observed in a gram positive *L. monocytogenes* upon cold, heat, chlorine, and starvation stress (Guillier *et al.*, 2005). However, no differences were observed in the growth pattern of a gram negative non-O157 STEC *E. coli* between chlorine stressed and non-stressed cells in cantaloupe or lettuce leaves (Yoo et al., 2015). In a reciprocal study by Cherchi and Gu (2011), chlorine was less effective on stationary phase cells of *E. coli* as compared to the lag or log phase cells. The stationary phase cells of *E. coli* when stressed with HOCI induced rpoS regulated genes *dps* (a DNA binding
protein), *katG*, and *ahp*, conferring resistance against HOCl (Dukan and Touati, 1996). In summary, the efficacy of chlorine as an antimicrobial or as a stressor depends on the growth phase of bacteria. So, in this study the cross-resistance development in different phases of *Salmonella* is studied separately.

**Development of rugose morphology in *Salmonella***

*Salmonella* is well known to produce a morphological change described as rugosity, which is referred to as a rough, dry, and red (*rdar*) morphotype because of its appearance on media containing congo red. This morphotype requires proteinaceous fibrils (Chapman et al., 2002), curli (Romling et al., 1998; Zogaj et al., 2001), and cellulose, which is basically comprised of exopolymeric substances (EPS) (Zogaj et al., 2001). *Salmonella* are known to display rugose morphotypes through extracellular appendages, and these morphotypes are known to be resistant to low pH or hydrogen peroxides and are usually associated with curli and cellulose (de Rezende et al., 2005). Curli are usually synthesized at a lower temperature (<30°C) and are known to have adherence properties. In addition to *S. Typhimurium* and *S. Enteritidis* other foodborne pathogens like *V. Cholarae* is also known to produce the rugose colonies, when cells are subjected to adverse environmental stressors (Rezende et al., 2005), nutrient deprivation (Wai et al., 2005). However, Rashid et al., (2003) suggested that the rugose morphotype of *V. cholerae* might be less pathogenic as compared to the wild type smooth morphotypes. In contrast to the rugose colonies of *V. cholerae* which are displayed at higher temperature (37°C), an incubation for 3 days at 25°C is known to induce the production of the rugose morphotype in *Salmonella Typhimurium* (Anriany et al., 2001). In another study, *Salmonella* Senftenberg rugose colony morphotype were isolated from a
high salt environment (Martinez-Urtaza et al., 2004). Jahid et al., (2015) reported disinfectant treatments caused a shift from smooth to rugose morphotypes in *S. Typhimurium*. All these unfavorable environments which lead to rugose morphotype formation in *Salmonella* are considered to be a threat to the food industry because of their harsh environmental conditions.

Rugose morphotypes are better biofilm formers, are better protected against stress, and have better cell to cell communication and gene exchange as compared to the smooth morphotypes (Romling et al., 2000). Studies reported that *Salmonella* rugose morphotypes are more resistant to chlorine, have higher biofilm forming ability and also have potential to revert back to the smooth colony type (Anriany et al, 2006). A rugose variant of *Salmonella Typhimurium* was spotted when the smooth variants were treated with cold oxygen plasma in planktonic form (Jahid et al., 2015). In one study by Karaca et al., (2013), who screened *Salmonella* isolates from beef, chicken and turkey products, reported a surprisingly high; 79 out of 140 isolates demonstrating the rdar morphotype with biofilm production.

**Biofilms and their role in food safety**

A biofilm is a community of microbes embedded in an organic polymeric matrix (EPS), adhering to a surface. The presence of a biofilm has enormous impact on food contamination during processing. Such biofilm cells in a food processing unit offer greater resistance to normal cleaning as compared to their planktonic counterparts (Joseph et al., 2001). They are resistant to anti-bacterial agents (Carpentier and Cerf, 1993), heat (Frank and Koffi, 1990), and bacteriophages (Hicks and Rowbury, 1987). One of the reports indicate that 80% of microbial diseases, including food borne
illnesses, are caused by microbes in biofilms (NIH, 1997). The process of biofilm formation poses a threat to public health as it becomes a continuous source of contamination in the food chain (Socransky and Haffajee, 2002). Biofilm formation in food processing facilities is also accountable for corrosion of metals and pipelines, mechanical blockades, and a decreases in the efficiency of heat exchanges (Mittelman, 1998).

**Salmonella biofilms in poultry processing environments**

The occurrence of *Salmonella* on food contact surfaces in the form of a biofilm plays a very important role in the spread and persistence of this organism. Studies have shown that *Salmonella* are capable of attaching and forming biofilms on common materials used by the food industry like metals, glass, rubber, stainless steel, plastic and cement (Joseph et al., 2001; Leriche and Carpentier, 2000, Wang et al., 2010; Chia et al., 2009). In poultry processing plants, the most common sites for biofilm formation are rubber 'fingers', plastic curtains, conveyor belt material and stainless steel surfaces on scald tank overflows. Stainless steel bolts and screws, which are difficult to dismantle and clean are areas where *Salmonella* forms biofilms (Morita et al., 2011). The highest bacterial counts were obtained from non-metallic surfaces such as rubber finger pluckers and plastic defeathering curtains (Lindsay et al., 1995). The attachment of the bacterial cells and biofilm formation are determined by physiochemical properties of cells and various structural determinants like flagella, fimbriae, pili, curli and surface lipopolysaccharides as well as cell surface interaction (Van Houdt and Michiels, 2010), and bacterial cellulose production (Jain and Chen, 2007).
Removal of biofilms in food processing environments

Controlling the formation of biofilm is not an easy task. In a poultry processing facility, due to continuous in line flow of nutrient rich meat, it is not easy to clean and disinfect at short intervals, however a careful and frequent cleaning and disinfection routine should be implemented.

The normal disinfectants and antimicrobials used against planktonic cells are not effective against biofilms (Shi and Zhu, 2009; Simoes, et al., 2010; Van Houdt and Michiels, 2010) which possibly is due to the extracellular polymeric substances (EPS) (Van Houdt and Michiels, 2010), a difficult diffusion barrier, and cell wall structures. Chlorine is the most common antimicrobial used in poultry processing facilities for sanitation and cleaning. In a study by Stewart and Costerton (2001) alkaline hypochlorite (1000 mg/L) was less effective against biofilm cells of *P. aeruginosa*, and *Klebsiella pneumonia*, as compared to their planktonic cells, which they explained is because of poor penetration of the chemical. In an another study with *L. monocytogenes*, the biofilm cells were 100 time more resistant against sodium hypochlorite and heat as compared to their planktonic forms (Frank and Kofi, 1990). Similarly, *Salmonella* biofilms are usually more resistant to the routine cleaning agents as compared to their planktonic forms (Bridier et al., 2011; Joseph et al., 2001) when treated with tri sodium phosphate (Scher et al., 2005), chlorine, and iodine (Joseph et al., 2001). Another possible reason given for resistance of biofilm against biocide is that biofilms sense the biocide stress rapidly and respond *via* stress proteins quicker than the planktonic cells (Szomolay et al., 2005).

The choice of material in processing facilities can make a difference in reducing biofilms. Materials which can reduce laminar product flow, decrease the static product,
and ease the cleaning and cleaning in place (CIP) processes can help in reducing bacterial attachment (Van Houdt and Michiels, 2010). By altering the intrinsic properties of the material used bacterial attachment can be reduced, as demonstrated by Dong et al. (2005) where coating stainless steel with antifouling layers reduced 81-96 % of listerial attachment and biofilm formation. The use of chemical cleaning agents should be formulated at the appropriate concentrations and used for the appropriate amount of time, at the desired temperature (Parkar et al., 2004; Antoniou and Frank, 2005). A new approach, ionization radiation, has been found to be effective at removing Salmonella biofilms from food and food contact surfaces (Niemira and Solomon, 2005). Atmospheric plasma which generates oxygen radicals also seems to be effective against biofilms (Vleugels et al., 2004).

**Factors affecting biofilm formation**

Various environmental factors that affect biofilm formation include: pH, contact time, temperature, nutrient condition, and bacterial type. Substratum surface (hydrophobicity) and bacterial flagellation, motility and hydrophobicity are among other factors that can affect biofilm formation by foodborne pathogens (Herald and Zottola, 1988; Hood and Zottala, 1995; Chavant et al., 2002; Moltz and Martin, 2005). Bacterial cell surface hydrophobicity is responsible for the cell attachment to the substratum. Hydrophilic surfaces with high free energy, like steel and glass, allow better bacterial attachment and biofilm formation as compared to hydrophobic surfaces like plastics. However, Chia, et al., (2009) reported that Salmonella attachment on stainless steel, glass, and teflon was not affected by hydrophobicity.
**pH**

The importance of pH has been described by Garrett et al. (2008) where it was stated that the optimal polysaccharide production by bacterial species depends on species, but for most bacteria it is near neutral pH. Xu et al., (2010) reported that *S. Enteriditis* biofilm formation was higher at a pH of 6 than at pH of 7. Similarly, *Stenotrophomonas maltophilia* produces more biofilm at a pH of 7.5-8.5 as compared to a pH of 5.5 (Di Bonaventura et al., 2007). Similarly, *Burkholderia pseudomallei* produced the highest biofilm at a pH of 7.2 as compared to an acidic or alkaline pH. In contrast, Giaouris et al., (2005) reported that *Salmonella Enteritidis* PT4 biofilm formation on stainless steel was not found to be affected by pH ranging from 4.5 to 7.4, when the biofilm formation was allowed to develop for 7 days at 20°C. In another study with *Streptococcus agalactiae*, an acidic pH strongly induced biofilm formation in a microtiter plate assay (D'Urzo et al., 2014).

**Nutrient level**

Bacteria in a food/poultry processing environment are exposed to nutrients with various levels of food components depending on the site and location of the plant (Djordjevic et al., 2002). However, at the same temperature, nutrition limitation induces higher biofilm formation. Hood and Zottola (1997) showed that *S. Typhimurium* in general produces more biofilm under nutrient limited condition. Yang et al., (2016) reported a significantly higher biofilm production for *S. Enteritidis* in 1/20 TSB as the growth medium when compared to normal TSB, on stainless steel. Also, Dewanti and Wong (1995) reported that *E. coli* develop faster and produced more biofilm when grown in a low nutrient medium.
**Temperature**

Biofilm formation depends on production of various enzymes and the expression of various proteins, which can easily explain the importance of temperature in biofilm formation (Garrettet et al., 2008). Stepanovic et al., (2003) reported that *Salmonella* spp. formed better biofilms at 30 and 22°C when incubated for 24 and 48 h, respectively as compared to 37°C. Similarly, Speranza et al., (2011) reported that *Salmonella* spp. were prone to form biofilms when the environmental temperature was between 30-32°C at a neutral pH and under nutritionally limited conditions. Also, 28°C was found to be more favorable for the expression of thin aggregative fimbriae in *S. Typhimurium* than at 37°C (Romling et al., 1998). Thin aggregative fimbriae are one of the major cellular components in the formation of the biofilm matrix for *Salmonella* (Zogaj, 2001).

Environmental temperatures have been shown to affect the bacterial attachment and biofilm formation in other bacterial spp. also. The expression and attachment of enterotoxin producing *E. coli* on plastic surfaces was better at 30°C as compared to 37°C (Szabo, 2005). A range of (4 to 45°C) incubation temperatures affects biofilm formation of *Salmonella* and *Listeria* species (Peel et al., 1988).

**Genetic role in biofilm formation**

The role of genetic factors involved in biofilm formation in bacteria are continuously being discovered, and the precise role of different genes and proteins are still under investigation. Microbes usually express a different set of genes and proteins when they form biofilms, to endure the unfavorable environment thereby ensuring their survival (Gandhi and Chikindas 2007; Fratamico et al., 2009).
The production of fimbriae and appendages are limited in the sessile phase of bacteria. The composition of individual components in *Salmonella* biofilms reveal that the LPS, EPS, and flagella composition varies depending on the substrate on which biofilms are formed (Prouty and Gunn, 2003). Cellulose is reported to be the main component of EPS in *Salmonella* biofilms for glass surfaces (Solano et al., 2002), whereas it is not a major component of the EPS for *Salmonella* biofilms on gall stones (Prouty and Gunn, 2003). Similarly, a cellulose biosynthetic gene, *yhiN*, in *Salmonella* Typhimurium disrupts biofilm formation in human epithelial cells, HEp-2 (Ledeboer and Jones, 2005). Expression of the *alg T* gene is known to enhance the production of alginate, a major component of EPS in *P. aureginosa* biofilms, while at the same time downregulating flagella synthesis (Davey and O'Toole, 2000). Roming et al. (1998) reported that rdar (red, dry and rough) morphotype of *Salmonella* Typhimurium has a tendency to adhere on glass and plastic surfaces. The inactivation of the *hns* gene in *S. Typhimurium* is known to reduce expression of the rdar morphotypes, and *hns* is also an activator of the *csgD* gene (Gerstel et al., 2003). *csgD* plays a role in the synthesis of curli and cellulose (Romling et al., 2000), thus assisting in biofilm formation. Another gene, *mlrA*, also regulates *csgD*, as demonstrated by the lack of curli production and rugose morphology in *mlrA* mutant *S. Typhimurium* (Brown et al., 2001). In *Salmonella* and *E. coli*, *RydC* prevents initiation of mRNA translation of *csgD*, hence impairing the biofilm formation by reducing curli biosynthesis (Bordeau and Felden, 2014). The pellicle formation by *S. Enteritidis* in broth is regulated by two operons responsible for cellulose biosynthesis: *bcsABZC* and *bcsEFG* (Solano, 2002), this proves the role of cellulose in pellicle formation.
Among the other genes responsible for biofilm formation, a virulence gene \( \text{spiA} \), in \textit{Salmonella} Enteritidis, is known to play a role in biofilm formation on microtiter plate assay and scanning electron microscopy (Dong et al., 2011). The \( \text{ycfR} \) gene in \textit{E. coli} is known to encode multiple resistance proteins and regulate biofilm formation (Zhang et al., 2007). Similarly, the \textit{E. coli} \( \text{bssS} \) gene controls indole transportation, which also affects biofilm formation. However, in contrast, Humphrey (2004) reported that deletion of \( \text{bssS} \) and \( \text{ycfR} \) in \textit{E. coli} lead to higher biofilm formation. In \textit{S. Enteritidis}, a large secreted protein, BapA has a role in biofilm formation and host colonization (Latasa, 2005). In yet another finding, Hamilton et al., (2009) reported that tryptophan biosynthesis genes \( \text{trpE} \) and an unknown gene \( \text{STM0341} \) were needed for biofilm growth in \textit{S. Typhimurium}. A histone like heterodimeric protein coding gene \( \text{ihf} \) in \textit{S. Typhimurium} is assumed to have some role in biofilm formation, as demonstrated by reduced biofilm formation in \( \text{ihf} \) mutants (Grestel, et al., 2003). Thus, the biofilm formation in \textit{Salmonella} could be attributed to the role of the putative biofilm forming genes such as, \( \text{bssS}, \text{ycfR}, \text{spiA}, \text{trpE} \) and \( \text{ihf} \).

\textbf{Role of sub-lethal biocide /chlorine concentration in induction and formation of biofilms}

When an antimicrobial treatment is not sufficient to kill bacteria (which can be called sub-inhibitory concentration), organisms tend to exhibit a response to sub-lethal stress (Montet et al., 2009). Sub-inhibitory concentration (SIC) is known to produce a structural change in bacteria when visualized under an electron microscope (Lorian, 1986). Salazar et al. (2013) reported that the expression of a stress regulatory gene, \( \text{ycfR} \), was significantly induced in \textit{S. enterica} upon exposure to chlorine treatment. They also
reported a differential regulation of the \textit{sirA} gene, which is responsible for \textit{Salmonella} to form biofilms under chlorine oxidative stress. In addition, the \textit{ycfR} gene in \textit{E. coli} showed increased expression in the presence of oxidative stressors like chlorine and hydrogen peroxide (Wang et al., 2009; Zheng et al., 2001). In a related study, it was reported that, the \textit{ycfR} gene was upregulated 12 fold in \textit{E. coli} biofilms as compared to its planktonic counterpart (Ren et al., 2004). Similarly, the expression of \textit{bssS} and \textit{ycfR} genes in \textit{S. Typhimurium} and \textit{S. Enteritidis} were increased, when they were exposed to sub-lethal chlorine concentrations (Wang et al., 2010). Free chlorine at SIC of 2 µg/ml (w/v) caused a two-fold increase in early biofilm formation of \textit{P. aeruginosa}, after two hours of incubation compared to the non-treated controls (Strempel and Overhage, 2011). Pagedar et al., (2012) reported that a non-antibiotic and biocide resistant strain of \textit{E. coli} when adapted to ciprofloxacin and benzylconium chloride displayed higher biofilm formation. Adaptation and previous exposure to sub-lethal concentration of sodium hypochlorite significantly improved the biofilm formation of \textit{E. coli}. (Capita et al., 2013). In another related study, a common laboratory disinfectant, ethanol, at 2 % and 4 % in TSB resulted in increased biofilm formation in low grade biofilm forming \textit{S. epidermidis} (Milisavljevic et al., 2008). Shemesh et al., (2010) discovered that the sub-lethal concentration of chlorine dioxide (4 µg/ml) accelerated the biofilm formation of \textit{B. subtilis} and \textit{P. aeruginosa} as compared to the cells grown in the absence of chlorine dioxide. Also, a SIC of chlorine caused an increase in the adherence (biofilm) of \textit{Acinetobacter} spp. and \textit{Pseudomonas aeruginosa} in polystyrene microtiter plates (Suman et al., 2013). Chlorine dioxide in a sub-lethal dose caused membrane damage to the cell. The potassium leakage and subsequent activation of \textit{Kinase C} is known to enhance the expression of an operon
in *Bacillus subtilis*, which leads to biofilm matrix production. Thus, the change in bacterial growth pattern and extension of lag phase due to various antimicrobial stress is known to enhance the bacteria’s ability to form biofilm which is due to the change in gene expression.

In another study, EPS synthesis was increased when antibiotics were exposed to biofilm cells of *E. coli* (Sailer et al., 2003). Sub-lethal doses of vancomycin and oxacillin induced biofilm formation of *Staphylococcus aureus* on various surfaces (Mirani and Jamil, 2011). Chlorine is one of the most common antimicrobials, in the food processing plant. *Salmonella* present on poultry carcasses and processing facility are prone to get exposed to a sub-lethal concentration of chlorine, due to improper sanitation of equipment as well as erroneous dosing of the antimicrobial. In this study, the effect of sub-lethal chlorine exposure to several *Salmonella* serotypes on their biofilm forming ability under various temperatures, substrates, and nutrient levels is determined.

**Thermal resistance of *Salmonella***

**D- value and Z-value**

Thermal treatment is very common practice in food the industry and at the consumer level. This treatment helps to reduce the microbial load. Bacterial destruction depends on temperature, time of exposure as well as the bacteria species. Thermal inactivation of bacteria is a logarithmic process *i.e.* for a thermal treatment at given temperature, where the same percentage of bacteria get destroyed in a fixed time (Juneja, 2007). Thermal lethality of a bacterial species can be measured using various parameters: D-value and Z-value.
D-value or decimal reduction time is defined as the time in minutes required to destroy 1 log (90%) of the total target bacteria at a given temperature. D-value is calculated as the negative reciprocal of the survival curve of bacteria or mathematically by:

\[ D = \frac{t}{\log N_0 - \log N_t} \]  

where, \( t \) is the time taken, \( N_0 \) and \( N_t \) are the bacterial count at the beginning and at the end of heat treatment.

In canned food, a 12-D approach is implemented to inactivate the majority of heat resistant \( \text{Clostridium botulinum} \) spores (Anderson et al., 2011).

\( Z \)-value, commonly used to calculate the microbial death time on the other hand is the change in temperature required to reduce D-value by 1 log10. To calculate the \( Z \)-value, we need to have at least two D-values then the following formula can be used:

\[ Z = \frac{T_1-T_2}{\log D_1 - \log D_2} \]  

where, \( T_1 \) and \( T_2 \) are initial and final temperatures, and \( D_1 \) and \( D_2 \) are two D-values.

**Sensitivity of different growth phases of \textit{Salmonella} on heat treatment**

Various studies reported that logarithmic and stationary phases of bacteria behave differently to thermal treatments (Buchanan and Edelson, 1999; Smith et al., 2001; Martinez et al., 2003). Cells in the stationary growth phase are generally known to be more heat resistant as compared to the log phase cells (Lou and Yousef, 1996). The higher resistance of stationary phase cells may be contributed to the higher production of stress resistant proteins and their response to various stresses (Hengge-Aronis, 2002).

\textit{Salmonella} in ground beef were found to be more heat resistant (higher D-value) while in stationary phase as compared to log phase (Smith et al., 2001). Ng et al., (1969)
reported that *S. Senftenberg* 775W attributed higher resistance in their stationary phase as compared to their log phase. Log phase cells of *Salmonella Typhimurium* STCC 878 were more susceptible to UV than the stationary phase cells (Gayan et al., 2012). In a similar study by Scher et al., (2005), stationary phase planktonic *S. Typhimurium* were shown to be more resistant to thermal treatment (70°C) as compared to the log phase cells. In 1978, Goodfellow and Brown studied thermal destruction of *Salmonella* in ground chicken and reported D-values of 61-62, 3.8-4.2, and 0.6-0.7 min at 51.6, 57.2, and 62.7°C respectively. Bacon et al., in 2003, using multi drug resistant *Salmonella*, in tryptic soy broth (TSB) reported D-values of 4.38, 1.47, 0.50, and 0.17 min for, 55, 57, 59, and 61°C, respectively. In another study by Yuan et al., (2012), *Salmonella Typhimurium* was reported to have D-values of 11.07, 6.35, and 4.13 min at 55.8, 57.8, and 59.8 °C respectively in TSB. Whereas, a range of D-values from 397.83 to 689.00, 11.35 to 260.95, and 1.12 to 6.81 seconds at 55, 60, and 65°C respectively were obtained for various strains of *Salmonella Typhimurium* when using TSB as medium (Burns et al., 2016).

Similar behaviors are displayed by other bacterial species. *E. coli O157:H7* showed higher acid and heat resistance in their stationary phase of growth which was attributed to the expression of *rpoS*-regulated genes (Cheville et al., 1996). *E. coli* O157:H7 causes a pronounced increase in heat resistance in stationary phase cells, which was not the case for log phase cells (Kaur et al., 1998). Farrel et al., (2010) reported that *E. coli* in stationary phase were more resistant to pulse light as compared to *E. coli* in log phase.
Apart from the variations in responses at different growth stages, microorganisms show enhanced thermotolerance upon prior treatment by various stressors. Starvation stressed *Salmonella* Typhimurium in a minimal medium with 0.02% glucose for 10 h at 37°C demonstrated a higher resistance at 52°C (Tolker-Nielsen and Molin 1996). Similarly, *S.* Typhimurium when acid adapted to a pH of 5.8 were able to survive longer at 50°C (Leyer and Johnson, 1993). Other gram negative bacteria such as *E. coli* O157:H7, non-pathogenic *E. coli*, and *Pseudomonas putida*, also displayed higher thermotolerance following prior exposure to starvation and acid (Leenanon and Drake, 2001; Givskov et al., 1994; Rowe and Kirk, 2000). *Salmonella* Typhimurium developed a thermal tolerance at a significant level after prior exposure to heat stress (Bunning et al., 1990). Similarly, *E. coli* which were exposed to chlorine oxidative stress were known to develop resistance against high temperature stress and oxidative stress (Wang et al., 2009). *S.* Typhimurium which were adapted to an oxidative antimicrobial hydrogen peroxide, displayed a higher thermal resistance as compared to the non-adapted *S.* Typhimurium (Christman et al., 1985). The oxidative effect of hydrogen peroxide treatment on *Salmonella* Typhimurium induces 30 proteins, including *Dnak*, a heat shock chaperone out of which five genes are also induced by heat stress (Morgan et al., 1986). These studies show that *Salmonella* might develop thermotolerance after prior exposure to chlorine or to an oxidative antimicrobial. In this study *Salmonella* will be exposed to short and long term chlorine stress and its subsequent response to thermal inactivation will be studied.

In summary, *Salmonella* spp. are known for posing a mechanism of resistance development against various stressors, strengthening their survival upon subsequent
exposure to either the same or to a different stressor. In the presence of chlorine stress, *Salmonella* are known to alter their genetic responses in terms of biofilm formation and thermal resistance. Therefore, measures should be taken to avoid *Salmonella* from being exposed to chlorine concentrations which do not kill the bacteria, avoiding the development of stress hardening in the organisms.
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CHAPTER III
EFFECT OF CHLORINE INDUCED SUB-LETHAL OXIDATIVE STRESS ON
THE BIOFILM FORMING ABILITY OF SALMONELLA AT DIFFERENT
TEMPERATURES, NUTRIENT CONDITIONS, AND
SUBSTATES

Abstract
The present study was conducted to evaluate the effect of chlorine induced
oxidative stress on biofilm formation of different Salmonella strains on polystyrene and
stainless steel (SS) surfaces at three different temperatures (30°C, room temperature, and
4°C) in tryptic soy broth (TSB) and 1/10th TSB. Fifteen Salmonella strains (6 different
serotypes) were exposed to a sub-lethal chlorine concentration (150 ppm) in TSB and for
2 h at 37°C. The biofilm forming ability of different Salmonella strains was determined
in 96 well polystyrene microtiter plates by a crystal violet staining method and on SS
coupons in 24 well tissue culture plates. All tested strains of Salmonella produced
biofilms on both surfaces tested in this study at room temperature and at 30°C. Out of 15
strains tested, none of the strains (chlorine stressed and non-stressed) formed biofilm at
4°C. At 30°C, S. Heidelberg (ID 72), S. Newport (ID 107) and S. Typhimurium (ATCC
14028) formed stronger biofilms as compared to the respective non-stressed controls on
polystyrene surface (P < 0.05). At room temperature, none of the chlorine stressed
Salmonella strains showed significant differences in biofilm formation as compared to
non-stressed control cells (P >0.05). The biofilm forming ability of *Salmonella* strains in nutrient deficient medium (1/10\textsuperscript{th} TSB) was quantitatively higher as compared to full strength TSB. At room temperature, chlorine stressed *S*. Heidelberg (ATCC 8326) and *S*. Enteritidis (ATCC 4931) formed stronger biofilms on SS coupons (P < 0.05) as compared to the non-stressed cells. These findings suggest that certain strains of *Salmonella* can produce significantly stronger biofilms on plastic and steel surfaces upon exposure to sub-lethal chlorine.

**Key words:** *Salmonella*, oxidative stress, chlorine, biofilm, stainless steel, plastic, tryptic soy broth

**Introduction**

Non-typhoidal *Salmonella* is the leading bacterial cause of foodborne illnesses and deaths in the United States (Scallan *et al.*, 2011). The top 10 *Salmonella* serotypes causing foodborne infections in the U.S. are: *Salmonella* Enteritidis, *S*. Newport, *S*. Typhimurium, *S*. Javina, *S*. I 4, [5],12: i: -, *S*. Poona, *S*. Muenchen, *S*. Heidelberg, *S*. Saintpaul, and *S*. Infantis (CDC, 2015). As per the CDC reports, there were 3 major *Salmonella* outbreaks in 2015 which were linked to poultry and various poultry products. Two *S*. Enteritidis outbreaks were linked to raw, frozen and stuffed chicken entrees whereas, one outbreak was linked to live poultry and involved multiple serotypes: *S*. Enteritidis, *S*. Hadar, *S*. Indiana, and *S*. Muenchen (CDC, 2015). In 2016, so far, two multi-state outbreaks, one linked to live poultry and another linked to eggs involving *S*. Oranienburg, have been reported (CDC, 2016).

In the food processing facilities, various surfaces and equipment such as plastic surfaces (Stepanovic *et al.*, 2004), stainless steel surfaces (Austin *et al.*, 1998), rubber
finger pluckers, and conveyor belt material (Lindsay et al., 1996) are prone to bacterial attachment and hence biofilm formation. *Salmonella* are capable of attaching to and forming biofilms on common materials used in the food industry like plastic, steel, glass, and rubber surfaces (Joseph et al., 2001; Leriche and Carpentier, 2000; Wang et al., 2010; Chia et al., 2009). Stainless steel bolts and screws, which are difficult to dismantle and clean are areas where *Salmonella* forms biofilms (Morita et al., 2011). It was reported that the plastic curtain surfaces along with rubber finger pluckers showed the highest biofilm formation demonstrated by bacterial count (Lindsay et al., 1996).

The presence of biofilm has an enormous impact on food contamination during processing. Such biofilm cells in a food processing unit offer greater resistance to normal cleaning and sanitation as compared to planktonic phase (Joseph et al., 2001). Bacterial cells in the biofilms are known to be more resistant to anti-bacterial agents (Carpentier and Cerf, 1993), heat (Frank and Koffi, 1990) and bacteriophages (Hicks and Rowbury, 1987).

The attachment of the bacterial cells and biofilm formation are determined by physiochemical properties of the cells along with various structural determinants like flagella, fimbriae, pili, curli and surface lipopolysaccharides as well as cell surface interactions (Van Houdt and Michiels, 2010) and bacterial cellulose production (Jain and Chen, 2007). Environmental factors such as temperature can affect bacterial attachment and biofilm formation. Many studies have shown that a range of (4 to 45°C) incubation temperatures affect biofilm formation of *Salmonella* and *Listeria* species (Peel et al., 1988). For example, 30°C was favored for the attachment of *Salmonella* spp. on plastic surfaces when compared to 37°C or 22°C (Stepanovic et al., 2003). Thin aggregative
fimbriae are one of the major cellular components in the formation of the biofilm matrix by *Salmonella* (Zogaj, 2011), and their expression is temperature dependent, 28°C being more favorable as compared to 37°C (Romling *et al*., 1998).

Chlorine is a well-known oxidizing agent and the most commonly used antimicrobial in the food industry including meat and poultry (Wang *et al*., 2010). Chlorine generates hydroxyl radicals and these oxidative species disrupt the cell’s membrane and metabolic processes. It is also reported that chemicals, including chlorine, baking soda, and potassium aluminium sulfate when used in water treatments, may render the surviving pathogens more resistant to antibiotic and possibly more virulent by expressing antibiotic resistant genes in gram negative bacteria like *E. coli* and *P. aeruginosa* (Shrivastava *et al*., 2004; Armstrong *et al*., 1982; Shi *et al*., 2013; Xi *et al*., 2009). Free chlorine exposure (4 ppm) is also known to cause up-regulation of antibiotic resistant genes in the gram negative bacteria, *Acinetobacter baumannii* (Karumathil *et al*., 2014).

Biofilm formation by *Salmonella* is regulated by expression of *ycfR, sirA* and *bssS* genes (Wang *et al*., 2010; Salazar *et al*., 2013). Salazar *et al*., (2013) reported up-regulation of the *ycfR* gene in *S. Typhimurium* and *S. Enteritidis* under chlorine oxidative stress. In addition, the *ycfR* gene in *E. coli* was upregulated in the presence of chlorine and hydrogen peroxide (Wang *et al*., 2009; Zheng *et al*., 200). The expression of *bssS* and *ycfR* genes in *S. Typhimurium* and *S. Enteritidis* increased when the cells were exposed to chlorine (Wang *et al*., 2010). Free chlorine at sub-inhibitory concentrations of 2 µg/ml (w/v) resulted in a two-fold increase in biofilm formation of gram negative *P. aeruginosa* after two hours of incubation compared to the non-treated controls (Strempel and
Overhage, 2011). In another study, it was found that previous exposure to a sub-lethal concentration of sodium hypochlorite resulted in homologous adaptation and significantly improved biofilm formation by *E. coli* on a polystyrene surface (Capita *et al.*, 2013). Shemesh *et al.*, (2010) discovered that a sub-lethal concentration of chlorine dioxide accelerated biofilm formation of *B. subtilis* and *P. aeruginosa* as compared to the cells grown in the absence of chlorine dioxide. In addition to chlorine stress, other environmental stressors such as temperature and pH can also affect biofilm formation by *S. Typhimurium* on stainless steel and acrylic surfaces (Nguyen, 2014).

Very limited scientific information is available on the effect of oxidative stress of chlorine or other antimicrobials on biofilm formation by *Salmonella*. The studies so far, have only reported the genetic expression of biofilm forming genes, however the information on production and attachment of biomass under different environmental conditions is lacking in *Salmonella*. Foodborne pathogens such as *Salmonella* can be exposed to sub-lethal concentrations of chlorine during cleaning and sanitation in a food processing facility. These cells can survive and possibly attach to variety of surfaces inside a processing facility. This study was designed to study the effect of a sub-lethal chlorine concentration on the biofilm forming ability of *Salmonella* spp. mimicking the environment of a food processing facility including different substrates (plastic vs stainless steel surface), nutrient environments (nutrient rich vs nutrient deprived), and temperatures (4°C, 30°C, and room temperature).
Materials and Methods

Salmonella-strains/serotypes

A total of 15 strains of Salmonella representing 6 serotypes (Table 3.1) were used in this research to study their biofilm formation after chlorine stress on polystyrene and stainless steel surfaces at three different temperatures (4°C, 30°C, and room temperature) under two different nutrient levels. Three serotypes (S. Enteritidis, S. Heidelberg and S. Typhimurium) were procured from American type culture collection (ATCC) and the other 12 strains were isolated from grocery meat samples. The serotyping of the strains was performed by National Veterinary Service Laboratory (NVSL), Ames, Iowa. All the strains were maintained in tryptic soy broth (TSB)-glycerol (7:3) at -80°C. Prior to use, the frozen cultures were streaked on tryptic soy agar (TSA) plates and incubated at 37°C for 24 h. A single colony of each Salmonella strain was inoculated in 10 ml of TSB and incubated at 37°C for 18 to 24 h.

Chlorine Source

Chlorox® (Oakland, California), commercially available bleach (7.85% active chlorine), was used as the source of chlorine. The total available chlorine in Chlorox was confirmed using HACH Aquachek® test strips and a HACH colorimeter test kit.

Minimum Inhibitory Concentration (MIC) Assay

The determination of MIC for planktonic cells was performed in TSB by the broth micro dilution method according to the Clinical and Laboratory Standards Institute (Cockerill et al., 2012). A volume of 100 µL of sodium hypochlorite solution consisting of twice the desired final concentration was dispensed in triplicate wells of a 96 well
plate. One hundred microliters of bacterial culture containing ~6 log CFU/mL (in TSB) was added to each well to make a final volume of 200 µL. A positive control consisted of *Salmonella* inoculum (no chlorine) and negative control was TSB without *Salmonella*. The microtiter plate was incubated at 37ºC for 24 h and visible growth in the wells was used to determine the MIC. The concentration range of chlorine was narrowed in subsequent replications so as to obtain the lowest concentration of chlorine required to inhibit growth. MIC was determined as the lowest concentration of chlorine that inhibited the visible growth of *Salmonella* after 24 h of incubation at 37ºC.

**Bacterial Growth Curve Analyses and Determination of Sub-lethal Concentration for Biofilm Assay**

Growth curves for three *Salmonella* serotypes (*S*. Enteritidis (ATCC 4931), *S*. Heidelberg (ATCC 8326), and *S*. Typhimurium (ATCC 14028) treated with chlorine concentrations (0 ppm (control), 25, 50, 100, 130, 150, 200, and 300 ppm) in TSB were constructed to determine the sub-lethal chlorine concentration that would stress *Salmonella* the most (to alter the bacterial physiology without killing the cells). The extension of lag phase was used as a measurement that the cells received oxidative stress of chlorine. Three wells per concentration, per strain (100 µl each of culture and chlorine) were loaded into a 96 well microtiter plate and placed in a Spectramax® plus 384 microplate reader. The OD$_{600}$ readings were recorded after every 30 min interval for 18 h, and the values were plotted against time interval and analyzed for extension of the lag phase. Sub-lethal injury as a result of exposure to antimicrobials like acetic and lactic acids in *S*. Enteritidis is known to extend the lag phase in *Salmonella* (Alexandrou *et al.*, 2010).
The duration of the lag phase was measured based on the assumption that bacterial cells enter the log phase when the OD$_{600}$ exceeds 0.2 (Wang et al., 2010).

**Biofilm Forming Ability of Chlorine Stressed Salmonella Strains**

**On plastic surface**

The crystal violet staining method was used to determine biofilm formation for all 15 strains of *Salmonella* (stressed and non-stressed) on the plastic surface in a 96 well microtiter plate (Patel and Sharma, 2010). Overnight (~18 h) cultures of individual *Salmonella* strains in TSB were diluted to a working concentration of ~ 7 log CFU/ml in TSB. The treatments (chlorine stressed *Salmonella*) consisted of cells exposed to 150 ppm of chlorine in half strength TSB for 2h at pre-determined temperatures, whereas controls were maintained in half strength TSB without chlorine. After 2 h of incubation at 4°C, 30°C, and room temperature, the control and treated cell suspensions were centrifuged at 10,000 rpm for 5 min. The supernatant was discarded and the pellets were re-suspended in fresh TSB followed by dispensing 200μl of the cell suspension into a sterile 96 well microtiter plate (eight wells for each strain). Positive controls consisted of *Salmonella* cultures (without chlorine stress), and negative controls consisted of TSB without culture. The plates were incubated at 4°C, 30°C, and room temperature for 48 h. After incubation, 200μl of the culture was completely removed, and the wells were washed three times with sterile distilled water. The plates were then air-dried for an additional 45 min, and 200μl of crystal violet solution (0.41% w/v) dye was added per well and incubated at room temperature for 45 min. Crystal violet solution was completely removed from wells by aspiration and washed three times with sterile distilled water. After allowing wells to air dry for 45 min, 200μl of 95% ethanol was added to
each well, and the contents of the wells were mixed to dissolve the crystal violet dye. Biofilm formation in each well was measured at an OD$_{600}$ using a microplate spectrophotometer (BioTek® Elx800). The material and methods for the nutrient deprived condition were similar, except incubation of the control and treated cells that occurred for 48 h was performed in 1/10 TSB instead of full strength TSB.

**On Stainless Steel**

In order to determine the effect of chlorine stress on *Salmonella* attachment to a steel surface, an established method with slight modifications was used (Hood and Zottola, 1997). Custom synthesized stainless steel (SS) coupons (#4 Finish, 26G, 1.5 cm X 1 cm) were procured from Stainless Supply ® (NC, USA). SS coupons were washed in order to remove fingerprints, oils, grease, and other soils (Hood and Zottola, 1997; Shen et al., 2012). Cleaned SS coupons were finally autoclaved at 121ºC for 15 min prior to use.

The *Salmonella* pellets of chlorine stressed (as mentioned above for the crystal violet assay) and non-stressed (control) cells were re-suspended in TSB, and then 1.6 ml of the culture (~ 7 log CFU/mL) was dispensed in duplicate wells of a 24 well tissue culture plate. Sterile SS coupons were then placed in each well, and the plate was incubated statically at 30ºC and room temperature for 48 h. At the end of the incubation, the coupons were removed from the wells with the help of sterile forceps, tapped on the edge of the well surface to drain excessive liquid and washed three times with sterile distilled water to remove non-attached or loosely attached cells. The coupons were then placed in 50 ml sterile centrifuge tubes containing 9.2 ml of sterile 0.1% peptone water and sterile glass beads. Centrifuge tube lids were tightly closed and then vortexed for ~3
min to detach the tightly attach cells and bring them into suspension. The suspension was then serially diluted (10 fold) and spread plated (0.1 ml volume) on TSA plates for *Salmonella* counts after 24 h of incubation at 37°C.

**Statistical Analysis**

The entire was replicated three times over three days. A randomized complete block design with days as blocks was used to determine difference in the treatment effects. The GLM procedure of SAS 9.4 version (SAS institute, Cary, NC) was used to perform analysis of variance tests. The means were separated using the Least Significant Difference test. The treatments and controls were found to be significantly different when $P \leq 0.05$.

**Results**

**Minimum Inhibitory Concentration (MIC)**

The MIC of chlorine in TSB against *S. Typhimurium* (ATCC 14028), *S. Heidelberg* (ATCC 8326), and *S. Enteritidis* (ATCC 4936) was found to be approximately 180 ppm, based on the visible growth in the 96 wells microtiter plates after 24 h of incubation at 37°C.

The growth of *S. Typhimurium*, *S. Heidelberg* and *S. Enteritidis* was affected in the presence of various chlorine concentrations (Fig. 3.1, 3.2, and 3.3). The growth inhibition of all three serotypes was profound with the increasing concentration of chlorine from 25 ppm to 300 ppm at 37°C. The $OD_{600}$ of $\geq 0.2$ was considered to be the beginning of logarithmic phase of bacterial growth (Wang *et al.*, 2010). For all three serotypes, the lag phase for the control (cells not exposed to chlorine) and treated cells
(treated with chlorine at 25 ppm, 50 ppm, and 100 ppm) was found to be approximately 2-2.5 h. However, the lag phase for the cells treated with 130 ppm and 150 ppm were extended for approximately 4 or 5 hours, respectively. Cells treated with chlorine concentrations below 200 ppm were able to revive and grow. S. Typhimurium and S. Heidelberg cells treated with 200 ppm chlorine showed an extended delay of ≥ 15 h before reaching an OD value of 0.2, whereas cells treated with 300 ppm chlorine never exhibited growth after 24 h. A chlorine concentration of 150 ppm, although not lethal to Salmonella, resulted in a significant effect on the growth of all three Salmonella serotypes. Exposure of S. Typhimurium, S. Heidelberg and S. Enteritidis to 150 ppm chlorine resulted in the longest lag phase as compared to other chlorine concentrations below 200 ppm, and for this reason, the 150 ppm chlorine treatment was selected as a sub-lethal concentration to induce oxidative stress to Salmonella and to study its effect on biofilm forming ability of the stressed Salmonella cells.

**Biofilm Formation on Plastic**

The screening for biofilm production of 15 strains of Salmonella in nutrient medium (TSB) at 4°C showed no significant biofilm production on polystyrene surface (Fig. 3.4a), which was not different than the negative control. Though, the OD$_{600}$ values for the nutrient deficient medium (1/10 TSB) were quantitatively higher than in TSB at 4°C, there were no significant differences in the OD values among the strains and negative control (Fig. 3.5a) and all these strains were non-biofilm producers at 4°C. At 30°C, in TSB, the OD$_{600}$ of the strains ranged from 0.244 to 1.135. The 3 ATCC serotypes demonstrated the highest biofilm formation, S. Typhimurium (ATCC 14028) formed the strongest biofilm with OD$_{600}$ of 1.135 followed by the other two ATCC
serotypes (S. Heidelberg and S. Enteritidis) as compared to all other strains (Fig. 3.6a). At 30°C, the biofilm production in nutrient deficient medium (1/10 TSB) was quantitatively lower, based on an OD$_{600}$ reading which ranged from 0.412 to 0.717. S. Heidelberg (IDs 52, 72, 92) from turkey origin and S. Typhimurium (ID 39) from turkey origin produced significantly less (P≤0.05) biofilm as compared to S. Typhimurium (ID 33) from chicken origin (Fig. 3.7a). At room temperature, the biofilm production in TSB after 48 h of incubation was less when compared to that at 30°C and ranged from a OD$_{600}$ of 0.133 for S. Heidelberg (ATCC 8326) to 0.590 for S. Typhimurium (ATCC 14028) (Fig. 3.8a). S. Typhimurium (ATCC 14028) demonstrated the strongest biofilm at room temperature. The biofilm production in 1/10 TSB at room temperature was quantitatively higher, with OD$_{600}$ ranging from 0.545 to 0.911, and S. Newport (ID 107) produced significantly stronger (P≤0.05) biofilm as compared to S. Typhimurium (ATCC 14028) (Fig. 3.9a).

The ability of biofilm formation by the chlorine stressed Salmonella were measured at three different temperatures. At 4°C, after 48 h of incubation of chlorine stressed cells in TSB as well as in 1/10 TSB there were no significant biofilm formation on polystyrene surface as the OD value of culture wells were no different than the negative control (Figs 3.4b and 3.5b). Similarly, as in non-stressed cells, the chlorine treated cells produced stronger biofilms at 30°C as compared to room temperature. At 30°C, the biofilm formation by chlorine stressed cells in TSB ranged from OD$_{600}$ of 0.355 to 1.395. S. Typhimurium (ATCC 14028) formed more biofilm with an OD$_{600}$ of 1.395 followed by two other ATCC serotypes, S. Heidelberg and S. Enteritidis as well as turkey isolate of S. Saintpaul (ID 43) (Fig. 3.6b). Similarly, OD$_{600}$ for the chlorine stressed cells in the nutrient deprived medium (1/10 TSB) ranged from 0.119 for S.
Saintpaul (strain ID 43) to 0.766 for S. Heidelberg (ATCC 8326) (Fig. 3.7b). The biofilm formation at room temperature for the chlorine stressed cells in TSB ranged from an OD$_{600}$ value of 0.134 to 0.573 with S. Typhimurium (ATCC 14028) producing the most biofilm (Fig. 3.8b). In 1/10 TSB the biofilm formation by chlorine stressed cells at room temperature ranged from an OD$_{600}$ of 0.570 to 1.062 (Fig. 3.9b). In general, the ATCC serotypes of S. Enteritidis, S. Heidelberg, and S. Typhimurium were better biofilm formers in both non-stressed as well as stressed condition at 30°C. At room temperature, S. Typhimurium (ATCC 14028) showed consistently stronger biofilm formation in stressed as well as non-stressed conditions.

When the biofilm forming ability of chlorine stressed Salmonella strains was compared to their respective controls, at 4ºC in TSB, neither of the 15 Salmonella strains (chlorine stressed as well as non-stressed controls) tested showed any significant attachment on the plastic (Fig. 3.4c). At 30ºC, S. Heidelberg (ID 72), S. Newport (ID 107), and S. Typhimurium (ATCC 14028) formed significantly higher biofilm as compared to their respective controls (P ≤0.05) in TSB (Fig. 3.6c). At room temperature, there were no significant differences (P >0.05) in the biofilm formation among the chlorine stressed and non-stressed Salmonella strains (Fig. 3.8c and 3.9c). At 30ºC (Fig. 3.6c and 3.7c) and room temperature (Fig. 3.8c and 3.9c) chlorine stressed Salmonella strains, in general formed stronger biofilms as compared to the control cells, however the differences were not significant (P ≥0.05) for some strains. These findings indicate that the nutrient deprived conditions favor stronger biofilm formation in Salmonella as compared to full strength nutrient medium (TSB).
**Biofilm formation in Stainless Steel (SS) Coupon**

The biofilm formation was quantified by enumerating the strongly attached cells on tryptic soy agar (TSA) plates. The results were reported as log CFU/mL and the difference in log values was used as a measurement of difference in biofilm formation or attachment of stressed vs non-stressed cells on stainless steel coupons (Fig. 3.10). There was no significant difference in the biofilm formation between chlorine stressed and non-stressed cells at 30°C (Fig. 3.10b) whereas, chlorine stressed *S. Heidelberg* (ATCC 8326) and *S. Enteritidis* (ATCC 4931) formed significantly (P ≤0.05) stronger biofilms as compared to their respective controls at room temperature (Fig. 3.10a).

**Discussion**

Wet and nutrient rich surfaces in food and poultry processing environments offer ideal conditions for bacteria to attach and form biofilms (Chmielewski and Frank, 2003). The existence of biofilm-forming foodborne pathogens including *Salmonella* is a real challenge for the food industry. A wide range of antimicrobials including chlorine and acidified sodium chlorite are commonly used by the food and poultry industry for regular cleaning and sanitation (Lillard, 1979; Tsai et al., 1992; Bauermeister, 2008). Because the resistance is conferred by the extracellular polymeric substances (EPS) of biofilms, the disinfectants which are effective against planktonic cells are usually ineffective or less effective against the cells within a biofilm (Korber et al., 1997; Xavier et al., 2005; Van Houdt and Michiels, 2010). Attachment of bacteria is affected by physical and chemical properties of the cell and the substratum surfaces as well as the environment of the medium (Chmielewski and Frank, 2003). It is well reported that *Salmonella* can attach and form biofilms on food processing surfaces like plastic, cement, and stainless
steel located in processing plants (Joseph et al., 2001; Helke and Wong, 1994; Jones and Bradshaw, 1997).

LeChevallier et al. (1981) reported that chlorination is negatively correlated with the presence of organic compounds in the medium. The organic compounds in TSB could rapidly neutralize free chlorine (HOCl and OCl⁻), which otherwise would have been utilized as an antimicrobial against bacteria in the medium. Wang et al. (2009) reported that the oxidation reduction potential (ORP) of chlorine decreased by 49% in Brain Heart Infusion (BHI) broth compared to that in a water solution after 5 min.

Growth curves of different Salmonella strains were analyzed and we found a similar trend in the growth pattern in all three serotypes (S. Enteritidis, S. Heidelberg, and S. Typhimurium) tested. The extension of the lag phase for all three serotypes was in proportion with the level of chlorine exposure, whereas cells exposed to a higher concentration of chlorine showed the longer lag phase. The lag phase is assumed to allow for the adaptation of bacterial cells in a new or hostile environment (Madigan et al., 1997). These findings suggest that chlorine at higher concentrations, 150 ppm, exerts greater oxidative stress on Salmonella, demonstrating an extended lag phase of 4-6 h. Alexandrou et al., (1995) discovered that sub-lethal injury caused by antimicrobials such as acetic acid and lactic acid in S. Enteritidis caused an extension of the lag phase in general purpose media for up to 4 h, making it consistent with our finding of a 4-6 h extension of the lag phase. Wang et al., (2010) reported that the extension of the lag phase in Salmonella after chlorine stress in BHI broth resulted in down regulation of genes associated with general cellular metabolism, ribosomal proteins, osmolarity and pH regulatory outer membrane proteins. Further, it was reported that S. Typhimurium
accumulates iron during its lag phase which is associated with their sensitivity to oxidative stress (Rolfe et al., 2012). In a recent study by Li et al., (2016), it was reported that the extension of the lag phase in gram negative bacteria offers a better survival advantage and a revival after antibiotic removal from the medium. Similar findings were observed for *L. monocytogenes* upon cold, heat, chlorine, and starvation stress (Guillier et al., 2005). The surge in the OD$_{600}$ value observed in all three strains (S. Enteritidis, S. Heidelberg, and S. Typhimurium) after 18 h under 200 ppm of chlorine stress in our study could be attributed to the revival of stressed cells. Researches have shown that the injured cells can repair the damage when optimum temperature is provided in a nutritionally adequate environment in the absence of selective agents (Ray, 1979; Wu, 2007).

The screening for biofilm formation from 15 strains of *Salmonella* including 12 poultry isolates in polystyrene revealed that these strains possess the capacity to form biofilms. There was variation in the extent of biofilm formation for different *Salmonella* serotypes. We didn’t find any consistent variation in biofilm forming ability of *Salmonella* based on the source of origin (ATCC source or poultry isolates) which was in accordance to the finding by Stepanovic et al. (2004). Based on the cut-off OD (ODc) for microtitre plate (Stepanovic et al., 2000), all the strains tested were either moderate, weak or no biofilm former at all three temperatures tested. None of them were strong biofilm formers.

Bacterial attachment to a substrate is a complex process involving electrostatic force, van der Waals forces, and hydrophobic interactions during initial attachment followed by specific irreversible attachment owing to exopolysaccharide (EPS)
production (Ledeboer and Jones, 2005; Bayoudh et al., 2006; Palmer et al., 2007). Veluz et al., (2012), reported different levels of attachment by Salmonella to polypropylene and stainless steel surfaces. The attachment of bacteria to a substrate depends on variety of factors including, surface charge and the hydrophobicity of bacteria (Sinde and Carballo, 2000), surface roughness and microtopography (Palmer et al., 2007), as well as cellular appendages like flagella (Tresse et al., 2006). Salmonella are known to attach more radially to hydrophobic materials like plastic as compared to hydrophilic material like stainless steel (Sinde and Carballo, 2000). Strain variations of Salmonella also offers different cell surface hydrophobicity (Chia et al., 2009), and thus most of the biofilm formation by Salmonella are on hydrophobic cell surfaces (Ukuku and Fett, 2006). Our study demonstrated that different serovars of Salmonella have different biofilm forming abilities which also vary between temperature, nutrient level, and substrate attachment. We reported a variability in the attachment property of chlorine stressed and non-stressed Salmonella on plastic surfaces that varied with nutrient composition and temperature of incubation. In general, biofilm formation in TSB at 30°C were higher than at room temperature. At 4°C, the biofilm mass on plastic surfaces was negligible when measured using OD$_{600}$, which could be due to Salmonella’s inability to multiply at refrigerated temperatures. The OD values of the wells with Salmonella culture were no different than negative controls. For cells to form biofilm, OD value of the culture should be higher than the OD cut-off (ODc) values, where a value three standard deviation above the mean OD of the negative control is considered as the OD cut-off (ODc) value Stepanovic et al. (2000). The variation in biofilm production among different strains could be attributed to different genetic make ups and attachment properties of the different strains.
The hardness of a substrate also affects the attachment of bacteria to some extent. Stainless steel is one of the most commonly used contact materials in the food processing environment (Olszewska, 2013), and it is considered to be the ideal material in food processing, as it is chemically and physiologically stable to various food processing temperatures, cleaning agents, and corrosion (Verran, 2001). The greater hardness of stainless steel offers less bacterial attachment as compared to that by polystyrene (Midelet and Carpentier, 2002; Tolvanén et al., 2007; Veluz et al., 2012). In addition, cleaning of stainless steel surfaces is more efficient (Tolvanén et al., 2007) making it a preferred material in food contact surfaces. In this study an incubation time of 48 h was used to allow *Salmonella* cells to attach to the stainless steel surfaces. The higher recovery rate of certain serotypes of chlorine stressed *Salmonella* cells from the SS coupons represents the higher attachment and biofilm formation by stressed cells. This result can also be correlated with the higher expression of biofilm forming genes such as *bssS* and *ycfR* in *S. Typhimurium* and *S. Enteritidis* upon chlorine exposure (Wang et al., 2010). There is also strain variation in the attachment rate of *Salmonella* on the SS coupons.

The choice of temperatures (4°C, 30°C, and room temperature) for the biofilm study is based on the different environmental conditions encountered during food and poultry processing and storage. The optimum temperature for bacterial attachment and biofilm formation has been reported to be 30°C, and the reason for choosing 4°C is because food is stored at refrigeration temperature (4°C) (Else et al., 2003). The room temperature (23°C-24°C) was selected to simulate the biofilm formation by *Salmonella* in a temperature abused conditions during food storage. All of the strains tested produced no
biofilms at the refrigerated temperature. The lower rate of biofilm formation at 4°C attributed to *Salmonella*’s ability to survive but not to multiply at this temperature (Pradhan, 2012). In a similar study by Giaouris *et al.*, (2005), *S. Enteritidis* formed poor biofilms at 5°C as compared to 20°C or at 37°C on stainless steel surfaces. However, in our study, nutrient limitation conditions at 4°C caused higher biofilm formation as compared to nutrient medium which is consistent with a finding by Hood and Zottola (1997). At 30°C in TSB, chlorine stressed *S. Heidelberg* (strain ID 72), *S. Newport* (ID 107) and *S. Typhimurium* (ATCC 14028) produced significantly more biofilm as compared to non-stressed cells. This finding can be attributed to how different *Salmonella* species, namely *S. Enteritidis* and *S. Typhimurium*, react to stress where their biofilm forming genes, *bssS* and *ycfR* are upregulated under sub-lethal oxidative stress by chlorine (Wang *et al.*, 2010). Chlorine is more efficient against *Salmonella* at higher temperatures (30°C) as compared to room temperature and 4°C (Lopez-Velasco *et al.*, 2012). The higher efficacy of sub-lethal chlorine during incubation of 2 hours at 30°C might have exerted oxidative stress to the *Salmonella* leading to stronger biofilm formation.

Bacteria in a food processing environment tend to remain in contact with different levels of food particles and food components depending on the location of the facility (Djordjevic *et al.*, 2002). Compared to the nutrient rich laboratory media, diluted TSB (1/10 TSB) in this experiment was chosen to simulate the environmental conditions such as places or locations inside a food processing premise which come in contact with limited food material (Leriche and Carpentier, 2000). *Salmonella* is known to produce stronger biofilm under nutrient limited condition (Hood and Zottola, 1997; Lagha *et al.*, 2010).
The greater biofilm formation at room temperature, both in stressed and control cells in a nutrient deprived condition (1/10 TSB) in our study is consistent with the study by Stepanovic et al. (2004) who reported an increased biofilm formation in Salmonella spp. in a starved condition of 1/20 TSB as compared to TSB. One of the probable reason for this can be the over expression of the biofilm formation gene, agfD, and aggregative fimbriae in the nutrient limited condition (Romling et al., 2000; Gerstel and Romling 2001). Increased biofilm formation by S. Enteritidis in 1/20 TSB compared to TSB on stainless steel (Yang et al., 2016) further supports the fact that biofilm formation is better induced under nutrient limited conditions. Our findings are consistent with the previous findings by Castelijn et al. (2012) who reported that S. Typhimurium strains, isolated from industrial outbreaks, formed significantly stronger biofilm in 1/20 TSB as compared to TSB at 25°C, however, there was no difference reported in the biofilm formation of clinical isolates in TSB vs 1/20 TSB. Also, Kroupitski et al., (2009) reported no significant difference in the amount of biofilm formation for several Salmonella serotypes except for S. Virchow when tested in TSB as compared to 1/20 TSB, whereas in 1/10 Luria Bertani broth (LB) the biofilm formation of all tested strains were significantly lower. This difference in biofilm properties indicates that genetic factor, nutrient composition or strain variation may have certain influences on biofilm formation in addition to starvation. Another possible explanation for this can be a mutation of genes controlling biofilm formation (Romling et al., 1998). Lagha et al., (2015) described the hyper-flagellation and elongation of the starved cells as a reason of higher biofilm formation. In reference to another gram negative bacteria, Pseudomonas aureofaciens, Monds et al., (2001) described why the starvation may lead to greater biofilm formation.
They stated that the attachment of cells to a substrate requires a threshold level of extracellular inorganic phosphate (Pi), which may be a limiting factor in starved cells. Chlorine induced oxidative stress can effectively stimulate stronger biofilm formation in certain strains of *Salmonella*. The higher biofilm formation by the chlorine stressed cells depends on temperature and substrate of attachment. On polystyrene, certain chlorine stressed strains demonstrated stronger biofilm formation at 30°C, whereas on SS coupons room temperature was more favorable for attachment of certain chlorine stressed strains.

**Conclusions**

Formation of biofilms by *Salmonella* on various food contact surfaces at various temperatures, and nutrient conditions has significant implication to the food processing industry. Biofilms formed on food processing equipment and food contact surfaces become a persistent source of contamination in food, threatening food safety and public health. The potential of some *Salmonella* strains to form stronger biofilms following a sub-lethal chlorine stress highlights the importance of through cleaning and sanitation.
Table 3.1 Information about the *Salmonella* strains used in the study

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Serotype</th>
<th>Sources</th>
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<tbody>
<tr>
<td>33</td>
<td>Typhimurium</td>
<td>Chicken Breast</td>
</tr>
<tr>
<td>39</td>
<td>Typhimurium</td>
<td>Ground Turkey</td>
</tr>
<tr>
<td>41</td>
<td>Saintpaul</td>
<td>Ground Turkey</td>
</tr>
<tr>
<td>43</td>
<td>Saintpaul</td>
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<tr>
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<td>Heidelberg</td>
<td>Ground Turkey</td>
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<tr>
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<td>Saintpaul</td>
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<td>107</td>
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<td>ATCC</td>
</tr>
<tr>
<td>8326</td>
<td>Heidelberg</td>
<td>ATCC</td>
</tr>
<tr>
<td>4931</td>
<td>Enteritidis</td>
<td>ATCC</td>
</tr>
</tbody>
</table>
Figure 3.1  Growth curves of *S. Typhimurium* (ATCC 14028) in the presence of various chlorine concentrations in TSB.
Figure 3.2  Growth curves of *S. Heidelberg* (ATCC 8326) in the presence of various chlorine concentrations in TSB.
Figure 3.3  Growth curves of *S. Enteritidis* (ATCC 4931) in the presence of various chlorine concentrations in TSB
Figure 3.4  Biofilm formation by various *Salmonella* strains after 48 h in a 96-well polystyrene microtitre plate in TSB at 4°C

a) controls (non-stressed cells) b) cells stressed with 150 ppm chlorine for 2h and c) stressed vs. non-stressed cells.
Figure 3.5  Biofilm formation by various *Salmonella* strains after 48 h in a 96-well polystyrene microtitre plate in nutrient deprived condition (1/10 TSB) at 4°

a) controls (non-stressed cells) b) cells stressed with 150 ppm chlorine for 2h and c) stressed vs. non-stressed cells.
Figure 3.6  Biofilm formation by various *Salmonella* strains after 48 h in a 96-well polystyrene microtitre plate in TSB at 30°C

a) controls (non-stressed cells) b) cells stressed with 150 ppm chlorine for 2h and c) stressed vs. non-stressed cells.
Figure 3.7  Biofilm formation by various *Salmonella* strains after 48 h in a 96-well polystyrene microtitre plate in nutrient deprived condition (1/10 TSB) at 30°C.

a) controls (non-stressed cells)  b) cells stressed with 150 ppm chlorine for 2h and c) stressed vs. non-stressed cells.
Figure 3.8  Biofilm formation by various *Salmonella* strains after 48 h in a 96-well polystyrene microtitre plate in TSB at room temperature

a) controls (non-stressed cells) b) cells stressed with 150 ppm chlorine for 2h and c) stressed vs. non-stressed cells.
Figure 3.9  Biofilm formation by various *Salmonella* strains after 48 h in a 96-well polystyrene microtitre plate in nutrient deprived condition (1/10 TSB) at room temperature.

a) controls (non-stressed cells) b) cells stressed with 150 ppm chlorine for 2h and c) stressed vs. non-stressed cells.
Figure 3.10  Comparison of biofilm formation of chlorine stressed vs non-stressed Salmonella spp. after 48 h on stainless steel coupons in tryptic soy broth (TSB)

(a) at room temperature and (b) at 30°C.
References


Peel, M., Donachie, W., & Shaw, A. (1988). Temperature-dependent expression of flagella of *Listeria monocytogenes* studied by electron microscopy, SDS-PAGE.


CHAPTER IV

EFFECT OF SHORT TERM AND LONG TERM SUB-LETHAL CHLORINE INDUCED OXIDATIVE STRESS ON THERMAL RESISTANCE OF SALMONELLA

Abstract

The effect of short (1h exposure) and long term (27 d) sub-lethal chlorine stress on the thermal resistance of Salmonella Heidelberg and S. Typhimurium was studied in tryptic soy broth (TSB) and chicken broth at 55°C, 58°C, and 61°C during the logarithmic and stationary growth phases. Long term exposure of Salmonella to sub-lethal chlorine concentration led to development of rugose morphology that was exhibited by both S. Typhimurium and S. Heidelberg. The short term chlorine stressed Salmonella cells (for 1h) were more sensitive to thermal treatments (P<0.05) as compared to the non-stressed cells. The $D_{55}$, $D_{58}$ and $D_{61}$ values of log phase short-term stressed S. Heidelberg and S. Typhimurium in TSB were 3.78 and 3.96 min for non-stressed versus 2.9 and 3.28 min for stressed cells. After long term chlorine stress, S. Typhimurium at 55°C and both Typhimurium and Heidelberg at 61°C were better protected against thermal inactivation. At 55°C, thermal resistance of the long-term stressed rugose morphotype of S. Typhimurium in TSB was higher (P<0.05) as compared to the non-stressed wild type smooth cells in log as well as stationary phases cell. The $D_{58}$ values for rugose and smooth S. Heidelberg and S. Typhimurium were similar in TSB (P>0.05) in both log and
stationary phases cell. The D$_{61}$ for long-term chlorine stressed (rugose morphotypes) log phase of S. Typhimurium and stationary phase S. Heidelberg, were higher (P<0.05) as compared to the non-stressed smooth wild type variants.

In chicken broth (at 55°C), short term chlorine stressed stationary phase S. Typhimurium cells exhibited higher thermal resistance as compared to non-stressed cells. No differences were observed in the thermal resistance between long-term stressed rugose and non-stressed smooth variants of stationary phase S. Typhimurium at 55°C in chicken broth. It was also observed that overall, stationary phase Salmonella were more resistant to heat treatment as compared to log phase cells. These findings suggest that short term chlorine stress makes log phase Salmonella sensitive to heat treatments, and long term chlorine stress provides a certain level of cross protection against heat.

Key Words: Salmonella, chlorine stress, rugose morphotype, thermal resistance, D-value, chicken broth

Introduction

In the United States, S. Heidelberg and S. Typhimurium are among the top 10 serotypes of Salmonella most commonly isolated from human foodborne illnesses (CDC, 2015). Since 2013, four outbreaks involving S. Typhimurium from poultry, beef, rodents and microbiological laboratory origin have been reported, and 3 outbreaks involving multi drug resistant S. Heidelberg from chicken origin have been recorded (CDC, 2014; CDC, 2016).

Thermal processing of food products is a common practice in food industries to maintain food safety and prolong shelf life of the product. Thermal treatment in the food processing plant should ensure that the product is free of any foodborne pathogens.
However, post-processing contamination, improper thermal treatment, or the presence of thermally resistant strains (Ng et al., 1969) can result in contamination of fully cooked and ready to eat products. Most of Salmonella related outbreaks in cooked products occur due to undercooking during thermal processing (Bean and Griffin, 1990; Juneja et al., 2013; Kimura et al., 2004; Marcus et al., 2007). Recall of a fully cooked chicken wing product in 2015 was possibly linked to some pathogen contamination based on the reported ‘off odor’ of the products (USDA-FSIS, 2015).

The thermal destruction of microorganisms is measured in terms of D-value, which is defined as the time in minutes required to destroy 1 log (90 %) of the total target bacteria at a given temperature in food. Z-value, which is used to calculate the microbial death time is the change in temperature required to reduce D-value by 1 log unit. A deviation in the first order kinetics displaying a sigmoidal curve or a shoulder or a tail indicates a poor heat condition in the mixture a heterogeneity of cell population, or a protective effect of fat, proteins, and dead cells (Juneja, 2002; McKellar and Lu, 2004). Current performance standards for lethality requires that all fully and partially cooked poultry product must achieve a 7-D or 7 log Salmonella reduction (USDA-FSIS, 1999). Most of the Salmonella serotypes pose a normal heat resistance, with the D-value of Salmonella in poultry meat between 30.1 min to 0.238 min at 55 to 70°C (D’Aoust, 1989). Amongst all the serotypes of Salmonella, S. Senftenberg is known to possess the highest thermal death time (Murphy et al., 1999). An optimal thermal treatment procedure should aim to destroy the pathogens in their most heat resistant state so as to secure an adequate level of safety in food.
Thermal lethality of a pathogen in meat during heat treatment depends on meat species, muscle type, fat content, and pH (Ghazala and others, 1995; Veeramuthu et al., 1998). Bacteria are thermally more resistant in meat as compared to 0.05 M phosphate buffer at a pH of 7 (Bell and Lacy, 1984).

Pre-exposure of pathogens to various antimicrobials and environmental stressors in the food processing facilities affect the behavior of Salmonella during thermal exposures. Increased thermal tolerance as well as resistance to high pH was demonstrated by S. Enteritidis which were pretreated with 1.5% tri-sodium phosphate (TSP) (Sampathkumar et al., 2004). Acid adaptation is known to induce cross resistance against heat, salt, and polymyxin B in S. Typhimurium (Leyer and Johnson, 1993). Increased solid content, low moisture or acidic food also render S. Enteritidis resistant against thermal treatments (Blackburn et al., 1997). S. Typhimurium which were adapted to hydrogen peroxide stress displayed a higher thermal resistance compared to non-adapted cells (Christman et al., 1985). The thermal tolerance of bacteria also depends on the growth phase of bacteria. S. Enteritidis in the stationary growth phase are known to be more resistant to thermal destruction as compared to the log phase of growth (Humphrey et al., 1995).

Chlorine is one of the most commonly used antimicrobials in poultry processing plants (Tsai et al., 1995). Chlorine and H₂O₂, share common modes of action, killing the bacterial cells by oxidative damage (Wang et al., 2010). The oxidizing effect of hydrogen peroxide treatment on S. Typhimurium induces 30 proteins, including Dnak, a heat shock chaperone, and out of these 30 proteins, five are also induced by heat stress (Morgan et
al., 1986). Hence, the cells which are stressed with peroxide produce a set of heat shock chaperones, which in turn can protect them upon subsequent thermal challenge.

*Salmonella* Typhimurium and *S. Enteritidis* are known to display rugose morphology under stressful environmental conditions such as low temperature (Anriany et al., 2001) and nutrient deprivation (Wai et al., 2005). Rugosity refers to a rough, dry, and red (*rdar*) morphotype owing to its appearance on media containing congo red. This morphotype requires proteinaceous fibrils (Chapman *et al*., 2002), curli (Romling *et al*., 1998; Zogaj *et al*., 2001), and cellulose, which are basically comprised of exopolymeric substances (EPS) (Zogaj *et al*., 2001). In a recent study by Jahid *et al*., (2015), it was reported that a disinfectant treatment, cold oxygen plasma (COP) causes a shift from smooth to rugose morphotypes in *S. Typhimurium*. In addition, rugose morphotypes in *Salmonella* are well known to be induced at a low incubation temperature (25°C) (Anriany *et al*., 2001). Rugose morphotypes are also known to be resistant against low pH and hydrogen peroxides which are usually associated with curli and cellulose (Rezende de *et al*., 2005). Rugose morphotypes of *Salmonella* are more resistant to chlorine, have more biofilm forming ability, and also have potential to revert to the smooth colony type (Anriany *et al*., 2006). These variants are more efficient in cell to cell communication (quorum sensing) and gene exchange (Romling *et al*., 2000). *radr* morphotypes were known to significantly resist starvation and desiccation (White *et al*., 2006). Hence, the rugose morphotype is better equipped to survive under adverse conditions as compared to smooth wild type cells. In the food processing facilities, foodborne pathogens are constantly exposed to various levels of antimicrobials and harsh environmental conditions (especially cold) which create the possibility of development of
such rugose variants. *S. Typhimurium* adapted to an oxidative antimicrobial, hydrogen peroxide, displayed a higher thermal resistance as compared to the non-adapted ones (Christman *et al.*, 1985). Similarly, Wang *et al.*, (2009) reported that chlorine stressed *E. coli* developed resistance against high temperature stress as shown by the over expression of heat-shock proteins such as *htpX, dnaK, dnaJ, htpG*.

However, to date none of the studies have reported the thermal cross protection of *Salmonella* after being stressed by various levels of chlorine. In this study, we investigated the thermal resistance of two most common *Salmonella* serotypes, *S. Heidelberg* and *S. Typhimurium*, after exposing the cells to sublethal chlorine (short-term and long-term stress). The heat resistant ability of stressed *Salmonella* was determined using D-values and Z-values.

**Materials and Methods**

**Salmonella-strains/serotypes**

*Salmonella* serotypes, *S. Heidelberg* (ATCC 8326) and *S. Typhimurium* (ATCC 14028), were used in this experiment to study the effect of sub-lethal chlorine exposure on thermal resistance of these serotypes. Both strains were maintained in tryptic soy broth (TSB)-glycerol at -80°C. Prior to use, the frozen culture was streaked on tryptic soy agar (TSA) plates and incubated at 37°C for 24 h. A single colony of each *Salmonella* strain was inoculated in 10 ml of TSB and incubated at 37°C for 18 to 24 h.

**Chicken broth**

Commercially available chicken broth (Campbell®) was used as a source of nutrient broth. The ingredients in the chicken broth were: chicken stock, water, chicken
fat, yeast extract, less than 2% of salt, monosodium glutamate, dextrose, flavoring and corn oil hydrolyzed soy protein. The sterility of the canned chicken broth was tested multiple times in both general purpose nutrient agar plates and selective plates for the presence of any possible bacterial contamination, Salmonella in particular. Before proceeding for each experiment, the broth was opened aseptically and a negative control was maintained to rule out the presence of possible background Salmonella. The pH of the chicken broth used in the study was approximately 5.4.

**Chlorine source**

Chlorox® (Oakland, California), commercially available bleach (7.85% active chlorine) was used as a source of chlorine. The total available chlorine in the Chlorox was confirmed using HACH Aquachek® test strips and HACH colorimeter test kit.

**Short term chlorine stress**

Overnight (~18 h) cultures of individual Salmonella serovars (S. Heidelberg and S. Typhimurium) in TSB were diluted to a working concentration of ~ 8 log CFU/ml in TSB. The treatments (stressed cells) consisted of cells exposed to 150 ppm of total chlorine in half strength TSB for 1h at 37°C. Controls were incubated in half strength TSB. Stationary phase cells were harvested after ~18 h of incubation at 37°C, followed by 1 h treatment in chlorine. For log phase cells, cells were harvested after 4 h of incubation at 37°C before subjecting them to chlorine stress for 1h as described earlier. After 1 h of chlorine stress at 37°C, the suspensions were neutralized with sodium thiosulphate (1 ml, 1%) and then centrifuged at 5000 rpm for 10 min. The cell pellets of stressed and non-stressed Salmonella cultures were re-suspended in fresh TSB. The
thermal resistance of the cells was determined using a microcapillary method as described by Bacon *et al.*, (2003) discussed later in the section. For the thermal experiment involving chicken broth, chicken broth (Campbell®, pH 5.4, 3% fat) was used for resuspension of the stressed cell pellets.

**Long term chlorine stress**

In order to determine the adaptation of *Salmonella* to chlorine, a method of exposing *Salmonella* to a long sequential increase in chlorine concentration in TSB was used. The cells were continuously exposed to chlorine stress in a proportionately increased concentration up to the point where cells demonstrated no visible growth after overnight incubation at 37°C.

As a starting concentration for the long term stress, 50 ppm of chlorine was selected for both the *Salmonella* serotypes. After adapting the cells at the 50 ppm concentration for 3 consecutive days, an increment of 50 ppm of chlorine was made for the next 3 days and so on till the maximum concentration was obtained that allowed for bacterial growth in the form of visible turbidity after 24 h incubation at 37°C. The chlorine stressed cells were plated on TSA plates to quantify the bacterial population and to observe their phenotypic behavior or colony morphology. *S.* Typhimurium and *S.* Heidelberg after exposure to 300 ppm of chlorine (17 days of stress) exhibited rugose morphology with rough, dry and filamentous appearance on tryptic soy agar. Only the rugose cells were selected as long-term chlorine stressed cells for determining the thermal resistance in comparison to non-chlorine stressed (smooth morphology) cells. The minimum inhibitory concentration (MIC) of chlorine against *S.* Heidelberg and *S.*
Typhimurium for both smooth and rugose type cells was determined using broth macro-dilution method in TSB (CLSI, 2015).

Control cells were generated in a similar fashion as treated cells but by adding sterile distilled water instead of chlorine in the subsequent days of treatments. Log and stationary phase cells were harvested as described previously. An overnight culture of long-term stressed rugose and non-stressed smooth wild morphotypes for each of the *Salmonella* serotypes were used in the thermal experiment. After centrifugation (5000 rpm, 10 min), the pellets were re-suspended in either TSB or chicken broth (Campbell®, pH 5.4, 3% fat).

**Thermal inactivation in TSB and chicken broth**

The thermal inactivation experiments for *Salmonella* were performed using a well-established microcapillary technique (Bacon *et al.*, 2003, Jung and Beuchat, 2000) in TSB and chicken broth. A total of four experiments were performed: short term stressed cells in TSB, long term stressed (or rugose cells) in TSB, short term stressed cells in chicken broth, and long term stressed cells in chicken broth. The thermal inactivation study in TSB, was performed at three temperatures (55°C, 58°C, and 61°C) for both log and stationary phase cells of two serotypes (*S. Typhimurium* and *S. Heidelberg*). Based on the thermal inactivation results in TSB, the thermal inactivation study in chicken broth was performed for stationary phase *S. Typhimurium* at 55°C. The microcapillary method was performed using sterile microcapillaries (KIMAX® capillary melting point tubes (0.8-1.1 by 100 mm with 0.25 wall thickness), Kimble Product, Vineland, NJ). A volume of 50µL of chlorine stressed and control *Salmonella* cultures was dispensed in duplicate microcapillaries. The capillaries were then manually sealed
using a Scripto® propane torch inside a biosafety laminar hood. Utmost care was taken to avoid heating of the cell suspension. The capillaries were then transferred to a digital circulating water bath (ANOVA®) with precise temperature controls. The temperatures chosen for thermal inactivation in the TSB study were 55°C, 58°C, and 61°C. The heating time interval for each temperature was determined based on preliminary experiments targeting at least a 5-log reduction of Salmonella. The capillaries were removed from the water bath at pre-determined equally spaced time intervals and immediately transferred onto ice chilled water. Tables 4.1 and 4.2 provide the time intervals used for thermal inactivation for short and long term chlorine stressed cells, respectively. After cooling, the capillaries were disinfected using 500 ppm of sodium hypochlorite (Bacon et al., 2003), followed by three washes with sterile distilled water. The capillaries were then transferred into 15 ml sterile plastic centrifuge tubes (Thermo -Scientific™) containing 450 µL of 0.1% sterile peptone water (PW). The capillaries were finely crushed using sterile glass rods. The crushed suspensions were then serially diluted in 0.9 ml of 0.1% PW and spread plated on duplicate tryptic soy agar plates supplemented with 0.6% yeast extract (TSAYE) and 1% sodium pyruvate (TSAYE+P). Plates were incubated at 37°C for 30 hours before the colonies were counted.

The thermal inactivation of the stressed and non-stressed cells was expressed in term of D-values, which is the time taken for a microorganism at a given temperature to be reduced by 90% (1 log). The D-values of bacteria also varies depending on the method of recovering the injured cells from thermal treatment thereby giving different estimates of thermal tolerance (Ng et al., 1969). Incubation of the injured or heat damaged cells at a lower temperature (Gibson, 1973) and in rich supplemented media has been shown to
enhance the recovery as compared to selective media (Stephens et al., 1997). Thus, to get the optimum recovery of the thermally injured cells, tryptic soy agar (TSA) plates containing yeast extract as a source of protein and a readily available source of energy in the form of sodium pyruvate were used. Sodium pyruvate acts as a non-enzyme peroxide degrading compound and increases the recovery of the stressed E. coli and Salmonella spp. (Calabrese, and Bissonnette 2009; Lee and Hartman 1989 and McDonald, 1983).

**Statistical analysis**

A two-way factorial arrangement was used with strains and stress level (stressed or not) as factors. The entire study was replicated 3 times over 3 days, therefore a RCBD was used with days serving as blocks. The survival cells were counted and converted into log CFU/mL. Using temperature versus log CFU/mL, a scatter plot was constructed with linear regression lines for each serotype, for each temperature, and for each growth phase. The decimal reduction time (D-values) were calculated using the slope of the linear regression line of the survivor curve. Z-values were determined as the negative inverse slope of the linear regression line of the log of D-values over the ranges of temperatures tested. The GLM procedure, SAS 9.4 version was used, and the treatments were considered significantly different compared to control when P ≤ 0.05.

**Results**

The MIC of chlorine against both non-adapted (smooth morphology) S. Heidelberg (ATCC 8326) and S. Typhimurium (ATCC 14028) in TSB was found to be 400 ppm. The MIC was determined based on the absence of visible growth in the glass test tubes after 24 h of incubation at 37°C. MIC of chlorine against the long-term stressed
rugose morphotypes was found to be 500 ppm for both the serotypes indicating that the
rugose morphotypes have developed some resistance against chlorine after a long term
chlorine stress.

During long term stress adaptation, the two serotypes of *Salmonella* were
subjected to a sequential increase in chlorine concentrations (50 ppm every fourth day).
The chlorine exposed cells started displaying a typical rugose type morphology on TSA
plates (Fig. 4.1) after the cells were exposed to 300 ppm of chlorine (after 18-day stress
treatment). There was a gradual increase in the proportion of the rugose to smooth cells,
as the chlorine concentration increased. At the highest tested chlorine concentration of
450 ppm, in which the *S*. *Heidelberg* and *S*. *Typhimurium* showed visible turbidity on
overnight incubation, almost all the cells on the TSA plate were rugose (Fig. 4.1). The
rugose cells appeared more rough, dry, flattened and filamentous. The exhibition of
rugosity by *S*. *Heidelberg* and *S*. *Typhimurium* cells was consistent in all three
replications of the long term stress study. The rugose morphology of *S*. *Heidelberg* and *S.*
*Typhimurium* was absent on selective media, xylose lysine-tergitol4 (XLT4).
Furthermore, the rugose cells were able to retain this phenotypic character after several
growth cycles on TSA plates. It was found that the overnight incubation of individual
rugose colonies in TSB broth leads to the formation of a jelly like pellicle at the air-liquid
interface (Fig. 4.2).

**Thermal resistance of stressed vs control cells**

The survivor population (log CFU/mL) were plotted against heating time intervals
in a scatter plot and a linear regression line was determined for each of the inactivation
studies for each serotype at each phase of growth at all the tested temperatures. The $R^2$ value for regression lines were greater than 0.9 in the study.

**Thermal inactivation of short term chlorine stressed Salmonella in TSB**

Figs. 4.3, 4.4 and 4.5 represent the scatter plots and linear regressions for the behavior of short term chlorine stressed *S.* Heidelberg and *S.* Typhimurium (log and stationary phase) to thermal treatments of 55°C, 58°C and 61°C. The D-values of *S.* Heidelberg and *S.* Typhimurium obtained from scatter plots in Figs. 4.3, 4.4 and 4.5 are presented in Table 4.3. The chlorine stressed (1 h) *S.* Heidelberg and *S.* Typhimurium cells in the log phase had lower $D_{55}$ values ($P \leq 0.05$) as compared to the control or non-chlorine stressed cells, which indicate that the short term exposure to sub-lethal chlorine makes these cells more sensitive to heat at 55°C. Similarly, chlorine stressed log phase *S.* Typhimurium cells had lower $D_{61}$ values (0.10 min) as compared to the $D_{61}$ values of non-stressed cells (0.12 min) ($P < 0.05$). There were no differences in the $D_{58}$ values for control and stressed log phase *S.* Heidelberg and *S.* Typhimurium cells. The thermal resistance of stationary phase non-stressed and stressed *S.* Heidelberg and *S.* Typhimurium cells was the same with no significant differences in their D-values at 55°C and 61°C. However, the $D_{58}$ values for stressed and non-stressed stationary phase *S.* Typhimurium cells were ($P < 0.05$) 0.83 and 0.72, respectively (Table 4.3), which indicates a higher thermal tolerance for chlorine stressed cells.

*Z*-value is a term used to calculate thermal death time of a microbe and is the number of degrees of the temperature required to achieve a ten-fold reduction of the D-value. *Z*-values were calculated separately for stressed and non-stressed *S.* Heidelberg and *S.* Typhimurium cells. The *Z*-values of both the serotypes in their log and stationary
phases during the short term stress experiment are shown in table 4.4. The Z-values for stressed and non-stressed log phase S. Heidelberg were recorded to be 4.66 min and 3.90 min, respectively, indicating that stressed S. Heidelberg requires a higher temperature to get one log reduction in D-value. There was no difference in the Z-values of stressed vs non-stressed S. Typhimurium in the log phase.

**Thermal inactivation of long-term chlorine stressed Salmonella (rugose morphotype) in TSB**

Table 4.5 summarizes the $D_{55}$, $D_{58}$ and $D_{61}$ values for S. Heidelberg and S. Typhimurium cells exposed to longer (27 d) chlorine stress. Figs. 4.6, 4.7 and 4.8 represent the scatter plots and linear regressions for the behavior of rugose type cells to the thermal treatments at 55°C, 58°C, and 61°C, respectively. The $D_{55}$ values for the rugose morphotype of S. Heidelberg in both log and stationary growth phases were not different ($P>0.05$) from the control (smooth morphology) although the $D_{55}$ values of rugose cells were numerically higher than smooth type cells. However, both log and stationary phase rugose S. Typhimurium cells had significantly higher $D_{55}$ values ($P\leq0.05$) as compared to their respective controls, which indicate, greater thermal resistance of rugose S. Typhimurium cells as compared to control cells with smooth morphology. The $D_{55}$ values of rugose S. Typhimurium cells in the log and stationary phase were 8.52 and 9.17, respectively as compared to $D_{55}$ values of 5.80 and 7.34 for log and stationary phase smooth (control/non-stressed) S. Typhimurium (Table 4.5). At 58°C, there were no differences ($P>0.05$) in thermal resistance (D-value) of the log and stationary phases of rugose and smooth S. Heidelberg and S. Typhimurium. At the higher temperature of 61°C, cells in the stationary phase of rugose S. Heidelberg showed a
significantly higher (P≤0.05) D-value as compared to its smooth wild type variant. The $D_{61}$ of the log phase rugose *S. Typhimurium* cells was 0.19 min, which was significantly higher (P≤0.05) than $D_{61}$ of 0.17 min for the smooth variant. However, there were no significant differences (P≥0.05) in D-values for rugose versus wild type smooth variant cells in the stationary phase for *S. Typhimurium*.

The Z-values of both serotypes in their log and stationary phases during the long term stress experiment are shown in table 4.6. The Z-values for both serotypes (*S. Heidelberg* and *S. Typhimurium*) in their log and stationary phase cells ranged in between 3.52 min to 3.93 min.

**Thermal inactivation of short and long term chlorine stressed Salmonella in chicken broth**

The thermal inactivation experiment was performed to determine the behavior of chlorine stressed, short and long term (rugose morphotype), *S. Typhimurium* in their stationary phase of growth in chicken broth at 55°C. *S. Typhimurium* cells that were exposed to chlorine for 1 hour were more resistant (P<0.05) to thermal inactivation at 55°C as compared to the non-stressed cells with $D_{55}$ values of 10.71 vs 9.76 min for stressed and control cells, respectively (Fig. 4.9, table 4.7). However, there were no significant differences (P>0.05) in the $D_{55}$ values of rugose cells as compared to their smooth control cells in chicken broth. The thermal experiment of *Salmonella* in chicken broth was performed to simulate the similar nutrient condition seen in the poultry processing scenario.
**Discussion**

Thermal treatment of food is an effective way to control *Salmonella* and other foodborne pathogens (Bermúdez-Aguirre and Corradini, 2012; Silva and Gibbs, 2012). *Salmonella* growth has been reported at temperatures as low as 5.5°C and as high as 45°C (Murphy *et al.*, 1999; Ng *et al.*, 1969). The lethal temperature for *Salmonella* depends on its growth phase (Humprey *et al.*, 1995), strains (Murphy *et al.*, 1999), food composition (Blackburn *et al.*, 1997), and the presence of other competitive microbes (Duffy *et al.*, 1995).

Microorganisms including *Salmonella* exposed to a high level of antimicrobials are usually destroyed. Some populations tend to have a natural resistance or acquire resistance against antimicrobials and therefore become adapted (Bower and Daeschel, 1999). Chlorine and chlorine compounds are one of the most common group of antimicrobials used in food industries. Because the precise mechanism of action of chlorine is not known, the exact mechanism of resistance development against this compound is not fully understood. Exposure of sub-lethal levels of chlorine might make bacteria resistant to a higher level of the antimicrobial. Among many, the use of chlorine at sub-lethal concentrations by error or residual chlorine left over during neutralization are instances where bacteria receive less than the lethal dose of chlorine. Mokgatla *et al.* (1998) isolated *Salmonella* from different sites of the poultry processing plant and found them to be resistant to as high as 72 ppm of chlorine in TSB, indicating *Salmonella* can adapt and grow at a concentration of chlorine which otherwise is inhibitory. Based on that study, the authors warned about any possible exposure of *Salmonella* to a sub-lethal dose of chlorine. Potential development of chlorine resistant strains of bacteria in fruits...
and vegetables while using chlorine as a sanitizer has also been reported (Beuchat, 1996; Cherry, 1999). In this study, *Salmonella* revealed resistance to thermal exposure (at 55°C and 61°C) after they were subjected to a long term exposure to an increasing dose of chlorine.

It is well documented that a previous exposure to a sub-lethal level of stress and/or antimicrobials makes *Salmonella* more resistant to subsequent harsh environments. *Salmonella* spp. which were acid adapted for 24 h were more resistant to subsequent thermal treatment as compared to non-adapted cells (Singh et al., 2010). *Salmonella* when challenged with tri-sodium phosphate (TSP) became more resistant to subsequent heat inactivation (Sampathkumar et al., 2004). A sub-lethal heat shock can induce thermal resistance in *S. Typhimurium* by the generation of a heat shock response (Neidhardt and VanBogelen, 1987; Yamamori and Yura, 1982). *S. Typhimurium* adapted to hydrogen peroxide showed better resistance against thermal challenge (Christman et al., 1985). However, in *E. coli O157:H7*, a challenge with a similar oxidative antimicrobial, peroxyacetic acid, didn’t confer any cross protection against thermal treatment (Zook et al., 2001). Similarly, various antimicrobials like carvacrol, cinnamaldehyde, sodium lactate, and oregano oil are known to facilitate the thermal destruction of pathogens in ground beef (Juneja, 2002; Juneja and Friedman, 2008 and Juneja et al., 2009).

In the current study, the initial appearance of the rugose morphotype of *Salmonella* on TSA plates was observed after exposure to 300 ppm of chlorine, which was after approximately 17 days of chlorine exposure. These cells were phenotypically distinct when compared to the normal smooth wild type cells. This morphotype was
observed in both serotypes of *Salmonella* used. The appearance of the rugose morphotype might be because chlorine stress caused the cells to over express its extracellular matrix and fimbriae. These morphotypes could be the result of acquired resistance development against chlorine, and they are known to tolerate extreme conditions (Romling *et al.*, 2000). The surface modification and production of extra cellular matrixes allow these cells to adhere better on food processing surfaces and food matrixes. Studies have reported the development of the rugose morphotype in *Salmonella* upon exposure to unfavorable conditions. In a recent study by Jahid *et al.* (2015) disinfectant treatment with cold oxygen plasma (COP) led to the shift from smooth to rugose morphotypes in *S.* Typhimurium. Similarly, Anriany *et al.* (2001) observed the rugose morphotype in *S.* Typhimurium when they were grown at a suboptimal temperature of 25°C for 3-4 days. The appearance of rugosity in chlorine stressed *Salmonella* cells at 37°C in our study supports the findings that rugosity develops in stressful environments. The rugose cells of *Salmonella* discovered in our study retained their rugosity upon re-inoculation into TSB and re-streaking onto TSA even after several passages. However, these morphotypes were not able to display the rugosity in XLT4 plates, possibly due to the selective nature of XLT4 media. These rugose morphotype were characterized by the formation of a typical pellicle with a tight bacterial network. The mature pellicles (after 48 h) totally block the surface of the culture and couldn’t be dispersed by gentle shaking. This observation was in accordance to the finding by Solano et al. (2002), who observed similar pellicle formations in *S.* Enteritidis. The matrix of the pellicle is composed of curlie, fimbriae, and cellulose (Zogaj *et al.*, 2001; Solano *et al.*, 2002) and a protein
encoded by the gene, *stm2689*, is responsible for the pellicle formation at the interface
(Latasa et al., 2000).

The higher MIC of chlorine against rugose morphotypes in the current study as
compared to the smooth wild variants might be because of the acquired resistant
development or genetic mutation in the cells against chlorine. This development might
have permitted the *Salmonella* to grow at a higher chlorine concentration, which
otherwise would be inhibitory. These findings correlated with the observations of
Mokgatla *et al.*, (1998), who were able to isolate resistant strains of *Salmonella* from the
poultry processing plants which could grow in up to 72 ppm of chlorine, at a time when
the maximum level of chlorine that was used in the plant was 50 ppm.

The R² values for all the linear regression lines throughout the experiments were
>0.90, and there was no sigmoidal, shoulder or tail in the curve indicating the
homogeneity of the heating and media (TSB as well as chicken broth). Results from our
study showed that the log phase *S*. Typhimurium after 1 h of stress with sub-lethal
chlorine had lower D-values compared to non-stressed cells, which indicate that the log
phase stressed cells became susceptible to thermal challenge regardless of the
temperature treatment: 55°C, 58°C, or 61°C. Similarly, log phase chlorine stressed *S.*
Heidelberg cells were sensitive to thermal exposure at 55°C but not at 58°C or 61°C. Our
findings are in accordance with the finding of Seward *et al.* (1986) who reported a heat
sensitizing effect of a commercial antimicrobial, TSP, on *S*. Typhimurium (ATCC
14028). A similar finding by Zoook *et al.* (2001) indicated that a 1 h stress of
peroxyacetic acid on *E. coli* failed to produce any cross resistance against thermal
challenge in TSB-YE and cells became thermally more sensitive.
Our results are in contrary to the study of Yuan et al., (2012,) who reported higher D-values for *S. Typhimurium* in TSB, upon prior adaptation to a common sanitizer, sodium lactate and sodium acetate for 24 h. This difference could be a result of the different exposure periods to the stress. For example, 1 h of chlorine stress in our study might have triggered a different stress response in *Salmonella* as compared to the 24 h stress in their study or the heat shock proteins were not produced during the short term stress. Additionally, the duration of stress can cause bacteria to elicit different responses. As Wolf et al. (2008) described in a report that a short term and a long term memory response occurs in bacteria, and short term response can be transient or forgettable. In another study, stationary phase *S. Typhimurium* in a lower water activity yielded a protective effect above 55°C, however it was sensitive at temperatures below 55°C (Aljarallah et al., 2007) in nutrient broth. In *L. monocytogenes*, Taormina and Beuchat (2001) reported a heat sensitive effect at 56°C, when they had been stressed with chlorine prior to heating. Serotype variation in our study may be a reason *S. Heidelberg* log phase cells did not show any differences in their thermal resistance between stressed and non-stressed cells after being thermally challenged at 58°C and 61°C. However, neither of the stationary phase cells of *S. Heidelberg* nor *S. Typhimurium* showed any significant differences in thermal resistance between stressed and non-stressed cells. The possible reason for this could be the variation in the synthesis of heat shock proteins and other chaperones in the stationary phase of the *Salmonella* as compared to the log phase cells. In addition, the prior effect of chlorine stress for stationary phase cells may have caused *Salmonella* to behave differently than log phase *Salmonella* as shown in a study by Liao
et al., (2003) that antimicrobial effects of peroxyacetic acid were more effective against log phase cells as compared to the stationary phase cells of *Salmonella* spp.

The thermal resistance of rugose morphotypes of both serotypes in general had higher D-values as compared to their smooth wild type variants. As stated earlier, the chlorine stress may have triggered a resistance mechanism in bacteria against subsequent stressors. Our findings are supported by a gene level study in which hydrogen peroxide, in *S. Typhimurium* induced 30 proteins, including a heat shock chaperone (*dnaK*), out of which five are also induced by heat stress (Morgan *et al.*, 1986). These heat shock proteins may play a role in protecting the bacterial cells from heat damage thereby giving them a better survival opportunity (higher D-value).

At a lower temperature of 55°C, rugose morphotypes of *S. Typhimurium* (ATCC 14028) displayed significantly higher thermal resistance as compared to their smooth variants. Thermal resistance between rugose and smooth variants at 58°C for both serotypes was not different in log as well as stationary phase cells. However, the appearance of significantly higher thermal resistance at 61°C for log phase rugose type cells of *S. Typhimurium* (ATCC 14028) and stationary phase rugose type cells of *S. Heidelberg* (ATCC 8326) is perplexing. The reason for the reversion of thermal resistance in rugose cells at a higher temperature needs to be studied further using a molecular approach with proteomic analysis to analyze the expression levels of various stress proteins at different temperatures.

The D-values of *S. Typhimurium* in chicken broth in our study were higher compared to that reported by Juneja *et al.*, (2001). This difference can be the result of different product compositions of broth, source of the isolate, and the thermal inactivation
method (submerged coil heating vs water bath). The overall higher D-values of S. Typhimurium in chicken broth as compared to TSB may be accounted for by the presence of fat (~3 %) in chicken broth which provides a protective effect to Salmonella against heat (Juneja, 2002). In a previous study it was reported that, bacteria are more thermo-tolerant when they are in meat as compared to a phosphate buffer (Bell and Lacy, 1984). The higher thermal tolerance of short-term chlorine stressed S. Typhimurium in chicken broth as compared to the TSB is something to be further studied. The possible reason for this can be the protective effect of food particles and fat to the stressed cells.

The higher D-values of stationary phase cells as compared to the log phase cells in our study could be explained by the different sets of protein expression in stationary phase as compared to the log phase cells. Stationary phase cells are thermally more resistant as compared to the log phase cells (Hoover et al., 1989; Heddleson et al., 1991), and in the case of E. coli, the stationary phase cells expressed the rpoS-regulated genes (Cheville et al., 1996). A short term (30 min) chlorine stress in log phase Salmonella caused the down regulation of phoQ genes, which usually respond to environmental stressors (Kakani, 2011), whereas the stress proteins rpoS and uspA were upregulated in stationary phase Salmonella at the same level of chlorine stress. (Kakani, 2011). A phage shock protein (PSP) is also found in Salmonella (Model et al., 1997) and E. coli. This protein is expressed at higher levels in stationary phase cells as compared to log phase cells in E. coli. It is expressed during environmental stressors like heat shock, osmotic shock or ethanol exposure (Weiner and Model, 1994). Expression of PSP may help stationary phase cells to resist thermal stress in a better way, giving a higher D-value.
Conclusions

The short term chlorine stress did not provide any cross protection to *Salmonella* against thermal stress in TSB but offered a significant cross protection against subsequent thermal exposure in chicken broth. The rugose morphotypes of *Salmonella*, in general, revealed higher thermal resistance in TSB as well as in chicken broth. These findings suggest that the display of the rugose morphology by *S. Heidelberg* and *S. Typhimurium* provides a certain level of protection against heat. These results further highlight the concern over the development of such rugose variants of *Salmonella* after consistent exposure to sub-lethal chlorine, and hence signifies the need for thorough cleaning and sanitation in the processing facilities.

Further studies are needed to determine the pathogenicity and invasiveness of such chlorine adapted rugose morphotypes. Another potential follow-up research of this study can be a transcriptome study for in depth understanding of potential roles of genes in the development of a cross protection.

Table 4.1  Time intervals used in the thermal experiment of *S. Heidelberg* and *S. Typhimurium* (short term stress)

<table>
<thead>
<tr>
<th>Time intervals</th>
<th>Temperatures (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>61</td>
</tr>
<tr>
<td>Log phase (min)</td>
<td>Stationary phase (min)</td>
</tr>
<tr>
<td>Log phase (sec)</td>
<td>Stationary phase (sec)</td>
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<td>T4</td>
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</tr>
<tr>
<td>T5</td>
<td>12 24</td>
</tr>
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<td>T6</td>
<td>15 30</td>
</tr>
<tr>
<td>T7</td>
<td>18 36</td>
</tr>
</tbody>
</table>
### Table 4.2 Time intervals used in the thermal experiment of *S. Heidelberg* and *S. Typhimurium* (long term stress)

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<th>61</th>
</tr>
</thead>
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<td></td>
<td>Log phase (min)</td>
<td>Stationary phase (min)</td>
<td>Log phase (sec)</td>
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<td>0</td>
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<tr>
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<td>9</td>
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<td>T5</td>
<td>28</td>
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<td>T7</td>
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<td>54</td>
<td>270</td>
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</table>

### Table 4.3 D- values\(^a\) (in min) of short term chlorine stressed *Salmonella* at different temperatures (°C)\(^b\) in TSB

<table>
<thead>
<tr>
<th>Serotype</th>
<th>55</th>
<th>58</th>
<th>61</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. Heidelberg</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strressed cells</td>
<td>2.9</td>
<td>5.99</td>
<td>0.43</td>
</tr>
<tr>
<td>Control cells</td>
<td>3.78</td>
<td>6.05</td>
<td>0.53</td>
</tr>
<tr>
<td>P value</td>
<td>0.0022</td>
<td>0.2448</td>
<td>0.6194</td>
</tr>
<tr>
<td>SEM</td>
<td>0.02310</td>
<td>1.57700</td>
<td>0.00386</td>
</tr>
<tr>
<td><em>S. Typhimurium</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strressed cells</td>
<td>3.28</td>
<td>5.49</td>
<td>0.46</td>
</tr>
<tr>
<td>Control cells</td>
<td>3.96</td>
<td>6.21</td>
<td>0.53</td>
</tr>
<tr>
<td>P value</td>
<td>0.0161</td>
<td>0.2117</td>
<td>0.4194</td>
</tr>
<tr>
<td>SEM</td>
<td>0.47400</td>
<td>0.22000</td>
<td>0.00046</td>
</tr>
</tbody>
</table>

\(^a\) Mean D- value of three replications

\(^b\) Thermal inactivation of *Salmonella* were studied at three challenge temperatures (55°C, 58°C, 61°C) (Stat. phase = stationary phase)
Table 4.4  
Z- values of short term stressed cells and non-stressed cells of *S.* Heidelberg and *S.* Typhimurium from linear regression line

<table>
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<tr>
<th></th>
<th>Log phase</th>
<th></th>
<th>Stationary phase</th>
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</thead>
<tbody>
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<td></td>
<td>stressed</td>
<td>non-stressed</td>
<td>stressed</td>
<td>non-stressed</td>
</tr>
<tr>
<td><strong>S. Heidelberg</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>(ATCC 8326)</td>
<td>4.6642</td>
<td>3.9063</td>
<td>3.6075</td>
<td>3.5236</td>
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<tr>
<td><strong>S. Typhimurium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ATCC 14028)</td>
<td>3.9588</td>
<td>3.9510</td>
<td>3.6140</td>
<td>3.5002</td>
</tr>
</tbody>
</table>

Table 4.5  
D- values \(^a\) (in min) long term chlorine stressed (rugose morphotype) *Salmonella* at different temperatures (\(^b\)\(^°\)C) in TSB

<table>
<thead>
<tr>
<th>Serotype</th>
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<th>58</th>
<th>61</th>
<th>55</th>
<th>58</th>
<th>61</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. Heidelberg</strong></td>
<td>Rugose type</td>
<td>9.58</td>
<td>10.23</td>
<td>1.05</td>
<td>1.6</td>
<td>0.19</td>
</tr>
<tr>
<td>(ATCC 8326)</td>
<td>Wild type</td>
<td>8.63</td>
<td>9.69</td>
<td>0.89</td>
<td>1.52</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.0641</td>
<td>0.473</td>
<td>0.7255</td>
<td>0.1383</td>
<td>0.0664</td>
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<tr>
<td></td>
<td>SEM</td>
<td>0.1928</td>
<td>0.1092</td>
<td>0.3387</td>
<td>0.08410</td>
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<tr>
<td><strong>S. Typhimurium</strong></td>
<td>Rugose type</td>
<td>8.52</td>
<td>9.17</td>
<td>0.92</td>
<td>1.48</td>
<td>0.19</td>
</tr>
<tr>
<td>(ATCC 14028)</td>
<td>Wild type</td>
<td>5.8</td>
<td>7.34</td>
<td>0.78</td>
<td>1.27</td>
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<td></td>
<td>P value</td>
<td>0.05</td>
<td>0.05</td>
<td>0.1028</td>
<td>0.8023</td>
<td>0.0052</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.00312</td>
<td>0.23070</td>
<td>0.00760</td>
<td>0.01690</td>
<td>0.00039</td>
</tr>
</tbody>
</table>

\(^a\) Mean D- value of three replications  
\(^b\) Thermal inactivation of *Salmonella* were studied at three challenge temperatures (55\(^°\)C, 58\(^°\)C, 61\(^°\)C) (stat.= stationary phase)
Table 4.6  Z-values of long term stressed cells and non-stressed cells of S. Heidelberg and S. Typhimurium from linear regression line

<table>
<thead>
<tr>
<th></th>
<th>Log phase</th>
<th>Stationary phase</th>
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<tr>
<td></td>
<td>stressed</td>
<td>non-stressed</td>
</tr>
<tr>
<td></td>
<td>cells</td>
<td>cells</td>
</tr>
<tr>
<td>S. Heidelberg</td>
<td>3.5236</td>
<td>3.4095</td>
</tr>
<tr>
<td>(ATCC 8326)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. Typhimurium</td>
<td>3.6324</td>
<td>3.9139</td>
</tr>
<tr>
<td>(ATCC 14028)</td>
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<td></td>
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</tbody>
</table>

Table 4.7  D-values (in minutes) of stationary phase Salmonella Typhimurium in chicken broth at 55°C

<table>
<thead>
<tr>
<th></th>
<th>Short Term</th>
<th>Long term</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stressed cells</td>
<td>10.71</td>
<td>Rugose type 9.09</td>
</tr>
<tr>
<td>Control cells</td>
<td>9.76</td>
<td>Wild type 10.63</td>
</tr>
<tr>
<td>P value</td>
<td>0.0124</td>
<td>P value 0.7038</td>
</tr>
<tr>
<td>SEM</td>
<td>0.2006</td>
<td>0.4090</td>
</tr>
</tbody>
</table>
Figure 4.1  Morphology of *Salmonella* Typhimurium normal (a) smooth morphotype (b) versus rugose morphotype.
Figure 4.2  Pellicle formation by rugose morphology *Salmonella* Typhimurium (a) in TSB broth on air-liquid interface after 48 h of incubation at 37°C as compared to smooth morphology of *Salmonella* Typhimurium (b).
Figure 4.3  Scatter plots of survival populations of chlorine stressed (trt)* and non-stressed (cont) *Salmonella* in TSB at 55°C.

(a) log phase *S. Heidelberg* (ATCC 8326) (b) stationary phase *S. Heidelberg* (ATCC 8326) (c) log phase *S. Typhimurium* (ATCC 14028) and (d) stationary phase *S. Typhimurium* (ATCC 14028).

*The stressed cells were treated with 150 ppm chlorine in half strength TSB for 1 h at 37°C and non-stressed were grown in half strength TSB only.
Figure 4.4  Scatter plots of survival populations of chlorine stressed (trt)* and non-stressed (cont) *Salmonella* in TSB at 58°C.

(a) log phase *S. Heidelberg* (ATCC 8326) (b) stationary phase *S. Heidelberg* (ATCC 8326) (c) log phase *S. Typhimurium* (ATCC 14028) and (d) stationary phase *S. Typhimurium* (ATCC 14028).

*The stressed cells were treated with 150 ppm chlorine in half strength TSB for 1 h at 37°C and non-stressed were grown in half strength TSB only.
Figure 4.5 Scatter plots of survival populations of chlorine stressed (trt)* and non-stressed (cont) *Salmonella* in TSB at 61°C.

(a) log phase *S. Heidelberg* (ATCC 8326) (b) stationary phase *S. Heidelberg* (ATCC 8326) (c) log phase *S. Typhimurium* (ATCC 14028) and (d) stationary phase *S. Typhimurium* (ATCC 14028).

*The stressed cells were treated with 150 ppm chlorine in half strength TSB for 1 h at 37°C and non-stressed were grown in half strength TSB only.
Figure 4.6  Scatter plots of survival populations of chlorine stressed (rugose)* and non-stressed (wild) *Salmonella* in TSB at 55°C.

(a) log phase *S. Heidelberg* (ATCC 8326) (b) stationary phase *S. Heidelberg* (ATCC 8326) (c) log phase *S. Typhimurium* (ATCC 14028) and (d) stationary phase *S. Typhimurium* (ATCC 14028).

*The stressed (rugose) cells were generated after a long term sub-lethal chlorine stress and the smooth wild type control cells after a similar treatment but in sterile distilled water.*
Figure 4.7  Scatter plots of survival populations of chlorine stressed (rugose)* and non-stressed (wild) *Salmonella* in TSB at 58°C.

(a) log phase *S. Heidelberg* (ATCC 8326) (b) stationary phase *S. Heidelberg* (ATCC 8326) (c) log phase *S. Typhimurium* (ATCC 14028) and (d) stationary phase *S. Typhimurium* (ATCC 14028).

*The stressed (rugose) cells were generated after a long term sub-lethal chlorine stress and the smooth wild type control cells after a similar treatment but in sterile distilled water.*
Figure 4.8  Scatter plots of survival populations of chlorine stressed (rugose)* and non-stressed (wild) *Salmonella* in TSB at 61°C.

(a) log phase *S. Heidelberg* (ATCC 8326) (b) stationary phase *S. Heidelberg* (ATCC 8326) (c) log phase *S. Typhimurium* (ATCC 14028) and (d) stationary phase *S. Typhimurium* (ATCC 14028).

*The stressed (rugose) cells were generated after a long term sub-lethal chlorine stress and the smooth wild type control cells after a similar treatment but in sterile distilled water.
Figure 4.9   Scatter plots of survival populations of chlorine stressed *Salmonella* in chicken broth at 55°C.  

(a) chlorine stressed (rugose)* and non-stressed (wild) and (b) chlorine stressed (trt)* and non-stressed (cont) stationary phase *Salmonella Typhimurium* in chicken broth at 55°C.  

*The stressed cells were treated with 150 ppm chlorine in half strength TSB for 1 h at 37°C and non-stressed controls were treated with sterile distilled water under similar condition.**The rugose cells were generated after a long term sub-lethal chlorine stress and the smooth wild type cells were the control cells after a similar treatment but in sterile distilled water.
References


Kimura, A. C., Reddy, V., Marcus, R., Cieslak, P. R., Mohle-Boetani, J. C., Kassenborg, H. D., ... & Emerging Infections Program FoodNet Working Group. (2004). Chicken consumption is a newly identified risk factor for sporadic Salmonella enterica serotype Enteritidis infections in the United States: a case-control study in FoodNet sites. Clinical Infectious Diseases, 38(Supplement 3), S244-S252.


CHAPTER V
SUMMARY

This research provided an understanding about the possible development of heterologous cross protection and biofilm formation in *Salmonella* due to chlorine induced oxidative stress. Production of a stronger biofilm mass by some serotypes of chlorine stressed *Salmonella* spp. on plastic as well as on stainless steel surfaces suggested that *Salmonella* can form stronger biofilms after chlorine stress.

The thermal resistance of *Salmonella* varied with the duration and type of chlorine stress. The development of the rugose phenotype by *Salmonella* (S. Heidelberg and S. Typhimurium) upon exposure to a long term chlorine stress revealed a higher thermal tolerance by the rugose morphotypes when compared to the normal smooth variant. Also, short-term chlorine stress caused *Salmonella* spp. to become more sensitive to the thermal inactivation.

Timely cleaning of the equipment in the facilities to prevent biofilm attachment is warranted. The premises which less likely get in contact with food materials should not be avoided in the cleaning protocols, as nutrient limited conditions are known to favor biofilm formation. In addition, the development of rugose morphotypes under chlorine stress condition underlines the importance of minimizing predisposing factors such as nutrient limitation and long-term chlorine exposure which are known to cause the morphology shift.