EFFECT OF ORGANIC ACIDS AND QUATERNARY AMMONIUM COMPOUNDS ON SURVIVAL OF *SALMONELLA* SEROVARS WITH SGI1- MEDIATED MULTI-ANTIBIOTIC RESISTANCE.

by

ANA RODRIGUEZ

(Under the Direction of Mark A. Harrison)

ABSTRACT

In this study, the fate of six multidrug resistant (MDR) and two non-MDR *Salmonella* were evaluated when exposed to organic acids and quaternary ammonium compounds. Acid adapted bacteria survived better than non-adapted bacteria when challenged with 2% of acetic or lactic acids at pH 3.5. Adjustment with organic acids did not confer cross-protection against further treatments with quaternary ammonium compounds. There was no significant difference ($p>0.05$) in survival between MDR and non-MDR *Salmonella* to organic acid-cross protection against quaternary ammonium treatments and to resistance in biofilms; however, there was a significant difference ($p<0.05$) in survival between MDR and non-MDR *Salmonella* after exposure to quaternary ammonium compounds and organic acid treatments.

INDEX WORDS: Multi-drug resistant *Salmonella*, organic acids, quaternary ammonium compounds, biofilms.

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DEDICATION

To my son, family and friends whose unconditional love and support encourage me to fulfill this long term dream.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Chapter 1

INTRODUCTION

It has been estimated that 1.4 million cases of salmonellosis, leading to 16,000 hospitalizations and about 600 deaths occur each year in the United States (*20*). In 2008, according to the Center for Disease Control and Prevention (CDC) (*1*) *Salmonella* species accounted for 7,444 foodborne infections per 100,000 people in the U. S. *Salmonella* serotypes Enteritidis, Typhimurium, Newport, Javiana and Saint Paul were the most frequently isolated during that year. *Salmonella* infections with nontyphoid serotypes usually appear 8 to 72 h after contact with the pathogen. Generally the illness is self-limiting and the use of antibiotics is not necessary; however, invasive cases such as meningitis and septicemia might require antimicrobial therapy (*3, 17*).

Over the last two decades, there has been an increase in the emergence and prevalence of antimicrobial resistant bacteria, among them *Salmonella* (*10, 14*). Infections with multidrug resistant bacteria represent a public health concern, since failure to treatment increases morbidity and mortality in humans (*18*). The intensive use of antimicrobials in different fields, such as human, veterinary medicine, and as food animal growth promoting agents has been associated with the emergence of antimicrobial resistant microorganisms (*9, 11, 14*).

Antibiotic resistance in *Salmonella* is attributed to mobile genetic elements such as plasmids, transposons and integrons. There are at least eight classes of integrons, with class 1 integrons the most frequently found in human and animal pathogens. Class 1 integrons usually contain a 5' conserved segment (5'-CS) with the *int* gene with a specific recombinase site (*IntI*), the *attI* where the gene cassette is inserted by the integrase, and the promoter. Class 1 integrons also have a 3' conserved region (3'-CS) consisting of *qacΔE* gene that encodes for partial resistance to quaternary ammonium compounds, the *sul1* gene which confers resistance to sulphonamide and two open reading frames, Orf5 and Orf6. Genes associated with class 1 integrons are connected with resistance to aminoglycosides, β-lactams, phenicols, macrolides, quaternary ammonium and trimethoprim (*2, 3, 8, 14*).

Salmonella is a ubiquitous microorganism and infections have been related to the consumption of contaminated products, such as poultry, beef, pork, eggs, milk, seafood, fresh produce, and direct contact with animals (*14, 19, 20*). Several interventions, such as chemical dehairing, washing, and sanitizing animal carcasses have been developed to minimize microbial contamination from food animals coming from abattoirs. Hot water, chlorine, short-chain organic acids, and multiple hurdles are frequently used as part of the treatments. (*6, 12*).

Antimicrobials such as organic acids and quaternary ammonium compounds are usually used to prevent, reduce, or eliminate contamination in food processing plants (*5*). However, there are some conditions such as slow growth rate, nutrient depletion, and surface attachment (biofilms) that modify the cell envelope of microorganisms and can modify resistance to antimicrobial agents (*7*).

Organic acids have been used as food additives, but not all of them have antimicrobial activity. Acetic, lactic, propionic, sorbic, and benzoic acids have been the most effective as antimicrobials (*4*). The antimicrobial effect of the organic acids is based on a decrease of the internal pH of the microorganism causing acidity of the cytoplasm if the cell is not able to maintain the proper internal pH it will die (*4, 5*).

Quaternary ammonium compounds (QACs) disturb the cellular cytoplasm and outer membrane lipid bilayers of microorgaisms. The positive charge of the nitrogen in the QACs binds electrostatically to the negatively charged sites on the bacterial cell wall, disrupting both the cell wall and the cytoplasmic membrane; consequently, causing cell lysis, leakage and death (*13*, *16*).

This research was intended to compare the effectiveness of organic acids against multidrug resistant (MDR) and non-MDR *Salmonella* when they have been adapted to acid conditions, to compare the effectiveness of quaternary ammonium treatment against MDR and non-MDR *Salmonella,* to determine whether organic acid treatments confer cross-protection against subsequent quaternary ammonium treatment on MDR and non-MDR *Salmonella,* and finally to determine survival of MDR and non-MDR *Salmonella* in biofilms after quaternary ammonium compound treatment.

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Chapter 2

LITERARURE REVIEW

Multidrug resistant *Salmonella* outbreaks

An estimated 1,400,000 cases of *Salmonella* infections occur annually in the USA (*77*). According to the Center for Disease Control and Prevention (CDC) foodborne outbreaks summary for 2007, 135 from 257 of the confirmed bacterial outbreaks were caused by *Salmonella*. *S.* serovar Enteritidis and *S.* serovar Typhimurium were most frequently isolated. Among the outbreaks, 6 were linked to the consumption of pork (*11*).

Antimicrobial therapy is not usually required for *Salmonella* infections; however, therapy may be used in systemic cases (*72*). The increase in *Salmonella* antimicrobial resistance strains is of public concern since failure to treatment increases the prevalence of infections (*76*).

Varna et al., (*76*) investigated data of antimicrobial-resistance *Salmonella* outbreaks from 1984 to 2002. From 48 of the community outbreaks only, 39 had available data about antimicrobial susceptibility. Eleven of the 39 outbreaks were resistant to antibiotics; among these, 7 outbreaks, involving 17,182 persons, had strains that were resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (ACSSuT). The number of people hospitalized and who died was higher for the outbreaks caused by resistant *Salmonella* strains compared with outbreaks caused by susceptible strains (*76*).

The National Antimicrobial Resistance Monitoring System (NARMS) report for 2006 addressed an increase in resistance in most of the antibiotics tested against non-Typhi *Salmonella* compared with 1996. From a total of 2,184 of human isolates of non-Typhi *Salmonella*, 2.6% were resistant to nalidixic acid, compared with 0.4% in 1996, 3.6% of the isolates were resistant to third-generation cephalosporins, compared with 0.2% in 1996, 5.5% of the isolates had the AcSSuT resistance pattern, compared with 8.8% in 1996, and 2.0% of the isolates had the MDR-AmnC pattern, phenotype that was not detected in 1996 (*58*).

In addition to the human component, the NARMS report for 2005 involved retail meats and food animals. One hundred percent of the *Salmonella* isolates tested for antibiotic sensitivity from retail meats (chicken breasts, ground turkey, ground beef and pork chops) had the AcSSuT resistance pattern (*59*).

Several studies have concluded that the use of antibiotics in veterinary medicine has contributed to the spread of antibiotic resistant strains among food animals, which might be transmitted to humans (*72*). In an effort to reduce the spread of multidrug resistant strains the World Health Organization (WHO) worked on a Global Strategy for the Containment of Antimicrobial Resistance which involved the participation of the United Nations Food and Agriculture Organization (FAO) and the Office International des Epizooties (OIE) (*80*).

Organic acids

Organic acids have been used as food additives, but not all of them have antimicrobial activity. Acetic, lactic, propionic, sorbic, and benzoic acids have been the most effective antimicrobials (*19*). As General Recognized As Safe (GRAS) food additives (*25*), organic acids may be used as sanitizers if the final concentration when used is not less than 150 parts per million (ppm) and not more than 300 parts per million (ppm) of acetic acid, and at least 69 parts per million (ppm) and no more than 138 parts per million (ppm) of lactic acid (21 CFR 178.1010) (*26*).

Acetic acid is a short chain acid (CH_3COOH) with a pK_a value of 4.75 that has been used as an antimicrobial; however, it can produce off-odors in meat products which limits its use (*31*).

Acetic acid can be produced by fermentation of ethanol using microorganisms such as *Acetobacter aceti*, *Gluconobacter*, and *Clostridium thermoaceticum*, or synthetically by oxidation of acetaldehyde, oxidation of hydrocarbons, methanol carbonylation, and ethylene direct oxidation (*81*).

Lactic acid in its dry form (2-hydroxypropionic acid) is a white powder with a melting point between 18°C to 26°C, and with a pK_a value of 3.85. It is produced mainly by homofermentative lactic acid bacteria. It is produced commercially either by fermentation of carbohydrates such as glucose, sucrose, or lactose, or by a procedure involving formation of lactonitrile from acetaldehyde and hydrogen cyanide and subsequent hydrolysis to lactic acid (*70*). Although heterofermentative bacteria are able to produce lactic acid, they are not frequently used on an industrial scale (*70*). Lactic acid is a short chain acid ($C_3H_6O_3$) that has a mild, creamy odor with a sour taste (*21*) and when it is used at the appropriate concentration (up to 2%), has demonstrated bactericidal properties without compromising the sensory characteristics in meat products (*24, 31, 69*).

The antimicrobial effect of the organic acids is based on a decrease of the internal pH of the microorganism *(18, 66*). Organic acids such as acetic and lactic are weak acids which in the protonated form can penetrate the lipid membrane of the bacterial cell and once inside it, dissociate into anions and protons ($RCOO^-$ and H^+) (*65*). The protons formed from the split of the organic acid acidify the cytoplasm. The cell in an effort to keep its homeostasis pumps those protons out at the expense of ATP which will be depleted soon causing exhaustion of the cell and death (*17, 18*).

Organic acids have been used in several food processes as antimicrobials, mainly in the meat and poultry processes. Foodborne pathogens such as *Salmonella* spp. could be introduced into the meat during the slaughter process representing a hazard to humans. One way to reduce the level of microbial contamination on the carcass is by washing and spraying it with organic acids (*19, 31, 44*).

Acid stress response

Salmonella spp. are considered among the neutrophilic bacteria and as such are able to grow over a wide range of pH (5 to 9); however, when they are not able to maintain their internal pH they die (*5*). Microorganisms have some mechanisms that help them to regulate their cytoplasmic pH to a constant level (7.6) when there are changes in their external pH (*28*). Microorganisms have passive and active mechanisms of pH homeostasis. The low permeability of the membrane to protons and the cell high buffering capacity make part of the passive mechanisms. Buffering capacity in the cell is provided by the protein content of the cytoplasm and from glutamate and polyamines accumulated in the cytoplasm (*38*). Proton antiport systems such as potassium and sodium antiporters are part of the active ones (*38*). *Salmonella* as most of Gram-negative microorganisms use the antiporter systems in order to generate a pH gradient and thus maintain a constant internal pH; however, these systems protect the cell only within certain pH limits *(63*).

Na^+/H^+ antiporters give cells the capacity of tolerating Na^+ (Li^+) and/or the ability of growing under alkaline pH in the presence of either sodium or lithium (*40*). NhaA has been described extensively as the main antiporter in *E. coli* and other enterobacteria and its activity is regulated by pH. NhaA brings into the cytoplasm 2 H^+ for each Na^+ / Li^+ that is expelled out, leading to acidification of the cytoplasm. NhaA is downregulated below pH 6.5 and is activated at pHs between 7 and 8, reaching its maximum activity at pH 8.5 (*8, 62*). Mutagenesis in the histidine residue 226 in *E. coli* changes the pH response profile suggesting that that site is involved in the control of NhaA activity. In addition to that residue, the glycine residue 338 is also involved in the NhaA pH response *(29*).

Conversely to sodium/proton antiporter system, potassium transport system increases its activity when the internal pH needs to be alkalinized (*41*). TrK is the main potassium/proton transport system in bacteria and is also involved in their pH homeostasis. Its function is the expulsion of monovalent cations such as potassium from the cytosol and is also a pH dependent system (*8*).

Under acid stress conditions, such as that encountered in the stomach and macrophages, *Salmonella* must adapt to a higher ΔH^+ in order to resist their antibacterial effects of the acidity, and in order to do so the microorganism counters with mechanisms such as the acid tolerance response (ATR) and some amino acid decarboxylases. These mechanisms allow the microorganism to endure harsh pH conditions in both log and stationary phases of growth (*38*).

During log phase and at pH 4.5, fifty acid shock proteins (ASPs) are released in order to help the cell survive. This at the same time activates several regulatory genes such as the alternative sigma factor (σ^s) encoded by *rpoS,* the iron regulator Fur, and the signal transduction system PhoPQ (*phoP* and *phoQ*). Each of those regulators induces the expression of a small number of acid shock proteins (*27, 28, 65*).

The acid tolerance response (ATR) during stationary phase is induced when the cells in this stage are exposed to low external pHs and is totally different from the general stress response system that is induced as soon the cells enter into stationary phase regardless of the pH of the medium and is dependent of the sigma factor σ^s (*3, 27*). Stationary cells in addition to the general stress response system also have an acid pH-inducible system that is independent of sigma factor σ^s, the OmpR/Envz regulatory system, and 15 acid shock proteins. All of them help the cell to cope with low pH in the environment. EnvZ is not important in the induction of the acid tolerance response, since EnvZ mutants had a normal acid-inducible acid tolerance response (*1*).

In addition to the regulatory proteins, there are some individual effector proteins such as those in charge of the cell-surface structure and maintenance (aas, pbpA and cld), stress response (dps and rna), and efllux pumps mar and emr that are involved in acid survival (*65*). Acid stress in *Salmonella,* also induces the activation of amino acid decarboxylases such as lysine and ornitine decarboxilases which convert lysine and ornitine to cadaverine and putrescine, respectively, and helps to buffer the microorganism's surroundings and thus enhances its survival (*15, 63*).

Acid adaptation and cross-protection

One way to control growth of spoilage and pathogenic microorganisms in foods is through acidification. Some pathogenic microorganisms such as *S.* Typhimurium*, E. coli* O157:H7 and *L. monocytogenes* can adapt and overcome the acid conditions in foods becoming a safety concern. In addition, acid adapted bacteria can survive better to further environmental stresses (*38, 49, 34, 51, 82*).

Leyer and Johnson (*50*) reported that *S.* Typhimurium that had been adapted to mild acid conditions (pH 5.8) for two cell doublings was more resistant to heat, osmotic stress, lactoperoxidase system, crystal violet and polymyxin. Yuk and Schneider (*82*) studied the acid resistance in simulated gastric fluid of *Salmonella* spp. that had been acid adapted in apple (pH 3.7), tomato (pH 4.4) and orange (pH 3.9) juices and found that all serovars that had been acid adapted had greater acid resistance during the time of exposure (100 s) to the simulated gastric fluid (pH 1.5) than the non-adapted controls.

Leyer and Johnson (*49*) showed that acid-adapted *Salmonella* survived better in cheese as compared with the non-adapted counterparts. Briefly, the strains were adjusted at pH 5.8 with HCl for one or two doublings, and then both adapted and non-adapted were inoculated with a concentration of 10^4 CFU/g on the surface of cheddar (pH 5.2), Swiss (pH 5.6), and mozzarella (pH 5.3) cheeses and stored at 5°C under aerobic and anaerobic conditions. Non-adapted *Salmonella* was absent from the cheddar cheese after 28 days, whereas the adapted *Salmonella* was still detectable at the day 74; similar patterns were observed with the other cheeses. Cells in cheeses that were incubated under anaerobic conditions died quicker than those that were incubated under aerobic conditions.

Quaternary ammonium

Quaternary ammonium compounds (QACs) are cationic agents that are frequently used as disinfectants in the medical and food industries. Quaternary ammonium compounds have in their molecular structure both a hydrophobic and a hydrophilic group, so they are categorized as surface-active agents (surfactants) (*75*).

Quaternary ammonium compounds are known for disturbing the cytoplasm and outer membrane lipid bilayers. The positive charge of the nitrogen in the QACs binds electrostatically to the negatively charged sites on the bacterial cell wall, disrupting both the cell wall and the cytoplasmic membrane; consequently, causing cell lysis, leakage, and death (*52, 75*).

Quaternary ammonium compounds also cause protein denaturation which leads to cell death (*69*). QACs are bacteriostatic at low concentrations and bactericidal at high concentrations, and Gram-negative bacteria are more resistant to bactericidal concentrations than Gram-positive ones *(54*). Certain microorganisms have shown to acquire resistance to QACs after they have been exposed to sublethal concentrations under laboratory conditions. In *E. coli*

(*54*), *L. monocytogenes* (*75*), and *Pseudomonas* spp. (*46, 72*), that resistance may be attributed to mutations, plasmids, transposons, efflux pumps, and enzymes (*46, 52*). Efflux pumps can remove QACs from the membrane core thus reducing their effectiveness. In *Staphylococcus aureus,* several multi-drug efflux pumps contribute to the inactivation of QACs making the microorganism more resistant to them (*67*).

QACs also confer cross-resistance to antibiotics. Langsrud et al., (*47*) observed that *E. coli* that were adapted to benzalkonium chloride were more resistant to chloramphenicol that the non-adapted bacteria. They found that the adapted cells had enhanced efflux and concluded that this mechanism of resistance was involved. Similar observations were made by Braoudaki and Hilton (*9*) when *S.* serovar Typhimurium and *E. coli* O157:H7 where adapted to benzalkonium chloride. They showed more resistance to chlorhexidine and triclosan, respectively.

QACs are in many of the sanitizing solutions utilized in food processing plants. They can be safely used on equipment, utensils, and other food-contact surfaces when the final concentration does not exceed 200 parts per million of the active quaternary compound (21 CFR 178.1010) (*27*).

Biofilms

Microorganisms can exist in nature in a free-living state (planktonic) or as part of communities called biofilms. Biofilms can be defined as an irreversible association between microbial cells and a surface which are surrounded by a matrix of extracellular polymeric substances (EPS) (*20, 37*). Cells in biofilm are more resistant to chemical, physical, and mechanical stresses in comparison to plantktonic cells (*22, 53*).

EPS is mainly composed of polysaccharides; some of them are neutral or polyanionic. In Gram-negative bacteria, the presence of D-glucuronic, D-galacturonic, and mannuronic acids gives the anionic property. This allows the association of calcium and magnesium which provide more strength to the formed biofilm. In the case of Gram-positive bacteria, the EPS is primarily cationic. EPS production is affected by the nutrient's availability. A surplus of carbon and a limitation of nitrogen, potassium, or phosphate increase the EPS synthesis (*20*). In some bacteria such as *S.* Typhimurium*, P. aeruginosa* and *E. coli*, the EPS is mainly comprised of cellulose, alginate, and colonic acid, respectively (*10*).

There are several steps involved in the formation of biofilms: attachment of plantktonic cells to a surface, microcolony and EPS production, and maturation (*14*). Attachment is influenced by cell surface properties such as flagella, pili, adhesion protein, capsules, and surface charge *(14*). Attachment occurs in two steps: initial reversible adherence to a surface by electrostatic forces, van der Waals, and hydrophobic interactions, and production of extracellular matrix that permits the irreversible attachment (*7*).

Hydrophobicity of the cell surface plays an important role in adhesion. Most bacteria are negatively charged; however, they have hydrophobic components such as fimbriae which contribute to cell surface hydrophobicity and attachment. Other components of the cell surface such as mycolic acid also contribute to the hydrophobicity of the cell surface. Microorganisms like *Corynebacterium*, *Nocardia*, and *Mycobacterium* which are rich in mycolic acid have been found to be more hydrophobic than those that do not contain mycolic acid. In contrast, the O antigen component of lipopolyssacharide (LPS) confers hydrophilic properties to Gram-negative bacteria (*20*). After irreversible attachment occurs, there is a simultaneous aggregation and growth of microorganisms and production of EPS that makes part of the microcolony formation (*14*).

Cell-to-cell communication (quorum sensing) and planktonic cells are involved during aggregation (*14*). There is evidence that regulation of some genes is involved in attachment of cells. In *P. aeruginosa,* the *algC* gene is transcribed during attachment, resulting in up-regulation of *algT* for the synthesis of alginate, which is the main component of their EPS (*14*). Other genes such as *algD, algU, rpoS,* and genes that control polyphosphokinase (PPK) are also up-regulated during biofilm formation of *P. aeruginosa* (*20*). In *S.* Typhimurium and *S.* Enteritidis two operons, bcsABZD and bcsEFG are required for cellulose biosyntheis, which is the main component of their EPS; and disruption of any of the two operons increases the susceptibility of the bacteria to disinfectants (*48*).

Bacterial pheromones, such as acylhomoserine lactones (AHL), are involved in cell aggregation and regulation of the formation of the biofilm structure in Gram-negative bacteria. Unlike Gram-negative bacteria, Gram-positive bacterial pheromones are composed of oligopeptides, amino acids, and fatty acids (*78*). Another type of molecule that is involved in cell-cell communication is the molecule autoinducer-2 (AI-2); this molecule is used for communication between Gram-negative and Gram-positive bacteria (*61*). Other surface structures called curli (amyloid protein fibers) is involved in surface and cell-cell aggregation in *E. coli* and *S. enterica* (*60*). In addition, BapA, a large secreted protein, seems to be required for biofilm formation by *S.* Enteritidis (*48*).

After enough number of cells aggregate to form microcolonies, the production of extracellular polymer substances (EPS) starts (*14*). The composition of EPS varies and depends on the bacterial species forming the biofilm and the surrounding environment, but mostly it consists of extracellular polysaccharides, surface and secreted proteins, and extracellular DNA (*14, 61*).

The biofilm matrix plays important roles in adhesion and protection. Through the adhesion mechanism biofilm integrity is kept by cell-cell and cell-surface interactions. The protective mechanism involves inactivation of antimicrobial agents, avoidance of the host immune response, and other environmental stresses such as UV radiation, pH shifts, osmotic shock, and desiccation (*16, 61*).

Finally, if conditions such as growth and aggregation are favorable within the biofilm, it matures into a more organized structure. The architecture of a mature biofilm is heterogeneous, sometimes formed of a monolayer of cells or multilayered microcolonies held together with EPS and separated from other microcolonies by water channels.

Water channels are a vital part of the biofilm structure and function; they allow the diffusion of nutrients, oxygen and even antimicrobial agents (*14, 20*).

***Salmonella* biofilms on meat surfaces and meat processing environments**

Spoilage and pathogenic microorganisms, such as *E. coli* O157:H7*, Salmonella* spp. *Pseudomonas* spp., *E. faecalis, L. monocytogenes, S. aureus,* and others, have the ability to attach, grow, and multiply to form biofilms on meat tissues and food surfaces. Thus, they become an important source of cross contamination and post-processing contamination and a potential food hazard (*30, 39, 45*).

Bacteria, through the use of lectins (fibronectin, laminin, and type IV collagen) have a specific binding to collagen fibers in meat; however, nonspecific electrostatic or hydrophobic interactions might be involved as well (*30*). Thomas and McMeekin (*74*) examined attachment of *Salmonella* spp. to chicken muscle surfaces and found that attachment occurred only when muscle tissue was immersed in water inoculated with bacteria and allowed to swell. The presence of sodium chloride in the suspension prevented attachment of cells to collagen fibers.

After attachment to the muscle fibers, bacteria can penetrate meat tissues making decontamination methods ineffective since these cells will be more difficult to remove; consequently, leading to food spoilage or transmission of diseases (*30, 71*). However, cells attached to beef muscle can be transferred to clean food contact surfaces, cutting boards, utensils, and thus serve as a source of contamination (*30, 59, 83*).

Multidrug resistant (MDR) *Salmonella*

According to the CDC *Salmonella* is the number one bacterial cause of foodborne disease and over the last two decades a high percentage of isolates are resistant to several antibiotics generating a public health problem (*43*). Non-typhoid salmonellosis is a self-limiting illness; however, some systemic infections might demand antibiotic therapy. Fluoroquinolones such as ciprofloxacin and third-generation cephalosporins are the antibiotics of choice (*2, 33*).

The intensive use of antimicrobials in different fields, such as human, veterinary medicine, and as food animal growth promoting agents have been associated with the emergence of antimicrobial resistant microorganisms (*36, 43, 57*). In addition, the use of disinfectants might also select for antibiotic resistance (*43, 68*). Antibiotic resistant in microorganisms can be intrinsic or acquired. Intrinsic mechanisms of resistance include impermeability of the cell membrane and efflux pumps (*68*). Acquired resistance may result from a spontaneous mutation or by the acquisition of a resistance gene from another microorganism.

Multi-drug resistant may imply: a) the acquisition of gene clusters (a single plasmid or transposon encoding resistance to a variety of antibiotics); b) a single gene that encodes a biochemical mechanism that confers resistance to a class of related antibiotics (*erm* confers resistance to macrolides, *aac, aph* and *ant* confers resistance to aminoglycosides and *bla* to the β-lactams); c) a single gene that encodes resistance to different class of antibiotics (*aad* confers resistance to streptomycin and spectinomycin); d) a disinfectant or salt that contributes to setting up genes that are genetically linked to antibiotic resistance; and e) multiple, independent target genes mutations (*55*).

Antibiotic resistance in *Salmonella* is attributed to mobile genetic elements such as plasmids, transposons, and integrons (*15*). There are at least eight classes of integrons, and all of them are made of three elements: a gene that encodes for an integrase, a recombination site, and a promoter. Class 1 integrons are most frequently found in human and animal pathogens and usually contain a 5' conserved segment (5'-CS) with the *int* gene with a specific recombinase site (*IntI),* the *attI* where the gene cassette is inserted by the integrase, and the promoter. Class 1 integrons also have a 3' conserved region (3'-CS) consisting of *qacΔE* gene that codes for partial resistance to quaternary ammonium compounds, the *sul1* gene which confers resistance to sulphonamide, and two open reading frames, Orf5 and Orf6. Genes associated with class 1 integrons are connected with resistance to aminoglycosides, β-lactams, phenicols, macrolides, quaternary ammonium, and trimethoprim (*12, 32, 57*).

Multi-drug resistant *S.* Typhimurium DT104 was first observed in parrots and sea gulls in the United Kingdom early in the 1980s and soon after that it was found in cattle, humans, poultry, pigs, and sheep. This strain is typically resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines (ACSSuT) (*4*); however, some isolates have shown resistance to fluoroquinolones, trimethoprin, and kanamycin as well (*6*). An antibiotic resistance gene cluster located in the *Salmonella* genomic island 1 (SGI1) is responsible for conferring resistance to the antibiotic profile. SGI1 was first described in *S. enterica* serovar Typrimurium phage type DT104 and is located between the *thdf* and *int2* genes of its chromosome; however, in other *S. enterica* serovars the location is different (*12*).

Other *Salmonella* serotypes have shown resistance to antibiotics. In 1998, multi-drug resistant *S. enterica* serotype Newport from cattle was first reported in the United States. This isolate produces a plasmid-mediated CMY-2 AmpC β-lactamase that inactivates third-generation cephalosporins. These particular isolates are known as Newport MDR-AmpC (*23, 79*).

Salmonella enterica serotype Paratyphi B strain of biovar java isolated from a tropical fish in Singapore, showed the same multidrug resistance profile of serotype Typhimurium DT104 and serotype Agona carrying the *Salmonella* genomic island 1 (SGI1). Further research with that particular strain confirmed the presence of SGI1 suggesting horizontal transfer of that region (*55*).

Resistance to fluoroquinolones in *Salmonella* is linked to mutations in the bacterial DNA gyrase (*gyrA* and *gyrB*), topoisomerase IV (*parC* and *parE*) genes, and active efflux pumps such as ATP-binding cassette (ABC), major facilitator superfamily (MFS), small multi-drug resistance (SMR), multi-antimicrobial resistance (MAR), resistance nodulation division (RND), and multi-drug and toxic compound extrusion (MATE) (*13, 35*).

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Chapter 3

EFFECT OF ORGANIC ACIDS AND QUATERNARY AMMONIUM COMPOUNDS ON SURVIVAL OF *SALMONELLA* SEROVARS WITH SGI1- MEDIATED MULTI-ANTIBIOTIC RESISTANCE[1]

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Abstract

Multi-drug resistant (MDR) *Salmonella* emerged early in the 1980s in the United Kingdom, and since then several infections and outbreaks have been reported worldwide. In this study, the fate of six multidrug resistant (MDR) *Salmonella* and two non-MDR *Salmonella* were evaluated when exposed to organic acids and quaternary ammonium compounds. Acid adapted bacteria survived better than non-adapted bacteria when challenged with 2% of either acid at pH 3.5. Lactic acid was more effective than acetic acid after 4 h of exposure. Adjustment with organic acids did not confer cross-protection against further treatments with quaternary ammonium compounds. There was no significant difference ($p>0.05$) in survival between MDR and non-MDR *Salmonella* to organic acid-cross protection against quaternary ammonium compound treatments and biofilms; however, there was significant difference ($p<0.05$) in survival between MDR and non-MDR *Salmonella* after exposure to quaternary ammonium compounds and organic acid treatments.

Key words: Multidrug resistant *Salmonella*, quaternary ammonium compounds, organic acids, cross-protection

Introduction

Salmonella is an ubiquitous microorganism and infections have been related to the consumption of contaminated products, such as poultry, beef, pork, eggs, milk, seafood, fresh produce, and direct contact with animals (*23*, *34*, *36*). Over the last two decades, there has been an increase in the emergence and prevalence of antimicrobial resistant in *Salmonella* (*14, 23*). Infections with multi-drug resistant bacteria represent a public health concern since treatment failure increases morbidity and mortality in humans (*33*). The intensive use of antimicrobials in different fields, such as human, veterinary medicine, and as food animal growth promoting agents has been associated with the emergence of antimicrobial resistant microorganisms (*13*, *16*, *23*).

Organic acids have been used as antimicrobials in the meat and poultry processing. Several interventions, such as chemical dehairing, washing, and sanitizing beef carcasses have been developed in order to minimize microbial contamination from food animals coming from abattoirs. Hot water, chlorine, short-chain organic acids, and multiple hurdles are frequently used as part of the treatments (*4, 17*).

Sanitizers, such as quaternary ammonium compounds are usually used to prevent and eliminate contamination in food processing plants. However, there are some conditions such as slow growth rate, nutrient depletion, and surface attachment and community development (biofilms) that modify the cell envelope of microorganisms. Thus resistance to antimicrobial agents may be altered (9).

This research was intended to compare the effectiveness of organic acids against multi-drug resistant (MDR) and non-MDR *Salmonella* when they have been adapted to acid conditions, to compare the effectiveness of quaternary ammonium treatment against MDR and non-MDR *Salmonella,* to determine whether organic acid treatments confer cross-protection against subsequent quaternary ammonium treatment on MDR and non-MDR *Salmonella,* and finally to determine survival of MDR and non-MDR *Salmonella* in biofilms after quaternary ammonium compounds treatment.

Materials and Methods

Bacterial strains

MDR resistant *Salmonella* used included: *S.* Typhimurium DT104S, *S.* Typhimurium DT104565, *S.* Typhimurium DT104S/960081, *S.* Newport 01-2174, *S.* Agona 1146SA97, and *S.* Newport (Athens). Non-MDR resistant *Salmonella* used included: *S.* Typhimurium DT104S/921495 and *S.* Agona 0059SA98. *Pseudomonas aeruginosa* ATCC 15442 was used as quality control for the quaternary ammonium studies. *S*. Newport (Athens) was provided by Dr. John Maurer (UGA) while all the other *Salmonella* strains were provided by Dr. Axel Cloeckaert (French National Center for *Salmonella*).

Stock strains were grown in brain heart infusion broth (BHI, pH 7.3; Becton Dickinson, Sparks, MD) containing 15% glycerol and were frozen at -80^{o}C until they were used. Prior to each experiment, loop inocula were transferred from the frozen stocks to tryptic soy agar (TSA, pH 7.2; Becton Dickinson) plates and incubated at 37^{o}C for 24 h.

Sanitizers and neutralizing solution

Food grade lactic and acetic acids (85 and 99.5%, respectively; SAFC, St. Louis, MO, cetylpyridinium chloride (100%); Sigma-Aldrich, St. Louis, MO, benzalkonium chloride (50%); Spectrum Laboratories, Gardena, CA, and SS4 (10%); Saratoga Food Safety, Northlake, Il) commercial quaternary ammonium were used. Quaternary ammonium compounds (QACs) were neutralized with D/E neutralizing broth, containing lecithin, polysorbate 80, sodium bisulfite, sodium thiosulfate, sodium thioglycollate, dextrose, yeast extract, and pancreatic digest of casein (pH 7.4; Becton Dickinson).

Adaptation of *Salmonella* to acid and determination of survival

For acid adaptation, *Salmonella* strains were grown statically in tryptic soy broth (TSB, pH 7.2; Becton Dickinson) without glucose at 37^{o}C for 24 h and sequentially transferred in TSB without glucose whose pH had been adjusted with concentrated lactic acid to pH 6.5 and 6.0. Acid adapted *Salmonella* were kept refrigerated (4^{o}C) on TSA slants which had been adjusted to pH 6.0 with concentrated lactic acid.

A loopful of acid adapted and non-adapted *Salmonella* were grown in 9 ml of TSB without glucose adjusted with concentrated lactic acid to pH 6.0 and 7.2, respectively, incubated at 37^{o}C for 24 h, and transferred to a covered, sterile beaker. The pH of each strain was measured (Accumet Basic, AB15 Fisher Scientific; USA) and then the pH was decreased to 3.5 with either 2% lactic or acetic acid. Beakers were continuously stirred on a magnetic stirrer, and samples were removed at different times from 0 to 4 h. Cellular viability was determined by spreading onto TSA (pH 7.2) containing 0.1% sodium pyruvate (Sigma–Aldrich) to restore injured cells (*19*) and incubated at 37^{o}C for 48 h. Acid adapted *Salmonella* (pH 6.0) and non-adapted (pH 7.2) were exposed with both acids.

Susceptibility to quaternary ammonium compounds (QACs)

All strains were grown in TSB (pH 7.2), incubated at 37^{o}C for 24 h, centrifuged (Beckman Coulter Allegra X-22R, Fullerton, CA) twice at 3,500 rpm for 5 min, and suspended in 5 ml of 0.85% saline solution (J.T. Baker; NJ). One ml of each bacterial suspension was mixed with 9 ml of QAC solution prepared to give a final concentration of 0 (no QAC), 100, and

200 ppm. Solutions were continuously shaken on an orbital shaker (VWR Scientific Product), and samples were removed at 20, 120, 300, and 600 s of exposure. Once removed, the samples were immediately mixed with 9 ml of D/E neutralizing broth (pH 7.4; Becton Dickinson). Surviving cells were enumerated on TSA (pH 7.2) plates containing 0.1% sodium pyruvate (Sigma-Aldrich) which were incubated at 37^{o}C for 48 h.

Organic acid cross protection to QACs treatments

The procedure to produce acid adapted *Salmonella* was followed as above. Acid adapted and non-adapted bacteria were grown in 9 ml of TSB without glucose at pH 6.0 (acid adapted) and pH 7.2 (non-adapted), incubated at 37^{o}C for 24 h, centrifuged (Beckman Coulter Allegra X-22R, Fulleton, CA) twice at 3,500 rpm for 5 min, and suspended in 3 ml of 0.85% saline solution. Portions (0.1 ml) of the suspensions were dispensed on two different sterile stainless steel coupons (type 304, No. 4 finish, 2 x 5 cm) inside a sterile Petri dish. One served as the control and the other as the treatment. The suspensions were mixed with 200 µl of 100 ppm of each QAC to obtain a final concentration of 33 ppm with an exposure time of 10 min. After exposure, samples were transferred to a tube containing 9 ml of D/E neutralizing broth (pH 7.4), serial dilutions were prepared, and surviving cells were enumerated for the control (no sanitizer) and treatment coupons by plating onto TSA (pH 7.2) with 0.1% sodium pyruvate (Sigma-Aldrich). Plates were incubated at 37^{o}C for 48 h.

Biofilms

The procedure of Ren and Frank (*26*) was followed for the planktonic cultures and biofilms with slight modifications. Stainless steel coupons (type 304, No. 4 finish, 2 x 5 cm) were used as the support for the biofilms. New stainless steel coupons were degreased with acetone (Sigma-Aldrich), washed with alkaline jet-clean detergent (Fisher-Scientific, USA), and rinsed thoroughly with distilled water. The coupons were placed into two (one control and one treatment) 25 x 180 mm culture tubes with 25 ml of TSB (pH 7.2) and autoclaved. Each tube was inoculated with 20 µl of an overnight TSB (pH 7.2) culture, and incubated at 37^{o}C for 24 h. The coupons were removed from the cultures tubes with sterile forceps, rinsed with sterile hemagglutination buffer (pH 7.4; Becton Dickinson), and placed into tubes containing fresh sterile TSB (pH 7.2), and incubated for another 24 h. After 48 h incubation, coupons rinsed with sterile hemagglutination buffer (pH 7.4) were ready for the inactivation experiments. The remaining suspension in the tube was used for the planktonic studies.

To determine the inactivation rates of planktonic cells, 1 milliliter of the cells was added to 9 ml of the correspondent QAC that was prepared to obtain a final concentration of 200 ppm. The solution was continuously shaken on an orbital shaker (VWR Scientific Product) for 10 min. After the 10 min, exposure time, 1 ml of the solution was removed and added to 9 ml of D/E neutralizing broth (pH 7.4). Control samples were mixed directly with D/E neutralizing broth (pH 7.4) without exposure to QAC. Surviving cells were calculated by spreading onto TSA plates containing 0.1% sodium pyruvate and incubated at 37^{o}C for 48 h. .

To determine inactivation rates of biofilm cells, stainless steel coupons were removed from the culture tubes with sterile forceps, rinsed with sterile hemagglutination buffer (pH 7.4) to remove unattached cells, dipped into 45 ml of 200 ppm QAC solution, and continuously shaken on an orbital shaker for 10 min. At the end of the exposure time coupons were placed into sterile D/E neutralizing broth (pH 7.4) for 1 min. Control coupons (no QAC) were rinsed with sterile hemagglutination buffer (pH 7.4) and placed immediately into sterile D/E neutralizing broth (pH 7.4) for the same time. Biofilm cells were removed from the neutralized coupons by swabbing both sides of the coupon 10 times with a sterile calcium alginate swab (Fischer Scientific). The swab was transferred to a 9 ml sterile D/E neutralizing broth (pH 7.4) tube, serial dilutions made, viable cells enumerated by plating onto TSA (pH 7.2) containing 0.1% sodium pyruvate (Sigma-Aldrich), and incubated at 37^{o}C for 48 h.

In order to observe the biofilms, each coupon was stained with Hoechst 33258 stain and then visualized under an epifluorescence microscope (*6*).

Statistical Analysis

Each experiment was replicated three times. The response variable (CFU/ml) in the four experiments had spread distribution, so it was log transformed. For the acid study, there was a difference in the initial population (time -1) among all the strains that were misleading the comparison between the two acids, so the data was analyzed as log (initial CFU count at time -1/CFU count after acid treatment) where t = 0, 1, 2, 3 and 4, and it was referred as diff log CFU. Similar to the acid study, the bacterial populations in the quaternary ammonium study, were calculated as log (initial CFU count at time - 1/CFU count after QAC treatment) where t = 20, 120, 300, and 600 sec. For the cross-protection and biofilm study the bacterial populations were calculated as log (control CFU count/treatment CFU count). Population means were analyzed using analysis of variance (ANOVA), and subsequently, Tukey's multiple comparison method was used to determine difference among means when significant effects were observed in ANOVA. The level of significance throughout the project was $p< 0.05$.

Results

Effectiveness of organic acids against multidrug resistant (MDR) and non-MDR *Salmonella* when they have been adapted to acid conditions

In this study, acid adapted and non-adapted *Salmonella* strains were treated with lactic acid and acetic acid. Overall, the acid adapted bacteria survived better ($p<0.05$) than non-adapted bacteria when challenged with 2% of both acids and after the final pH was decreased to 3.5 (Table 3.1, Table 3.2); however, this data needs to be interpreted carefully since comparison by pH depends upon other variables such as time, microorganism and acid.

Acetic and lactic acid, were both effective on decreasing the initial microbial population; however, it was better observed after 4 h of exposure. Lactic acid was more effective than acetic acid ($p<0.05$). (Table 3.3; Fig. 3.1).

There was significant difference ($p<0.0001$) in the overall response of *Salmonella* strains to the challenge pHs (6.0 and 7.2). *S.* Typhimurium DT104565, *S.* Typhimurium DT104S/921495, and *S.* Typhimurium DT104s were more susceptible to the acid treatments than the other strains (Fig. 3.2).

The comparison between MDR and non-MDR *Salmonella* and the acid treatments did not depend on the interactions pH or time ($P>0.05$); meaning that such comparison was straightforward by group, where the MDR group was more resistant to the acid treatments ($p<0.05$) (Table 3.4).

Effectiveness of quaternary ammonium treatments against MDR and non-MDR ***Salmonella***

In order to analyze the interaction between individual strains and each disinfectant ($p<0.0001$) the data for population reduction for the different exposure times and different concentrations was combined (Fig. 3.3). Overall, inactivation of the *Salmonella* strains was rapid with at least a 5.0 log decrease within the first 20 sec (Fig 3.4) and increasing over time and with increasing concentration; however, this reduction did not change significantly between 300 s and 600 s of exposure (Table 3.5). *Pseudomonas aeruginosa* ATCC 15442 which is more resistant to QAC exposure than *Salmonella*, was used as a positive quality control organism for the QAC studies and was included for the individual strains comparative purposes; however, this strain was excluded when comparing strains vs. time.

Overall, there was a significant ($p<0.05$) difference in inactivation between the QACs. Benzalkonium chloride and SS4 were more effective than cetylpyridinium chloride ($p<0.05$) (Fig. 3.5). There appears to be slight differences in the inactivation of the strains over time, but the *Salmonella* strains responded in similar fashions (Fig. 3.6).

From a statistical stand point non-MDR *Salmonella* were more resistant ($p<0.05$) to quaternary ammonium compounds than MDR *Salmonella* (Table 3.6); however, from a microbiological standpoint such a difference was considered insignificant as the QACs were able to reduce the populations of both groups by at least 5.9 logs from the initial populations.

The interaction between concentration of disinfectant (ppm) and time was also significant ($p<0.001$), but higher reduction was achieved at 200 ppm (Table 3.7).

Organic acids adaptation conferring cross-protection against subsequent quaternary ammonium treatment on MDR and non-MDR *Salmonella*

In order to determine a possible difference in the survivor rate in this portion of the project, lower than recommended levels (33 ppm rather than 200 ppm) of the QACs were used. Overall, there was no significant difference ($p>0.05$) between the two pH levels (Table 3.8), meaning that pre-adjustment with organic acids (lactic or acetic) did not confer cross-protection against further treatment with quaternary ammonium compounds. In addition, there was no significant difference ($p>0.05$) between MDR and non-MDR *Salmonella* in response to QAC treatment after pre-adjustment to acid conditions (Table 3.9). Overall, cetylpyridinium chloride

(CPC) was the less effective ($p<0.05$) of the three QACs used in this part of the study (Table 3.10). Each *Salmonella* strain behaved differently with each quaternary ammonium compound, but overall, cetylpyridinium chloride was the less effective ($p<0.05$) (Fig. 3.7).

Survival of MDR and non-MDR *Salmonella* in biofilms after quaternary ammonium compounds treatments

Planktonic

Overall there was no significant difference ($p>0.05$) in the survival of MDR and non-MDR *Salmonella* planktonic cells after treatment with 200 ppm QACs (Table 3.11). There was no individual significant difference ($p>0.05$) among *Salmonella* strains. Planktonic cells were killed more effectively ($p<0.05$) with benzalkonium chloride and cetylpyridinium chloride than when SS4 was used (Table 3.12).

Biofilms

There was no significant difference ($p>0.05$) in the survival of MDR and non-MDR *Salmonella* biofilm cells after treatment with QACs (Table 3.13). There was no individual significant difference ($p>0.05$) among *Salmonella* strains; however, there was difference ($p<0.05$) between *Salmonella* strains and the control strain, *P. aeruginosa* (Fig. 3.9). Biofilm cells were equally resistant to the benzalkonium chloride, cetylpyridinium chloride, and SS4 (Table 3.14).

Discussion

Efectiveness of organic acids against multidrug resistant (MDR) and non-MDR *Salmonella* when adapted to acid conditions

Salmonella spp grown at pH 5.5 to 6.0 (acid adaptation) induces an acid tolerance response which protects the cells against lower pH (3.0 to 4.0) (*7*). In this study, the procedure of Dickson and Kunduru (*5*) with some modifications was followed for acid adaptation. Briefly, eight *Salmonella* strains were grown at pH 6.0 for 24 h (pH observed on the outside carcass surface after slaughter) and subsequently the pH was decreased to 3.5 (pH observed the outside carcass surface after spraying with lactic acid 2%) with concentrated lactic acid, and samples were taken from 0 up to 4 h exposure.

Acid adapted and non-adapted *Salmonella* were both sensitive to the organic acid treatments; however, the overall log reductions of the acid adapted bacteria were smaller than those of the non-adapted strains, meaning that acid adapted bacteria were more resistant to the acid treatments. The range of log reductions for the acid adapted bacteria was from 2.1 to 2.9 CFU/ml, and the range for the non-adapted bacteria was from 1.8 to 4.0 CFU/ml. Similar findings were reported by Selvan et al., (30), Dickson (*4*), Fu et al., (*11*), Van Netten et al., (*32*). However, Dickson and Kunduru (*5*) evaluated the effectiveness of lactic acid (1.5 and 3.0%) on *S.* Typhimurium using beef tissue, and they found that at 3% acid, the acid adapted strain was more sensitive than the nonadapted strain.

S. Typhimurium DT104565, *S.* Typhimurium DT104S/921495, and *S.* Typhimurium DT104s were more susceptible to the acid treatments at the challenged pHs (6.0 and 7.2) than the other *Salmonella* serovars; however, the adapted *S*. Typhimurium strains were more resistant than the nonadapted counterparts.

In the present study, the microbial populations decreased gradually after exposure to the challenged acids (acetic and lactic) with time through the 4 hours of exposure; however, lactic acid was slightly more effective than acetic acid. This difference in effectiveness might be explained by the lower pH of the lactic acid, to a higher concentration of undissociated acid (*3, 30*, *35*), and to the ability of lactic acid to disintegrate the outer membrane of gram negative bacteria (*1).*

Organic acids can induce cross-protection to subsequent treatments (*3, 7*). Leyer and Johnson (*19*) reported that *S. Typhymurium* strains that had been adapted to acid conditions (pH 5.8) were more resistant to heat, salt, crystal violet, lactoperoxidase system, and polymyxin B; however, heat shock did not confer acid resistance. Even though acid adaptation can induce cross-protection against other environmental stresses it can also induce sensitivity to them; Greenacre et al (*12*) reported that *S. Typhimurium* that had been adapted with lactic acid was more sensitive to hydrogen peroxide than the non-adapted strains. In our findings, acid adaptation did not appear to induce cross-protection to exposure to subsequent quaternary ammonium compounds treatments, and a speculation behind it could be attributed to the fact that mechanisms of protection are different to those expressed during QACs response.

Effectiveness of QACs against multidrug resistant (MDR) and non-MDR *Salmonella*

Sallam and Donnelly (*29*) stated that a sanitizer is considered to be effective if it reduces in at least 5 log cycles the initial bacterial population in 30 s exposure. Based on this criterion, the results in this study show that the QACs were effective against *Salmonella* strains at 200 ppm and 20 s exposure. In this part of the study it was found that benzalkonium chloride and SS4 were more effective than cetylpiridinium chloride in reducing bacterial populations. Bactericidal effects of QACs have been related to their carbon chain length being C_{16} more effective than shorter chain compounds.

It has been speculated that some microorganisms, including *Salmonella*, might acquire adaptive resistance to quaternary ammonium compounds (*2, 20, 21, 25*). Several genes have been implicated in conferring resistant to cationic biocides such as quaternary ammonium compounds. Some of them are *qacA, qacB, qacG, qacH, smr, qacE, and qacΔE1* (*28*). The *qacΔE1* gene is present in integrons class 1 that make part of MDR *Salmonella*. Our findings show that this particular gene was not involved in conferring resistant to QACs since there was little, if any, difference in susceptibility to the QAC treatments between the MDR and non-MDR *Salmonella* that were tested.

Pseudomonas aeruginosa was included in the QACs experiments as a quality control strain due to its intrinsic resistance to QACs (*30*). In all cases, it was significantly more resistant to QACs than the *Salmonella* strains.

Survival of MDR and non-MDR *Salmonella* in biofilms after quaternary ammonium compounds treatments

Results of this study agree with previous reports of bacterial capability to attach to surfaces such as stainless steel and their ability to be more resistant to sanitizers than planktonic bacteria (9, *26, 27*). In our findings, planktonic cells were more effectively reduced (5.9 log CFU/ml) than the biofilm cells (2.6 log CFU/cm^2), after treatment with the quaternary ammonium compounds tested. Frank and Koffi (*9*) reported total inactivation of planktonic cells of *Listeria monocytogenes* after 30 s when using 100, 400, and 800 ppm of benzalkonium chloride, and 3 log decrease on biofilm cells after 10 m treatment when the same disinfectant was used at 200 ppm. Frank and Chmielewski (*10*) reported 5 log unit reductions in populations of *Staphylococcus* that were allowed to attach for 4 h to different stainless steel surfaces and then treated with 200 ppm QAC.

Mosteller and Bishop (*24*) reported that sanitizers do not work with the same efficacy over attached bacteria and that it would be desirable for a sanitizer to reduce the number of surface attached bacteria by 3-log cycles. Based on this the results in this study were similar to that target since the highest reduction was 2.9 log and the lowest reduction was 2.6 log.

Not all the QACs tested against planktonic cells were equally effective. Benzalkonium chloride and cetylpiridinium chloride were more effective ($p<0.05$) than the commercial QAC (SS4). In contrast, there was no significant difference ($p>0.05$) among the effectiveness of quaternary ammonium compounds when they were used on biofilm cells. Meyer (*22*) reported that nonoxidative biocides such as quaternary ammonium compounds are the least effective against treatment of biofilm cells, and oxidizing agents such as chlorine and peroxacetic acid are the most effective. In addition, it has been reported that QACs are more effective against Gram positive microorganisms than against Gram negative ones (*24, 29*). The lethal effect is attributed to reactions with cell membranes, denaturation of proteins, and inactivation of enzymes.

In this study, we used stainless steel as the attachment surface since this material is widely used in food processing plants and if there are favorable conditions, microorganisms attach easily to it (*8, 37*). Finish of the stainless steel surface seems to play important role in the effectiveness of sanitizers over biofilms. A rougher finish is more difficult to clean (*10, 18*).

In general, there was no significant difference ($p>0.05$) between the MDR and non-MDR *Salmonella* populations survival rates. Benzalkonium chloride was the most effective QAC followed in most cases by SS4, the commercial QAC, and then cetylpyridinium chloride.

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Chapter 4

CONCLUSION

The results in this study showed that treatments with organic acids and quaternary ammonium compounds were equally effective against MDR and non-MDR *Salmonella* strains, so pork producers should not be concerned about the practices in place. Quaternary ammonium compounds were less effective against bacteria that were attached to the stainless steel surface than when they were free in suspension. This agrees with what has been reported in literature regarding to biofilms and their resistance to antimicrobials, so it would be recommended for food producers to keep good manufacture practices in order to avoid their formation and possible health hazards.

Table 3.1. Population decrease of acid adapted (pH 6.0) and non-adapted (pH 7.2) *Salmonella* after subsequent exposure to either acetic or lactic acids 2%.

Treatment	Log reduction
Adapted (pH 6.0)	2.59^{a}
Non-adapted (pH 7.2)	2.76^{b}

^{ab} Values with different superscripts were significantly different ($p<0.05$) by Tukey's multiple comparison method. Initial population 10^{8} CFU/ml

Table 3.2. Population decrease for acetic and lactic acid at pH 6.0 and 7.2.

Treament	pH	Log reduction
Acetic	6.0	2.63
Lactic	6.0	2.54
Acetic	7.2	2.54
Lactic	7.2	2.97

Initial population 10^{8} CFU/ml

Table 3.3. Population decrease of acid adapted (pH 6.0) and non-adapted (pH 7.2) *Salmonella* after 4 h exposure to 2% acetic and 2% lactic acid.

Acid at both pHs	Log reduction
Acetic	2.58^{a}
Lactic	2.75^{b}

^{ab}Values with different superscripts were significantly different ($p<0.05$) by Tukey's multiple comparison method. Initial population 10^{8} CFU/ml

Table 3.4. Population decrease of multidrug resistant (MDR) and non- MDR *Salmonella* after treatment with either acetic or lactic acids 2%.

Group	Log reduction
MDR	2.59^{a}
Non-MDR	2.89^{b}

^{ab}Values with different superscripts were significantly different ($p<0.05$) by Tukey's multiple comparison method. Initial population 10^{8} CFU/ml

Table 3.5. Population decrease for MDR and non-MDR *Salmonella* after treatment with quaternary ammonium compounds over time.

Time (sec)	Log reduction
20	5.0^{c}
120	6.1^{b}
300	6.6^{a}
600	6.8^{a}

[abc] Values with different superscripts were significantly different ($p<0.05$) by Tukey's multiple comparison method. Initial population 10^{8} CFU/ml

Table 3.6. Population decrease for MDR and non-MDR *Salmonella* populations to quaternary ammonium compounds.

Group	Log reduction
MDR	6.2^{a}
Non-MDR	5.9^{b}

[ab] Values with different superscripts were significantly different ($p<0.05$) by Tukey 's multiple comparison method. Initial population 10^{8} CFU/ml

Table 3.7. Population decrease of MDR and non-MDR *Salmonella* after exposure to quaternary ammonium compounds 100 and 200 ppm.

Concentration (ppm)	Log reduction
100	5.8^{b}
200	6.4^{a}

[ab] Values with the same superscripts were significantly different ($p>0.05$) by Tukey's multiple comparison method. Initial population 10^{8} CFU/ml

Table 3.8. Population decrease of acid adapted (pH 6.0) and non-adapted (pH 7.2) MDR and non-MDR *Salmonella* after treatment with quaternary ammonium compounds (33 ppm).

Treatment	Log reduction
Adapted (6.0)	2.79^{a}
Non-adapted (7.2)	2.98^{a}

[a] Values with the same superscripts were not significantly different ($p>0.05$) by Tukey's multiple comparison method. Initial population 10^{8} CFU/ml

Table 3.9. Population decrease of MDR and non-MDR *Salmonella* after treatment with quaternary ammonium compounds (33 ppm).

Group	Log reduction
MDR	2.85[a]
Non-MDR	2.99[a]

[a]Values with the same superscripts were not significantly different ($p>0.05$) by Tukey's multiple comparison method. Initial population 10^8 CFU/ml

Table 3.10. Population decrease of MDR and non-MDR *Salmonella* populations after exposure to the different quaternary ammonium compounds (33 ppm).

QAC	Log reduction
BK	3.57[a]
SS4	3.15[a]
CPC	1.93[b]

[ab] Values with different superscripts were significantly different ($p<0.05$) by Tukey's multiple comparison method. Initial population 10^8 CFU/ml

BK: benzalkonium chloride, CPC: cetylpyridinium chloride, SS4: commercial QAC

Table 3.11. Population decrease of planktonic MDR and non-MDR *Salmonella* populations after treatment with quaternary ammonium compounds (200 ppm).

Group	Log reduction
MDR	6.00[a]
Non-MDR	6.01[a]

[a]Values with the same superscripts were not significantly different ($p>0.05$) by Tukey's multiple comparison method. Initial population 10^8 CFU/ml

Table 3.12. Population decrease of planktonic MDR and non-MDR Sa*lmonella* populations to different quaternary ammonium compounds (200 ppm).

QAC	Log reduction
BK	6.24[a]
SS4	5.20[b]
CPC	6.11[a]

[ab] Values with different superscripts were significantly different ($p<0.05$) by Tukey's multiple comparison method. . Initial population 10^8 CFU/ml

BK: benzalkonium chloride, CPC: cetylpyridinium chloride, SS4: commercial QAC

Table 3.13. Population decrease of MDR and non-MDR *Salmonella* populations in an established biofilm after quaternary ammonium compounds treatment (200 ppm).

Group	Log reduction
MDR	2.73[a]
Non-MDR	2.75[a]

[a] Values with the same superscripts were not significantly different ($p>0.05$) by Tukey 's multiple comparison method. Initial population 10^6 CFU/cm^2

Table 3.14. Population decrease of MDR and non-MDR *Salmonella* populations in an established biofilm after exposure to the different quaternary ammonium compounds (200 ppm).

QAC	Log reduction
BK	2.61^a
SS4	2.45^a
CPC	2.64^a

^{a}Values with the same superscripts were not significantly different ($p>0.05$) by Tukey's multiple comparison method. . Initial population 10^6 CFU/cm^2

BK: benzalkonium chloride, CPC: cetylpyridinium chloride, SS4: commercial QAC

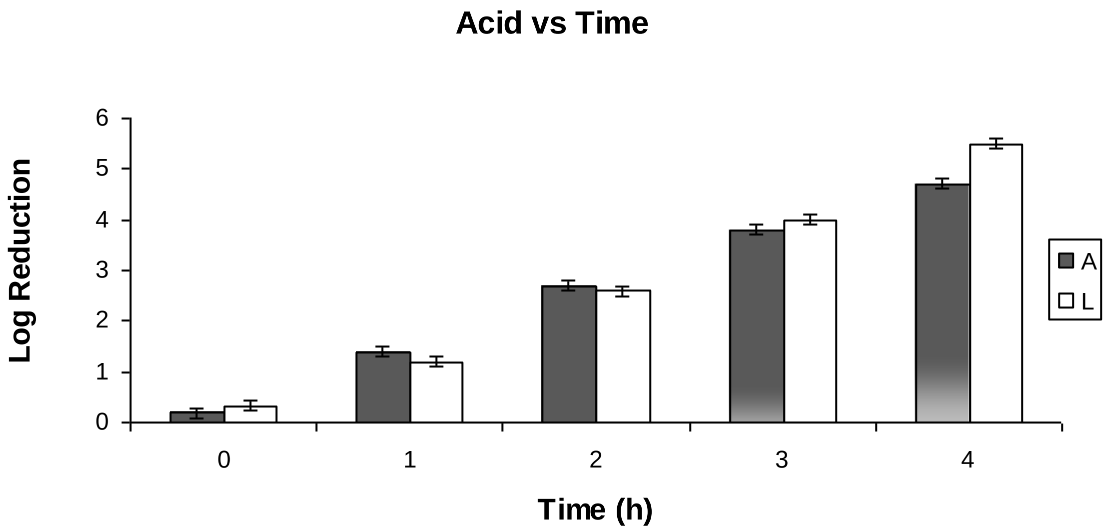

Figure 3.1. Population decrease of acid adapted and non-adapted *Salmonella* (MDR and non-MDR) after 4 h exposure to 2% acetic or 2% lactic acids. A: Acetic acid, L: Lactic acid.

Initial population 10^8 CFU/ml

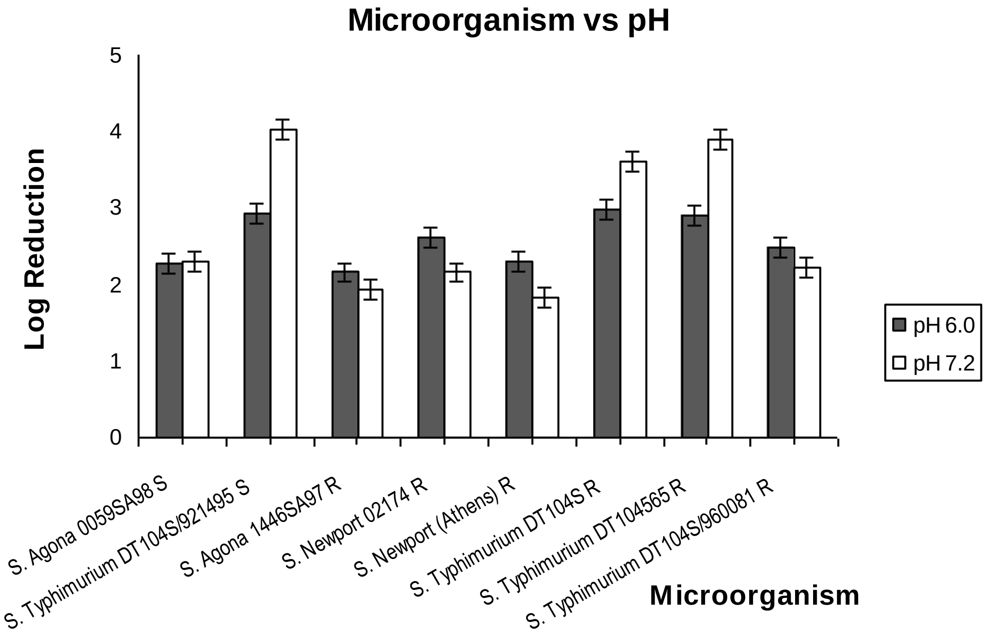

Figure 3.2. Population decrease for individual MDR and non-MDR *Salmonella* isolates that were acid adapted (pH 6.0) and non-adapted (pH 7.2) in response to acids (2% acetic or 2% lactic acids) exposure.

Initial population 10^8 CFU/ml

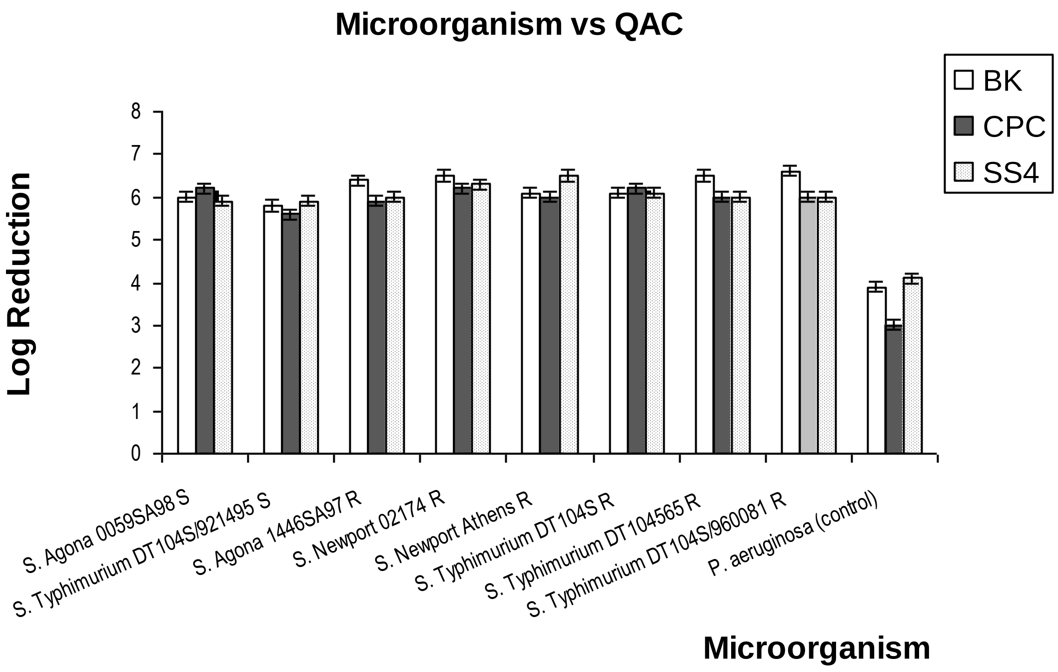

Figure 3.3. Population decrease of *Salmonella* (MDR and non- MDR) after quaternary ammonium compound treatments (100 and 200 ppm). BK: benzalkonium chloride, CPC: cetylpyridinium chloride, SS4: commercial QAC. Initial population 10^8 CFU/ml

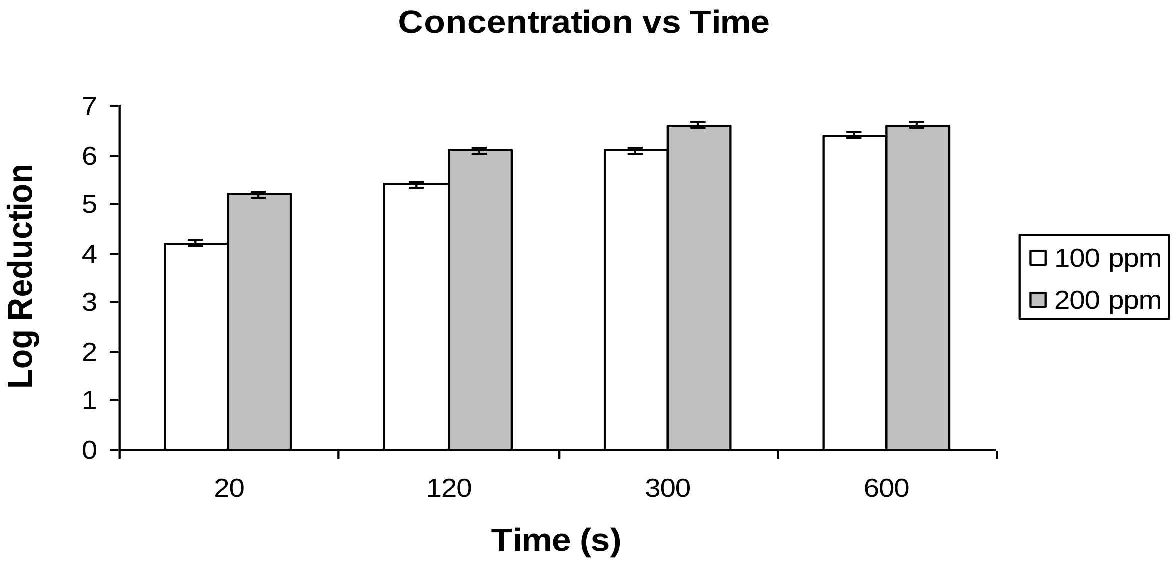

Fig 3.4. Population decrease of MDR and non-MDR *Salmonella* after exposure to 100 ppm and 200 ppm QACs over time.

Initial population 10^8 CFU/ml

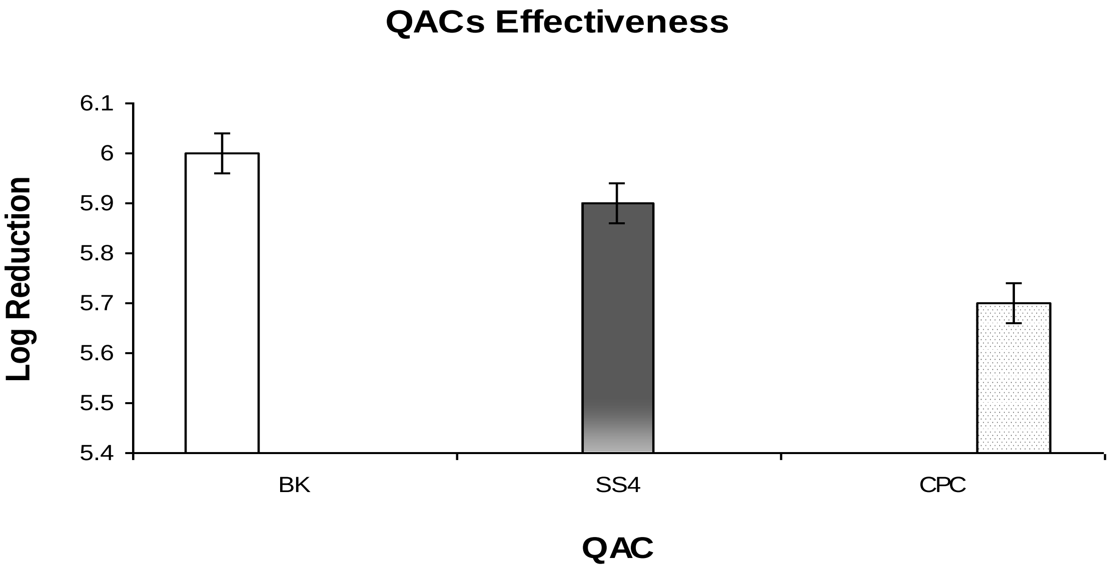

Figure 3.5. Population decrease of MDR and non-MDR *Salmonella* after 600 s exposure to belzalkonium chloride (BK), a commercial quaternary ammonium compound (SS4), and cetylpyridinium chloride (CPC).

Initial population 10^8 CFU/ml

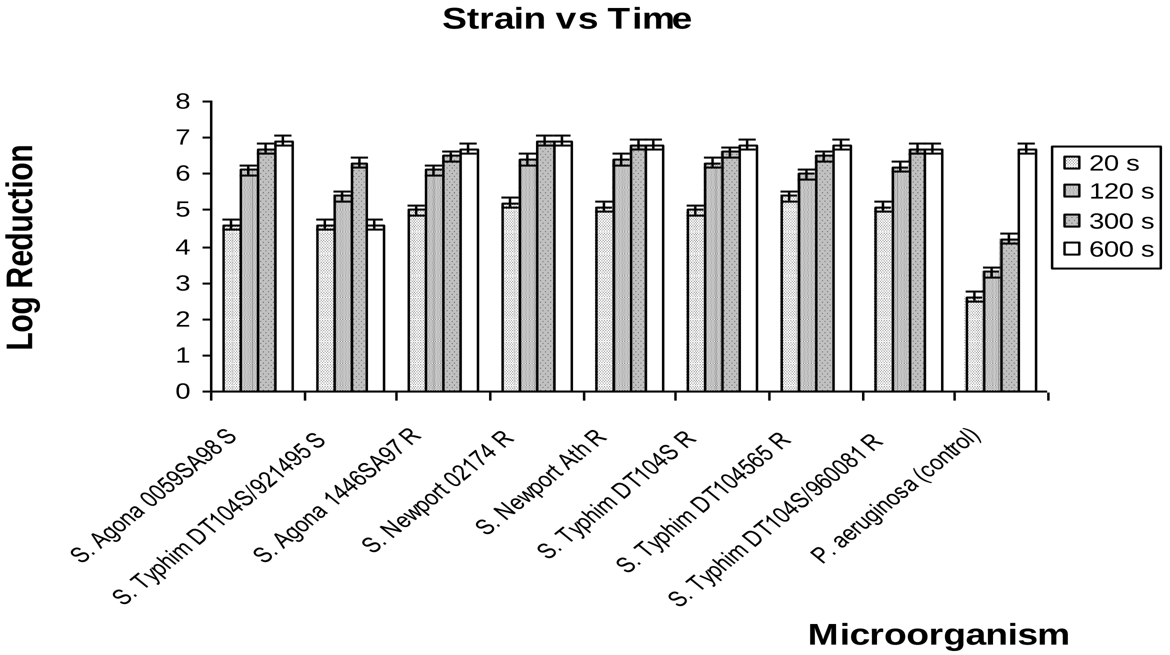

Figure 3.6. Population decrease of MDR and non-MDR *Salmonella* when exposed to benzalkonium chloride (BK), a commercial quaternary ammonium compound (SS4), and cetylpyridinium chloride (CPC) over time (s).

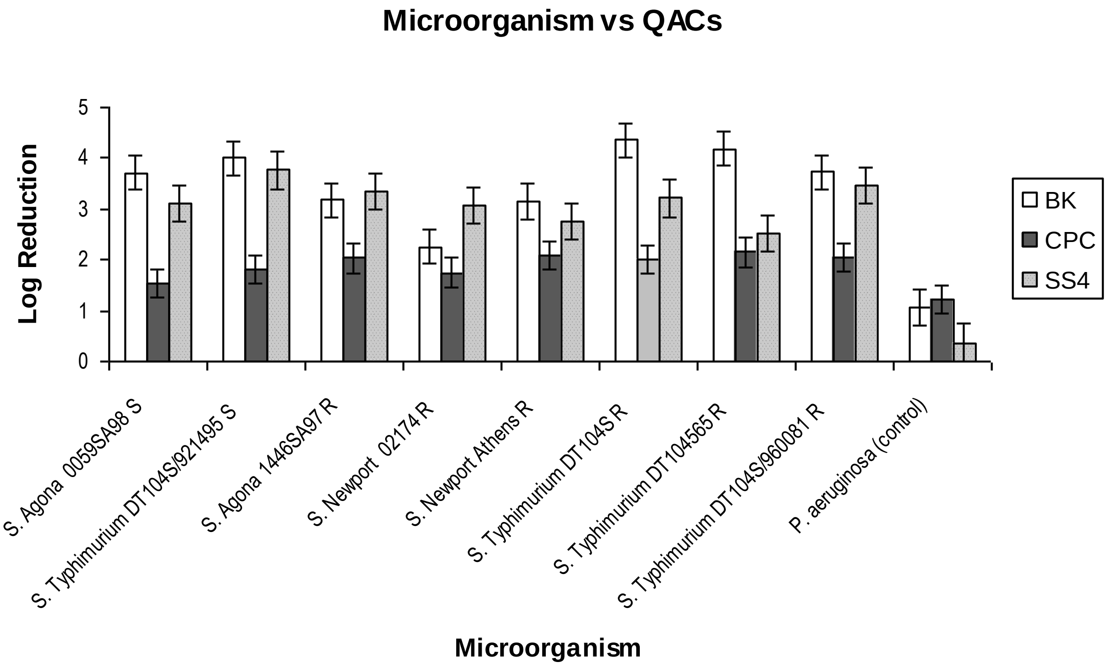

Fig 3.7. Population decrease of individual MDR and non-MDR *Salmonella* when acid adapted (pH 6.0) and non-adapted (pH 7.2), and after quaternary ammonium compounds (33 ppm) exposure. BK: benzalkonium chloride, CPC: cetylpyridinium chloride, SS4: commercial QAC.

Initial population 10^8 CFU/ml

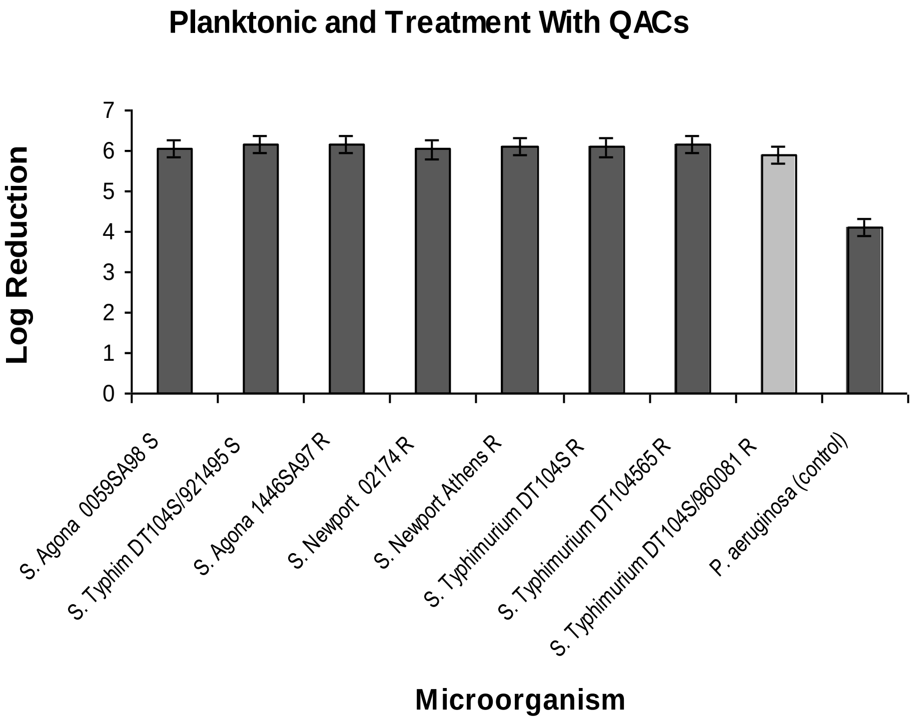

Figure 3.8. Population decrease of individual strains of planktonic MDR and non-MDR *Salmonella* after treatment with quaternary ammonium compounds (200 ppm). Initial population 10^8 CFU/ml

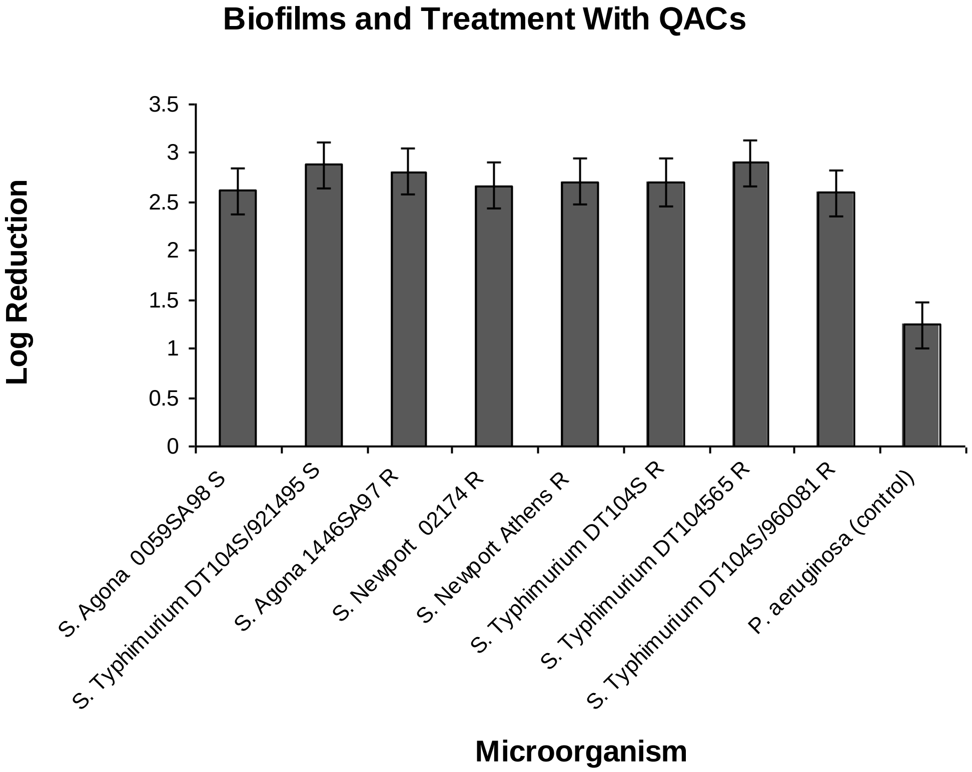

Figure 3.9. Population decease of individual strains of MDR and non-MDR *Salmonella* in an established biofilm to quaternary ammonium compounds (200 ppm). Initial population 106 CFU/cm^2