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Effect of Sub-Lethal Chlorine Stress on the Homologous Stress Adaptation, Antibiotic Resistance, and Biofilm Forming Ability of Salmonella Enterica

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Effect of sub-lethal chlorine stress on the homologous stress adaptation, antibiotic resistance, and biofilm forming ability of Salmonella enterica

By

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The effect of exposure to sub-lethal chlorine stress on *Salmonella enterica* serotypes Typhimurium and Heidelberg was examined in this study. Both serotypes demonstrated an acquired tolerance to chlorine with the adapted cells growing in concentrations above the MIC. Chlorine induced a morphological change to the rugose variant in *Salmonella*. The biofilm formation of the adapted and control cells was tested on food-contact surfaces at room temperature and 37°C. The chlorine-adapted rugose formed stronger biofilms ($P < 0.05$) when compared to smooth (adapted and control) on both surfaces tested and at both temperatures. The possibility of cross-adaptation to antibiotics and low pH was evaluated. Adapted rugose showed reduced susceptibility against some of the antibiotics tested. Chlorine does not aid in the survival of *Salmonella enterica* at low pH. Chlorine stress can select for tolerant *Salmonella* cells that attach strongly to food-contact surfaces and after some time may become less susceptible to antimicrobials.

DEDICATION

To God and my savior Jesus Christ.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Foodborne illnesses continue to remain a public health concern not only in developing countries but also in developed nations such as the United States. There have been numerous reported cases of illness, hospitalization, and even death as a result of foodborne infection. There are hundreds of infections that are known to be transmitted through contaminated food, and according to the Centers for Disease Control and Prevention (CDC), each year one out of six Americans become sickened by consuming contaminated food or water (Bryan, 1982; Mead et al., 1999; CDC, 2015a). It was estimated that in the United States only, 48 million people will get sick, out of which there will be 128,000 hospitalizations and 3000 deaths from foodborne diseases annually (CDC, 2014).

Salmonella is a major foodborne pathogen of public health importance. When diarrhea is diagnosed in the laboratory, *Salmonella* is the pathogen that is often isolated and identified (Hohmann, 2001). In the most recent nomenclature by the CDC, the genus *Salmonella* is divided into 2 species, which are *S. enterica* and *S. bongori* (Brenner et al., 2000). While *Salmonella enterica* is further divided into subspecies with several serotypes, *Salmonella bongori* has no subspecies. Also, *Salmonella* can be defined by 2 types of strains, typhoid and non-typhoid *Salmonella* (Sanchez-Vargas et al., 2011). The types of typhoidal *Salmonella* are *S. enterica* sub specie *enterica* serotype Typhi and

Paratyphi, they are the causative agent of enteric fever (Brenner et al., 2000; Sanchez-Vargas et al., 2011). Whereas the nontyphoidal *Salmonella* includes all other sub species, they are the causative agent of gastroenteritis and bacteremia (Hohmann, 2001; CDC, 2016a). In the United States, non-typhoid *Salmonella* is estimated to cause 1,200,000 illnesses and 450 deaths each year (Scallan et al., 2011).

Foodborne salmonellosis is the infection caused by *Salmonella* species. This microorganism is rod-shaped, non-spore forming, facultative anaerobes, belonging to the family enterobacteriaceae. Some of the symptoms experienced by an infected person include abdominal cramps, fever, and diarrhea, which manifest 36 to 72 hours after ingestion of the bacterium. The sickness persists and can last up to 7 days after signs of infection (Su and Chiu, 2007). Some of the ways *Salmonella* can be transmitted to humans are: by direct consumption of contaminated food, cross contamination with an infected food product, and contact with an infected animal (Agbaje et al., 2011; CDC, 2015a).

The poultry industry is continually faced with the challenges of foodborne pathogens, amongst which *Salmonella* is of major importance. Production of food containing poultry products have significantly increased and contamination with *Salmonella* may result in human illness (Morris and Wells, 1970). During poultry processing, an individually infected live bird that enters the processing plant, can become a major source of contamination to all of the final poultry products (Morris and Wells, 1970; Ramesh et al., 2002). *Salmonella* is known to colonize the intestinal tract of animals, such as birds, it may not harm the bird but can be transmitted to the carcass during processing (Mead, 2004; Morris and wells, 1970). In 2016, the CDC in

conjunction with the U.S Department of Agriculture's Animal and Plant Health Inspection Service (USDA-APHIS) investigated seven separate multistate outbreaks of human *Salmonella* infections that were linked to live poultry from a backyard flock. There were a total of 324 cases reported with 66 hospitalizations and 1 death that may have been a result of the outbreak/infection (CDC, 2016b). Furthermore, another outbreak related to frozen poultry products occurred in 2015, this resulted in a recall of over 2 million pounds of frozen, raw, stuffed, and breaded chicken products that may have been contaminated with *Salmonella* Enteritidis (CDC, 2015b). As a result of the food safety burdens posed by this foodborne pathogen, the U.S Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) recently finalized a new food safety measures to reduce *Salmonella* in poultry. According to the safety measures issued in 2016, the maximum acceptable percent of broiler carcass that is *Salmonella* positive is 9.8% or less. Meanwhile the minimum number of broiler samples to assess for *Salmonella* in a particular period is 11 carcasses, which has changed tremendously from the safety measures issued in 2015 (USDA-FSIS, 2015).

However, in order to achieve these safety measures, the USDA-FSIS approved the use of antimicrobials during poultry processing. Some of the antimicrobials approved, that are commonly used during poultry processing are cetylpyridinium chloride, acidified sodium chlorite, peracetic acid, chlorine, and chlorine-releasing compounds. These chemicals can be used in the water during processing, in the poultry chiller water, on the surface of the whole poultry carcass, on visceral, and chicken parts (either as dips or sprays) as stated in the FSIS Directive 71201 (USDA-FSIS, 2015). Chlorine and chlorine-based compounds are previously known to be used during poultry processing as

an antimicrobial. Their efficacy has been adequately investigated by several authors (Mead et al., 1975; Lillard, 1979; Tsai et al., 1992; Bolder, 1997). The majority of the research, however, found that the antimicrobial efficacy of chlorine has been significantly reduced over the years (Byrd and McKee, 2005; Nagel et al., 2013). Furthermore, other studies that are focused on antimicrobials such as cetylpyridinium chloride and peracetic acid have observed a remarkable reduction in the microbial load (Kim and Slavik, 1996; Arritt et al., 2002; Nagel et al., 2013).

In poultry processing, proper cleaning and sanitation coupled with adequate water treatments are important for food safety, as a result, processing plants are required to have a hazard analysis and critical control points (HACCP) plan, which includes standard operating procedures (SOP's) for all part of production. In addition, the use of cleaning agents and sanitizers have been incorporated into good manufacturing practices (GMP) in the food industry (Cruz and Fletcher, 2012). In a poultry processing operation, the plant runs three shifts per day with the third shift dedicated mainly for cleaning and sanitization. There has been some research on the effectiveness of chlorine as a sanitizer in the food industry, most especially for the sanitization of food contact surfaces and processing equipment. A major concern for the industry is the attachment of foodborne pathogen such as *Salmonella* on a food contact surface and perhaps equipment (Han et al., 1999; Weissinger et al., 2000; Beuchat et al., 2004). Improper cleaning and sanitization procedure of a poultry processing facility or equipment can become a significant contributor to foodborne infections particularly salmonellosis and listeriosis (Chmielewski and frank, 2003). This is a public health issue because a food-contact surface that is not cleaned appropriately will have food deposits and soils, which will

help bacteria to accumulate on such a surface (Boulange-Peterman et al., 1993). The inaccurate use of sanitizing agent at a sub-lethal concentration could select for a tolerant pathogenic bacteria strain, which may add to the formation of a film (biofilm) on food-contact surfaces (Braoudaki and Hinton, 2004a). The sanitizing agent may over time become ineffective as bacteria within the biofilm matrix become more resistant to the antimicrobial agent (Costerton and Lashen, 1984; Bower et al., 1996). The survival of *Salmonella* in food processing to a sanitizing agent may aid its resistance. An additional concern is cross-resistance to other stress factors such as low pH, other sanitizing agents and to a range of antibiotics that are of public health importance especially those used in the treatment of foodborne infections (Braoudaki and Hinton, 2004b). More precisely, Tattawasart et al., (1999) reported the development of a stable cross-resistance to antibiotics when *Pseudomonas stutzeri* was exposed to gradual sub-inhibitory concentrations of cetylpyridinium chloride. Similarly, other research observed a biocide-adapted *E. coli* O157 showed cross-resistance patterns to a variety of antibiotics (Braoudaki and Hinton, 2004b).

Therefore, the main objectives of this study was: (1) to determine the homologous stress adaptation of *Salmonella enterica* to sodium hypochlorite (chlorine) by measuring changes in minimal inhibitory concentration (MIC) before and after exposure. (2) to determine the difference in biofilm forming ability of stress adapted vs non-adapted cells on different food-contact surfaces, and (3) to determine the antibiotic susceptibility patterns coupled with the acid resistance of stress adapted and non-adapted cells.

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CHAPTER II

LITERATURE REVIEW

Food Safety Concerns in the United States

In the United States, illnesses caused by foodborne pathogens have been of national concern. Between 1999 and 2010, the Centers for Disease Control and Prevention (CDC) estimated that foodborne infections caused approximately 76 million illnesses that resulted in 325,000 hospitalizations and 5000 deaths each year (Mead et al., 1999). However, with a better epidemiological tool in 2011, the CDC adjusted their estimates and stated that 48 million people become sick, 128,000 are hospitalized, and 3000 die annually in the US due to foodborne infections (CDC, 2011a). Foodborne illnesses severely impact the United States economy, it was estimated that out of all foodborne illnesses occurring each year in the United States, only 20% were attributed to a particular pathogen, and that imposed a burden over $15.5 billion annually (Hoffman et al., 2015). Furthermore, the CDC reported non-typhoidal *Salmonella* as one of the top five pathogens causing most of the illnesses, hospitalizations, and deaths reported in the United States. In fact, each year, non-typhoidal *Salmonella* alone causes over 1 million illnesses, 19,000 hospitalizations, and 300 deaths in the United States (CDC, 2011b).

The food implicated the most in many outbreaks are of animal origin, which includes poultry, beef, eggs, and fish, however, fruits and vegetables can also be considered a source (Davies et al., 1997; Uyttendaele et al., 1998). Moreover, meat and

poultry related outbreaks are responsible for approximately 22% of all illnesses as well as 29% of the deaths (CDC, 2013). In addition, a CDC report in 2011 revealed that among all the food sources associated with foodborne illnesses, poultry can be attributed to 19% of most deaths with many of the infections commonly caused by *Salmonella* (CDC, 2011b). *S.* Enteritidis is the most common strain identified in foodborne illnesses followed by *S.* Typhimurium, *S*. Newport, *S*. Javiana, and *S*. Heidelberg. The *Salmonella* serotypes mostly associated with poultry and poultry products however are *S.* Enteritidis, *S.* Typhimurium, and *S.* Heidelberg causing 32%, 13%, and 8% of all the *Salmonella* outbreaks, respectively (CDC, 2013).

Salmonella Pathogenicity

A *Salmonella* infection is manifested by different clinical signs. Typhoidal *Salmonella,* which includes *Salmonella* Typhi and Paratyphi A, B, or C is the causative agent of enteric fever (Sanchez-Vargas et al., 2011). It is more common in developing countries, because of poor hygiene, sanitation, and contaminated water (Sanchez-Vargas et al., 2011). It is estimated to result in over 21 million illnesses and approximately 217,000 deaths annually worldwide (Crump et al., 2004; Meltzer and Schwartz, 2010). Nontyphoidal *Salmonella* infection however is evident by gastroenteritis (watery diarrhea) and bacteremia (Mandal and Brennand, 1988; Gal-Mor et al., 2014). Gastroenteritis alone is estimated to result in approximately 94 million cases with 155,000 deaths occurring each year all over the world (Majorwicz et al., 2010). In typhoidal infections, the pathogenicity of *Salmonella* includes binding to the receptors on the surface of the gut epithelial cells after which the bacteria invade the cells by rearranging the cytoskeleton (Giannella et al., 1973b). This invasion can be a reversible

adhesion by means of the fimbriae or irreversible docking through the Type III secretion system 1 (T1; Misselwitz et al., 2011). The T1 system delivers some effector proteins, which are virulence factors to the host cell. The effectors including SopE, SopE2, SopB, and SipA are responsible for the polymerization of actin, which functions to help aid in the invasion process (Zhou et al., 1999; Schlumberger and Hardt, 2006). The maturation of the bacteria is facilitated in a vacuole (this includes macrophages and dendritic cells) found inside the host cell. The mature bacteria inside the vacuole evoke another set of virulence factors (Type III secretion system 2, T2) that are encoded on the *Salmonella* pathogenicity island 2, which aid in the systemic infection of *Salmonella* (SP1-2; Schlumberger and Hardt, 2006). *Salmonella* pathogenicity island namely SP1-1 that houses the T1, functions to promote bacteria engulfment after the effector proteins mentioned earlier are released (Zhou and Gallan, 2001; McGhie et al., 2009).

Upon invasion of the epithelial cells, the bacteria grow rapidly in number, thereby spreading into the mesenteric lymph nodes and subsequently all over the body before they are captured by the reticuloendothelial cells, which work to control the spread of the bacteria (Giannella, 1996; Misselwitz et al., 2011). The majority of nontyphoidal infection involves *Salmonella* invasion of the intestinal mucosa, which results in the induction of an acute inflammatory response that causes ulceration and release of cytokines including NF-2, IL-1, IL-2, and IL-6. These inflammatory responses are evident in nontyphoidal *Salmonella* symptoms such as fever, abdominal cramps, and diarrhea (Giannella et al., 1979).

Salmonellosis

The infection caused by *Salmonella* species associated with animals is known as non-typhoidal salmonellosis. This infection is usually caused by the consumption of infected food, water or through cross-contamination by another infected person or animal (Piu et al., 2011). The clinical symptoms experienced by an infected person include diarrhea, fever, and abdominal cramps, which occur about 12 to 72 hours after infection by the bacteria (CDC, 2014a). The clinical symptoms of this foodborne pathogen last between 4 to 7 days. Even though it is possible for an infected person to recover without any treatment other than consuming fluids, in some cases with severe diarrhea, the infected person may be hospitalized (CDC, 2014a). Individuals infected with *Salmonella* have also had *Salmonella* isolated from major part of the body such as the synovial fluid of the joints, the nervous system, the blood and urine (Sirinavin et al., 1999). When infected with a pathogen like *Salmonella,* it is essential for individuals to seek treatment immediately because *Salmonella* infections can lead to death. The FoodNet reported that the incidence of *Salmonella* infection between 2006 and 2013 was 15.2 illnesses per 100,000 individuals each year in the United States (CDC, 2014c). The group of people that are at most risk of infection include the elderly, young children, and individuals with a compromised immune system (Sirinavin et al., 1999). Nontyphoidal *Salmonella* species have been reported to cause several foodborne outbreaks (CDC, 2011a).

Salmonella Outbreaks related to Poultry and Other Food Products

According to the CDC, a foodborne disease outbreak occurs when more than two people become sick with indistinguishable symptoms and have consumed the same contaminated food or drink (CDC, 2011a). The majority of outbreaks reported by the

CDC have occurred in more than one state. Foodborne illnesses associated with *Salmonella* have been on the rise. In 2013, the number of outbreaks caused by nontyphoidal *Salmonella* increased by 39% and hospitalizations increased by 38% when compared to the previous year (CDC, 2015). Moreover, the food most implicated from these outbreaks has been related to poultry and poultry products. The recent nontyphoidal *Salmonella* outbreaks in the United States that are related to poultry and poultry products are listed in table 2.1, while table 2.2 represents the trend in non-typhoidal *Salmonella* outbreaks related to other food products in the United States from 2006-2016

Table 2.1 Recent *Salmonella* outbreaks related to poultry and poultry products

Year	Source	Serotype	Cases (No.)	State (No.)	Hospitalizations (%)
2010	Cheesy Chicken Rice Frozen Entree	*S.* Chester	44	18	37
2011	Ground Turkey	*S.* Heidelberg	136	34	39
2011	Chicken Livers	*S.* Heidelberg	190	7	19
2012	Live Poultry	*S.* Montevideo	93	23	25
2012	Live Poultry	*S.* Hadar	46	11	28
2012	Live Poultry	*S.* Infantis, *S.* Newport, *S.* Lille	195	27	34
2013	Live Poultry	*S.* Typhimurium	356	39	62
2013	Live Poultry	*S.* Infantis, *S.* Lille, *S.* Newport, *S.* Mbandaka	158	30	31
2013	Branded Chicken	*S.* Heidelberg	134	30	28
2014	Branded Chicken	*S.* Heidelberg	9	1	22
2014	Live Poultry	*S.* Infantis, *S.* Newport, *S.* Hadar	363	43	33
2015	Raw, Frozen, Stuffed Chicken	*S.* Enteritidis	15	7	40
2016	Live Poultry	*S.* spp.	611	45	23

(CDC, 2015)

Table 2.2 *Salmonella* outbreaks related to other food products from 2006-2016

Year	Source	Serotype	Cases (No.)	State (No.)	Hospitalizations (%)
2006	Tomatoes	*S.* Typhimurium	183	21	12
2007	Peanut Butter	*S.* Tennessee	425	44	20
2007	Pot Pie	*S.* I 4[5],12:i:	272	35	23
2008	Cantaloupes	*S.* Litchfield	51	16	30
2008	Raw Produce	*S.* Saintpaul	1442	43	39
2009	Peanut Butter	*S.* Typhimurium	714	46	24
2009	Raw Alfalfa Sprout	*S.* Saintpaul	235	14	3
2010	Alfalfa Sprout	*S.* Newport	44	11	19
2010	Red and Black Pepper/Italian Style Meat	*S.* Montevideo	272	44	26
2011	Ground Beef	*S.* Typhimurium	20	7	47
2011	Whole, Fresh Imported Papayas	*S.* Agona	106	25	10
2012	Mangoes	*S.* Braenderup	127	15	25
2012	Ground Beef	*S.* Enteritidis	46	9	19
2013	Ground Beef	*S.* Typhimurium	22	6	50
2014	Raw Cashew Cheese	*S.* Stanley	17	3	20
2014	Cucumbers	*S.* Newport	275	29	34
2015	Pork	*S.* Infantis *and S.* I 4[5],12:i:	192	5	15
2016	Wonderful Pistachios	*S.* Montevideo and *S.* Senftenberg	11	9	18

(CDC, 2015)

Control of *Salmonella* during Food Processing: Cleaning and Sanitation

As seen above, there is a pressing need to manage foodborne outbreaks in the United States. In order to reduce the incidence of foodborne salmonellosis, foodborne pathogens such as *Salmonella* must be controlled both before and during food processing. *Salmonella* is well known to colonize the intestinal tract of birds and can become a source of contamination (Van Immerseel et al., 2009). Furthermore, breeder flock and layer flock can contaminate their hatching eggs with *Salmonella* through vertical transmission (trans-ovarian transmission) due to the infection of the ovary and oviduct (Snoeyenbos et al., 1969; McIlroy et al., 1989; Timothy et al., 1989; Weirup et al., 1995; Poppe et al., 1998). Trans ovarian transmission has not only been reported in *Salmonella* serotypes Gallinarum and Pullorum but also in Typhimurium, Enteritidis and Heidelberg (Snoeyenbos et al., 1969; Timothy et al., 1989; Barnhart et al., 1991). This emphasizes the importance of a good control program on the farm. In addition, during poultry processing, live chicken entering the plant with *Salmonella* infection can cause cross contamination during any steps of processing. It can also lead to cross-contamination of food-contact surfaces and processing equipment such as feather pickers, shackles, debone knives, and transport containers (Morris and wells, 1970). The high level of illnesses resulting from foodborne pathogens suggests the need for adequate antimicrobial intervention from the farm to the processing plant (Callaway et al., 2004; Plym and Wierup, 2006; Bucher et al., 2012). Although the USDA-FSIS approved the use of various antimicrobials such as peroxyacetic acid, quaternary ammonium compounds (cetylpyridinium chloride) and organic acids to control foodborne pathogen during

processing, a proper cleaning and sanitation protocol for the food processing facility is still paramount (USDA, 2015).

Cleaning is a process that removes deposit layers of dirt, food, soil and chemical residue amongst other from equipment and surfaces by either chemical of physical means (Wilson, 2005). Prior to establishing a Hazard Analysis and Critical Control Point (HACCP) plan, proper preventive measures that include practices and procedures that effectively control the introduction of pathogens, chemicals, and physical objects into food, also known as Good Retail Practices (GRP), should be practiced in a processing plant (Lowry, 2010; Ryther, 2013). The procedures to follow in order to clean a food processing plant can be (i.) clean-in-place: this is cleaning food processing equipment that requires little dismantling. Dismantling is done before starting the cleaning operation. (ii.) clean-out-place: this is the removal of equipment or parts of the equipment to be cleaned, which are then moved to a specialized area before cleaning and reassembling, and (iii.) external surface cleaning: this is the manual cleaning of all external surfaces of the processing equipment, as well as the entire production facility (Schmidt, 1997; Lowry, 2010; Ryther, 2013). Therefore, a good cleaning system should be achieved before sanitation.

Sanitization is a process used to reduce the microorganisms located on a surface or area. It can be thermal i.e. the use of hot water or steam at a particular temperature for a specified period of time, or chemical i.e. the use of an approved chemical sanitizer at a specific concentration and contact time (Schmidt, 1997). In a food processing plant, if the cleaning and sanitization process do not result in the adequate removal of soils and dirt from food contact surfaces, processing equipment, and the environment, it may lead to

the accumulation of microbial agents thereby resulting in a potential source of contamination to the final product (Ryther, 2013). Also, the food processing plant should have in place a control measure that follows an adequate Sanitation Standard Operating Procedures (SSOP's) and implementation of a HACCP plan with proper validation requirements (Arvanitoyannis, 2009). Moreover, the SSOP's are the major steps taken in order to perform the sanitation procedures. The SSOP will allow food processor to have a properly documented protocol for cleaning and sanitizing equipment or an area in the processing plant (Arvanitoyannis and Kassaveti, 2009). It is also important to have a verification plan that will make sure the cleaning and sanitization program was properly completed and well documented (Arvanitoyannis and Kassaveti, 2009). It is, therefore, critical to have an effective cleaning system that includes appropriate cleaning agents and sanitizers in order to control foodborne microorganisms.

Cleaning Agents

A cleaning agent may either be a physical activity or chemical agents and in some cases, it may be the combination of both. The physical activity involves manual rubbing and/or scrubbing with heavy machinery or a hard brush to remove any dirt or food soil located on the surface of equipment or contact surfaces. Chemical agents, on the other hand, can be found in the form of a liquid or powder (Ryther, 2013). They are used to completely remove any visible or invisible dirt and food soil from equipment or contact surfaces. Common cleaning agents used on equipment or food-contact surfaces are listed in table 2.3.

Table 2.3 Cleaning agents, uses and their targets

Type	Uses	Target
Alkaline cleaners (Degreaser)	In areas with heavy grease, removes organic soil	Break down and solubilize fats, protein and starches
Acidic cleaners (Phosphoric acid, Hydrofluoric acid, Sodium bisulfate)	Remove inorganic scale, used on mineral deposits	Provide hydrogen cations to a solution so as to dissolve inorganic scale deposit
Chelants and Sequestrans (*EDTA, Citrate, Phosphonates)	Dissolve scales on equipment surfaces, keep hardness ions from precipitating out e.g. Ca, Mg	Binds dissolved metal salts
Surfactants (Soap)	Dissolve food soils and emulsify oils, keep oily soils from redepositing during cleaning	Bind water, oil, and solubilize ionic soils
Caustic – Oxidizer (Chlorine, Hydrogen Peroxide)	Protect equipment surface from oxidation damage	Break down and solubilize food protein molecules
Enzymes based cleaners	Use on equipment such as membrane	Break down biological molecule, and specific soils such as proteases in case of protein, lipases for fats and amylase for starches

(Ryther, 2013) *EDTA: Ethylenediamine tetra-acetic acid

Sanitizer and Disinfectant Definition

According to the US Environmental Protection Agency (US EPA), antimicrobial agents are substances or mixtures that can be used to slow down or completely destroy the growth of harmful microorganisms, i.e. bacteria, virus, and fungi, on any inanimate object or surfaces (EPA Fact Sheet, 2016). Disinfectants and sanitizers are antimicrobial agents used by the food and poultry industry.

A disinfectant can be a chemical or physical agent that destroys or irreversibly inactivates many or all microorganism (virus, bacteria and fungi) on an inanimate object,

but may not necessarily kill the bacterial endospores. When disinfectants are applied on food contact surfaces, it is important to perform a final rinse (Patterson, 1932; Schmidt, 1997; McDonnell and Russel, 1999; Powitz, 2008; Ryther, 2013).

A sanitizer is an agent used in order to reduce the microbial load to a level that is safe (from the public health perspective) without having any detrimental effect on the safety or quality of the product. It usually reduces but may not necessarily eliminate the number of microorganism from an inanimate object or the environment (food contact and non-food contact surfaces) to a safe level as determined by the public health codes or regulations. The sanitizers used in the United States for food processing are non-rinse agents that are safe for food-contact surfaces when they are used per manufacturer's recommendation (Schmidt, 1997; Ryther, 2013). These sanitizers have a different mode of action and those approved for use in food processing are described below.

Classification of Approved Sanitizing Chemicals

Peroxides

Hydrogen peroxide and peroxyacetic acid (PAA) are 2 peroxides effective against a wide range of microorganisms including bacteria, virus, fungi, yeast, and spores (CDC, 2008). The antimicrobial activity of hydrogen peroxide is a result of oxidation, which leads to the generation of superoxide, or by producing destructive free hydroxyl radicals that can attack membrane lipids, DNA, and other essential cell components (McDonnell and Russell, 1999). PAA is more effective on bacteria and their spores as compared to hydrogen peroxide. The antimicrobial activity of PAA functions by denaturing proteins, disrupting cell wall permeability, and oxidizing sulfur bonds in proteins, enzymes, and other metabolites (McBain et al., 2004). Hydrogen peroxide is odorless and requires a

higher concentration to be effective as a sanitizer. It is usually applied at a concentration between 80ppm to 600ppm (Schmidt, 1997). PAA has an odor, it is effective at a lower concentration compared to hydrogen peroxide but is costlier (Ryther, 2013). Industrial application of PAA as an antimicrobial is usually at a concentration of 100ppm to 200ppm (Schmidt, 1997).

Quaternary ammonium compounds (QAC's)

QAC's are amphoteric surfactants containing a positive charge, which becomes active when bound to the acidic phospholipids of the bacterial cell wall (McDonnell and Russel, 1999; McBain et al., 2004). They are mostly active against gram-positive, non-spore forming bacteria but are considered to be poorly active on gram-negative bacteria (McBain et al., 2004). Also, the QAC's antimicrobial activity can be attributed to the inactivation of energy-producing enzymes, denaturation of essential cell proteins, and disruption of cell membrane (Schmidt, 1997; CDC, 2008). They are known to inhibit the outgrowth of spores but not the actual germination process (Russel 1990; McDonnel and Russel, 1999). QAC's are stable, odorless in solution, non-staining, non-corrosive, non-irritant to skin, non-toxic to the environment, and can leave a bacteriostatic coating on surfaces so as to inhibit microbial growth even after application (Dean-Raymond and Alexander, 1977; Knight and Cooke, 2002; McBain et al., 2004). They are also active at high pH, warm temperatures, light organic loads, and are fairly tolerant to hard water (Maillard, 2002; McBain et al., 2004; CDC, 2008). As a sanitizer, QAC's are commonly applied at 200ppm on a food-contact surface (Schmidt, 1997).

Iodine based compounds: Iodine and Iodophors

Iodine and iodophors are considered to be an effective antimicrobial agent, they can be both bactericidal and sporicidal. Iodophors react with the S-H group of proteins, the N-H group of nucleotides, which disrupts protein structures (Block, 1991; McDonnell and Russel, 1999). Iodine can penetrate the cell wall of microorganisms, and affect protein and nucleic acid synthesis (CDC, 2008). These compounds are more active at an acidic pH and are effective at lower concentrations. They do not cause any significant effect on the environment, but are known to stain equipment surfaces easily (Gottardi, 2001; EPA, 2006). The recommended concentration for iodine is 12.5ppm to 25ppm for a contact time of 1-minute (Schmidt, 1997).

Chlorine-based compounds

Chlorine compounds may be present in different forms, as (i.) chlorine gas, (ii.) hypochlorite i.e. sodium, calcium, magnesium, and (iii.) chlorine releasing agent i.e. chloramines (Knight and Cooke, 2002; Harms and O'Brien, 2010; Ryther, 2013). Hypochlorite is the most commonly used chlorine-based compound because it is known to be effective, relatively cheap and readily available (Ryther, 2013). Chlorine compounds are broad spectrum antimicrobial agents, they can be active against bacteria and their spores, by causing damage to the microbial outer membranes (Schmidt, 1997). Additionally, these compounds inhibit cellular enzymes important for glucose metabolism, oxidize cellular protein, and degrade DNA (Venkobachar et al., 1977; Schmidt, 1997). The antimicrobial activity of chlorine compounds can be affected however, by various factors such as temperature, pH, and organic matter. Chlorine is one of the most widely used sanitizing agents in the food industry and the USDA allows up to

200 ppm chlorine on food-contact surfaces and equipment (Ryther, 2013, USDA-FSIS, 2015).

Sodium Hypochlorite: Chlorine

Chlorine, in the form of sodium hypochlorite, is the most frequently used sanitizing solution in the food and poultry processing plant (CDC, 2008). It is used as a sanitizing solution either in the form of aqueous sodium hypochlorite or hypochlorous acid (Ryther, 2013). Chlorine products that are most prevalent in the United States are aqueous solutions that contain 5.25% - 6.15% sodium hypochlorite, also commonly known as household bleach (CDC, 2008). As a sanitizer, hypochlorite has a broad spectrum antimicrobial activity, it is generally inexpensive and fast acting (Gordon et al., 1993; CDC, 2008; Ryther, 2013).

Chemical Characteristics of Chlorine

Sodium hypochlorite, (chlorine) can be produced by (i.) Dissolving salt in water, the resulting solution is electrolyzed to form a sodium hypochlorite solution in water. (ii.) Mixing elemental chlorine (Cl_2) with caustic soda (NaOH), which result in the production of sodium hypochlorite, salt, and water (Gordon et al., 1995). This reaction is described below

$$Cl_2 + 2NaOH \rightarrow NaOCl + NaCl + H_2O \quad (2.1)$$

The amount of active chlorine in a solution is usually expressed as free or available chlorine. Other terms that are used to describe chlorine in solution is combined chlorine and total chlorine (Dychadala, 1991). Free or available chlorine is the relative concentration of elemental chlorine Cl_2 that is present in hypochlorous acid (HOCl) and a

hypochlorite ion (OCl^-). The concentration of hypochlorite in a solution is known by determining the electrochemical equivalent amount of Cl_2 in the solution (Hass, 1999). If 1- mole of elemental chlorine react with 2 electrons, the result will be the formation of inert chloride, as described below

$$Cl_2 + 2e = 2Cl^- \quad (2.2)$$

The reaction of 1 mole of hypochlorite (OCl^-) with 2 electrons results in the formation of a chloride

$$OCl^- + 2e^- + 2H^+ = Cl^- + H_2O \quad (2.3)$$

Combined chlorine, also known as chloramines is formed when chlorine in solution is mixed with organic matter such as nitrogenous compounds in the water. The reaction of chlorine with ammonia is expressed below (Yee at al., 2008).

$$HOCl + NH_3 \leftrightarrow NH_2Cl \text{ (monochloramine)} + H_2O \quad (2.4)$$

$$HOCl + NH_2Cl \leftrightarrow NHCl_2 \text{ (dichloramine)} + H_2O \quad (2.5)$$

$$HOCl + NHCl_2 \leftrightarrow NCl_3 \text{ (trichloramine)} + H_2O \quad (2.6)$$

Total chlorine is the combination of free chlorine and chloramine (Yee at al., 2008).

As a sanitizer, when chlorine (present in the form of sodium hypochlorite) is added to water, hypochlorous acid (HOCl) is formed.

$$NaOCl + H_2O \rightarrow HOCl + NaOH^- \quad (2.7)$$

Sodium hypochlorite in water is expressed as either hypochlorous acid (HOCl) or a hypochlorite ion (OCl^-), but hypochlorous acid is more effective than the hypochlorite ion. The variation in pH will determine the concentrations of hypochlorous acid versus the hypochlorite ion (CDC, 2008; Yee at al., 2008). Besides pH, other factors that impact

the stability of sodium hypochlorite are temperature and light. The variation in hydrogen ion concentration allows hypochlorous acid to dissociate rapidly thus, shifting the concentration of HOCl versus OCl^- (CDC, 2008). The dissociation of hypochlorous acid is expressed below

$$HOCl \leftrightarrow OCl^- + H^+ \quad (2.8)$$

In order to have more of hypochlorous acid, the pH must be maintained between 4 and 7 (McBain et al., 2004).

Antimicrobial Properties of Chlorine

As an antimicrobial, sodium hypochlorite (NaOCl) reacts with water to form the active agent hypochlorous acid (HOCl). This reaction has been previously described. NaOCl is active against both the bacteria and their spores due to the oxidizing potential of undissociated hypochlorous acid (HOCl). However, the degree of dissociation often affects the antimicrobial properties of sodium hypochlorite because a higher pH may result in reduced antimicrobial activity (Rosenthal et al., 1992; CDC, 2008). High organic loads in the solution can also adversely affect the antimicrobial activity of chlorine, however, hypochlorous acid, the active agent in sodium hypochlorite, was found to completely inhibit the growth of *E. coli* at 2.6 ppm within 5 minutes (McKenna and Davies, 1988; CDC, 2008). A possible hazard when using chlorine is the production of carcinogenic compounds such as trihalomethanes (THM) due to its reaction with organics, and bis(chloromethyl) ether when hypochlorite in solution come in contact with formaldehyde (CDC, 2008; Ryther, 2013)

Mode of Action on Microorganisms

The particular mechanism of action of chlorine is not fully understood (McDonnel and Russel 1999; CDC, 2008). However, chlorine is known to act on microbial cell membranes by disrupting oxidative phosphorylation and other outer membrane-associated activities (McDonnel and Russel, 1999). It causes oxidization of sulfhydryl enzymes and amino acids to destroy the cellular activity of proteins (Schmidt, 1997; CDC, 2008). Also, chlorine decreases ATP production and depresses DNA synthesis (Camper and McFeters, 1979; McDonnel and Russel, 1999). It was reported that the actual mechanism of how chlorine destroys microorganisms might be one or a combination of any of these factors (CDC, 2008). Even though chlorine is an effective antimicrobial, bacteria in biofilms are not easily killed by most antimicrobials including chlorine.

Biofilm Formation in Food Processing Environments

Biofilms as defined by Shi and Zhu (2009) is a group of microorganisms that attach to a surface thereby acting as a prevalent mode of growth for microorganisms in nature. When this group of bacteria colonizes a surface, it is not only the bacteria that forms a biofilm, rather there is an extracellular material produced by the bacteria including other materials that helps in anchoring the biofilm matrix (Hood and Zottola, 1995). Initially, when microorganisms are deposited on a surface, they often attach to that surface by growing and later multiply to form a colony of cells (Allison and Sutherland, 1987; Kumar and Anand, 1998). The process of biofilm formation is dynamic, prior to microorganisms forming a matrix of biofilm, events such as bacterial sticking and adhering must occur. As the process continues, the microorganisms become firmly

attached and at this point attached biofilms can be used to describe the process (Hood and Zottola, 1995). Similarly, Marshall et al., (1971) described biofilm formation as a two-step process. First, the bacteria contaminant come into close proximity with the surface so as to be adsorbed. Second, the bacteria then produce extracellular material which in turn helps with the attachment process. Overtime, attached bacteria on a surface will result in the formation of a fully mature biofilm, at this stage the process becomes irreversible. The extracellular material produced by bacteria in a biofilm is referred to as extracellular polymeric substances or exopolysaccharide (EPS). The EPS is mainly composed of polysaccharides, proteins, phospholipids, teichoic and nucleic acids (Shi and Zhu, 2009). The EPS serves as a shield that protects the bacterial colony or biofilm matrix from any harmful agents such as an antimicrobial. The first step described (i.e. adsorption of bacteria on a surface) is not uncommon in the food industry such as a poultry processing plant due to cross-contamination. However, at that point a thorough cleaning and perhaps sanitization can remove any adhered or adsorbed bacteria cells or colonies before the production of any extracellular material, which may enhance attachment and growth. It, therefore, becomes a public health problem when bacteria adhesion is not promptly removed, and leads to cross-contamination with any product that is in contact with such surface. The attachment of microorganisms to surfaces can be affected by a number of factors, that includes availability of nutrients in the growth medium, pH, temperature, physiochemical properties (i.e. hydrophobicity) of the surface, production of extracellular polysaccharides, presence of cellular structure, and microbial cell interaction (Hood and Zottola, 1995; James et al., 1995; Davies et al., 1998; Dourou et al., 2011; Chmielewski and Frank, 2003).

Bacteria attachment and subsequent biofilm formation in a food processing environment are a major concern since it may lead to a foodborne outbreak which can significantly affect the economy as a result of losses incurred through food spoilage (Hood and Zottola, 1995; Holah and Kearney, 1992). It was reported that a biofilm matrix might be composed of a single bacteria specie and/or multispecies of bacteria that forms either a single layer or a three-dimensional structure (Shi and Zhu, 2009). Several scientists have reported biofilm formation by several different microorganisms including *Listeria monocytogenes*, *E. coli*, *Campylobacter*, *Vibrio,* and *Salmonella*, (Stern and Kazmi, 1989; Faber and Peterkin, 1991; Dewanti and Wong, 1995; Ramesh et al., 2002; Somers and Wong, 2004; Ryu et al., 2004; Rivas et al., 2007; Dourou et al., 2011; Nguyen et al., 2014; Yang et al., 2016). Some of these studies observed the attachment of pathogenic microorganism like *Salmonella* on food processing equipment, which acts as a stable source of contamination to food products (Joseph et al., 2001; Nguyen et al., 2014; Yang et al., 2016).

Salmonella **Biofilms**

Salmonella is known to persist in the environment; it can be present as either a single or multispecies in a biofilm matrix (Soni et al., 2012). *Salmonella* species in a biofilm matrix have been reported to persist longer and were more difficult to inactivate when compared to planktonic (i.e. free) cells (Joseph et al., 2001; Vestby et al., 2009). This is because the biofilm matrix allows *Salmonella* cells to tolerate adverse conditions such as temperature, pH, oxidative and sanitizing agents (Yang et al., 2016). Furthermore, *Salmonella* may be exposed to different kinds of stress such as cleaning agents that are either acidic or alkaline, deprived of nutrients, exposal to temperature

abuse, or non-lethal concentrations of disinfectants and sanitizers during food processing, preservation, and storage (Belessi et al., 2011; Nguyen et al., 2014; Yang at al., 2016). *Salmonella* has been reported to have the ability to survive some of the stresses mentioned earlier and form resistance to other stressful conditions (Giaouris and Nychas, 2006; Nguyen et al., 2014). Nguyen et al., (2013) reported the resistance of *Salmonella* biofilm to industrial sanitizers, and suggest factors such as temperature, pH, and age of biofilm may have a profound influence. Another study reported the inability of some chemical disinfectants to inactivate *Salmonella* cells embedded in a biofilm (Moretro et al., 2009).

Salmonella have the ability to form biofilm on different surfaces including glass, rubber, cement, stainless steel, and plastic, which are the most common material used in food processing (Karunasagar et al., 1996; Joseph et al., 2001; Prouty and Gunn, 2003; Stepanovic et al., 2004; Nguyen and Yuk, 2013; Nguyen at al., 2014). For instance, Stepanovic et al., (2004) observed the biofilms of different *Salmonella* isolates on a plastic microtiter plate and found a high number of attached cells of *Salmonella* on the plastic surface. Similarly, *Salmonella* Enteritidis was found to form biofilms on a stainless steel surface (Giaouris and Nychas, 2006).

Other studies report the preference of *Salmonella* biofilm for different surfaces (Joseph et al., 2001; Nguyen et al., 2014; Yang et al., 2016). *Salmonella* Typhimurium adhered more to stainless steel at a neutral pH and elevated temperature with a high number of cells when compared to acrylic surfaces (Nguyen et al., 2014). Yang et al., (2016) observed a high biofilm density in *Salmonella* Enteritidis formed at 25°C on stainless steel coupons. In food processing particularly poultry processing, plastic and

stainless steel are the most common materials used for processing. Biofilms could form on the feather plucker, which is made of rubber, conveyor belts, steel cone lines, and plastic transport containers. All of these constitute a potential source of contamination and poses a significant risk to public health when *Salmonella* is present in the biofilm. When designing the cleaning and sanitation program of any food processing plant, consideration should be imposed on the areas where biofilms could be formed on the equipment and the influence of *Salmonella* cells that may be present in biofilms (Joseph et al., 2001). In addition, it is important to consider the type and effectiveness of the antimicrobial agent needed to sanitize food-processing equipment. It has been previously discussed earlier that sub-lethal concentrations of antimicrobials could represent a stressful condition that bacteria adapt to, which can make antimicrobials not effective especially for bacterial cells in a biofilm matrix.

Bacterial Stress Adaptation and Cross-Adaptation

Foodborne pathogens such as *Salmonella* and *Listeria* have been reported to possess the ability to tolerate stressful conditions and confer such tolerance to similar or different stress factors (Buchmeier and Heffron, 1990; Linton et al., 1992; Lou and Yousef, 1996). Stress was described by Yousef and Courtney (2003) as a detrimental factor or condition that causes a negative effect on the growth and survival of a pathogen. In any food processing environment, the processing and preservation techniques may impose stress to any bacteria present. There is a number of stresses encountered by bacteria during food processing. These includes starvation, heat, low temperature, acids, and biocides, cleaning and sanitizing agents (Lou and Yousef, 1997; Denyer and Maillard, 2002). The response and survival of the exposed pathogen to the stresses

mentioned above is dynamic, the bacteria may adapt by increasing their tolerance to the stress condition, produce some proteins that will repair the damage or ultimately eliminate the threat (Foster, 2005; Marles-Wright and Lewis, 2007). A common practice in food processing is the use of antimicrobials to inhibit the growth of bacteria either for processing purposes or on equipment surfaces after proper cleaning. However, using the antimicrobials at a non-lethal level (i.e. a concentration that is not enough to kill the bacteria) may retard growth and ensure the survival of some bacterial populations. There is a possibility of an induced tolerance in the surviving bacteria population, which then become the stress adapted bacteria. This phenomenon has been well elucidated by researchers (Foster, 1991; Murphy et al., 2003; Capita et al., 2014). It was suggested that the adapted bacteria pose a great threat to food safety (Capita et al., 2014). The impact of stress adapted bacteria can be severe when the bacteria encounter and survive greater challenge such as a lethal level (i.e. a concentration that should kill many if not all the bacteria) of the antimicrobial or a more severe environmental stress (Sheridan et al., 2012).

The incidence of cross-protection or cross-adaptation in foodborne pathogens have been well studied (Vester et al., 2001; Murphy et al., 2003; Braoudaki and Hinton, 2004; Chung et al., 2006). Some of these studies suggest that induced resistance was a result of bacterial exposure to a stress condition, which then confers cross-resistance to other stress conditions (Diez-Garcia et al., 2012; Alonso-Calleja et al., 2015). When bacteria are exposed to the same type of stress for a prolong duration of time and the same bacteria is later exposed to and survive similar stress either at sub-lethal or lethal levels, it is referred to as homologous stress adaptation and cross-adaptation. However,

when previously adapted bacteria are exposed to and survive a different stress condition, it is then considered to be a heterologous stress adaptation and cross-adaptation (Yousef and Courtney, 2003, Plaks et al., 2004).

Salmonella Stress Adaptation and Cross-Adaptation

Salmonella, reportedly displays adaptation in response to stresses posed by the high osmotic environment, acidification, food processing heat stress, and oxidative stress (Christman et al., 1985; Csunka, 1989; Foster, 1991). *Salmonella* strains adapted to induced stress posed by an antimicrobial benzalkonium chloride was observed to display resistance to chlorhexidine a quaternary ammonium compound and cross-resistance to the following antibiotics: amoxicillin, amoxicillin-clavulanic acid, chloramphenicol and trimethoprim (Braoudaki and Hinton, 2004). Similarly, sub-lethal exposure of *Salmonella* to sodium nitrite and sodium hypochlorite induce adaptation and reduce susceptibility to a range of antibiotics (Molina-Gonzalez et al., 2014). In another study, acid adapted *Salmonella* was observed to tolerate exposure to high temperature and salt stress (Leyer and Johnson, 1993). Induced acid tolerance response (ATR) in *Salmonella* protects against exposure to acid shock, strong acidic environment with a pH ≤ 3 and weak acids similar to the ones encountered in the human gastrointestinal system (Baik et al., 1996). Potenski et al., (2003) also found that mutant *Salmonella* Enteritidis cells that possess a great tolerance for sodium nitrite and sodium benzoate showed greater tolerance to tetracycline when compared to the cells that were not previously exposed.

The use of antimicrobials as a disinfectant or sanitizer has been well investigated. *Salmonella* is also known to possess the ability to adapt and survive different stressful conditions including those posed by sub-lethal concentrations of antimicrobials (like

chlorine), thereby increasing the ability to survive similar or different stress. Therefore, it is important for food processors to develop a control program that includes a routine check of sanitizer efficacy and the use of appropriate concentrations of antimicrobials that will prevent the development of resistant *Salmonella* populations and inhibit any bacteria in the biofilm.

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CHAPTER III

RUGOSE MORPHOTYPE OF *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM ATCC14028 EXHIBITS CHLORINE RESISTANCE AND STRONG BIOFILM FORMING ABILITY

Abstract

In this study, *Salmonella enterica* serovar Typhimurium (ATCC 14028) was tested for its ability to adapt to increasing increment of chlorine starting at 125 ppm in tryptic soy broth (TSB). *Salmonella* Typhimurium demonstrated an acquired tolerance to chlorine in TSB with adapted cells growing in concentrations up to 600 ppm whereas the non-adapted cells did not grow beyond 500 ppm. After 4 days of incubation, *S.* Typhimurium exposed to sub-inhibitory concentrations of chlorine displayed a distinct rugose morphology on tryptic soy agar (TSA) plates incubated at 37°C. The chlorine tolerant *S.* Typhimurium cells contained a mixture of both rugose and smooth morphology. The rugose cells, in contrast to smooth morphology (both adapted and control), showed the ability to form very strong biofilms ($P < 0.05$) in polystyrene microtiter plate at room temperature and 37°C. The antibiotic susceptibility patterns of adapted (rugose and smooth) and control cells were tested using different antibiotics according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines. There was not much difference observed in antibiotics resistance of either

adapted cell type as compared to control. The findings of this study suggest that the incorrect application of chlorine during cleaning and sanitation could select for adapted *Salmonella* cells, which may attach strongly to plastic surfaces.

Key words: *Salmonella,* chlorine, adaptation, rugose, biofilm

Introduction

Around the world, nontyphoidal *Salmonella* is a major foodborne pathogen causing numerous reported cases of foodborne infections (Hohmann, 2001, WHO, 2013). It was reported to result in over 40,000 cases leading to about 500 deaths each year by the centers for disease control and prevention (CDC, Hohmann, 2001). Gastroenteritis and bacteremia are caused by nontyphoidal *Salmonella* (CDC, 2013). In the past year, the CDC estimated that nontyphoidal salmonella, which is identified as the leading cause of diarrhea in most parts of the world, caused 94 million cases of gastroenteritis and 115,000 deaths globally (CDC 2013). Due to the food safety concerns imposed by *Salmonella* and other foodborne pathogens, the USDA-FSIS has a list of substances (antimicrobials) that are approved to be used during the production of poultry, meat, and egg products (FSIS directive 7120.1; USDA-FSIS, 2015). The poultry industry uses the antimicrobials directly in the chiller water and by spraying or dipping poultry products. In addition, a thorough cleaning, disinfecting and sanitization program for contact surfaces, equipment, and poultry processing facility are incorporated into good manufacturing practices (GMP) by the industry.

However, bacteria can still aggregate on surfaces to form biofilms, which are a considerable food safety concern. It was reported that about 80% of all the infections caused by microorganisms including foodborne illnesses are caused by microorganisms

that are present as a biofilm (NIH, 1997; Jahid and Ha, 2014). A biofilm is composed of several microorganisms, they interact with each other and become attached to an exposed surface by a matrix produced by the organisms (Costerton et al., 1995; Shi and Zhu, 2009). The film produced by the microbes is covered by an exopolysaccharide layer consisting of cellulose and curli fimbriae (Watnick and Kolter, 2000; O'Leary et al., 2012). Austin et al., (1998) suggests that curli fimbriae are the main protein nutrient of the biofilm matrix. They function by aiding in the aggregation and adhesion of bacteria cells to surfaces thus forming a full mature biofilm (O'Leary et al., 2012). Several studies have reported the microbial composition of biofilms that can include species such as *Vibrio spp., Listeria monocytogenes, E. coli,* and other spoilage microbes in different food processing environments (Bagge-Ravn et al., 2003; Gunduz and Tuncel, 2006). Some of these studies observed that the pathogens in biofilms could be isolated even after cleaning and sanitization (Bagge-Ravn et al., 2003).

In food processing, the ability of foodborne pathogens to attach to and subsequently form a biofilm on food contact surfaces may constitute a potential source of post-processing cross contamination of products (Nguyen et al., 2014). Chemical treatment like chlorine and chlorine-based compounds, quaternary ammonium compounds, organic acids (peroxyacetic and acetic acid), hydrogen peroxide, and iodine compounds are commonly used to remove any attached cells (Nguyen et al., 2013). However, several studies have shown that bacteria in biofilms are more resistant to antimicrobial treatment and sanitation procedures when compared to planktonic cells (Somers et al., 1994; Joseph et al., 2001; Ramesh et al., 2002; Chavant et al., 2002; Furukawa et al., 2010; O'Leary et al., 2012; Nguyen et al., 2013).

Among the approved chemical sanitizers for food-contact surfaces, chlorine and choline-based compounds are the most widely utilized. Chlorine is not costly and provides a broad-spectrum bactericidal activity (Liu et al., 2006; Yang et al., 2016). The recommended level of chlorine for sanitation purposes is 200ppm and it is most effective at a pH of 6.5 or below (Chen at al., 2014; USDA-FSIS, 2015). However, the use of antimicrobials including chlorine at a sub-lethal concentration may constitute a crucial public health risk.

The increase in pathogenic bacterial resistance towards antibiotics and antimicrobials is a persistent public health challenge all over the world (Braoudaki and Hilton, 2004; Capita and Alonso-Calleja, 2013). Exposure of foodborne pathogens to sub-lethal concentrations of sanitizing agents like chlorine may create a potential challenge to the bacteria (Sheridan et al., 2012). The possibility of such pathogenic bacteria adapting to the stress and using the adaptive resistance to induce cross-resistance to antibiotics has been elucidated by others (Davidson and Harrison, 2002; Braoudaki and Hilton, 2005; Capita et al., 2014; Alonso-Calleja et al., 2015). The fact that chlorine is the most common sanitizing agent used in food processing and bacteria can survive when chlorine is used at a sub-lethal level, is concerning. However, the greater concern is when the exposed pathogens confer cross-adaptation to clinically important antibiotics.

In this study, exposure of *Salmonella enterica* serovar Typhimurium to sub-lethal concentrations of chlorine caused a morphological change to the rugose variant of *Salmonella*. Anriany et al., (2001) observed the rugose phenotype in *S*. Typhimurium DT104 on TSA after three days of incubation at 25°C. It was defined as a corrugated colony morphology associated with the formation of exopolysaccharide (EPS) and cell

aggregation (Morris et al., 1996). Rugosity usually develops in response to unfavorable conditions such as stress and starvation (Johnson et al., 1992; Mizunoe et al., 1999; Morris et al., 1996; Wai et al., 1998; Anriany et al., 2001). Studies have shown that the rugose variant in *Vibrio cholerae* displayed increased resistance to chlorine treatment, which may have aided in the survival of *Vibrio* in various foodborne outbreaks (Wai et al., 1998; Mizunoe et al., 1999; Yildiz et al., 1999; Anriany et al., 2001). Nevertheless, the ability of rugose cells to adapt to chlorine treatment has not been well reported in *Salmonella* Typhimurium ATCC14028.

Therefore, the main aim of this study was to determine homologous stress adaptation by measuring the change in the minimum inhibitory concentration (MIC) both pre and post exposure to chlorine. When this aim was achieved, the difference in biofilm forming ability of stress adapted compared to non-adapted cells were determined. Also, the possibility of cross-adaptation to different antibiotics and acidic stress was assessed.

Materials and Methods

Salmonella Strain and Inoculum Preparation

One loop full of frozen *Salmonella* Typhimurium ATCC14028 culture was obtained and streaked on a tryptic soy agar plate (TSA; Sigma-Aldrich Co., St. Louis, MO, USA). The plate was subsequently incubated at 37°C for 24 h. The cells were maintained through monthly transfer on TSA slants that were stored at 4 ± 1°C. The broth culture was prepared by transferring a single colony from a TSA plate into sterile 10 ml tryptic soy broth (TSB; Sigma-Aldrich Co., St. Louis, MO), which was incubated at 37°C for 24 h to achieve a total plate count of approximately 10^9 CFU/ml. Cells were harvested by centrifugation (Eppendorf Biotech company Hamburg, Germany) at 5500 rpm for 10

minutes at 4°C. The supernatant was discarded and the pellet was re-suspended in fresh 10 ml sterile TSB to make a stock culture of ~10^9 CFU/ml.

Sanitizing Agent

Chlorine, in the form of sodium hypochlorite, containing 5% available chlorine (ACROS Organics, New Jersey USA), was used in this study. The concentration of free chlorine was validated using the HACH (chlorine test kit) Pocket Colorimeter (HACH Company, Loveland, CO, USA) according to the manufacturer's instructions.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Assay

The MIC and MBC of chlorine were determined for *Salmonella* Typhimurium with a 96-well polystyrene microtiter plate (Thermo Fisher Scientific, Fair Lawn, NJ, USA) using the broth microdilution method according to the Clinical and Laboratory Standards Institute, CLSI (2008) guidelines with minimal modifications. A volume of 200µl of sodium hypochlorite in TSB containing double fold concentrations was added to the first well of the microtiter plate as an initial concentration. Then 100µl of sterile TSB was added to all other wells in the same row. The sanitizing agent was diluted two-fold by transferring 100µl of sodium hypochlorite from the first well through the last well and the excess was discarded. After diluting the sanitizing agent, 100µl of inoculum prepared by serial dilution of the stock culture (final concentration of ~10^7 CFU/ml) was added to each well. A positive control (i.e. 100µl TSB + 100µl inoculum) and negative control (i.e. 200µl of TSB without inoculum) were maintained throughout the experiment. Bacterial growth for each well was determined by turbidity after incubation at 37°C for 24 h. The MIC was determined to be the lowest concentration of the sanitizing agent in

which there was no bacterial growth. The sanitizer was tested in all the 96-wells for *Salmonella* Typhimurium. To determine the MBC of sodium hypochlorite against *Salmonella* Typhimurium, the wells showing no visible bacterial growth after 24 h of incubation at 37°C were selected. An aliquot of 50µL from the clear wells was spread plated on TSA plates and the plates were incubated at 37°C for 24 h. The highest dilution of chlorine that showed no bacterial growth on TSA plates after incubation was then considered to be the MBC.

Exposure to Increasing Concentrations of Chlorine

For the stress adaptation study, the overnight stock culture was prepared to a final concentration of ~10^9 CFU/ml. An aliquot of 100µl of the stock culture was added to 9.9 ml TSB containing a starting concentration of 125 ppm chlorine in a (15ml) sterile polypropylene flat cap tube (Thermo Fisher Scientific, Fair Lawn, NJ) to make a final inoculum concentration of ~10^7 CFU/ml. The tube was incubated at 37°C for 24 h, bacterial growth in the tube was observed for turbidity and when growth was observed, 100µl of the suspension was aseptically transferred into a sterile tube, which contained 9.9 ml TSB with the next highest concentration of chlorine. The suspension was then diluted and plated on TSA and incubated overnight at 37°C to observe *Salmonella* growth.

The procedure continued with daily increase (25 ppm) of chlorine until a concentration of chlorine was reached where there was no visible bacterial growth observed after incubation at 37°C. This required a total of 18 days. The suspension from the last tube with visible growth was plated on TSA plates without chlorine, and the cells obtained after incubation at 37°C were considered to be adapted *Salmonella* cells. The

agar plates were kept at 4 ± 1°C with weekly transfer. Non-exposed cells were grown in TSB without chlorine and were subsequently diluted, and plated on TSA plates to serve as the control. The stress adaptation study was replicated three times over the course of 3 months.

Confirmation Test

A selective media (i) Xylose-Lysine-Tergitol (XLT4) agar, (ii) Brilliant green sulfa agar followed by gram staining procedure (Becton, Dickinson and Company, Franklin Lakes, NJ) and polymerase chain reactions were used to identify and confirm the different morphotypes of *Salmonella* Typhimurium ATCC14028 observed.

Stability of Adapted Cells to Homologous Stress

To determine the stability of the adapted cells, the MIC and MBC of chlorine was determined against the adapted cells of *Salmonella* Typhimurium ATCC14028 with disposable borosilicate glass round bottom culture tubes (Thermo Fisher Scientific, Fair Lawn, NJ) using the broth macrodilution method in accordance with the CLSI (2012) guidelines. A colony of adapted *Salmonella* cells was inoculated into glass tubes containing TSB with chlorine at (i) a concentration below MIC, (ii) a concentration equivalent to MIC, and (iii) two concentrations that are 50 ppm and 100 ppm respectively above MIC. Non-adapted (control) *Salmonella* cells were also tested at the same concentrations. The adapted and control cells were tested after storage on TSA plates without chlorine. The experiment was replicated three times in duplicate glass tubes. The glass tube showing no turbidity was considered to be the MIC of the adapted and control cells. For MBC determination, the glass tube showing no bacterial growth after

incubation at 37°C for 24 h was selected. An aliquot of 100µl from the clear tube was spread plated on TSA plates and was incubated for approximately 24 h at 37°C. The lowest concentration of chlorine that showed no visible colonies on TSA plates after incubation was considered to be the MBC of adapted and control cells.

Biofilm Formation Study

The biofilm forming ability of *Salmonella* Typhimurium (ATCC14028) cells was performed using a previously described method (Patel and Sharma, 2010). Adapted cells were grown at 37°C for 24 h, and appropriate dilutions in TSB were prepared to obtain a final inoculum concentration of ~10^6 CFU/ml. TSB containing 550 ppm chlorine (the highest concentration that supports bacterial growth) was used for the growth of previously adapted cells, and TSB without chlorine was used for the control cells. The microtiter plate was prepared by adding 200µl of the culture and was then incubated at both room temperature and 37°C for 48 h. After incubation, the inoculum was completely removed from each well by aspiration, and the wells were washed five times with sterile distilled water in order to remove any loosely attached bacteria. The plate was allowed to air dry for 45 minutes. Afterwards, 200µl of crystal violet solution (0.41% w/v dye, ACROS Organics New Jersey, USA) was added to each well and incubated at room temperature for 45 mins. Following incubation, the crystal violet solution was completely removed from the wells by aspiration and the wells were washed five additional times with sterile distilled water. The plate was air dried for 45 mins and then 200µl of 95% ethanol (Thermo Fisher Scientific, Fair Lawn, NJ) was added to each well and the content of the wells was mixed. The biofilm formation in the well was measured by taking optical

density readings at 600 nm (OD_{600}) using a micro-quant microplate spectrophotometer (BioTek Instruments, Winooski, VT).

Enumeration of Attached Cells

The biofilm formation of chlorine adapted and control cells were also determined by enumerating the number of strongly attached cells on a plastic surface using the 24-well polystyrene plate. Similar to the crystal violet assay, ~10^6 CFU/ml of adapted and control cells were obtained and 200µl of the inoculum was put into each well. The plates were incubated at room temperature and 37°C for 48 h. Afterwards, the wells were emptied by removing the inoculum and each well was washed with sterile distilled water three times to remove any loosely attached cells. The strongly attached cells in each well was subsequently scraped into 0.1% peptone water. The suspension was then vortexed for 2 min, subjected to a 10-fold serial dilution and a volume of 100µl was spread plated on TSA plates. The number of cells after incubation at 37°C for 24 h was enumerated.

Antibiotic Susceptibility Testing

The chlorine stressed and control cells were screened for susceptibility to a range of antibiotics in Mueller-Hinton broth and on Mueller-Hinton agar (Oxoid) by the disk diffusion and MIC broth microdilution method as described in the CLSI (2008) guidelines. The discs used were: sulphamethoxazole/trimethoprim (SXT, 25 µg), gentamicin (GN, 10 µg), streptomycin (S, 10 µg), amoxicillin/clavulanic acid (AMC, 30 µg), nalidixic acid (NA, 30 µg), ciprofloxacin (CIP, 5 µg), ceftriaxone (CTX, 30 µg), and ampicillin (AMP, 10 µg). The zones of inhibition were measured and recorded as susceptible, intermediate, and resistant according to the CLSI (2008) guidelines. The

broth microdilution for the antibiotics was performed in a 96-well polystyrene microtiter plate. Each panel contains approximately 6 dilutions using the MIC breakpoints recommended by the CLSI (2008) guidelines. One well represented the positive control (i.e. broth and inoculum), and one served as the negative control (broth only). The results were observed and recorded as the least concentration of antibiotics that inhibit the growth of *Salmonella* either as susceptible, intermediate, or resistant according to the CLSI (2008) guidelines. All combinations of *Salmonella* cells (adapted and control) with antibiotics were conducted on separate days.

Acid Resistance Testing

Cross-adaptation of the chlorine adapted and control cells to low acidity was determined. One hundred microliters of the overnight culture was added to 9.9 ml sterile TSB (adjusted to pH 3 with 1N HCl) to achieve a final concentration of ~10^7 CFU/ml. The culture was then incubated at 37°C for 12 h. One milliliter was removed from the suspension every 3 h and was serially diluted in 0.1% peptone water. One hundred microliters of each dilution was spread plated on TSA plates and incubated at 37°C for 24 h to observe the surviving *Salmonella* cells.

Statistical Analysis

All experiments were replicated three times sequentially. Analysis of variance (ANOVA) in the General Linear Model (GLM) of SAS v. 9.4 (SAS Institute, Cary, NC, USA) was used to analyze the data. The means were separated by Fisher's Least Significant Difference test. The treatments and controls were considered to be significant when $P \leq 0.05$.

Results and Discussion

Observations during Stress Adaptation Study

When *Salmonella* Typhimurium reached a 200 ppm concentration of chlorine in TSB which was exactly 4 days of incubation from the start of the stress adaptation, a mixed colony with two different morphologies were observed on TSA plates. One morphology appeared to be rough and dry, it is referred to as the "rugose" morphotype of *Salmonella* Typhimurium. The rugose and normal smooth *Salmonella* morphotypes continued to grow together on each day of transfer until the concentration of chlorine in TSB reached 550 ppm. This concentration was identified as the highest concentration that supports bacterial growth. The rugose and smooth *Salmonella* cells harvested at this concentration were considered to be the adapted *Salmonella* cells. In suspension, the rugose morphotype remained as a cell aggregate, whereas the smooth morphotype was dispersed in the broth making a homogenous solution.

In the current study, the rugose variant of *S.* Typhimurium was observed in response to the exposure of the smooth variant to sub-inhibitory concentrations of chlorine starting from 200 ppm at 37°C. Similarly, the rugose phenotype has been observed in both *Vibrio cholerae* (O1 and non-O1 groups) and *Salmonella enterica* serovar Typhimurium DT104 and non-DT104 (White, 1938; Rice et al., 1992; Morris et al., 1996; Anriany et al., 2001). However, in the case of *Vibrio cholerae*, the rugose variant was observed after several passages of the smooth variant in alkaline peptone water at 37°C and in response to nutrient starvation when grown at 16°C for several days (Morris et al., 1996; Wai et al., 1998; Mizunoe et al., 1999). In *Salmonella* Typhimurium DT104, the rugose phenotype was observed after 4 days of extended incubation of the

smooth colony at 19 to 28°C on TSA plates (Anriany et al., 2001). Contrary to the observations in the current study, Anriany et al., (2001) suggested that the rugose phenotype was either temperature or media dependent because it was not identified upon incubation at 37°C and it required more incubation time to grow on brilliant green agar (BGA). In the current study, the rugose morphotype was observed on brilliant green sulfa agar (BGSA) but could not be observed on XLT4 agar. There was no difference in the appearance of rugose and smooth *Salmonella* morphotype on (XLT4) agar plates, which is in agreement with the observations of Anriany et al., (2001). The findings in this study suggest that chlorine is a stressor, which induces the formation of the rugose morphotype in *Salmonella* Typhimurium ATCC14028.

Adaptation to Chorine Stress

The MIC of chlorine against *Salmonella* Typhimurium (ATCC14028) prior to exposure to sub-inhibitory concentrations of chlorine was observed to be below 500 ppm using the micro broth dilution method. After subsequent tests at lower concentrations, it was determined that 400 ppm was the MIC. When the glass tube macro broth method was used however, 500 ppm was determined to be the MIC for *Salmonella* Typhimurium (ATCC14028) due to the higher organic load in the tube. The MBC of chlorine against *S.* Typhimurium (ATCC14028) was observed to be 500 ppm and this value was the same for all replications **(Table 3.1).** After several passages through increasing concentrations of chlorine, the maximum concentration of chlorine that allowed growth after 18 days of incubation at 37°C was 550 ppm. This showed that the adapted cells were able to grow in concentrations of chlorine that are 1.375 times higher than the non-adapted cells.

Homologous Stress Adaptation

The MIC and MBC of adapted rugose and smooth *Salmonella* cells were determined to understand their stability to chlorine. The adapted *Salmonella* cells (rugose and smooth) were stored on TSA plates without chlorine. Single colonies were transferred weekly to a fresh chlorine-free TSA plates to ensure storage. The adapted rugose and smooth cells were able to grow at 450, 500, 550, and 600 ppm chlorine concentrations whereas, the non-adapted cells did not grow beyond 500 ppm. Thus, the MIC changed from 500 ppm before adaptation to 650 ppm after adaptation for the chlorine adapted *Salmonella* cells, which means homologous stability lasted even without chlorine **(Fig 3.1).**

This study attempts to investigate the significant effect of *Salmonella* Typhimurium adaptation to an antimicrobial which has been used in food processing for a long period of time, either directly or as a sanitizing agent on food-contact surfaces. Chlorine in the form of sodium hypochlorite is reported to be more frequently used by food processors (Ryther, 2013). There are previous reports on the adaptation of pathogenic microorganisms to food-grade biocides and antimicrobials used in food production (Tattawasart et al., 1999; Braoudaki and Hilton, 2005; Chung et al., 2006; Randall et al., 2007). In order to establish and know the extent of homologous adaptation in the studies, changes in MIC were determined. A change in MIC following the adaptation of *E. coli* to various antimicrobials such as sodium nitrite and sodium hypochlorite have been previously reported by Capita et al (2014). In that study, the MIC after adaptation was observed to be respectively 2.5313 and 1.6862 times higher than what it was before adaptation (Capita et al., 2014). Similarly, Braoudaki and Hilton

(2004) reported the stability of adapted *Salmonella enterica* to benzalkonium chloride after sub-culturing in antimicrobial-free broth. An increase in MIC was observed for the adapted cells as compared to the non-adapted cells. Alonso-Calleja et al., (2015) also reported a higher MIC for adapted *E. coli* cells that were exposed to different biocides after re-culturing in a TSB devoid of biocide. Similarly, the results of the current study agree with other studies on changes in MIC following adaptation. Furthermore, the rugose variant in *V. cholerae* has been observed to be chlorine tolerant (Morris et al., 1996). According to Yildiz and Schoolnik (1998), the smooth variant of *Vibrio cholerae* O1 E1 Tor was completely inactivated upon exposure to 3 ppm NaOCl for 5 min. However, under the same experimental conditions, the rugose variant survived and about 5 log CFU/ml cells were recovered. Another report consistent with these observations was the findings of Rice et al., (1993). It was suggested that the smooth variant is chlorine sensitive, and in contrast, the rugose variant was observed to be chlorine resistant. In addition, the smooth variant of *Vibrio* was reported to be inactivated with 0.5 mg/L free chlorine when exposed for less than 20 s. Contrarily, the rugose variant was exposed to a higher concentration of free chlorine (2 mg/L), under the same growth conditions and cells were still recovered after 30 mins (Morris et al., 1996). The majority of these studies supports the findings of the current study, which demonstrated the ability of chlorine-adapted *Salmonella* cells (rugose and smooth) to withstand the homologous stress of chlorine by surviving higher chlorine concentrations than was previously exposed. In addition, it is important to note that the majority of the studies on the rugose phenotype of *Vibrio* suggested that the formation of exopolysaccharide (EPS) was responsible for the resistance patterns to chlorine.

Biofilm Formation on Plastic Surface

The adapted *Salmonella* Typhimurium cells as well as control cells were tested for their ability to form biofilms on a polystyrene plastic surface (using 96-well polystyrene microtiter plate) under the experimental conditions tested**.** In the current study, exposure to sub-inhibitory concentrations of chlorine and subsequent adaptation was determined to influence the strength of biofilms formed by adapted cells as compared to non-adapted *Salmonella* Typhimurium cells. The adapted *Salmonella* cells were cultured in TSB containing 550 ppm chlorine. The optical density (OD) reading at 600 nm of the adapted rugose and smooth cells, as wells as the control was observed. Adapted rugose cells had the highest OD_{600} values with an average of 3.4 and 3.64 at 37°C and room temperature respectively. Whereas, the adapted smooth and non-adapted cells had average OD_{600} values of 0.8 and 0.47 at 37°C and 0.63 and 0.48 at room temperature respectively (**Fig 3.2a and 3.2b**). As expected, the negative control, which is TSB without bacteria or chlorine had the lowest OD_{600} value averaging 0.14 and 0.15 at 37°C and room temperature respectively (**Fig 3.2a and 3.2b**). At 37°C, the biofilm forming ability of all the *Salmonella* Typhimurium morphotypes tested was significantly different ($P < 0.05$) compared to the negative control (**Fig 3.2a**). The adapted rugose cells formed the strongest biofilms ($P < 0.05$) on the plastic surface followed by adapted smooth cells which were also a better biofilm former compared to the non-adapted cells (**Fig 3.2a**). Although, adapted rugose cells formed better biofilms at room temperature ($P < 0.05$), there was no significant difference ($P > 0.05$) observed in the biofilm forming ability of the adapted smooth as compared to the non-adapted *Salmonella* cells at 37°C (**Fig 3.2b**). This is because during the stress adaptation study, as the concentration of

chlorine in TSB increased, there appeared to be more rugose cells on TSA plates than the smooth cells and by the end of the study, the rugose cells had completely outgrown the smooth adapted cells. The strongly attached cells in the plastic plate were enumerated. The results demonstrated that a difference ($P < 0.05$) could be observed between all the cell types tested at both temperatures. The rugose cells concentration was more on the plastic surface when compared to the smooth adapted and non-adapted cells at 37°C and room temperature (**Fig 3.3a and 3.3b).**

Biofilms formation of microorganisms such as *E coli, Listeria monocytogenes, Salmonella enterica, Salmonella* Typhimurium ATCC14028, DT104, and *Vibrio cholerae* have been well documented (Yildiz and Schoolnik, 1998; Rodriguez et al., 2008; Dourou et al., 2011; O'Leary et al., 2012; Capital et al., 2014; Nguyen et al., 2013; Yang et al., 2016; Ziech et al., 2016). In *Vibrio cholerae,* it was reported that the smooth variant has a poor attachment as compared to rugose variant (Yildiz and Schoolnik, 1998). *Salmonella* Typhimurium has been reported to have the ability to adhere to different surfaces including stainless steel and acrylic, and better biofilms are formed on stainless steel compared to other surfaces tested (Chia et al., 2009). Regardless of the attachment surface, the great concern must be afforded to chlorine adapted *Salmonella* Typhimurium which are capable of forming strong biofilms. The presence of *Salmonella* in biofilms in a food processing plant may not be easily inactivated through the conventional sanitization process. In a previous study, the biofilms of *Salmonella* species formed on a plastic surface was completely inactivated after exposure to 100 ppm chlorine concentration for 20 mins (Joseph et al., 2001). However, in the current study, *Salmonella* cells that formed biofilms were previously adapted to a higher concentration

of chlorine even up to 550 ppm, which is substantially higher than the 200ppm allowed by USDA-FSIS for use during sanitation. Furthermore, a stronger biofilm formation for the adapted rugose cells observed in this study and other studies on *Vibrio cholerae* suggests that, the formation of exopolysaccharide help with the aggregation of cells and can act as a protective covering for the cells (Morris et al., 1996; Wai et al., 1998). It further assists in preventing the bacterial cells from being inactivated by the sanitizer. Additionally, another study presents a different perspective on the rugose morphotype. In the study, rugosity was explained to be correlated with curli and cellulose. The curli represents adhesion, and when it combines with cellulose, it allows the cells to adhere to one another (Prigent-Combaret et al., 2000; Zogaj et al., 2001; Eriksson de Rezende et al., 2005). The findings in the current study support the suggestions of Morris et al., (1996), that the production of exopolysaccharide mainly promotes attachment of bacteria as observed in both the crystal violet assay and the enumeration of strongly attached cells.

One of the aims of this study was to determine the ability of chlorine tolerant *Salmonella* cells to form biofilms. The finding from this study suggests that more cells of the chlorine adapted rugose variant of *Salmonella* Typhimurium attached to the plastic surface tested compared to the smooth variant (adapted and non-adapted), which is worth pointing out because plastic materials are often used in the processing of poultry carcasses. These findings will provide significant information to poultry processors on the right use of chemicals for the purpose of sanitization.

Antibiotic Cross-Adaptation

Salmonella Typhimurium was screened for susceptibility to different antibiotics after exposure to increasing sub-lethal concentrations of chlorine. The antibiotic resistance patterns of the adapted (rugose and smooth) and control cells are shown in **Table 3.2** and **3.3**. The results showed that there was no difference in the antibiotic susceptibility patterns of the adapted cells when compared to the control cells, for any of the antibiotics tested. However, the rugose cells showed a slight reduction (≤ 2mm) in susceptibility to streptomycin, nalidixic acid, ciprofloxacin, and ceftriaxone when compared to smooth and control. A change in MIC (antibiotic susceptibility) was observed for the adapted cells (rugose and smooth), which exhibited resistance to amoxicillin (**Table 3.3**). In addition, a slight increase in MIC was observed for rugose cells against all the antibiotics tested except ciprofloxacin compared to smooth (adapted and non-adapted). Over the years, there are concerns pertaining to the possibility of cross-adaptation between a previous adaptation to an antimicrobial and antibiotic resistance (Braoudaki and Hilton, 2004; Capita and Alonso-Calleja, 2013). The global growing concern for the resistance of foodborne pathogens to antibiotic calls for great attention, especially in nations where infections to the pathogens are rampant. Although, much difference was not observed in the susceptibility patterns of adapted and control cells to the majority of the antibiotics tested, a minimal reduction in susceptibility observed for antibiotics such as penicillin (AMX), cephalosporin (CTX), aminoglycoside (S and GN), quinolones (NA), tetracycline, and fluoroquinolones (CIP) is worth pointing out. This is because some of these are the antibiotics of choice in the treatment of *Salmonella* infections especially in adults (Sirinavin and Garner, 1999). Some studies

have reported a frequent occurrence in the cross-resistance patterns for fluoroquinolones, quinolones, and aminoglycosides against gram-negative bacteria (Gutmann et al, 1995; Braoudaki and Hilton, 2004). Contrary to the findings in this study, other studies have observed and reported cross-resistance between some antimicrobial agents and antibiotics in *E. coli*, *P. aeruginosa*, and some strains of *Salmonella* (Lambert et al., 2001; Lee et al., 2001; Braoudaki and Hilton, 2004; Capita et al., 2014). The majority of these studies suggested the presence of a common resistance mechanism between adaptations to an antimicrobial and antibiotic resistance (Suller and Russell, 2000; Braoudaki and Hilton, 2004; Capita et al., 2014).

The broad objective of this study was to examine whether *Salmonella* adaptation to chlorine stress would induce cross-adaptation to antibiotics. The current study did not find much interactions between chlorine adaptation and antibiotic resistance. However, the findings of this study suggest that the previously adapted rugose variant of *Salmonella* Typhimurium may possess a slight reduction in susceptibility to antibiotics. The rugose variant should be examined further to determine the depth and mechanism of adaptation. This will provide information that would be beneficial to food safety and public health.

Acid Resistance Assay

Chlorine adapted *Salmonella* (rugose and smooth) and non-adapted cells were exposed to a low pH environment for a 12 h period of time. There was no differences among the number of recoverable cells between any of the treatments. One of the aims of this study was to explore the possibility of chlorine acquired tolerance to confer resistance to acidic stress in *Salmonella*. Since other authors have suggested that

adaptation to acid could induce cross-protection to other environmental stress, including salt and heat stress (Lindquist, 1986; Csonka, 1989; Leyer and Johnson, 1993). Baik et al., (1996), reported that the acid tolerance response of *Salmonella* Typhimurium could probably allow the bacteria to survive extreme acidic conditions. In the current study, adaptation to chlorine does not induce cross-protection to acid stress. There is need to further examine the cells recovered after exposure to pH 3 to better understand the survival mechanism and observe their ability to survive other stress conditions.

Conclusion

The findings of this study show that *Salmonella* Typhimurium was adapted to chlorine after being exposed to sub-inhibitory concentrations and consequently was tolerant to higher concentrations even above the MIC. This resulted in the formation of rugose and smooth adapted cells, which are both able to form better biofilms than the non-adapted *Salmonella* cells. The ability of the rugose variant to attach to and subsequently form biofilms on a plastic surface may pose a potential threat to food safety In addition, sub-inhibitory exposure of foodborne pathogens to antimicrobials could occur when disinfecting chicken carcasses or sanitizing the processing equipment. This may result in the reduced efficacy of such antimicrobial and reduce the susceptibility of the foodborne pathogen to antibiotics as well. Therefore, the findings of this study suggest that emphasis should be laid on proper cleaning and sanitation. A routine check of sanitizer efficacy and the correct application of sanitizing agent is also recommended.

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Table 3.1 Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of chlorine (ppm) for *Salmonella* Typhimurium [a] (ATCC14028) before adaptation

	MIC (ppm)	MBC (ppm)
Micro	400	500
Macro	500	500

[a] *Salmonella* Typhimurium culture prior to chlorine exposure. Data represent the average of 3 replications.

Table 3.2 Disk diffusion assay of antibiotic cross-resistance patterns of adapted and control *S.* Typhimurium

Salmonella Typhimurium cultures previously adapted to chlorine [b]	Antibiotic [a]							
	SXT	GN	S	AMC	NA	CIP	CTX	AMP
Adapted rugose	20/S	13/I	**8**/R	21/S	**15**/I	**23**/I	**22**/I	18/S
Adapted smooth	20/S	13/I	**8**/R	21/S	**15**/I	25/I	23/I	18/S
Control	20/S	13/I	10/R	21/S	18/I	24/I	23/I	18/S

[a] SXT, sulphamethoxazole/trimethoprim (25 µg), GN, gentamicin (10 µg), S, streptomycin (10 µg), AMC, amoxicillin/clavulanic acid (30 µg), NA, nalidixic acid (30 µg), CIP, ciprofloxacin (5 µg), CTX, ceftriaxone (30 µg), AMP, ampicillin (10 µg). Zones of inhibition (millimeters) are reported as S, susceptible strain; I, intermediate susceptible strain; R, resistance strain. Boldfaced data indicate reduced susceptibility relative to unexposed (control) strains; data not bolded indicate exposed strains with no difference in susceptibility patterns relative to unexposed (control) strains. An increase in resistance was defined as a change in S (before chlorine exposure) to R (after chlorine exposure). [b] For adaptation, cultures were previously exposed to increasing sub-lethal concentrations of chlorine, control represent unexposed *Salmonella* culture.

Table 3.3 Broth microdilution assay of antibiotic cross-resistance patterns of adapted and control *S*. Typhimurium

Salmonella Typhimurium cultures previously adapted to chlorine [b]	Antibiotic [a] (μg/mL)						
	AMP	GN	S	NA	T	CIP	AMX
Adapted rugose	**4**/S	**4**/S	**64**	**16**/S	**4**/S	1/S	**32**/R
Adapted smooth	2/S	2/S	**32**	**16**/S	**4**/S	1/S	**32**/R
Control	2/S	2/S	16	8/S	2/S	1/S	16/I

[a] AMP, ampicillin; GN, gentamicin; S, streptomycin; NA, nalidixic acid; T, tetracycline; CIP; ciprofloxacin; AMX, amoxicillin. Data are reported as S, susceptible strain; I, intermediate susceptible strain; R, resistance strain. Boldfaced data indicate reduced susceptibility relative to unexposed (control) strains; data not bolded indicate exposed strains with no difference in susceptibility patterns relative to unexposed (control) strains. An increase in resistance was defined as a change in S (before chlorine exposure) to R (after chlorine exposure). [b] For adaptation, cultures were previously exposed to increasing sub-lethal concentrations of chlorine, control represent unexposed *Salmonella* culture.

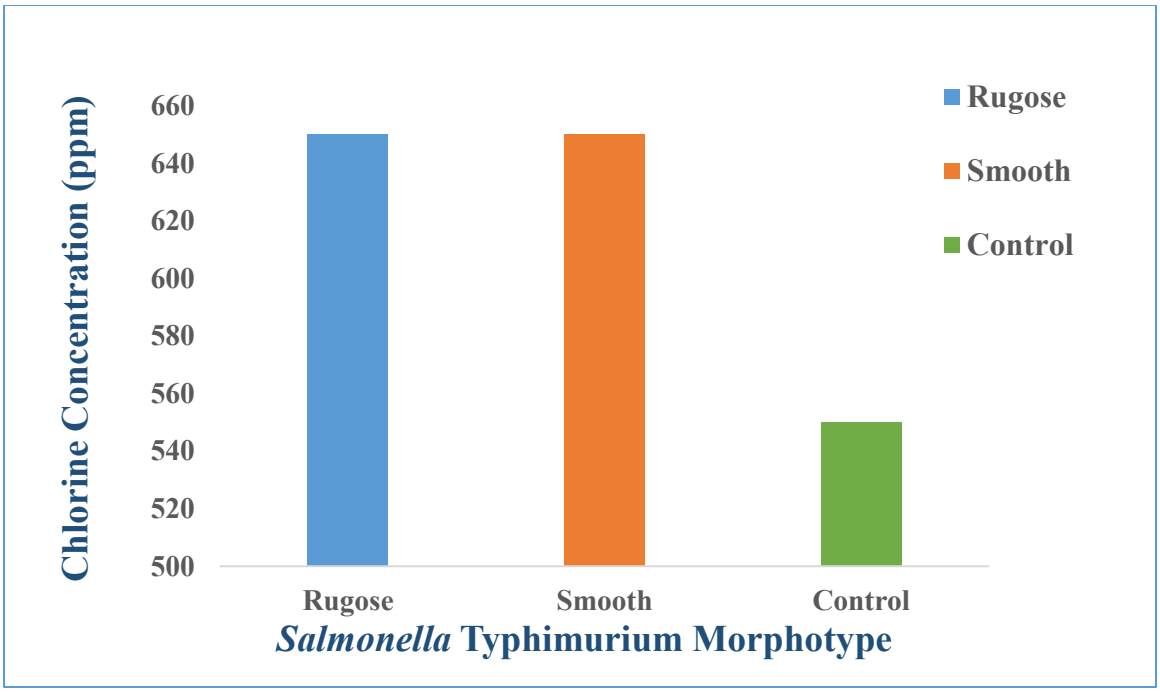

Figure 3.1 MIC for chlorine adapted and control *Salmonella* Typhimurium after stress adaptation

Stable chlorine-adapted *Salmonella* Typhimurium morphotype (rugose and smooth), non-exposed (control) after chlorine exposure

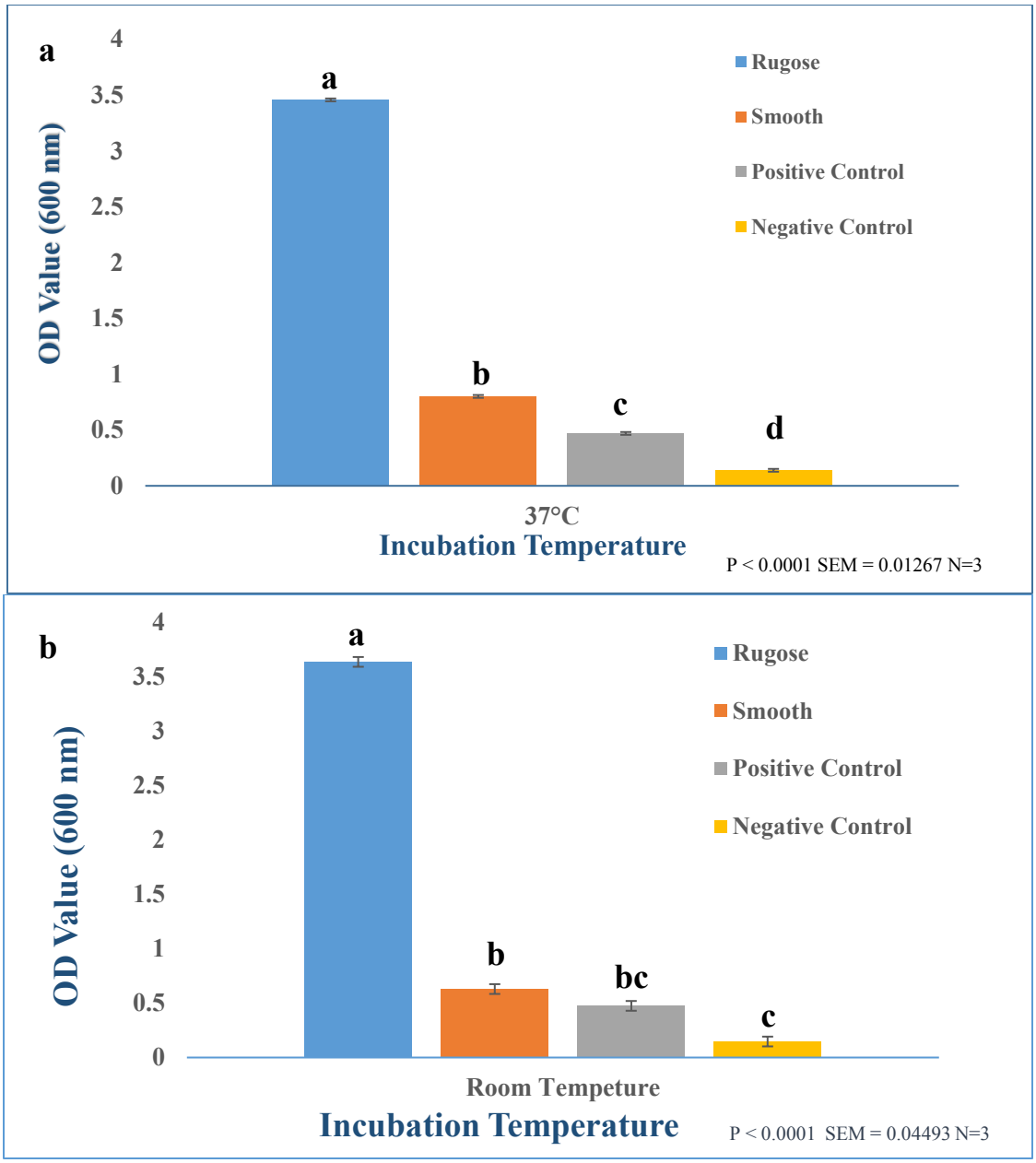

Figure 3.2 Biofilm formation by *Salmonella* Typhimurium after 48 h on plastic surface using 96-well polystyrene microtiter plate

(a) at 37°C (b) at room temperature. Treatments with different superscripts indicate significant differences ($P \leq 0.05$). Adapted cells (rugose and smooth), non-adapted (positive control), broth only (negative control)

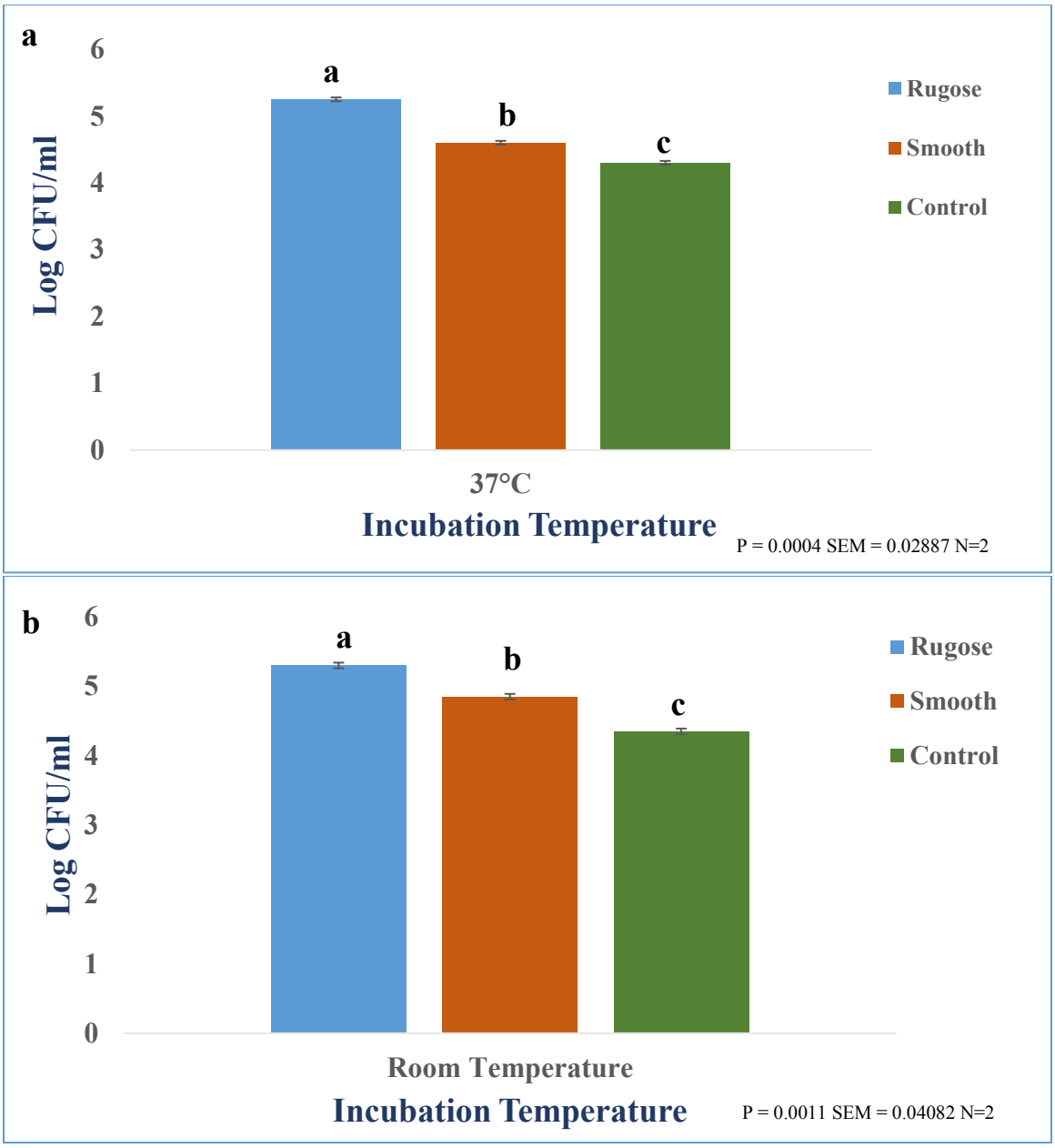

Figure 3.3 Enumeration of *Salmonella* Typhimurium after 48 h attached to 24-well polystyrene plate

(a) at 37°C (b) at room temperature. Treatments with different superscripts indicate significant differences ($P \leq 0.05$). Adapted cells (rugose and smooth), non-exposed (control)

CHAPTER IV

HOMOLOGOUS STRESS ADAPTATION, ANTIBIOTIC RESISTANCE, AND BIOFILM FORMING ABILITY OF *SALMONELLA ENTERICA* SEROVAR HEIDELBERG ATCC8326 ON DIFFERENT FOOD-CONTACT SURFACES FOLLOWING EXPOSURE TO SUB-LETHAL CHLORINE

Abstract

Salmonella enterica serovar Heidelberg (ATCC 8326) was examined for the ability to adapt to the homologous stress of chlorine through exposure to increasing chlorine concentrations (25 ppm daily increments) in tryptic soy broth (TSB). The tested strain exhibited an acquired tolerance to chlorine in TSB with the tolerant cells growing in concentrations up to 400 ppm. In addition, the chlorine stressed cells displayed rugose morphology on tryptic soy agar (TSA) plates at 37°C. The MIC of chlorine for adapted (rugose and smooth) cells was determined to be 550 ppm and 500 ppm, respectively whereas the MIC for the control was 450 ppm. The biofilm forming ability of the adapted and control cells were examined on both plastic and stainless steel surface at room temperature and 37°C. The rugose variant, in contrast to the smooth (adapted and control) showed the ability to form strong biofilms ($P \leq 0.05$) on a plastic surface at room temperature and 37°C. Rugose cells compared to smooth and control attached more ($P \leq 0.05$) to steel surfaces as well. The possibility of cross-adaptation was examined by

exposing the adapted and control cells to different antibiotics according to the Clinical & Laboratory Standards Institute (CLSI) guidelines and low pH. Adapted cells exhibited reduced susceptibility to some of the antibiotics tested as compared to control. There was no difference in the acidic tolerance of the adapted and control cells. The findings of this study suggest that exposure to sub-lethal chlorine concentration during the sanitization procedure can result in tolerant *Salmonella* cells. Chlorine may confer cross-protection that aids in the survival of the tolerant population to other environmental stresses.
Keywords: *Salmonella*, adaptation, chlorine, rugose, biofilm

Introduction

Salmonella enterica is well known as the leading cause of foodborne infections globally (Painter et al., 2013). The serotypes of *Salmonella enterica* are reported to result in over a million cases of illnesses, thousands of hospitalizations, and a few hundred deaths each year in the United States and also around the world (Mead et al., 1999; CDC, 2011a; Scallan et al., 2011). According to an estimate by the Centers for Disease Control and Prevention (CDC) in 2011, nontyphoidal *Salmonella*, which includes all *Salmonella* serotypes except Typhi and Paratyphi was among the top five pathogens in the United States causing the most reported foodborne illnesses, hospitalizations, and deaths annually (CDC, 2011b). In addition, nontyphoidal *Salmonella*, which is second to norovirus is estimated to cause 11% illnesses, 35% hospitalizations, and 28% deaths each year in the United States alone (CDC, 2011b). Moreover, of all the *Salmonella enterica* serotypes, the top four serotypes associated with animal products and are most often reported to cause human infections are Typhimurium, Enteritidis, Newport, and Heidelberg (Hur et al., 2012).

There are several food commodities implicated in foodborne illnesses, however, between 1998 and 2008, poultry accounted for 9.8% of the illnesses, 11.5% of the hospitalizations, and 19.1% of the deaths (Painter et al., 2013). Poultry is considered a major source of *Salmonella* contamination that results in human illness (Morris and Wells, 1970). In 2013, a multistate outbreak of multidrug-resistant *Salmonella* Heidelberg was linked to branded chicken. This outbreak was reported to result in 634 cases of illnesses and 38% hospitalizations (CDC, 2014). Due to the burdens attached to the control of foodborne pathogens, poultry processors employ many methods to prevent pathogens from contaminating poultry meat. One of the methods include the use of USDA approved antimicrobials for disinfecting poultry meat and sanitize food-contact surfaces coupled with the poultry processing facility (USDA-FSIS, 2015). The antimicrobials are capable of destroying both spoilage and pathogenic bacteria. Examples of antimicrobials used to disinfect, decontaminate or preserve food products include peracetic acid, calcium hypochlorite, sodium nitrite, trisodium phosphate, and sodium hypochlorite (Capita and Alonso-Calleja, 2013, Alonso-Calleja et al., 2015). The misuse of these compounds has allowed pathogenic bacteria to develop adaptation (Capita et al., 2014). Previous studies have shown that using an antimicrobial at a sub-lethal concentration may potentially aid in the survival of foodborne pathogens by enabling them to survive challenging conditions such as antimicrobial inactivation (Sheridan et al., 2012; Capita et al., 2014). This increase in bacteria tolerance to antimicrobials is a global public health concern (Braoudaki and Hilton, 2004)

Several studies have documented the ability of bacteria to adapt to stress posed by antimicrobials which they use to induce cross-protection against other stressful conditions

(Davidson and Harrison, 2002; Braoudaki and Hilton, 2005; Capita et al., 2014). The majority of these studies provides significant evidence that reduced susceptibility to one antimicrobial can cause cross-adaptation to another (Braoudaki and Hilton, 2005). Since chlorine is the antimicrobial agent that is most common in sanitizing solutions, adaptation to chlorine could constitute a potential threat to food safety by inducing cross-protection to antibiotics that are clinically important (Braoudaki and Hilton, 2004; Molina-Gonzalez et al., 2014). Antibiotic resistance has continued to be a global public health issue; research has been conducted that examines different mechanisms used by pathogens, such as *Salmonella*, *Listeria*, and *E.coli*, to adapt to disinfectants and induce cross-resistance to antibiotics (Buchmeier and Heffron, 1990; Lou and Yousef, 1996; Braoudaki and Hilton, 2004; Capita et al., 2014; Molina-Gonzalez et al., 2014). Some of this research demonstrates that an adapted bacteria will most likely use similar resistance mechanism to offer cross-protection to antibiotics (Braoudaki and Hilton, 2004).

In addition, it was reported that a frequent strategy used by pathogenic microorganisms to adapt to antimicrobials, is the formation of biofilm (IFT, 2006). Biofilms are bacteria that cluster together on a surface and are surrounded by an exopolymeric matrix that makes the biofilm matrix impenetrable to any harmful substance (Costerton et al., 1995; Iturriaga et al., 2007; Bae et al., 2012). An important challenge faced by food processors is the control of bacteria present in the biofilm. It was documented that biofilms are more difficult to destroy because they possess tolerance to environmental stresses when compared to free cells (Frank and Koffi, 1990; Joseph et al., 2001). In poultry processing, attachment of bacteria to surfaces of equipment can be a source of product contamination (Barnes et al., 1999, Giaouris et al., 2005). Some studies

have reported the incidence of cross-contamination due to bacterial attachment to equipment surface such as utensils used in food preparation (Scott and Bloomfield, 1990; Jiang and Doyle, 1999; Kusumaningrum et al., 2003). Other studies suggested that bacteria could attach to surfaces such as polystyrene, glass, rubber, acrylic, and cement (Czechowski, 1990; Mafu et al., 1990, Krysinski, 1992; Barnes et al, 1999; Nguyen et al., 2013).

Therefore, in this study, the homologous stress adaptation of *Salmonella* Heidelberg exposed to chlorine at a sub-lethal concentration was determined by observing changes in MIC. The biofilm formation of the adapted cells compared to non-adapted cells on different food-contact surfaces was measured. Antibiotic susceptibility patterns and acid resistance of adapted cells were further determined.

Materials and Methods

Salmonella Heidelberg Strain and Culture Preparation

Salmonella Heidelberg ATCC 8326 was maintained on tryptic soy broth (TSB; Sigma-Aldrich Co., St. Louis, MO). Prior to experiments, frozen cells were streaked on tryptic soy agar plates (TSA; Sigma-Aldrich Co., St. Louis, MO, USA) and incubated at 37°C for 24 h. A single colony was subcultured in TSB at 37°C for 20 to 24 h. Working cultures were stored at 4 ± 1°C on TSA slants and were subcultured monthly.

Chlorine Source

Sodium hypochlorite that contained 5% available chlorine (ACROS Organics, New Jersey USA), was used as the source of chlorine in this study. The amount of free chlorine in the sodium hypochlorite was confirmed using the HACH (chlorine test kit)

Pocket Colorimeter (HACH Company, Loveland, CO, USA) in accordance with the manufacturer's instructions. A sterile solution of chlorine was prepared in an appropriate media before each experiment.

Determination of Minimum Inhibitory Concentration (MIC) of chlorine

The MIC value was established using the broth microdilution method in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008) with minimal modifications. One colony of the planktonic cells was obtained from a TSA plate, inoculated in 10 ml TSB, and incubated at 37°C for 20 to 24 h. It was determined that 20 to 24 h incubation of the bacterial culture contained approximate 1 X 10^9 CFU/ml. To start the experiment, 96-well microtiter plates (Celltreat Scientific Product, Pepperell, MA) were used. A volume of 100µl of chlorine in TSB that contained double the desired concentration was added to each well of the plate. Afterwards, 100µl of inoculum prepared to a final concentration of ~ 10^7 CFU/ml was added to make the final volume in each well 200µl. A positive control that contained (100µl TSB + 100µl inoculum devoid of chlorine) and a negative control (200µl TSB) were included in the experiment. The microwell plate was incubated at 37°C for 24 h and visible growth of bacteria in each well was determined by turbidity. The MIC was established as the lowest chlorine concentration that is necessary to inhibit the growth of *Salmonella* Heidelberg after 24 h of incubation. The exact MIC was determined by narrowing the range of chlorine concentrations in each well for the subsequent experiment.

Stress Adaptation Study

This study was performed by preparing *Salmonella* stock culture to a final concentration of ~10^9 CFU/ml. The starting concentration of chlorine was 125 ppm, an aliquot of 100µl of the inoculum was transferred to a polypropylene tube (Fisher Scientific, Fair Lawn, NJ) that contained 9.9 ml (TSB + chlorine). After the transfer, the tube contained a final inoculum concentration of ~ 10^7 CFU/ml. Upon incubation for 24 h at 37°C, turbidity in the tube was used to examine bacteria growth. The suspension was then diluted and plated on TSA plates to enumerate *Salmonella* growth. An additional aliquot of 100µl was transferred from the same turbid tube to a sterile tube that contained 9.9 ml (TSB + higher sub-lethal chlorine concentration). This procedure was continued by increasing the concentration of chlorine by 25 ppm daily until no visible growth was observed in the tube. This required 12 days of incubation. The suspension in the last tube with visible bacteria growth was again diluted for *Salmonella* enumeration. The cells obtained on TSA plates after incubation for 24 h at 37°C were re-streaked and stored on TSA plate that did not contain chlorine. The cells were considered adapted *Salmonella* Heidelberg cells. The TSA plates containing the adapted cells were stored at 4 ± 1°C with the cells being transferred weekly to fresh non-chlorinated TSA plates.

Adaptive Stability to Chlorine

The adaptive tolerance to chlorine of the adapted *Salmonella* Heidelberg cells was tested by determining the MIC for the adapted cells against chlorine using the broth macro dilution method according to the CLSI guidelines (CLSI, 2012).This was done after storage on TSA plate that did not contain chlorine. A colony of the adapted cell was inoculated into a borosilicate round bottom glass culture tube (Fisher Scientific, Fair

Lawn, NJ) containing 10 ml TSB with chlorine at concentrations that were below, equivalent and above the MIC. Non-adapted *Salmonella* Heidelberg cells, which served as the control, were also tested at the same concentrations. Turbidity was used to observe bacteria growth, the glass culture tube showing no visible growth after incubation at 37°C for 24 h was considered the MIC of the adapted and control cells.

Biofilm Formation

Plastic Surface

The standard crystal violet assay as previously described by Patel and Sharma, (2010) was used to determine the biofilm forming ability of the chlorine adapted (rugose and smooth) and control cells. An overnight culture (~ 20 h) of adapted and control *Salmonella* cells grown in TSB were diluted to achieve a final inoculum level of ~ 10^6 CFU/ml. The treated cells (rugose and smooth) were cultured in TSB that contained 400 ppm chlorine which is the highest concentration that supports bacteria growth, whereas the control cells were cultured in chlorine-free TSB. To begin the experiment, 96-well polystyrene cell culture plates were prepared by dispensing 200µl of the inoculum into duplicate wells for each type of adapted *Salmonella* Heidelberg morphotypes and the control. TSB devoid of inoculum was used as a negative control. The plates were incubated at room temperature and 37°C for 48 h. After incubation was complete, 200µl of the inoculum was aspirated from each well of the plate, and each well was washed five times with sterile distilled water so that all loosely attached cells may be removed. The plates were later air-dried for 45 mins, and 200µl of crystal violet solution (0.41% w/v dye, ACROS Organics, NJ) was dispensed into each well. The plates were incubated at room temperature for an additional 45 mins, after which the dye was aspirated from each

well, and the well was washed five times using sterile distilled water. Each well was allowed another 45 mins to air dry, and then 200µl of 95% ethanol (Fisher Scientific, Fair Lawn NJ) was added to each well. The content of each well was mixed to dissolve the crystal violet dye. Biofilm formation in each well was measured by an optical density (OD_{600}) reading using a micro quant microplate spectrophotometer (BioTek Instruments, Winooski, VT).

The attached cells of adapted (rugose and smooth) and control on the plastic plate were enumerated. Similar to the crystal violet assay, 200µl of the inoculum prepared to ~ 10^6 CFU/ml was dispensed into each well of a 24-well tissue culture plate (Celltreat Scientific Product, Pepperell, MA) in duplicate. The plate were then incubated at room temperature and 37°C for 48 h. After incubation, each well was aspirated and washed three times with sterile distilled water to remove any loosely attached bacteria. The strongly attached cells were scraped into 0.1% peptone water, vortexed and serially diluted. After dilution, an aliquot of 100µl was plated on TSA plates, incubated at 37°C for 24 h in order to count the number of attached cells.

Stainless Steel (SS) Surface

To examine the influence of chlorine stress on the ability of *Salmonella* Heidelberg to attach to stainless steel (SS), a previously described method with minimal modifications was used (Hood and Zottola, 1997). Stainless steel coupons (#4 Finish, 26G, 1.5cm x 1 cm) and scoops were purchased from Stainless Supply® (Monroe, NC) and The Chefs' Warehouse, Inc. (Ridgefield, CT) respectively. The SS coupons and scoops were washed with detergent upon arrival, sanitized in 70% ethanol and autoclaved at 121°C for 15 mins. For the SS coupons, 2 ml of previously prepared inoculum (~ 10^6

CFU/ml) of adapted and control cells were dispensed into duplicate wells of a 24-well polystyrene tissue culture plate. Sterile SS coupons were gently placed in each well with sterile forceps, and the plate was incubated at room temperature and 37°C for 48 h. After incubation, the coupons were gently removed from each well using sterile forceps, tapped on the edge of the well to remove any excess fluid. The coupons were washed three times with sterile distilled water to remove any loosely attached bacteria. The coupons were subsequently placed in a 50 ml polypropylene centrifuge tube that contained 8.8 ml of 0.1% peptone water and sterile glass beads (Sigma-Aldrich Co., St. Louis, MO, USA). The centrifuge tube was vortexed for 2 min to remove all strongly attached cells into suspension. The resulting suspension was subjected to a 10-fold dilution and an aliquot of 100µl was plated on TSA plates for cell count after incubation at 37°C for 24 h. The method for quantifying the attached cells on the SS scoops was similar to the coupons. After the loosely attached cells were removed by washing, the strongly attached cells on the scoop were scraped into a solution of 0.1% peptone water and vortex for 2 min before being subjected to a 10-fold dilution in TSB. 100µl from each dilution was plated on TSA plates for quantification.

Determination of Antibiotic Susceptibility

Chlorine adapted *Salmonella* Heidelberg and control cells were screened against different antibiotics on Mueller-Hinton broth and Mueller-Hinton agar (MHB, MHA; Oxoid Co., Nepean, ON, Canada) to determine susceptibility. Adapted cells were grown in TSB containing chlorine (400 ppm) and the control cells were grown in TSB devoid of chlorine. MIC broth microdilution and disk diffusion method as described by the CLSI (2008) guideline with slight modifications were used in the study. The following

antibiotic disks (Fisher Scientific Fair Lawn, NJ) were used: sulphamethoxazole/trimethoprim (SXT, 25 µg), gentamicin (GN, 10 µg), streptomycin (S, 10 µg), amoxicillin/clavulanic acid (AMC, 30 µg), nalidixic acid (NA, 30 µg), ciprofloxacin (CIP, 5 µg), ceftriaxone (CTX, 30 µg), ampicillin (AMP, 10 µg). The inhibition zones were measured and recorded as susceptible, intermediate, or resistant according to the guidelines by CLSI (2008). Cultures of *Escherichia coli* 25922 with known antibiotic resistance patterns were used as control reference strain. The MIC for the antibiotics was determined using a 96-well cell culture plate. Each panel in the plate contained 5 dilutions using the MIC breakpoints provided by the CLSI (2008) guidelines for each antibiotic tested in this study. Positive control (TSB + inoculum) and negative control (TSB only) were maintained throughout the experiment. The plate was incubated at 37°C for 24 h, and the results were recorded as the least concentration of antibiotic that prevent the growth of bacteria either as susceptible, intermediate, or resistance as stated in the CLSI (2008) guidelines.

Determination of Acid Tolerance

The possibility of cross-protection between adaptation to chlorine and acid stress was examined. One hundred microliters of overnight culture (~ 20 h) of the adapted and control cells were added to a polypropylene tube containing 9.9 ml sterile TSB that had been adjusted to a pH of 3 using 1N HCL (Fisher Scientific Fair Lawn, NJ) to make a final bacteria concentration of ~ 10^7 CFU/ml. The tube was incubated at 37°C for 12 h. One milliliter of the incubated culture was then removed from the suspension and diluted (10-fold) in 0.1% peptone water every 3 h. From each dilution, 100µl was plated on TSA

plates and incubated at 37°C for 24 h. The amount of surviving *Salmonella* Heidelberg cells were then counted.

Statistical Analysis

The whole experiment was replicated twice on different days sequentially. All data analysis in this study was carried out using analysis of variance (ANOVA) in the General Linear Model (GLM) of SAS v. 9.4 (SAS Institute, Cary, NC, USA). Means separations were acquired using Fisher's Least Significant Difference test. The treatments and controls were determined to be significant at the 5% ($P \leq 0.05$) level.

Results

Homologous Stress Adaptation to Chorine

It was observed in previous research (Obe et al., 2016) that *Salmonella* Typhimurium undergoes a morphological change upon a prolong exposure to sub-lethal chlorine concentration. In the present study, *Salmonella* Heidelberg changes it morphology to the rugose variant after 4 days exposure to chlorine stress at 37°C. Rugose and smooth *Salmonella* Heidelberg continued to grow together on TSA plate until the chlorine concentration in TSB reached 400 ppm. That was after 12 days of incubation. This concentration was recorded as the maximum chlorine concentration that allowed the growth of stressed *Salmonella* Heidelberg and the cells recovered at this concentration was referred to as the chlorine-adapted *Salmonella* cells (**Fig. 4.1**).

The MIC of chlorine for *Salmonella* Heidelberg before sub-lethal chlorine exposure was determined to be approximately 400 ppm (**Table 4.1**). The adaptive tolerance to chlorine was later measured by sub-culturing the stressed cells in a

nonselective broth (TSB) after storage on TSA plates devoid of chlorine. The stressed cells (rugose and smooth) were able to grow above the MIC values up to 500 ppm and 450 ppm respectively, compared to the non-exposed cell that could not grow above 400 ppm (**Fig. 4.2**). This suggests that *Salmonella* Heidelberg ATCC 8326 possesses a stable homologous adaptation to chlorine.

Biofilm Formation on Plastic Surface

To determine whether adaptation to chlorine would influence the strength of biofilms formed, *Salmonella* Heidelberg was cultured in the presence of chlorine (400 ppm) for the adapted cells and in the absence of chlorine for the non-adapted (control) cells. The optical density reading (OD_{600}) of the adapted and control cells were observed and used to determine biofilm forming ability of each of the *Salmonella* variant. In the present study, *Salmonella* Heidelberg formed biofilms on polystyrene plastic surface both at room temperature and at 37°C. At room temperature, there was a significant difference ($P < 0.05$) in the biofilms formed by the adapted cells versus control. Adapted rugose formed the strongest biofilm with OD_{600} value of 3.4, followed by adapted smooth with OD_{600} value 1.13 representing a moderate biofilm former, compared to control that formed the weakest biofilm with OD_{600} value of 0.68 (**Fig. 4.3a**). At 37°C, similar to room temperature, the morphotypes tested were significantly different ($P < 0.05$) in their ability to form a biofilm. Adapted rugose showed a strong biofilm forming ability with OD_{600} value of 3.4. There were no differences observed for smooth morphologies (both adapted and control) which showed OD_{600} values of 0.7 and 0.57, respectively (**Fig. 4.3b**). Biofilm formation on the plastic surface was also determined by enumerating the strongly attached cells on TSA plates. The difference in log values was used to determine

the difference in biofilm forming ability of chlorine-adapted cells against control. The result were reported in Log CFU/ml. At room temperature, there was no significant difference ($P > 0.05$) observed for adapted cells (rugose compared to smooth) when compared to control (**Fig. 4.4a**). A significant difference ($P < 0.05$) was determined for the attachment of chlorine-adapted cells compared to control at 37°C (**Fig. 4.4b**). In addition, at 37°C, rugose attached more to the plastic surface compared to smooth (adapted and non-adapted) cells.

Biofilm Formation on SS Surface

To determine the biofilm formation of chlorine adapted and control *Salmonella* Heidelberg on a different food-contact surface other than plastic, the strongly attached cells on both stainless steel (SS) coupons and scoops were measured on TSA plates. Similar to plastic quantification, the results were reported in Log CFU/ml and the difference in log values were used to determine the difference in biofilm formation between adapted and control cells. For SS coupons, there was no difference ($P > 0.05$) in biofilm formation of adapted and control *Salmonella* cells at room temperature. Adapted rugose had higher cell concentration on the coupons at 37°C when compared to smooth (adapted and control) cells (**Fig. 4.5a and 4.5b**). However, more cells were determined to be present on the coupons at room temperature than at 37°C. For SS scoops, the adapted rugose cells were in higher concentrations when compared to the smooth (adapted and control) at room temperature (**Fig. 4.6a**). Whereas at 37°C, there was no difference in the concentration of adapted cells but the concentrations were significantly higher when compared to the concentration of the control cells (**Fig. 4.6b**). *Salmonella* Heidelberg

cells attached more to the SS scoop surface at 37°C than that observed at room temperature.

Antibiotic Cross-Resistance

Sensitivity to antibiotics was determined for *Salmonella* Heidelberg following adaptation to chlorine through exposure to sub-lethal chlorine concentrations. Zones of inhibition were measured (in millimeters) around an impregnated antibiotic disk. A broth microdilution assay was also performed to determine if there was a change in MIC between the non-exposed and the chlorine tolerant cells. The results for the antibiotic susceptibility patterns of adapted (rugose and smooth) and control cells is shown in **Table 4.2** and **4.3**. The results showed no cross-resistance to antibiotics was demonstrated for adapted cells when compared to control, against the majority of the antibiotic tested, however, a reduced susceptibility was observed in some of the antibiotics. A ≤ 2-mm inhibition zone difference was observed for adapted rugose to GN, S, AMC and CIP. The same difference was observed for adapted smooth against SXT and S. Similarly, a slight increase in MIC was recorded for adapted rugose against S, NA, T, and AMX, which moved from the susceptible to the intermediate category. In addition, the adapted (rugose and smooth) cells moved from the susceptible to the intermediate category for T.

Acid Cross-Protection

Cross-protection to low pH was determined for the chlorine adapted cells. There was no difference in the number of cells recovered from the adapted cells compared to control after 3 h of incubation at 37°C. At the end of 6 h, the cells recovered for the

adapted and control were not within the countable range. There was no cross-protection to acidic stress observed in this study because by the end of 9 h of incubation, all cells (adapted and control) were completely inhibited by the low pH (**Table 4.4**).

Discussion

After exposure to increasing sub-lethal concentrations of chlorine, *Salmonella* Heidelberg demonstrated the ability to acquire resistance. The ability of the adapted cells to tolerate higher chlorine concentrations continued even after repeated storage on chlorine-free TSA plates, which suggests the stability of adapted cells to the homologous stress of chlorine. In the current study, the MIC for the chlorine adapted (rugose and smooth) cells was 1.2 and 1.1 times higher than non-adapted control. This increase in MIC observed for chlorine-stressed *Salmonella* Heidelberg was in line with a previous study on *Salmonella* Typhimurium (Obe et al., 2016) and other studies on *Salmonella enterica* strains that exhibited adaptation to sub-lethal stress posed by different antimicrobials (Braoudaki and Hilton, 2004; Kim and Day, 2007; Alonso-Hernando et al., 2009; Stanojevic et al., 2010; Molina-Gonzalez et al., 2014). The inappropriate use of antimicrobials either directly or indirectly in the food processing establishment can expose any bacteria present to sub-lethal doses of disinfectants and sanitizers that are supposed to reduce the growth of bacteria (Capita et al., 2014). Another important finding from this study is the ability of the chlorine-adapted *Salmonella* Heidelberg to grow in the presence of high chlorine concentrations even above the concentration approved by the USDA for sanitation purposes on food-contact surfaces (200 ppm). The increased in bacteria tolerance following sub-lethal antimicrobial concentrations observed in this study have been well reported by others (Braoudaki and Hilton, 2004; Sheridan et al.,

2012). Additionally, sub-lethal doses of antimicrobials have been observed to induce resistance in foodborne pathogens (Braoudaki and Hilton, 2005; Alonso-Calleja et al., 2015). This is because bacteria respond to stressful conditions either by adaptation or elimination, a common means that bacteria use to adjust to strenuous environmental conditions is by changing their morphology (Foster, 2005; Young, 2007). Other authors have reported these changes as a survival mechanism for bacteria (Justice et al., 2004; Young, 2007; Capital et al., 2014). In this study, *Salmonella* Heidelberg undergoes a morphological change to the rugose morphotype in order to cope with the stress posed by increasing sub-lethal chlorine concentrations. The change in morphology of the smooth *Salmonella* Heidelberg to the rugose variant in TSB was observed through the formation of a pellicle that is composed of cell aggregates, thereby allowing the rugose cell to remain as an aggregate in solution. Similar changes have been reported for *Vibrio cholerae* O1 E1 Tor and *Salmonella* Typhimurium DT104 (Morris et al., 1996; Anriany et al., 2001). The rugose variant was reported to be more virulent and not easy to kill compared to the smooth variant (Rice et al., 1993; Morris et al., 1996). In addition, other studies have reported similar findings on the ability of some microorganisms including *E. coli*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa* to tolerate different environmental stress. These microorganisms are reported to survive by changing their morphology through degradation of the cell wall, elongation of cells, aggregation of damaged bacteria cells and disturbances during cell division (To et al., 2002; Giotis et al., 2009; Capita et al., 2014; Shalamanov, 2005).

The conditions at which rugose developed in this study is by daily transfer of an aliquot of 100µl of culture containing sub-lethal chlorine concentration. This is different

from the observations of other authors on the formation of the rugose variant in both *Vibrio* and *Salmonella.* In *Salmonella* Typhimurium DT104, rugose variant was observed after 4 days storage on TSA at room temperature and in *Vibrio cholerae* O1 strain TSI-4, rugose was observed 2 months post inoculation when the smooth variant was re-cultured under a starved conditions at 16°C (Wai et al., 1998; Anriany et al., 2001). Whereas in the current study, the rugose variant was observed after exposure to increasing sub-lethal chlorine concentration (200 ppm) but fails to develop when cultured at room temperature with or without exposure to chlorine under similar experimental conditions. Another important finding was the ability of the rugose variant in *Salmonella* Typhimurium ATCC14028 (Obe et al., 2016) and currently in *Salmonella* Heidelberg ATCC8326 to retain their morphology even after storage on chlorine-free TSA.

The adhesion and subsequent attachment of foodborne pathogens to food processing equipment and environment can result in a major food safety challenge when the pathogen contaminate food products thus causing a foodborne outbreak (Dourou et al., 2011). The attachment of *Salmonella* Heidelberg to different food-contact surfaces was examined in this study. The ability of the chlorine-adapted and control cells to adhere to both plastic and stainless steel surface was assessed at room temperature and 37°C. The results demonstrates that *Salmonella* Heidelberg possess the ability to attach to both surfaces tested. It was previously reported that *Salmonella* has the ability to colonize and attach to different surfaces including plastic, rubber, stainless steel, and glass (Helke et al., 1993; Sinde and Carballo, 2000; Nguyen et al., 2014; Yang et al., 2016). The rugose cells attached and formed stronger biofilms at both temperatures tested and showed no preference to a particular surface. The expression of rugose in *Salmonella* was

previously reported to be due to aggregation of cells and formation of exopolysaccharides (EPS), which might have aid in the strong attachment to food-contact surfaces (Marshall, 1992; Morris et al., 1996; Yildiz and Schoolnik, 1998; Wai et al., 1998). The smooth cells both adapted and control seems to attach better on the steel surface. The properties of the surface that *Salmonella* cells attached to, helps with their survival on such surface (O'Leary et al., 2013). Although *Salmonella* was reported to attach strongly to a hydrophobic surface, the ability to attach to a particular surface has been reported to be dependent on the strain of *Salmonella* (Chia et al., 2009; Sinde and Carballo. 2000). Nguyen et al., (2014) reported that *Salmonella* Typhimurium possess the ability to attach to both stainless steel and acrylic surface but showed greater attachment on stainless steel surfaces. This is because stainless steel is hydrophilic and bacteria attach more to such surfaces as compared to hydrophobic surface (Mafu et al., 1990; Sinde and Carballo. 2000). The results from this study shows that chlorine-adapted *Salmonella* Heidelberg exhibited no preference to a particular temperature. *Salmonella* generally grow well and has been observed to form biofilm at 37°C (Nguyen et al., 2013). Room temperature was used in this study to examine the ability of *Salmonella* to form biofilm when exposed to an unfavorable condition such as temperature abuse. In addition, the adapted cells attached well on both surfaces tested in this study. Plastic and stainless steel surface was used because they are the most common surfaces encountered in food processing (Chmielewski and Frank, 2006; Ismail et al., 2013). Residues of food processing left on these surfaces can contribute to the formation of film if they are not promptly removed during cleaning (Joseph et al., 2001; Chmielewski and Frank, 2006). The application of effective sanitizers on food-contact surfaces following cleaning is important to inactivate

and prevent the development of resistance in any pathogenic bacteria present. This will help prevent the acquisition of cross-protection to any other stress conditions encountered in processing

The chlorine-adapted cells were examined for their ability to exhibit cross-adaptation to antibiotics and low pH. From the results of this study, it appears there were some interactions between adaptations to chlorine and cross-adaption to antibiotics. *Salmonella* Heidelberg cells that were adapted to sub-lethal concentrations of chlorine exhibited a certain degree of reduced susceptibility to some of the antibiotics tested. For the adapted cells, a reduced zone of inhibition was observed when compared to non-adapted (control), but the reduction was not significant enough to move above the limit set by the CLSI (2012) guidelines on antibiotic susceptibility testing for the "susceptible" category. Other authors have reported similar observations, Molina-Gonzalez et al., (2014), observed some *Salmonella enterica* strains that were previously exposed to various antimicrobials including sodium hypochlorite exhibited a lower zone of inhibition when compared to non-exposed strains, but are still susceptible to the antibiotics tested. In another study on the cross-adaption patterns of *Salmonella enterica*, the authors suggested that a slight reduction in susceptibility is noteworthy; this is because the pathogen may not be inhibited in the presence of the antibiotic over time (Braoudaki and Hilton, 2004). Chlorine-adapted rugose showed reduced susceptibility to fluoroquinolones (CIP), quinolones (NA), aminoglycosides (S, GN), penicillin (AMX/AMC) and tetracycline (T). In addition, the MIC for the rugose variants increased and moved from the "susceptible" (i.e. bacterial infection will most likely respond to antibiotic treatment) category to the "intermediate" (bacterial infection may or may not

respond to antibiotic treatment) category when tested against amoxicillin. A similar trend has been observed in *Salmonella* Typhimurium (Obe et al., 2016). Other studies have reported similar changes in antibiotic susceptibility patterns of previously adapted foodborne pathogens to different classes of antibiotics (Suller and Russell, 2000; Braoudaki and Hilton, 2004; Capita et al., 2014). Some of these studies suggested that the adaptive nature of foodborne pathogens to antimicrobials like chlorine is directly associated with a broad spectrum mechanism of resistance which includes the presence of an active efflux and alterations to cell permeability. This mechanism makes it difficult for different chemical molecules to enter the adapted cells (Tattawasart et al., 1999; Suller and Russell, 2000; Braoudaki and Hilton, 2004; Capita and Alonso-Calleja, 2013; Molina-Gonzalez et al., 2014). Even though, some authors have reported that sub-lethal dosage of antimicrobials could select for resistance to antibiotics in *Salmonella*, some studies do not observe similar findings. The results of this study in part agree with those authors that do not observe a change in susceptibility to antibiotics after exposure to sub-lethal concentrations of antimicrobials (Thomas et al., 2000; Ledder et al., 2006).

Furthermore, adaptation to sub-lethal chlorine concentrations does not aid in the survival of the adapted cells at low pH. Several studied have examined the ability of previously adapted *Salmonella* strains to confer cross-protection to either a similar or a different stress (Yousef and Courtney, 2003, Braoudaki and Hilton, 2004; Plaks et al., 2004; Capita and Alonso-Calleja, 2013). The results of this study showed that chlorine-adapted *Salmonella* cells do not possess the ability to survive exposure to a heterologous stress such as low pH. This result could also suggest that the adapted cells recovered after 6 h exposure to acidic conditions may be able to withstand other environmental stress

conditions such as thermal stress. The mechanism of cross-protection in some foodborne pathogens particularly *Salmonella* Typhimurium and *E. coli* was identified to involve the synthesis of heat shock proteins (Christman et al., 1985; VanBogelen et al., 1987; Leyer and Johnson, 1993). The induction of heat shock proteins was reported to aid in the survival of the pathogens when exposed to oxidative stress, acid stress and amino acid starvation (Grossman et al., 1973; VanBogelen et al., 1987; Foster, 1991). Leyer and Johnson, (1993) also reported that acid tolerant *Salmonella* Typhimurium demonstrated high tolerance to heat stress. However, another study reported that exposure to heat stress does not increase resistance to acid stress in *Salmonella* Typhimurium (Foster and Hall, 1990). The induction of heat shock proteins mechanism of protection may not be so evident in the stressed cells (Leyer and Johnson, 1993). In addition, there is a possibility that a previous adaptation to acidic stress may offer cross-protection to oxidative stress (chlorine) but may not be the same when the situation is reversed, which was the observations in the current study. This is because acid tolerant *Salmonella* cells were reported to possess outer membrane proteins that aids in their survival against any stress factor that targets cell surface (Leyer and Johnson, 1993). Overall, chlorine adaptation does not induce cross-protection at low pH in the current study. This may be because the changes in cell surface was not adequate to ensure the survival of the exposed cells (particularly the chlorine-adapted cells).

Conclusion

In summary, the findings of the current study support the initial hypothesis that *Salmonella* Heidelberg would adapt to sub-lethal concentrations of chlorine. The adaptive tolerance to chlorine resulted in the formation of a more virulent *Salmonella* variant. The

adapted cells were better biofilm formers on both food-contact surfaces tested and exhibited a minimal reduction in susceptibility to different classes of antibiotics. This shows that chlorine stressed *Salmonella* may not be easily inactivated with high concentrations of chlorine. The possession of similar broad mechanism of adaptation may eventually enable the adapted cells to become resistant to certain antibiotic treatment. The findings in this study signify a possible challenge to food safety and suggest that the misuse of the antimicrobial agent at a sub-lethal concentration could represent a potential public health risk.

Table 4.1 Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of chlorine (ppm) for *Salmonella* Heidelberg [a] (ATCC8326) before adaptation

	MIC (ppm)	MBC (ppm)
Micro	400	500
Macro	500	500

[a] *Salmonella* Heidelberg culture prior to chlorine exposure. Data represent the average of 3 replicates

Table 4.2 Disk diffusion assay of antibiotic cross-resistance patterns of adapted and control *S*. Heidelberg

Salmonella Heidelberg cultures previously adapted to chlorine [b]	Antibiotic [a] (µg)							
	SXT	GN	S	AMC	NA	CIP	CTX	AMP
Adapted rugose	21/S	**15**/S	**8**/R	**19**/S	18/I	**26**/S	23/S	20/S
Adapted smooth	**19**/S	16/S	**8**/R	21/S	18/I	28/S	23/S	20/S
Control	21/S	16/S	10/R	21/S	18/I	28/S	23/S	20/S

[a] SXT, sulphamethoxazole/trimethoprim (25 µg), GN, gentamicin (10 µg), S, streptomycin (10 µg), AMC, amoxicillin/clavulanic acid (30 µg), NA, nalidixic acid (30 µg), CIP, ciprofloxacin (5 µg), CTX, ceftriaxone (30 µg), AMP, ampicillin (10 µg). Zones of inhibition (millimeters) are reported as S, susceptible strains; I, intermediate susceptible strains; R, resistance strains. Boldfaced data indicate reduced susceptibility relative to unexposed (control) strains; data not bolded indicate exposed strains with no difference in susceptibility patterns relative to unexposed (control) strains. An increase in resistance was defined as a change in S (before chlorine exposure) to R (after chlorine exposure). [b] For adaptation, cultures were previously exposed to increasing sub-lethal chlorine concentrations, control represent unexposed *Salmonella* culture.

Table 4.3 Broth microdilution assay of antibiotic cross-resistance patterns of adapted and control *S*. Heidelberg

Salmonella Heidelberg cultures previously adapted to chlorine [b]	Antibiotic [a] (µg/ml)						
	AMP	GN	S	NA	T	CIP	AMX
Adapted rugose	4/S	4/S	**64**	**16**/S	**8/I**	1/S	**16/I**
Adapted smooth	4/S	4/S	32	8/S	**8/I**	1/S	8/S
Control	4/S	4/S	32	8/S	4/S	1/S	8/S

AMP, ampicillin; GN, gentamicin; S, streptomycin; NA, nalidixic acid; T, tetracycline; CIP; ciprofloxacin; AMX, amoxicillin. Data are reported as SS, susceptible strains; I, intermediate susceptible strains; R, resistance strains. Boldfaced data indicate reduced susceptibility relative to unexposed (control) strains; data not bolded indicate exposed strains with no difference in susceptibility patterns relative to unexposed (control) strains. An increase in resistance was defined as a change in S (before chlorine exposure) to R (after chlorine exposure). [b] For adaptation, cultures were previously exposed to increasing sub-lethal chlorine concentrations, control represent unexposed *Salmonella* culture.

Table 4.4 Acid cross-resistance of adapted and control *S*. Heidelberg

Salmonella Heidelberg previously adapted to chlorine [b]	Cell count [a] / h			
	3h	6h	9h	12h
Adapted rugose	33	6	0	0
Adapted smooth	31	0	0	0
Control	48	11	0	0

[a] Cell count below 30 or above 300 were not suitable for analysis. [b] cultures were previously exposed to increasing sub-lethal chlorine concentrations, control represent unexposed *Salmonella* culture.

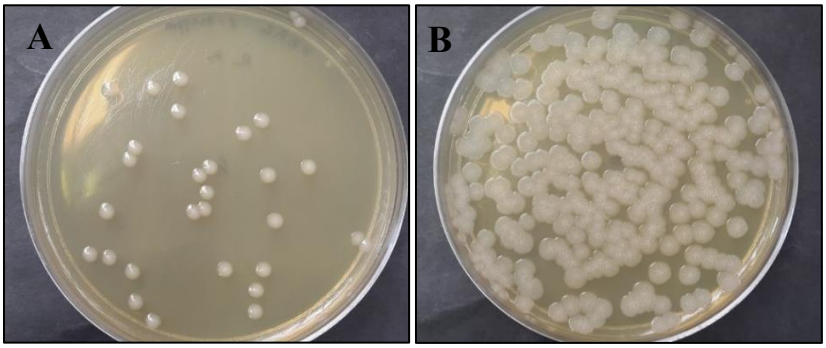

Figure 4.1 Comparison of chlorine-adapted smooth and rugose *Salmonella* Heidelberg morphotype

(A) chlorine adapted smooth morphotype, (B) chlorine adapted rugose morphotype.

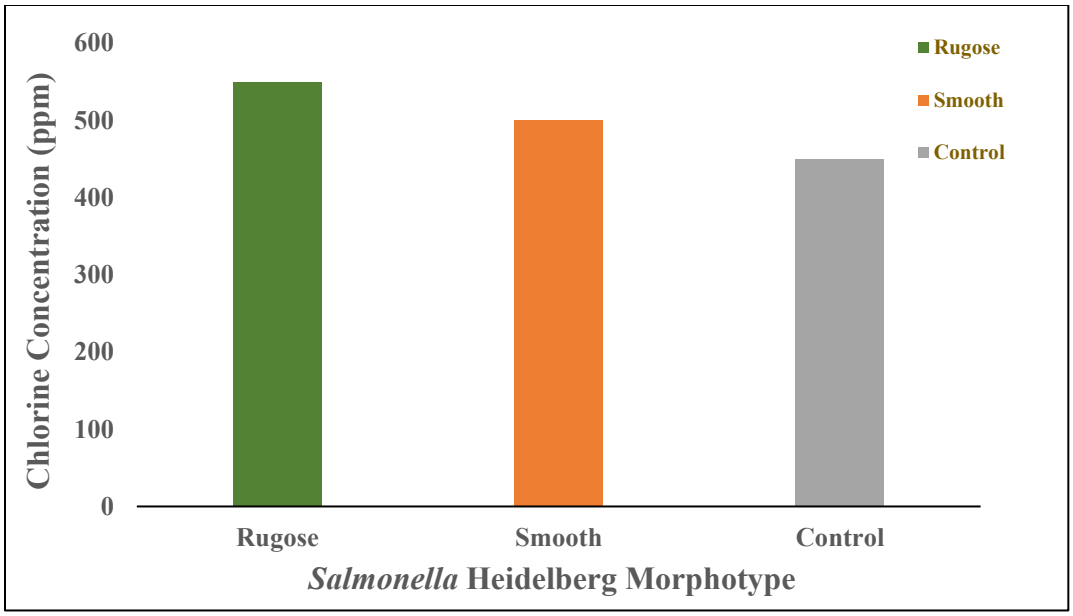

Figure 4.2 MIC for chlorine adapted and control *Salmonella* Heidelberg after stress adaptation

Rugose and smooth (chlorine-adapted cells), control (non-exposed cells)

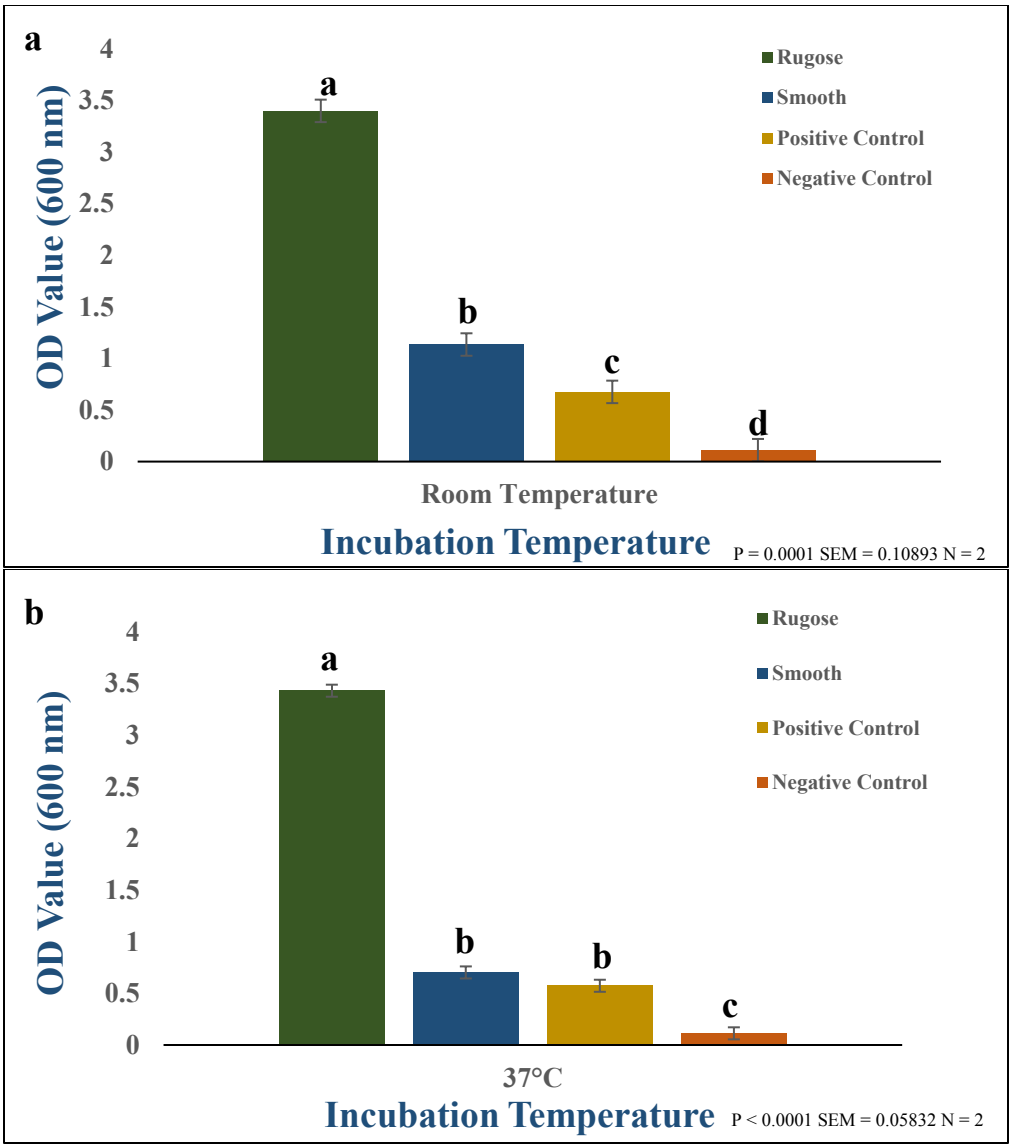

Figure 4.3 Biofilm formation by *Salmonella* Heidelberg after 48 h on plastic surface using 96-well polystyrene microtiter plate

at room temperature (b) at 37°C. Treatments with different superscripts indicate significant differences ($P \leq 0.05$). Adapted cells (rugose and smooth), non-exposed (positive control), broth only (negative control)

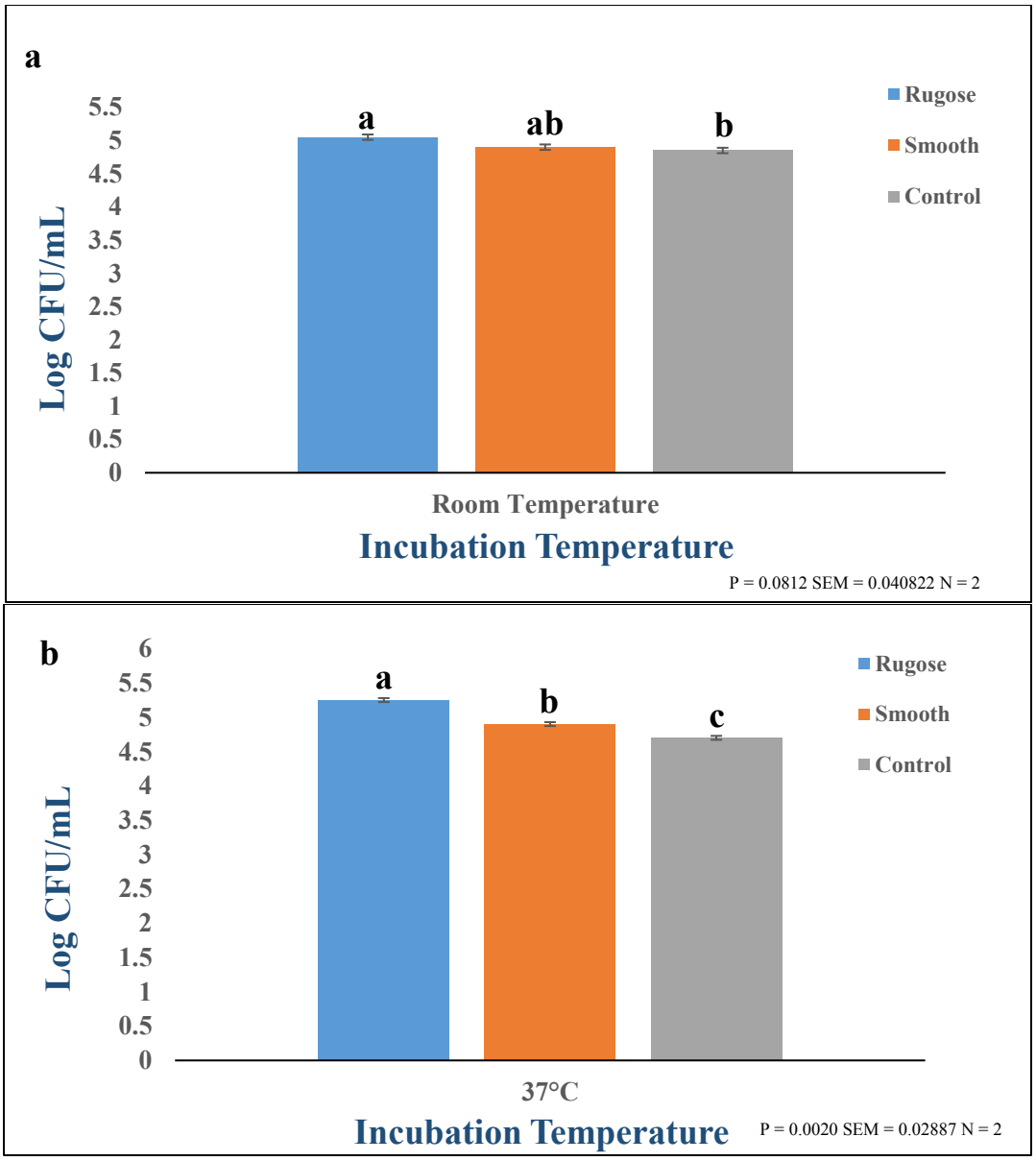

Figure 4.4 Quantification of *Salmonella* Heidelberg after 48 h attached to 24-well polystyrene plate

(a) at room temperature (b) at 37°C. Treatments with different superscripts indicate significant differences ($P \leq 0.05$). Adapted cells (rugose and smooth), non-exposed (control)

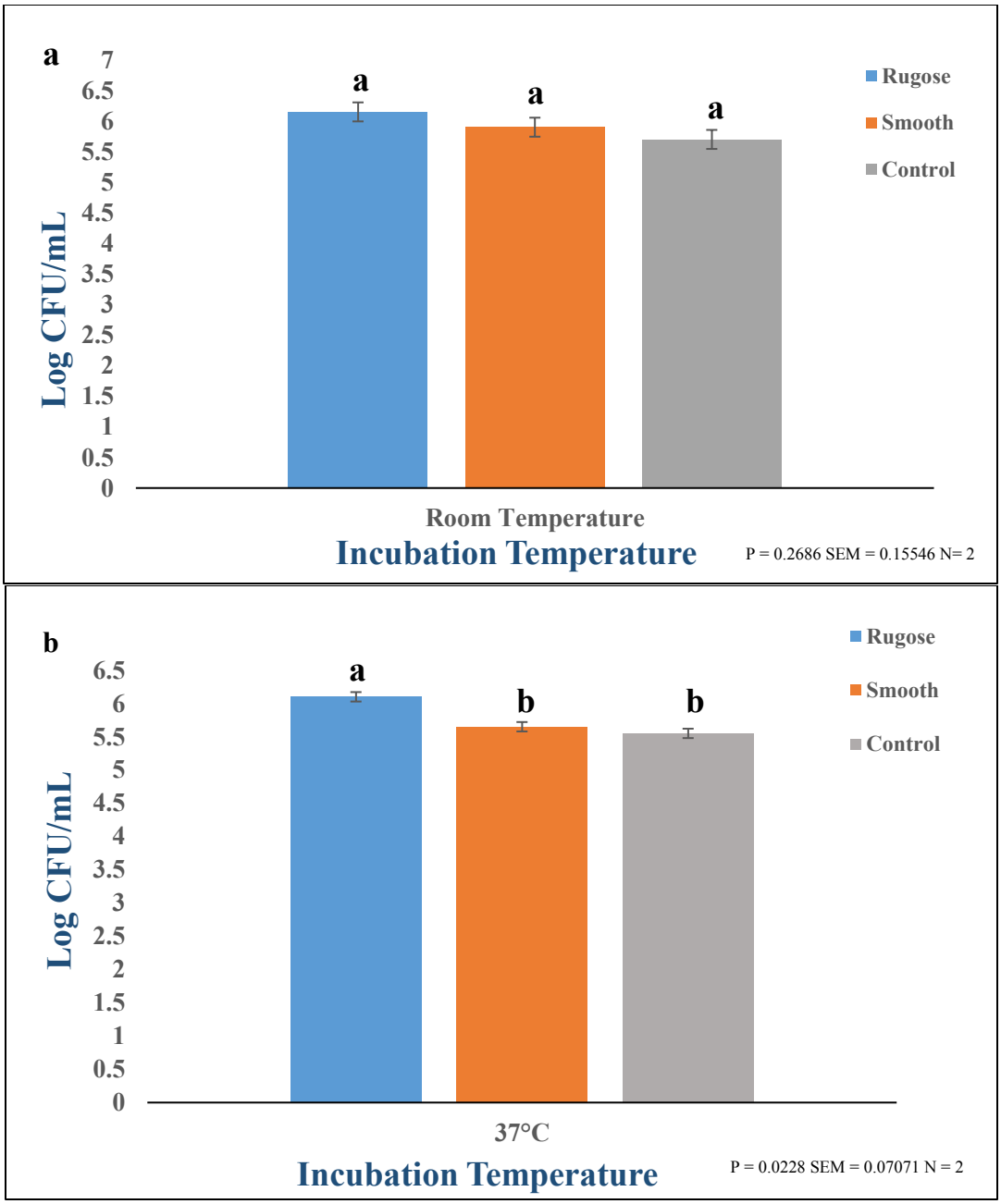

Figure 4.5 Quantification of *Salmonella* Heidelberg after 48 h attached to stainless steel coupons in tryptic soy broth

(a) at room temperature (b) at 37°C. Treatments with different superscripts indicate significant differences ($P \leq 0.05$). Adapted cells (rugose and smooth), non-exposed (control)

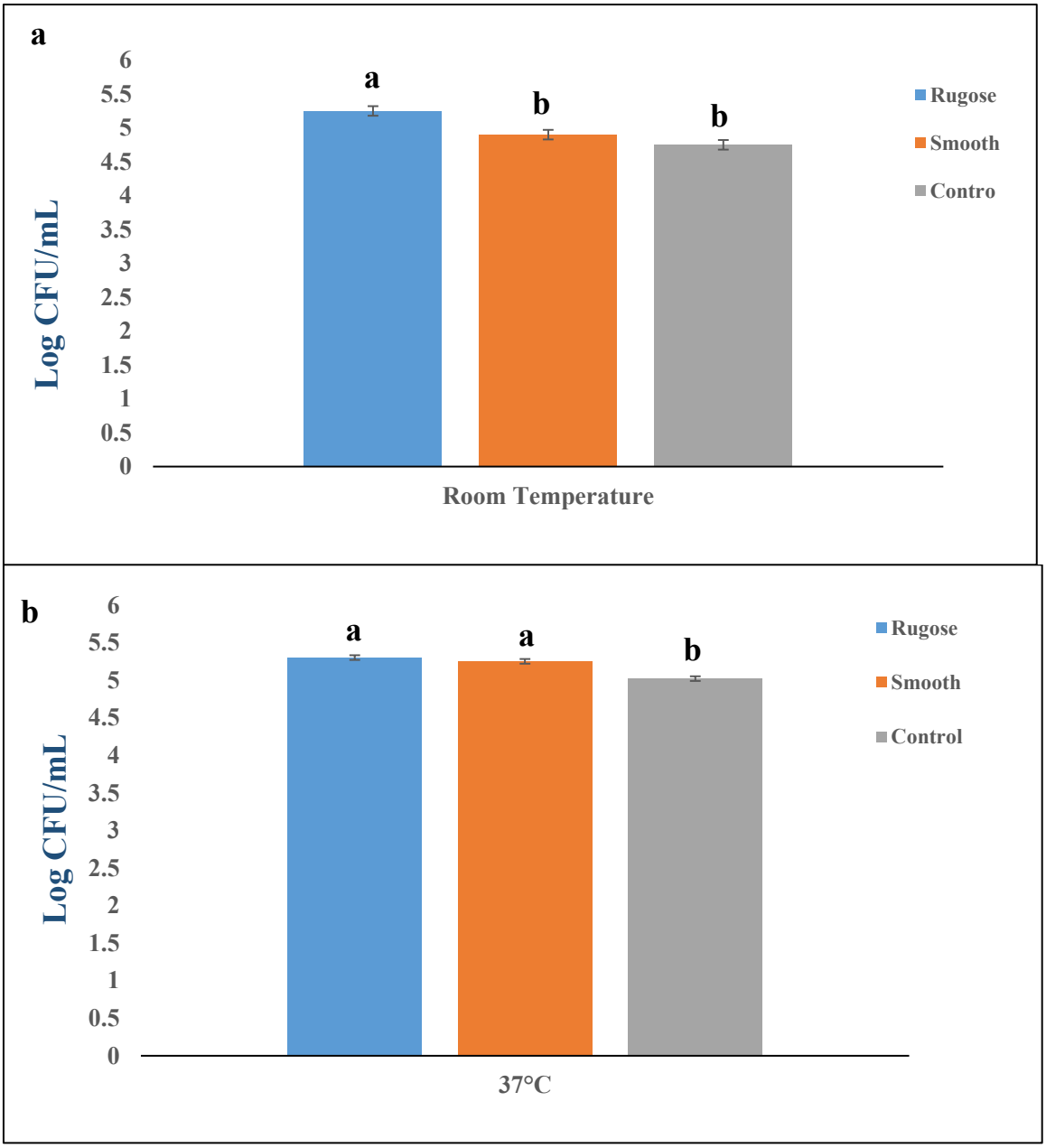

Figure 4.6 Quantification of *Salmonella* Heidelberg after 48 h attached to stainless steel scoop

(a) at room temperature (b) at 37°C. Treatments with different superscripts indicate significant differences ($P \leq 0.05$). Adapted cells (rugose and smooth), non-exposed (control)

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CHAPTER V

SUMMARY

The results of this research showed the possibility of adaptation in foodborne pathogens, especially *Salmonella* to antimicrobial stress. The development of rugose variant by *Salmonella enterica* (*Salmonella* Typhimurium and *Salmonella* Heidelberg) suggested a means the bacteria use to adapt to the stress posed by chlorine. In addition, the production of stronger biofilms by the rugose variant of *Salmonella* Typhimurium and *Salmonella* Heidelberg on both plastic and stainless steel surfaces revealed its ability to withstand the chlorine stress. There is a need for further studies, for example, scanning electron micrograph (SEM) of the rugose variant of *Salmonella* to provide a better understanding of the biofilm formed by rugose compared to smooth *Salmonella* morphotype.

Chlorine-adapted *Salmonella* in this study showed reduced susceptibility to some antibiotics, which suggests the possibility of cross-protection in *Salmonella* to either a homologous or heterologous stress. Adapted rugose in particular showed the greatest reduction and ability to induce cross-protection to clinically important antibiotics. Chlorine exposure does not induce cross-protection for the adapted *Salmonella* enterica and control to stress posed by low pH.

This research highlights the need for food processors to have a strong control protocol for cleaning and sanitation of food processing equipment and contact surfaces

(both food and non-food) including any hard to reach surfaces in order to prevent the formation of the film. The protocol should put into consideration the effectiveness of different sanitizing agents and applications according to manufacturer's recommendation to avoid sub-lethal dosage that may induce morphological change in foodborne pathogens, as have been observed in *Salmonella* serotypes investigated in this project.