Evaluating the Antimicrobial Mechanism of Neutral Electrochemically Activated Water on Foodborne Pathogens and Their Biofilms

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Dedication

This dissertation is dedicated to my family and teachers, who supported my career development and personal life.

Abstract

Sanitizing is a key step in ensuring food safety. Neutral electrochemically activated water (NECAW) not only has antimicrobial effects but also is relatively friendly to handlers and foods, and it complies with organic food practices. However, its antimicrobial effects on different pathogens and strains have not been examined and its antimicrobial mechanisms are not fully understood. The goal of this project was to investigate the use of NECAW as a sanitizer in inactivating foodborne pathogens.

The first objective of this study was to determine the effectiveness and broad-spectrum activity of NECAW against foodborne pathogens. The sanitizing efficacy of NECAW against 40 different strains of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* as liquid cultures, dried cells on stainless steel (SS) surfaces, and biofilms on SS was examined. It was found that NECAW with 100 mg/l free available chlorine (FAC) caused more than 5 log CFU/ml reductions for all strains in liquid culture, more than 3 log CFU/coupon reductions for 92.5% of the strains dried on stainless steel (SS) surfaces and for 27.5% of biofilms. Among all the strains, *S.* Newport B4442CDC was the most resistant strain to NECAW on surfaces while *E. coli* O157:H7 ATCC 43895 was the most resistant strain in biofilms and liquid pure cultures. Overall, NECAW was effective and had a broad-spectrum activity against foodborne pathogens.

The second objective was to evaluate the sanitizing effect of NECAW and other commercial 'active water' technologies on foodborne pathogens. Five individual strains of each foodborne pathogen including *E. coli* O157:H7, *L. monocytogenes,* and *Salmonella* as liquid cultures and dried cells on stainless steel surfaces were studied by

comparing the sanitizing effect of NECAW and available commercial technologies on these pathogens. The results showed that 100 mg/l ECAW had sanitizing effects of at least 5 log CFU/ml reductions on liquid culture and more than 4 log CFU/coupon reductions for pathogens dried on SS surfaces. In contrast, the other commercial technologies tested were not effective in sanitizing. They, however, washed the bacteria off the surface into rinse water, which would lead to a significant safety concern of cross contamination.

The third objective of this study was to examine the three dimensional morphology of foodborne pathogen biofilms using atomic force microscopy (AFM). AFM experiments were conducted by directly imaging the three-dimensional morphology of the foodborne pathogens biofilms (five strains for each pathogen), and imaging the biofilms pre- and post-NECAW treatment. Images of AFM showed tree-like structures as well as individual cells. *L. monocytogenes* biofilms had a higher percentage of tree-like structures than *E. coli* O157:H7 and *Salmonella*. The biofilm structures and microbial cells on SS were destroyed by the treatment with NECAW according to the AFM study, providing morphological evidence that NECAW was effective in controlling surface contamination of pathogenic bacteria and biofilm growth.

The fourth objective of this study was to assess the role of *sig*B and *inlA* genes in *L. monocytogenes* biofilm formation and the antimicrobial efficacy of NECAW treatment on *L. monocytogenes.* The expression levels of *sigB* and *inlA* genes were investigated by using qPCR, and sanitizing effects of NECAW on wild-type (WT) and *sigB*/*inlA* mutant *L. monocytogenes* strains were determined by the direct plating method. After NECAW

treatment, expression of both genes increased for the WT. While *sigB* gene expression of the Δ*inlA* strain increased to a level comparable to that of the WT, *inlA* gene expression of the Δ*sigB* strain did not significantly increase. Both genes were expressed more in biofilms than in liquid cultures. The level of *inlA* gene expression in WT increased by 4.28 and 5.51-fold by treatment with 4 mg/l NECAW for 10 min in liquid cultures and biofilms, respectively, while the corresponding values were 5.91 and 10.05-fold for the *sigB* gene. Mutant strains were more sensitive to NECAW treatment than the WT strain. The *sigB* gene was more important than *inlA* for the pathogen's survival under NECAW treatment. Surviving *L. monocytogenes* cells post-sublethal NECAW treatment might become resistant to further sanitizer treatment.

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CHAPTER I

INTRODUCTION AND STATEMENT OF THE PROBLEM

1. 1. Introduction

Sanitizing is a key step in food processing. Due to strict regulations by the National Organic Standards Board (NOSB), only a limited number of synthetic sanitizers can be applied to organic produce. Considering that these sanitizers may not be suitable for all organic produce processing, organic fresh produce faces potential safety issues. Therefore, it is very necessary to develop organic industry-compatible sanitizers to meet needs. Electrochemically activated water (ECAW), also referred to as electrolyzed water, has antimicrobial effects and does not violate the NOSB principles for sanitizers used for organic foods *(98, 181, 247)*. However, a thorough elucidation of its antimicrobial effects has not been conducted and the mechanisms of antimicrobial inactivation are not fully understood *(60, 166, 293, 341)*. The latter is greatly needed to make significant progress enhancing the antimicrobial effects of ECAW as a viable sanitizer. Further determination of the effects of neutral electrochemically activated water (NECAW) against foodborne pathogens and elucidation of the mechanism of its effects would improve the application of NECAW in the sanitization of food processing equipment and fresh produce.

1.2. Statement of the problem

Sanitizing is an important step for guaranteeing the safety of the food supply, however for organic foods there are very few approved sanitizers. Current producers of organic foods have to resort to a limited number of synthetic sanitizers to kill pathogens on food equipment surfaces as well as on fresh produce *(99, 163, 178, 216)*. For most of the few sanitizers allowed for direct use for organic products, only relatively low concentrations are permitted at which antimicrobial effects are limited. In addition, prohibited sanitizers should be rinsed off the treated surface to prevent contamination of food. This practice significantly increases the risk of further contamination during rinsing.

Due to the strict regulation of sanitizer usage, the safety of organic products becomes critical compared to that of their conventional counterparts. Potentially, ECAW is a relatively environmentally friendly sanitizer that is promising for sanitizing organic fresh produce, and application of ECAW is compatible with the principles of organic processing. However, the broad-spectrum effect of ECAW has not been studied and the antimicrobial mechanism is not clear. In addition, there are a few products whose marketers said the products had enough sanitizing effect while being small and cheap. However, their statements do not have scientific support. It is essential to accurately compare the sanitizing effects between ECAW and these commercial products. This project took a unique approach to comprehensively test the broad-spectrum effects of NECAW on three important foodborne pathogens. By elucidating in-depth biofilm morphology and gene transcriptional changes upon NECAW treatment, the results could be used to improve NECAW antimicrobial effects by optimizing equipment design.

Since only water and sodium chlorine is used for generating sanitization *(98, 124)*, NECAW technology is fairly safe to workers and relatively friendly to the environment. The results of this dissertation could also provide directions for better control of foodborne pathogens and developing reasonable sanitizers for the food industry. In addition, the data could be useful for in-depth understanding of the sanitizing mechanism of NECAW, from which corresponding technology could be applied to optimize sanitation equipment, and gene-targeting solutions can be proposed as well.

1.3. Goals, Research hypotheses (H_0), and Objectives

Goals:

The goal of this project was to investigate the application of NECAW as a sanitizer in inactivating foodborne pathogens.

Null Hypothesis I:

Different foodborne pathogens have the same sensitivity to NECAW;

Null Hypothesis II:

The sanitizing effects among NECAW and other commercial “active water” technologies on foodborne pathogens are not significantly different;

Null Hypothesis III:

Different foodborne pathogens have similar biofilm morphology and characteristics;

Null Hypothesis IV:

sigB and *inlA* gene do not play a role in *L. monocytogenes* biofilm formation and the antimicrobial efficacy of NECAW treatment on *L. monocytogenes.*

Objectives:

1. Determine the effectiveness and broad-spectrum activity of NECAW against foodborne pathogens.
2. Evaluate the sanitizing effect of NECAW and other commercial 'active water' technologies on foodborne pathogens.
3. Examine the three dimensional morphology of foodborne pathogen biofilms using atomic force microscopy.
4. Assess the role of *sig*B and *inlA* gene in *L. monocytogenes* biofilm formation and the antimicrobial efficacy of NECAW treatment on *L. monocytogenes.*

CHAPTER II

A REVIEW OF FOOD SAFETY, FOODBORNE PATHOGENS, ORGANIC FOODS, FOOD SANITIZATION & RESISTANCE

2.1. Food safety concerns

It has been estimated that contaminated foods cause the sickness of roughly 48 million people, the hospitalization of 128,000, and the death of 3,000 every year, according to recent estimates from the Centers for Disease Control and Prevention (CDC) *(273, 274)*. These figures pose a major challenge to public health in the U. S.

Food safety generates more and more concern nowadays. While an increasing number of food companies allege they have invested more money in increasing the safety of food that they produce, some surveys indicate that most consumers still lack confidence in food safety. A recent survey revealed that less than 20 percent of consumers trust food companies in developing and selling food products that are safe and healthy, and around 60 percent of consumers are concerned about the safety of food they purchase *(43)*. Another recent study done by the Center for Food Integrity's (CFI) "2008 Consumer Trust Survey" revealed that food safety is one of the top concerns— surprisingly ranking higher than even concerns about the Iraq War *(54)*. The study also showed that consumers believe farmers and food processors should take the main responsibility of maintaining a safe food supply, reducing and eventually eliminating foodborne illness

outbreaks.

Even though many advanced technologies can be applied to prevent foodborne diseases, incorrect personal hygiene can still be a serious problem compromising the safety of foods. A case occurred several years ago in which at least 96 students at Georgetown University fell ill after eating burritos at a cafeteria. The most probable reason was a deficiency in personal hygiene, as sinks used for handwashing in the service area lacked hand soap *(111, 252).*

Along with these above mentioned issues, there are also other concerns and different opinions with regard to how to improve the safety of foods in the U.S. For instance, should the current regulatory system be changed? Should a single food safety agency regulate all foods? What should be prioritized with a limited budget in order to be more efficient? How to prevent a bioterrorist attack through the food supply? Should more funds be spent on consumer education? Should more investment be put on research to develop more advanced techniques to control pathogenic organisms?

2.2. Fresh produce safety

Due to increased *per capita* consumption of fresh produce and long-distance transportation, fresh produce is increasingly recognized as a vehicle of foodborne outbreaks in the US as well as in many other countries. They are often consumed raw and easily contaminated by many pathogenic bacteria through water, soil, insects and other environmental sources that might have been in contact with animal feces or manure *(208).* Their consumption can cause nausea, vomiting, abdominal cramps and fever as well as

chronic diseases.

S. enterica and *Escherichia coli* O157:H7 are the two main agents of outbreaks associated with fresh produce. From the 1970s to 1990s, outbreaks associated with fresh produce increased from 1% to 6% among the cases in which the vehicle was confirmed *(53)*. Leafy vegetables (17%) and fruits/nuts (16%) ranked as the second and third commodity groups among outbreaks caused by a single food vehicle in 2006 *(13)*. Since fresh produce is widely consumed, the affected population could be very large if contamination happens. Recent large outbreaks of *E. coli* O157:H7 due to bagged baby spinach, and *Salmonella* Saintpaul infections linked to peppers or tomatoes are two representative cases *(53)(13)*.

Recently, fresh produce has been linked to *E. coli* O157:H7 outbreaks *(25, 97, 100, 107, 250)*. One example is the 2006 North American *E. coli* O157:H7 outbreak from spinach. At least 276 people illnesses and 3 deaths were due to the consumption of the contaminated spinach *(48)*.

2.3. The current safety issues of *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes*

Foodborne diseases are a major health problem in the United States. Foodborne diseases caused by fresh produce continue to be a major concern for industry, government and consumers as a result of recent outbreaks due to a variety of fresh fruits and vegetables. In 2006, data collected by the Foodborne Disease Outbreak Surveillance System of the CDC indicated that *Samonella* is the second most common cause of

confirmed foodborne disease outbreaks. In addition, *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* were responsible for the largest number of deaths linked to foods (82%)*(192)*. *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* are three of the most important infectious bacteria targeted for reductions in the CDC's Healthy People 2010 plan, but only the target for *E. coli* O157:H7 was met in 2009 *(192)*. As compared to the preceding 3 years, however, the estimated incidence of infections caused by *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* in 2008 did not decrease significantly *(317)*.

2.3.1. *E. coli* O157:H7 and its current safety issues

E. coli is a common bacterium that normally lives in the intestines of human beings and other animals. Though most of *E. coli* strains are avirulent and normally beneficial to their hosts, some *E. coli* strains have the capability of causing human illness. *E. coli* O157:H7 is an enterohemorrhagic strain able to produce Shiga toxins that cause severe damage to the intestine and kidneys of the host. The acute disease caused by *E. coli* O157:H7 is hemorrhagic colitis, and its typical symptoms usually include severe cramping and diarrhea with no or only slight fever, and occasional vomiting. Some infected persons, especially children and the elderly, may develop hemolytic uremic syndrome (HUS), characterized by thrombocytopenia, intravascular hemolytic anemia, and acute kidney injury *(31, 109, 301, 331)*, which can lead to kidney failure and death. It was estimated that *E. coli* O157:H7 is responsible for 2,138 hospitalizations and 20 or more deaths each year in the U.S. *(274)*, justifying increased efforts for controlling this pathogen.

E. coli O157:H7 can easily be transmitted from contaminated soil and water to food *(173, 227, 338)*. Ground beef has been implicated as the main source of *E. coli* O157:H7 infections *(10)*. The consumption of ground beef per year in the U.S. is approximately 2.0 billion lbs, which accounts for 48.9% of all beef products with approximately $17 billion gross retail values *(47)*. *E. coli* O157:H7 contamination of ground beef frequently leads to outbreaks and recalls.

Two recent examples of *E. coli* O157:H7 outbreaks occurred in 2007. One happened at the end of September 2007, in which the largest amount of ground beef recalled in the latest ten years, 21.7 million pounds of frozen hamburger, was announced by Topps Meat, Inc. because of possible infection of *E. coli* O157:H7. USDA reported that 27 illnesses with 3 confirmed were associated with this outbreak *(276)*. The second case occurred just several days later when four Minnesota children and four Wisconsin residents were infected by consuming ground beef patties from Sam's Club stores in the Twin Cities. During this outbreak, two patients developed hemolytic uremic syndrome (HUS) *(83)*.

It is urgent that more preventive measures are taken to improve the safety of the nation's ground beef supply as inspections are not sufficient. Freezing doesn't effectively kill *E. coli* O157:H7 *(82)*. Though high temperature cooking is an effective way to kill *E. coli* O157:H7, consumers frequently prefer not fully cooked meat. In addition, the use of thermometers has not been widely adapted. Therefore, it is necessary to find an alternative way to control foodborne pathogens including *E. coli* O157:H7, especially for ground beef.

2.3.2. *S. enterica* and its current safety issues

Salmonella are a group of pathogenic bacteria commonly found in the gastrointestinal tract of animals. In the U.S., *Salmonella* nontyphoidal strains are the main concern for *Salmonella* outbreaks with hospitalization of 19,336 and 378 deaths annually *(274)*. In Minnesota, for instance, there were 39 confirmed salmonellosis outbreaks due to contaminated product during 1995-2003 *(122, 196)*. Foodborne *Salmonella* outbreaks often were due to contaminated chicken products, meat *(108)*, egg products *(44, 298)*, dairy products *(38)*, peanut butter *(195, 280)*, and food workers *(197)*. One critical source of foodborne outbreaks of salmonellosis was infected food workers, especially workers in restaurants, since *Salmonella* can be transferred from infected people's hands to food *(122, 146)*.

Recently, raw produce has been increasingly recognized as the vehicle for salmonellosis. In 2008, an outbreak of *Salmonella* Saintpaul affected approximately 1500 sick people, caused 2 deaths, and was associated with jalapeño peppers by epidemiologic and microbiologic evidence *(21)*. Interestingly, foodborne outbreaks of *Salmonella* are sometimes serovar dependent on specific food, such as in the case of tomatoes *(281)*.

2.3.3. *L. monocytogenes* and its current safety issues

2.3.3.1. General properties and safety issues of *L. monocytogenes* outbreaks

L. monocytogenes is a ubiquitous, facultative and non-spore forming intracellular foodborne pathogen that causes an invasive infection named listeriosis in both humans

and animals. This pathogen can be more severe and even deadly for pregnant women, newborns, the elderly, and people with weakened immune systems *(76, 136, 188)*. *L. monocytogenes* can tolerate different disadvantageous environments in human bodies, including but not limited to bile salts, organic acids and osmotic conditions *(149, 203)*. In the U.S., *L. monocytogenes* infection causes annually 1455 hospitalizations and 255 deaths *(274)*.

This pathogen can colonize the surface of food utensils or processing equipment *(139)*, and also form biofilms which are more resistant to treatments and cleaning than their planktonic states *(94, 161, 308)*. Previous studies indicated that *L. monocytogenes* strains vary in their ability to adhere to stainless steel surfaces and form biofilms *(94)*. When forming biofilms, *L. monocytogenes* microbial cells are enclosed in a matrix fundamentally made up of polysaccharides, which leads to a greater resistance to sanitizers compared to planktonic cells *(94, 179)*.

L. monocytogenes outbreaks are often linked to meat, dairy and egg products *(41, 64, 133, 155, 156, 176, 177, 200, 210, 228, 229)*, especially when they are in the category of ready-to-eat foods (RTE foods). “RTE foods” are generally consumed without cooking, or reasonably appear to be suitable for consumption without cooking by consumers. Due to modern lifestyles, people consume more RTE foods than before to save time on food preparation and cooking *(155)*. One example of *Listeria* outbreak associated with RTE foods is the 2008 Canadian listeriosis outbreak associated with cold cuts produced by a Maple Leaf Foods plant in Toronto, Ontario *(100)*. Deaths of 23 and 57 total confirmed cases were related to this listeriosis outbreak. This outbreak once again aroused safety

concerns of *L. monocytogenes* outbreaks.

2.3.3.2. Role of *sigB* and *inlA* genes in *L. monocytogenes* stress resistance

L. monocytogenes is generally pathogenic, with a genome size of approximately 2.94 Mbp *(106, 211)*. It survives well and resists processing conditions, and is capable of existing on stainless steel either as planktonic cells or as biofilm-forming communities. Interventions imposed by industrial cleaning agents upon *L. monocytogenes* are critical for its control. However, sometimes, biofilms can be found on conveyer belts and stainless steel surfaces of food equipment after sanitizing treatments. These biofilm residues on stainless steel make sanitization a big safety challenge *(94)*. In addition, the survival of *L. monocytogenes* after the utilization of cleaning agents may lead to easy transfer to food and an increased potential for foodborne pathogen contamination *(270)*. It is of great interest to determine whether some genes may play a role in *L. monocytogenes* biofilm formation and sanitizer resistance.

Sigma factors are dissociable protein subunits that direct bacterial RNA polymerase holoenzymes for promoter sequence recognition upstream of genes prior to transcription initiation. Sigma factor σ^B, encoded by *sigB*, is an essential molecule in stress responses in many Gram-positive bacteria, including *L. monocytogenes (256)*. σ^B protects *L. monocytogenes* and other microorganisms against many environmental stresses *(89, 94, 150)*, and is essential for the resistance of *L. monocytogenes* to some sanitizers at lethal *(270)* or sublethal levels *(112)*. In addition, it was reported that σ^B was critical for biofilm formation and sporulation in *L. monocytogenes (270, 309)*. However,

Schwab et al. *(277)* reported that σ^B was not essential for initial surface attachment of *L. monocytogenes (55)* and σ^B-directed genes were not necessary for the spread of *L. monocytogenes* in mice *(323)*. It was also found that the activation of σ^B in response to osmotic stress in *L. monocytogenes* was rapid but transient, and was proportional to stress strength *(306)*.

The gene *inlA* is a key virulence factor specific to *L. monocytogenes (253)*. It encodes internalin (InlA), an 800-amino acid surface protein which can facilitate the entry of *L. monocytogenes* into epithelial cells expressing specific forms of E-cadherin. InlA is a cell-wall-anchored protein which is necessary for efficient Caco-2 and HepG2 invasion *(144, 261)*. Infection of hepatic cells by *L. monocytogenes* in vivo does not require the protein products of the *inlAB* operon *(113)*. It is believed that InlA contributes to *L. monocytogenes'* invasion of intestinal epithelial cells, an important step in the pathogenesis of systemic listeriosis *(218)*.

L. monocytogenes strains differ in their virulence and/or transmission characteristics and thus their ability to cause human diseases *(219)*. The level of *inlA* gene expression is associated with virulence and/or transmission characteristics. A positive correlation between the expression of the *inlA* gene and the strength of *L. monocytogenes* attachment to glass surfaces was recently reported *(57)*. Multiple nonsense mutations in *inlA* were reported led to the production of a truncated and secreted gene product. *L. monocytogenes* strains carrying these mutations were not only associated with reduced invasiveness in human intestinal epithelial cells but also represented a significant percentage of *L. monocytogenes* isolated from food samples

(218). In addition, several invasion-attenuated strains of *L. monocytogenes* showed reduced *inlA* transcript levels *(261)*. Furthermore, *inlA* premature stop codons are commonly found in *L. monocytogenes* isolated from foods *(217)*.

The *inlA* gene might be functionally related to the *sig*B gene of *L. monocytogenes*, although they have different functions. While *sig*B is a stress response gene, *inlA* is a virulence gene *(134)*, a σ^B (sigma factor B, encoded by *sig*B)-dependent promoter was identified upstream of *inlA*, and a loss of σ^B resulted in reduced levels of *inlA* expression and InlA in stationary-phase cells *(143)*. In addition, σ^B was found to contribute to *L. monocytogenes* invasion by controlling *inlA* expression *(144)*. The *inlA* gene might have a similar role as *sig*B, involved in the capability of *L. monocytogenes* to attach to food equipment surfaces and form biofilms *(56)*. Different strains of *L. monocytogenes* involved in outbreaks had significantly different invasion efficiencies and may utilize different mechanisms *(261)*. The regulation of *L. monocytogenes* virulence is very complex since the bacterium is still virulent even when some nonfunctional virulence genes are mutated *(263)*. Though different mechanisms may contribute to the varied ability of *L. monocytogenes* in causing listeriosis, transcription levels of *inlA* and *sigB* may play important roles. Figure 2.1 shows the function of σ^B and its relationship to *inlA* *(140)*. It indicates that *inlA* is transcribed by a promoter that is σ^B-dependent, and *inlB* is also possibly transcribed by the promoter *(140)*.

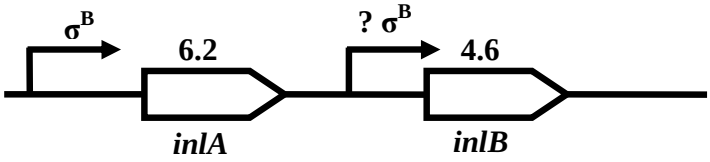


Figure 2.1. Possible relationship between *inlA* and σ^B in *Listeria monocytogenes (140)*. Numbers 6.2 and 4.6 in the Figure denote the expression ratios of wild type to mutant strains of *Listeria monocytogenes*. Arrows represent transcriptional start sites of the genes *inlA* and *inlB (29, 92, 168)*.

Source: Adapted from *(140)*

Studying *inlA* and *sigB* may lead to better elucidation of the molecular mechanism of *L. monocytogenes* biofilm formation and the effects of sanitizer on *L. monocytogenes (52)*. Additionally, it could be used to direct food processing and technology improvement. For instance, a lowered salt content of liver pâté did not significantly change the transcription level of virulence genes including *inlA*, which may indicate that it is safe to develop low salt content meat products *(225)*. However, one should be cautious when interpreting the experimental data since the virulence of *L. monocytogenes* is very complicated. The bacterium is still virulent even when some nonfunctional virulence genes are mutated *(263)*. Currently, both in vivo bioassays and in vitro cell assays used in evaluating *L. monocytogenes* virulence are not specific, as some targets exist in both virulent and avirulent *L. monocytogenes* strains *(171)*.

2.4. Foodborne pathogen biofilms

Biofilm formation is a mode microorganisms use to protect themselves and survive in disadvantageous environments *(116)*. Although different mechanisms exist for biofilm formation for many microbial cells *(150)*, it is thought that biofilms might be related to quorum sensing and the connections in sociomicrobiology *(245)*. In the food industry, the ability of a pathogen to attach to surfaces and further develop biofilms is in need of careful investigation. However, information about the initial attachment of microbial cells to a surface is still limited *(232)*. Different *L. monocytogenes* strains may differ in their abilities to attach to surfaces and many single strains are not capable of forming biofilms *(137)*.

L. monocytogenes biofilm growth and sanitizing treatment have been studied by different methods including crystal violet staining, ATP bioluminescence and total viable count *(160)*. ATP bioluminescence is a more appropriate method than crystal violet staining for biofilm bacterial growth considering a high correlation between ATP bioluminescence and biofilm growth *(268)*. For biofilm detection, with the help of fluorescein isothiocyanate- and tetramethyl rhodamine isothiocyanate-conjugated probes and confocal laser scanning microscopy (CLSM), the chemical compounds and spatial arrangements in extracellular polymeric substances (EPS) of a *Bacillus coagulans* biofilm were found to be nonuniformly distributed *(217, 330)*. Microbial cells in biofilms were also investigated. *E. coli* O157:H7, for instance, could be detected by a bacteriophage-based bioluminescent bioreporter using *lux* reporter genes *(37)*, bacteriophage-amplified bioluminescent sensing *(37, 260)*, and ATP bioluminescence immunoassay *(128)*. An alternative is to detect specific genes or proteins, for instance using the expression of the

Outer membrane protein F (OmpF) and OmpC variants which are specific to *E. coli* O157:H7 *(189)*.

2.5. Foodborne pathogens' morphology

2.5.1. Atomic force microscopy (AFM)

Microscopy is a common technology for investigating microstructure and morphology of target features. In food science, microscopy has been widely used for investigating morphology of foods and could be applied for improving processing technologies. Currently, transmission/scanning electron microscopy (TEM/SEM) *(120)*; light microscopy (LM) *(42)*, and confocal laser scanning microscopy *(70, 118, 184)* have been widely used. These techniques could provide different advantages for investigating detailed microstructures of food materials. However, in general, some of these techniques require complicated preparation steps which might affect the original state of the samples.

In 1986, a new microscopic technique was invented, which was named atomic force microscopy (AFM). The principle of AFM is that images are achieved by calculating variations in interactions between the AFM probe and the sample surface when the AFM probe scans the sample surface. Fig. 2.2 is a schematic image representing the AFM imaging process.

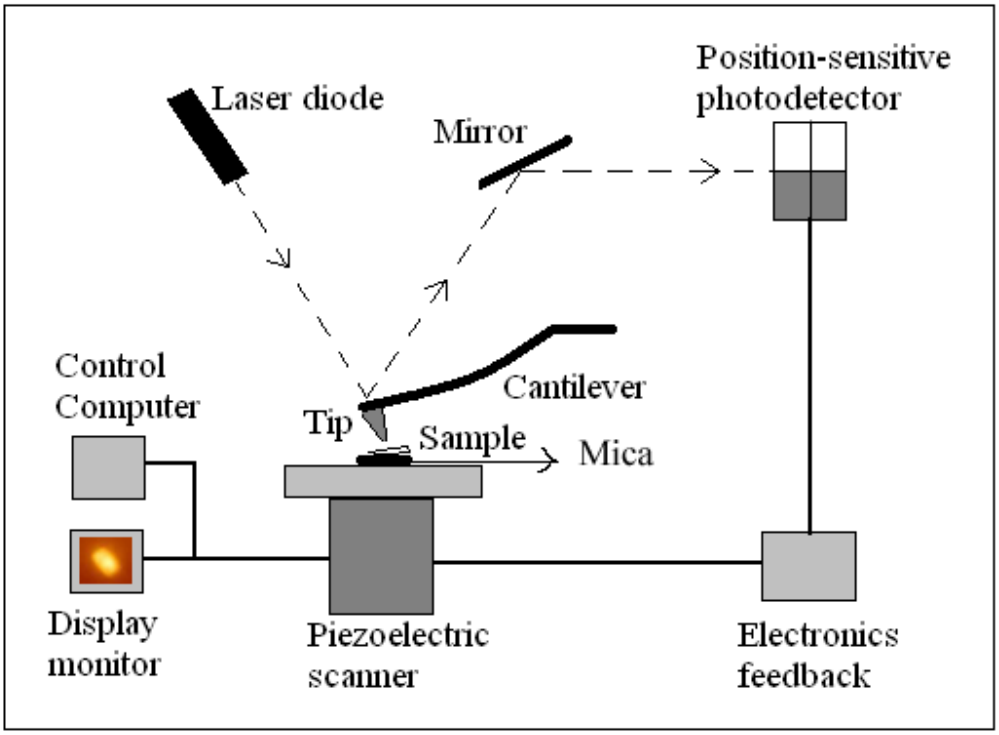


Figure 2.2. Principle of AFM imaging process. Reprinted with permission from Journal of Food Science 2008, 73, N44-50. Copyright (2008), John Wiley and Sons, Inc. *(334)*.

In brief, a beam of laser is transmitted from a laser diode onto the back of the AFM cantilever, reflected by a mirror and then reflected onto a photodiode detector. During scanning, the angle of the laser beam fluctuates due to variation in the interaction between the AFM tip and sample surface. The light signal is recorded by a photodiode, then an electrical signal is generated which quantifies the motion of the AFM tip. The surface morphology and other physical properties are displayed on a computer monitor. A second monitor shows the control screen, which can be modified for the next scanning

step *(206)*.

The distinct advantages of AFM mainly include high resolution and magnification, mild and simple sample preparation, and multimode imaging including three-dimensional imaging. One of the significant differences of AFM compared to other microscopic techniques is that it does not use any photons or lens for generating images. Thus the imaging is independent of the sample's transparency. Depending on the distance and fluctuation between the AFM tip and sample surface, AFM uses different operation modes. One of the most widely applied modes is the 'tapping mode', which is mainly used for imaging relatively soft materials in the field of biological science, including food science. Interestingly, the name given this mode may differ by different companies but the principles are similar. Table 2.1 compares AFM with other microscopic techniques. It should be noted that AFM image resolutions include lateral (X, Y) and vertical (Z) axes. For an AFM image, the scale and resolution in the lateral dimension is different from those in the vertical dimension.

Table 2.1. Comparison of the advantages and disadvantages of microscopy techniques. Reprinted with permission from Journal of Food Science 2007, 72, R65-75. Copyright (2007), John Wiley and Sons, Inc. *(336)*.

Characteristics	Microscopy				
	AFM	LM	SEM	TEM	CLSM
Advantages	High resolution, nanoscale	Large scan area	Nanoscale	Nanoscale	Study dynamic process
	Minimal sample preparation, near native status	Fast scan speed	High resolution	High resolution	Fast scan speed
	2D and 3D	Cheap	Fast scan speed	Fast scan speed	2D and 3D
	In air/liquid, in situ, continuous process				In situ
	Can be manipulated				
Disadvantages	Small scan size	Only 2D	Only 2D	Only 2D	Complicated operation
	Slower scan speed	Need pretreatment	Need pretreatment	Need pretreatment	Need pretreatment
	Difficult for soft material	Low resolution and magnification	Not native status	Not native status	

Abbreviations: AFM, atomic force microscopy; LM, light microscopy; SEM, scanning electron microscopy, TEM, transmission electron microscopy; CLSM, confocal light scanning microscopy; 2D, two dimensional; 3D, three dimensional.

Figure 2.3 shows an example of AFM images of individual microbial cells with

different imaging modes.

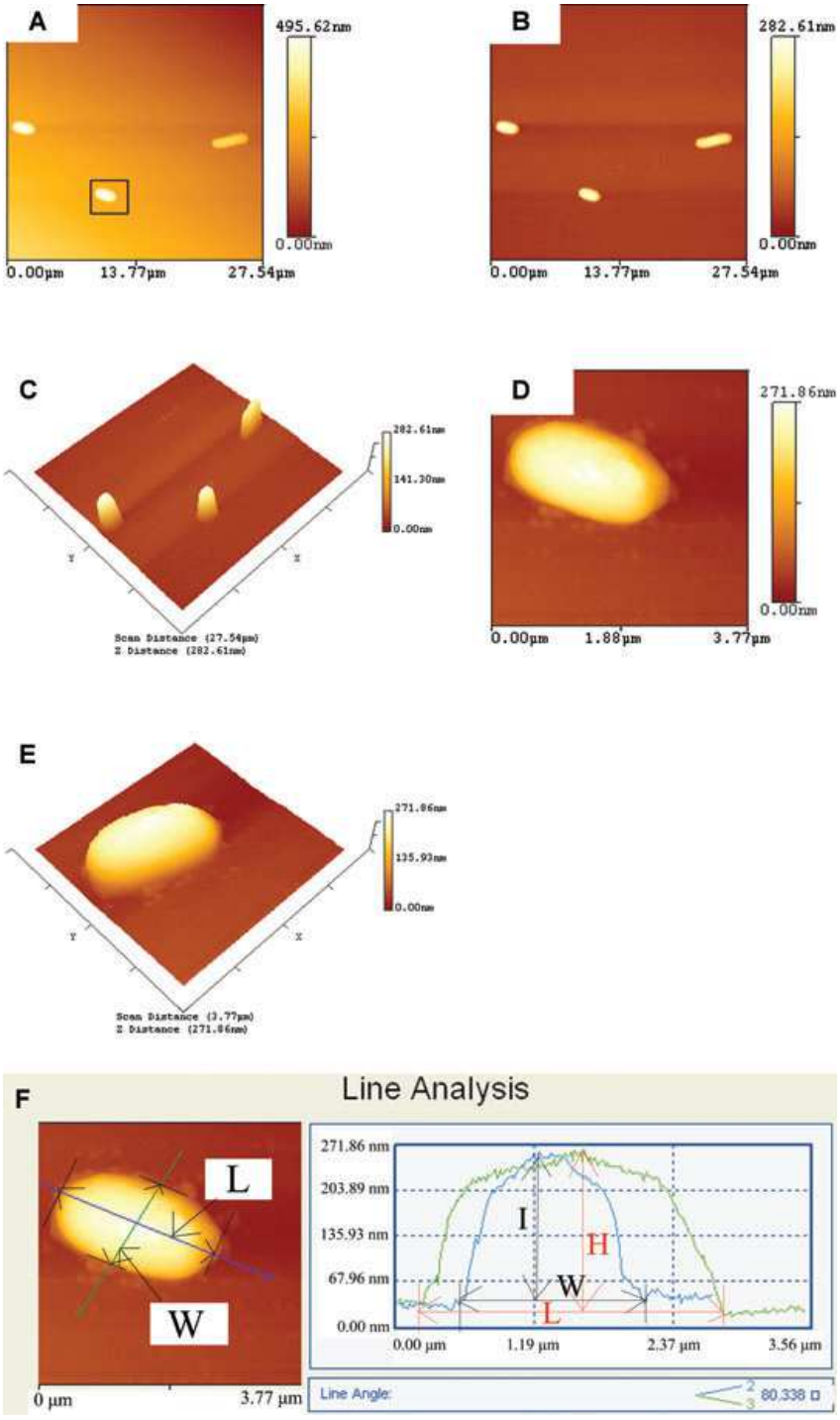


Figure 2.3. Atomic force microscopy (AFM) images of B strain microbial cells of *E. coli*. Reprinted with permission from Journal of Food Science 2008, 73, N44-50. Copyright (2008), John Wiley and Sons, Inc. *(334)*.

(A) unprocessed height image; (B) corresponding height image after leveling;

(C) corresponding 3D image after leveling; (D) enlarged image of a;

(E) corresponding 3D images of d; (F) dimension calculation of the microorganism.

L = length; W = width; H and I represent the heights of the directions of length and width, respectively.

2.5.2. Biofilm morphologies of foodborne pathogens

Morphological studies can provide direct evidence showing the effects of sanitizers on microorganisms. They also provide additional evidence to support observations of physical, chemical and physiological changes of microorganisms under various conditions *(101-105, 325-327)*. Morphological studies of pathogens may be useful in elucidating delicate changes in microorganisms during inactivation, as wells as the mechanism of inactivation *(20, 65, 213, 339)*. Many methods have been applied to characterize microorganisms in planktonic cells or biofilms. The morphology (especially for biofilms) obtained was much dependent on sample preparation or imaging conditions. The microtiter plate assay method can estimate the growth of bacteria in situ *(20, 37, 115)*, but is an indirect method and visualization of morphology is difficult *(73)*. Transmission/Scanning electron microscopy *(6, 7, 16, 38, 39, 52, 64)(74, 88, 212, 223, 272)*, epifluorescence microscopy *(58, 110, 236)*, and (confocal) laser scanning

microscopy *(23)* are the most often used techniques for biofilm characterization. However, these microscopic techniques are focused on microbial cells rather than EPS, a component of biofilms. Also, pretreatment steps including staining or vacuuming required for these techniques could affect the native status of the biofilm surface, mainly EPS *(36, 307, 336) (6, 7, 70, 77)*. EPS structure might be affected by draining of culture medium and the washing process during sample preparation for wide-field fluorescence microscopy (WFM) as well *(187)*, which is very important for understanding biofilms *(186, 320)* and the treatment of antimicrobial agents *(304)*. Due to the above reasons, information about the morphological organization and pattern of biofilms, especially that of *E. coli* O157:H7, *S. enterica* and *L. monocytogenes*, is very limited.

Atomic force microscopy (AFM) generates images by measuring changes in the interaction between probe and sample surface. The advantages of this instrument include minimal sample preparation, independence of the substance's light transparency and the ability to measure a sample's dimensions *(235, 307, 334, 336)*. A special advantage of AFM for biofilms is that it can characterize EPS to a large degree in its natural status since only minimal pretreatment steps for imaging are needed for obtaining the images *(157, 158)*. It is capable of characterizing the delicate structure of biofilm EPS without affecting the original morphology. AFM has been used to characterize foodborne pathogens including on stainless steel surfaces *(264)*, single microbial cells *(16, 41, 55, 90, 92, 118, 119, 139, 161, 197, 200)*, biofilms *(135, 183, 224, 265, 266)*, and corresponding physical properties of EPS of biofilms *(238, 240)*. AFM can also be used for differentiating surface differences between wild-type and mutant bacterial strains,

which is helpful for understanding the properties of these microbial cells in biofilm formation *(63)*. In all, AFM is a powerful tool in microbiology *(5, 78, 79, 209)* and an appropriate technique for the characterization of microbial cells and biofilms.

2.6. Organic foods

Organic agriculture is a holistic production management system. According to the Codex Alimentarious, this system promotes and improves agro-ecosystem health including biological cycles, biodiversity and soil biological activity. This practice was proposed by Albert Howard, an English soil scientist , in the 1930s, and the term 'Organic ' was used in the U.S. by Jerome Rodale almost at the same time. Organic agriculture does not allow using synthetic fertilizers and pesticides. However, due to general contamination by low level pollution of air or soil, organic agriculture does not guarantee that foods are completely free of residues *(72, 121)*.

In the U.S., organic production was relatively negligible before 1990. Since then, the Organic Foods Production Act (OFPA) was enacted and in 1992, the National Organic Standards Board (NOSB) was formed, and as a result the development of organic agriculture and organic foods was ensured. The Board has 15 members with 5 year tenure, one of whose main functions is reviewing and developing the National List of Approved and Prohibited Substances. In recent years, sales of organic foods have increased approximately 20% every year. A survey in 2007 reported that about 30% of Americans buy organic food at least occasionally. In addition, most of the consumers who buy organic food think it is safer *(30)*.

In the U.S., for any foods to be sold as ‘organic’, labeling indicates its status. Currently, there are 4 kinds of labeling for organic foods based on the relative percentage of the organic ingredients: ‘100% organic’ is for organic ingredients only; ‘organic’ is for foods with greater than 95% organic ingredients; ‘Made with organic ingredients’ is used for foods with greater than 70% organic ingredients, and ‘some organic ingredients’ is used for foods with less than 70% organic ingredients *(72, 121)*.

Organic foods are not absolutely safe, as many consumers think. While organic vegetables contain less pesticide residues compared to conventional vegetables, it does not mean that they pose no risk. Since synthetic fertilizers are not allowed in organic agriculture, manure is an important fertilizer, which can be a vehicle for transmission of zoonotic foodborne pathogens. Some studies have found little evidence that organic produce might pose a greater risk of foodborne pathogen contamination than conventional produce *(208)*. This was confirmed by a recent study showing there were no significant differences in foodborne pathogens between organic and conventional lettuce samples, as both were negative for *E. coil* O157:H7, *L. monocytogenes* and *Salmonella enterica (226)*.

Until now, there are very few cases of foodborne disease outbreaks that were due to organic food in the U.S. even though many cases of foodborne disease outbreaks were traced to fruits and vegetables and pinned to specific farmers. However, recent outbreaks have suggested that there might be potential risks of *E. coli* O157:H7 and *Salmonella* contamination on organic foods, with several cases of foodborne disease outbreaks in other counties that were linked to organic produce. One case was in Germany, in which

parsley was contaminated with verotoxigenic *Citrobacter freundii (300)*. Two other cases were outbreaks of *E. coli* O157:H7 of contaminated lettuce *(2, 123)*. Partial reasons for these cases may be due to limited number and efficacy of currently approved sanitizers.

2.7. Inactivation of foodborne pathogens

Sanitization is a critical step for foods, especially in fresh produce processing and handling in ensuring product safety. In general, methods for inactivating foodborne pathogens can be divided into three categories: physical, chemical and biological methods.

2.7.1. Physical methods for inactivating foodborne pathogens

2.7.1.1. Heat treatment: Pasteurization

Pasteurization is named after a scientist, Louis Pasteur, who first discovered that heating wine below its boiling point could extend its shelf-life by killing the organismsresponsible for spoilage during storage. Currently, this process is widely used in the food industry for inactivating food microorganisms to extend shelf-life. Pasteurization can kill foodborne pathogens that lead to listeriosis, typhoid fever, tuberculosis, diphtheria, and brucellosis. *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* can be completely inactivated by pasteurization. However, for fresh produce, this technique is seldom applied considering this heat treatment would affect physical properties, especially texture.

2.7.1.2. Food irradiation

Food irradiation is an effective technology for controlling food pathogens including *E. coli* O157:H7, *L. monocytogenes* and *Salmonella*, insects and parasites. It reduces the risk of foodborne diseases while avoiding impairment of the nutritional values of foods *(14, 81, 160, 180, 204, 231, 283, 284)*. It has a long history of use in the U.S., starting from 1905 when a patent using ionizing radiation to kill bacteria was approved. Later, in 1921, another patent of application of X-ray technology in meat was approved. FDA first approved food irradiation to control insects in wheat and flour in 1963. The application of irradiation of fruits and vegetables was approved by the FDA in 1986 and of fresh and frozen red meats in 1997 *(50, 62)*.

Food irradiation utilizes low levels of radiant energy including gamma rays, X-rays, and electrons. For ground beef, irradiation can greatly reduce potential hazards. The populations of *E. coli* O157:H7 in ground beef can be dramatically reduced by low-dose treatments with ionizing radiation. Additionally, the shelf-life of ground beef can be greatly extended after the application of irradiation *(81, 231)*. Food irradiation can also be very effective in controlling foodborne pathogens for fresh produce *(254)*. Moreover, it can extend the shelf-life of foods by inhibiting the sprouting of vegetables *(271, 297)*. In 2008 the Food and Drug Administration (FDA) announced that fresh spinach and iceberg lettuce may be irradiated. The purpose is to control pathogens such as *E. coli* O157:H7 and *Salmonella*, protecting consumers from foodborne diseases. Even though the FDA was petitioned to allow irradiation for many types of produce, only fresh spinach and

iceberg lettuce were approved in 2008 due to the 2006 *E. coli* outbreak from spinach and many previous outbreaks and recalls of lettuce *(303)*.

Food irradiation effectively kills or inactivates food pathogens including *E. coli* O157:H7 by transmitting radiant energy into the molecules of microorganisms. The energy generated by reactive chemicals could break or inactivate DNA of the pathogens, leading to failure in later reproduction *(214, 278)*. However, the effects of food irradiation depend on the amount of irradiation absorbed and the food status *(160)*. FDA approved different legal dosages for different foods: 30 kiloGray (kGy) for spices and dry vegetable seasoning; 10 kGy for dry or dehydrated enzyme preparations; 4.5 and 7 kGy to control food pathogens for refrigerated and frozen red meat, respectively, and 4.0 kGy to control foodborne pathogens and extension of shelf-life for fresh iceberg lettuce and fresh spinach *(50, 302)*.

In addition, the relative sensitivity of foodborne pathogens to ionizing radiation is largely dependent on their respective D_{10} values, which is the dose required for reducing the microbial population by 90%. Larger D_{10} values represent lower sensitivity of a microorganism to treatment. Table 2.2 shows the D_{10} values of the foodborne pathogens *E. coli* O157:H7, *L. monocytogenes* and *Salmonella*.

Table 2.2. Irradiation D_{10} values of foodborne pathogens on food products

Pathogen	Temperature (°C)	Product	D_{10}value (kGy)	Reference
Salmonella Heidelberg	0	Poultry (air packed)	0.24	Licciardello et al. (1970) *(167)*
	0	Poultry (air packed)	0.39	
Salmonella Enteriditis	5	Egg powder	0.6	Matic et al. (1990) *(191)*
	3	Ground beef	0.55-0.78	Tarkowski et al. (1984) *(294)*
Salmonella spp.	5	Turkey breast meat	0.71	Thayer et al. (1995) *(296)*
Listeria monocytogenes	5	Beef	0.45	Thayer et al. (1995) *(296)*
Escherichia coli O157:H7	5	Ground beef patties	0.27-0.38	Lopez-Gonzales et al. (1999) *(174)*

Source: Adapted from *(283)*

Irradiation technology is safe and not expensive. The source energy is weak and can not generate radioactivity. FDA alleges that irradiated food is safe. “There is no residue, there’s nothing left and certainly no radioactivity left,” said Dr. Laura Tarantino,

director of FDA's Office of Food Additive Safety *(18)*. The World Health Organization has announced that no toxic hazard was found for any food samples with irradiation at dosage under 10 kGy *(71)*. Currently, nearly 40 countries have approved the usage of food irradiation *(129)*.

2.7.2. Chemical methods for inactivating foodborne pathogens

Chemical methods for inactivating foodborne pathogens include, but are not limited to, organic acids *(149, 157, 201, 340)*, chlorine compounds *(84, 221, 262)*, iodine*(145)*, ozone *(28, 46, 248, 319)*, quaternary ammonium compounds *(35, 199)*, and hydrogen peroxide *(127, 175, 324)*. An AOAC official method is generally applied to evaluate the effectiveness of chemical sanitizers. According to the method, an effective sanitizer should be able to kill 99.999% of planktonic microbial cells, that is, 5 log CFU (colony forming unit) reductions of the microorganism population in 30 s *(8)*.

2.7.2.1. Neutral electrochemically activated water (NECAW)

Though traditional chemical sanitizers can effectively inactivate foodborne pathogens, chemical disposal, impact to produce quality, and worker safety are great concerns associated with the use of chemical sanitizers. There is an increasing demand for developing environmentally friendly sanitizers for fresh produce sanitization, especially for organic food.

2.7.2.1.1. Fundamentals of ECAW and NECAW

Electrochemically activated water (ECAW) is a water-based sanitizer for food processing equipment, which uses an electrolyzed diluted salt solution to generate distinct fractions. One fraction (anolyte) is generated at the anode containing chlorine (acidic or neutral), and has antimicrobial properties. The second fraction (catholyte) is generated at the cathode (alkaline), which can be used for cleaning food utensil surfaces or directly on food. Neutral ECAW (NECAW) generates anolytes with pH values near neutrality *(124)*.

The fundamental process underlying the generation of both fractions of NECAW is the electrolysis of diluted salt solution and dissociation into ions. Ions either move to the anode or cathode in an electrical field. At the same time chlorine in different forms is generated during the process. Sodium chlorine dissociates into negatively charged chlorine (Cl^-) and positively charged sodium (Na^+), and at the same time hydroxide (OH^-) and hydrogen (H^+) ions are formed when the water solution is electrolyzed. Cl^- moves to the anode and becomes chlorine gas (Cl_2), hypochlorite ion (OCl^-), hypochlorous acid (HOCl), and hydrochloric acid. It is believed that these different forms of chlorine are responsible for the antimicrobial properties of ECAW and NECAW *(124)*.

ECAW is often referred to as electrolyzed oxidant water (EOW). Generally, the term ECAW is used when a Russian technology is utilized while EOW refers to processes that use a Japanese design. The difference is that ECAW is produced by applying a short-term mild electrical charge to a diluted brine solution (<1% salt) when it passes through a module that converts the brine solution into a stream of reactive oxidants. It is believed that the oxidant composition of ECAW can be precisely controlled over a wide pH range. However, it is reported that there is no fundamental difference

between EOW and ECAW in sanitizing factors that include free available chlorine (FAC), oxidative-reduction potential (ORP) and pH *(60, 185, 267)*. The term ECAW will be used throughout this thesis denoting this type of water sanitizer.

NECAW has bactericidal efficacy similar to other common chemical sanitizers, but has several advantages *(66-69)*. The major advantages of ECAW include: 1) source materials contain no harmful chemicals, only water and NaCl are needed for generating ECAW; 2) no need to handle, distribute, or store toxic and corrosive chemicals *(142)*; and 3) slower rate of degradation of active chlorine compared to chemical chlorine sanitizers *(12, 13)*. Based on these characteristics, the usage of NECAW as a sanitizer appears to be safer for workers and the environment *(142)*. The neutral pH of NECAW minimizes the loss of chlorine due to evaporation and typically maintains antimicrobial activity longer than acidic ECAW *(11, 32, 48)*.

2.7.2.1.2. Sanitizing effects of ECAW and NECAW

Previous reports about the antimicrobial activity of ECAW on foodborne pathogens varied significantly *(124, 126, 243)*. In general, ECAW is more effective on liquid cultures than on dried surfaces *(125, 312, 313)*. A recent report indicated that treatment of *E. coli* NBRC 3301 for 0.5 min with ECAW (21.2 mg/l, pH 5.8, ORP 948 mV) achieved 3.85 log CFU reductions. Higher concentrations of FAC and ORP (45.3 mg/l, pH 2.6, ORP 1140) resulted in 5.27 log CFU reductions *(130, 131)*. Another report provided evidence that 5 min treatment with NECAW (89 mg/l, pH 8.0, ORP 760) achieved greater than 6 log CFU reductions of *E. coli* O157, *Salmonella* Enteritidis, and

L. monocytogenes (66). The antimicrobial effects of ECAW on bacteria dried on surfaces usually result in less killing than t in liquid suspensions. For *L. monocytogenes* dried on surfaces, ECAW (40 mg/l, pH 2.65, ORP 1155) resulted in 1.91 log CFU reductions per chip of dirty stainless steel, and it should be noted that tap water alone resulted in 1.03 log CFU reductions *(169)*.

For biofilms formed on utensil surfaces, only studies involving *L. monocytogenes* have been published *(11, 12, 141)*. The antimicrobial effects on biofilms had high variation under similar ECAW treatment conditions. ECAW treatment of *L. monocytogenes* biofilms for 1 min (85 mg/l, pH 2.29, ORP 1163) produced 3.69 log CFU reduction, however lower FAC concentrations (47 mg/l, pH 2.40, ORP 1163) resulted in larger reduction (4.65 log CFU). In another report with similar ECAW conditions (85 mg/l, pH 2.38, ORP 1169), bacterial cells were decreased by 4.81 log CFU *(11, 12)*.

There have been several reports about the antimicrobial effects of ECAW on fresh produce. Among fresh produce, the antimicrobial effects of ECAW were greater on the surface of tomatoes than on lettuce, cabbage and alfalfa sprouts *(16, 17, 66, 142, 151, 237)*. With similar parameters of ECAW, results from different groups were more variable compared with pure cultures of bacteria. For instance, NECAW (306 mg/l, ORP 880, pH 7.0) dipping of lettuce for 5 min resulted in approximately 2 log CFU/g reductions *(89)*, while dipping in NECAW for 10 min (120 mg/l, ORP 850, pH 6.3) resulted in 0.24 log CFU/ml reductions of *E. coli* O157:H7, and in almost 3 CFU/ml log reductions for *L. monocytogenes* and *S*. Typhimurium *(21)*. ECAW did not significantly affect the quality of treated foods. More than 2 log CFU reductions/g for lettuce or fresh-

cut lettuce were achieved by ECAW without significantly affecting quality attributes *(241, 333)*.

ECAW has also been used in other fields, for fresh-cut flowers *(132)* or in dental cleaning *(115, 285)*. However, reported effectiveness varied among different publications. The effect of ECAW on root canal cleaning indicated that a combination of anolyte (300 mg/L NECAW) and catholyte together was more effective than that of deionized water, 3% NaOCl, and 300 mg/l NECAW *(79)*. Another group reported that NECAW (pH 7.0 and 9.0) was not effective. The limitation of that report was that the authors failed to provide the FAC and ORP data *(185)*. NECAW at more than 300 mg/l was effective for *Bacillus anthracis* spore inactivation, and higher than 7 log CFU reductions could be achieved *(69)*. There are also reports showing limited bacterial inactivation on foods. Meat products generally had less than 1 log CFU reduction with ECAW treatment *(16-19)*. ECAW can also be applied for inactivating foodborne pathogens on meat products, but the sanitizing effects were much limited. For instance, the reduction of *L. monocytogenes* was less than1 log CFU/g *(87)*.

Some researchers have discussed factors that limit antimicrobial effects of ECAW *(169, 170, 230)*. It is believed that organochloramines are formed when chlorine compounds react with organic compounds resulting in weaker ECAW antimicrobial activity *(51, 171, 230, 242)*. ECAW had limited ability to penetrate into the protective layer of microbial polymers and some microbial cells could not be inactivated *(1, 74)*. Chlorine loss decreases with an increase of pH from acidic to alkaline, similar to chlorinated water *(164)*. Without food residue or organic compounds, ECAW (around 90

g/l, pH 2.5, ORP 1160) generated 3.5 to 4.0 log CFU reductions compared to deionized water, but this value decreased when food residue existed *(83, 84)*. Biofilms are more resistant than planktonic cells due to quorum sensing *(94)* or EPS *(50)*.

Agitation, temperature, and soaking can affect the antimicrobial activity of ECAW. Higher temperature promotes the penetration of ECAW into microbial cells but also facilitates the loss of chlorine *(153, 154)*. Agitation also facilitates the penetration of ECAW into microbial cells, and improves the efficiency of contact between chlorine and microbial cells *(124, 244)*. Soaking cutting boards in EOW at higher temperatures decreased the time needed to achieve the same reduction in bacterial counts obtained at lower temperatures *(124)*. Many researchers have demonstrated that NECAW can generate 2 to 6 log CFU reductions of some bacteria such as *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes.* However, other researchers have found that NECAW does not effectively reduce foodborne pathogens *(124)*. In general, NECAW is a promising sanitizer but little is known about its effectiveness and broad-spectrum activity against pathogenic bacteria.

2.7.2.1.3. Antimicrobial mechanism of ECAW and NECAW

In order to be effective, bactericides must penetrate into microbial cells and attain sufficient concentration at the target site *(59)*. But the action modes of different sanitizers differ *(159)*. For traditional chemical chlorine sanitizers, the mechanism has been well studied. For hydrogen peroxide, the antimicrobial effect is due to nondiscriminating oxidization, but hydrogen peroxide also attacks DNA, proteins, and lipids of microbial

cells. Hydrogen peroxide, however, does not affect the integrity of cell membranes *(91, 102)*. For hypochlorite, the antimicrobial effect is due to its interaction with lipids, slow oxidization of glutathione (GSH), toxic product generation, and disruption of nucleic acids *(62-64)(93)*. Chlorite disrupts microbial membranes, inhibits synthesis of proteins and nucleic acids, and oxidizes GSH. Chlorite does not modify membrane lipids *(34-36, 76)*.

FAC, ORP, and pH are three factors that are responsible for the sanitizing effects of ECAW. For NECAW, only two factors, chlorine (Cl_2, HOCl, and OCl^-) and ORP, determine its antimicrobial effects. However, there is no agreement about which of the two factors is the most important. Effects of NECAW chlorine include destroying microbial membranes, reacting with amino acids and nucleic acids of microbial cells, and affecting metabolism due to the destruction of key enzymes *(43, 45, 53, 54)*. It should be noted that according to reports from Japanese researchers, ECAW did not have sufficient disinfectant ability when FAC was less than 20 mg/l, and even if the ORP was high (> 800) *(152)*.

No significant difference ($P \geq 0.05$) was found between traditional chlorine sanitizers and NECAW *(68, 69, 117)* or acidic ECAW *(48, 49, 70, 71, 89)* with the same concentration of FAC. It seems that FAC was the determining factor of antimicrobial effects *(3, 26)*. Different FAC with similar ORP and pH had significantly different antimicrobial effects. NECAW had almost the same antimicrobial effects as acidic ECAW with the same concentration of FAC. Among FAC, HOCl is the most active component. A more widely accepted mechanism of antimicrobial effects of ECAW was

that active chlorine compounds destroy the membranes of microorganisms, while some other researchers proposed that chemical reactions between active chlorine components and important microorganism chemical molecules (amino acids, nucleic acids, or enzymes) are responsible for the antimicrobial effects *(124)*. Other researchers believed that high ORP was the determining factor. Higher ORP corresponds to greater oxidizing strength. ORP damaged cell membranes, oxidized cell surfaces, and disrupted cell metabolism and inactivated microbial cells *(166)*. One confounding factor in these reports was that chlorine or other chemical sanitizing components coexisted together with ORP when studying the sanitizing effects of ORP.

In some promising results on ECAW mechanism obtained recently *(16, 341)*, only limited indicators of microbial cells were investigated, and those indicators can be affected by many exterior factors. Current morphological results, for instance, could be easily affected by sample preparation processes *(335, 336)*. DNA degradation analysis did not exclude possible degradation due to DNase during the experiments *(147)*, and protein analysis is problematic since newly appeared subunits of proteins after ECAW treatment were not explained *(16, 341)*. It was proposed that the disinfection mechanism of ECAW were due to several factors including penetrating the protective sphere of bacteria, increased permeability of the microbial outer membrane, inactivated cellular compounds and some key enzymes *(341)*.

It should be noted that the inactivation mode of Gram-positive bacteria might differ from that of Gram-negative bacteria *(50)*. *E. coli* O157:H7 and *Salmonella* are Gram-negative bacteria while *L. monocytogenes* is a Gram-positive bacterium. The

antimicrobial mechanism of NECAW on *E. coli* O157:H7 and *Salmonella* might differ from that of *L. monocytogenes*. Partial reasons for this difference may be due to the existence of glutathione (GSH). GSH exists in almost all prokaryotes but not in mitochondria or chloroplasts *(45, 111, 123)*. In Gram-negative bacteria, glutathione is critical for maintaining the oxidation state of thiols and can be used by bacterial cells to cope with stresses. Furthermore, glutathione can undergo glutathionylation, a protein post-translational modification regulating protein function under stress *(190)*. A second reason could be related to outer membrane protein A (OmpA). With respect to *E. coli* O157:H7 and *S. enterica*, for instance, understanding the sanitizing mechanism of NECAW could be helped by studying the effects of NECAW on OmpA *(255, 256, 328)*.

2.7.2.2. Traditional chlorine and chlorine-based compounds

Chlorine-based compounds are still the most widely used sanitizers in the food industry due to their wide-spectrum antimicrobial property *(215)*. Their sanitizing effect is determined by the FAC of the solution. FAC consists of chlorine gas (Cl_2), hypochlorous acid (HClO), or hypochlorite ions (ClO^-), in which HClO has the strongest antimicrobial activity. The pH of the solution can change the relative contents of these three compounds. A mildly acidic pH (approximately pH 5) chlorine-based compound could have the maximum content as HClO, thus resulting in strong sanitizing effects *(27)*. Commonly used chlorine-based sanitizers are sodium hypochloride or calcium hypochlorite. They can react with water and generate HClO, with strong antimicrobial activity. In fresh produce processing, a general sanitizing treatment is 200 mg/l at a pH <

8.0 with 1-2 min treatment. When FAC was smaller than 50 mg/l, sanitizing effect of chlorine on *L. monocytogenes* was not observed. A very high (>200 mg/l) concentration of chlorine is required to achieve 1 log CFU reduction of pathogens on fresh produce in washing solution *(34)*.

Chlorine dioxide is another commonly used chlorine-based sanitizer. It is a volatile molecule and remains in water as a dissolved gas. Chlorine dioxide can not be used directly as a commercial product since it is highly explosive. Therefore, the application of this compound requires onsite generation. A common way of generating chlorine dioxide is by reacting sodium chlorite with gaseous chlorine. An alternative way is by using hypochlorous acid to replace Cl_2 *(299, 310, 321)*. Compared to chlorine, one advantage of chlorine dioxide for sanitizing fresh produce is that it generates fewer carcinogenic byproducts such as trihalomethanes. Plus, it is less affected by pH than chlorine *(299, 310, 321)*.

2.7.2.3. Ozone

Ozone (O_3) has a high oxidation potential and is a versatile sanitizing agent. It can be generated from oxygen (O_2) with the exertion of high energy using photochemical, electric discharge, thermal or electrolytic methods *(85, 138, 222)*. Ozone is stable in air but is potent and unstable in water. Ozone in water can decompose into oxygen spontaneously and quickly without any residues *(138, 318)*.

Ozone can be used as a disinfectant or sanitizer for food processing including organic foods *(19, 85, 138)*. This can be done using different forms of ozonated water or

by adding gaseous ozone to a storage room *(119, 138, 222, 246)*. The efficiency of ozone is mainly determined by ozone demand of the medium's residual ozone, that is, the ozone remaining on the surfaces treated *(138)*. Organic debris can compromise the effectiveness of ozone treatment; therefore, it is necessary to clean equipment surfaces before ozone sanitizing treatment. It was reported that ozone did not change the organoleptic properties of foods under appropriate conditions. For example, 0.1 mg/l ozone treatment for 6 h did not affect the organoleptic properties of whole and ground black peppers *(85)*. Ozone did not affect the quality properties of treated lettuce as well *(119)*.

2.7.3. Biological sanitizers

2.7.3.1. Phages

Phages, or bacteriophages, are viruses, which can infect bacteria. They exist widely in natural environments using bacteria as their hosts. However, they have their own DNA or RNA. Phages can be isolated from waste water, sewage, and human or animal fecal samples. Compared to chemical sanitizers, most phages have less spectrum killing effects on bacteria. Therefore, for inactivating various strains of pathogens, one solution to overcome this disadvantage is to use cocktails of several phages *(61, 95)*.

Phages are considered to be a potential alternative to chemical sanitizers, and because of the emergence of antibiotic resistance, there is significant research devoted to developing viable alternatives for many different pathogenic bacteria. Phages have been approved for use on foods and at least three different commercially available phage

preparations are offered by different companies. Currently, one of the most widely used phages for foodborne pathogens is commercially available *Listeria* phage, P 100 *L. monocytogenes* phage. It was reported that this phage is effective on many strains of *L. monocytogenes* on different foods *(45, 148, 205, 275, 287-289)*. The limitation is that the strains tested are much limited and should be designated by the manufacturing company.

In our laboratory, a phage cocktail was examined for inactivating *E. coli* O157:H7 mixture at or above room temperature on different commonly-used hard surfaces including stainless steel coupons and directly on food surfaces. *E. coli* O157:H7 on romaine lettuce and spinach were found to be inactivated by phage *(314-316)*.

CHAPTER III

EFFECTIVENESS AND BROAD-SPECTRUM ACTIVITY OF NEUTRAL ELECTROCHEMICALLY ACTIVATED WATER AGAINST FOODBORNE PATHOGENS

The objective of this study was to determine the effectiveness and broad-spectrum activity of neutral electrochemically activated water (NECAW) against *Escherichia coli* O157:H7, *Listeria monocytogenes,* and *Salmonella enterica*. Liquid bacterial cultures, bacterial cells dried on stainless steel (SS) surfaces and biofilms of 40 strains of *E. coli* O157:H7, *L. monocytogenes,* and *S. enterica* were treated with NECAW. Sanitizing effects were determined by direct plate counting and enrichment testing. NECAW containing 50 or 100 mg/l FAC caused more than 5 log CFU/ml reductions for 70% and 100% of the strains in liquid culture, respectively. Treatment with NECAW (100 mg/l FAC) resulted in more than 3 log CFU/coupon reductions for 92.5% and 27.5% of the strains dried on SS surface and as biofilms, respectively. Among all the strains, *S. enterica* serovar Newport B4442CDC was the most resistant to NECAW on surfaces while *E. coli* O157:H7 ATCC 43895 was the most resistant in biofilms and liquid pure cultures. NEACW was effective and had a broad-spectrum activity against foodborne pathogens. Different species and strains of foodborne pathogens had variable sensitivity to NECAW. NECAW can be potentially used as a sanitizer for food and food processing

utensils.

3.1. Introduction

Foodborne diseases are a major health problem worldwide. *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* are three of the most important infectious bacteria, responsible for the largest percentage (82%) of food-related deaths in the U. S. *(192, 274)*. They were targeted for reductions in the Center's Disease Control and Prevention's Healthy People 2010 plan *(192)*. Compared to the preceding 3 years, the estimated incidence of infections caused by the three bacteria in 2008 did not decrease significantly *(317)*.

Food and food processing equipment are important carriers of foodborne pathogens. In addition, pathogens on food equipment surfaces can easily form biofilms. Sanitization is a critical step to ensure food safety during food processing and handling. Traditional chemical sanitizers can effectively inactivate foodborne pathogens, but chemical disposal, impact on food quality and worker safety are great concerns associated with their use. Thus, developing environmentally friendly sanitizers for food sanitization is a critical need, especially for organic food production due to limited choices and efficacy of currently approved sanitizers *(198)*.

Electrochemically activated water (ECAW), often referred to electrolyzed oxidizing water (EOW), is a water-based sanitizer for food and food processing equipment that uses an electrolyzed diluted salt solution to generate two distinct fractions, referred as the anolyte and the catholyte. The anolyte is generated at the anode containing

chlorine (acidic or neutral) and is the fraction with antimicrobial properties. This antimicrobial activity is due to formation of free available chlorine (FAC) and relatively high oxidation-reduction potential (ORP). In contrast, the catholyte is generated at the cathode, which can be used for cleaning food utensil surfaces or food directly *(60, 185, 267)*. The sanitizing effect of ECAW is determined by the parameters ORP, FAC, and pH of the anolyte. Neutral ECAW (NECAW) is ECAW of the anolyte with a pH near neutrality *(124)*.

A number of researchers have demonstrated that NECAW is an effective sanitizer, resulting in 2 to 6 log colony forming unit (CFU) reductions of some bacteria such as *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes (66, 130).* However, some researchers observed different results suggesting NECAW did not effectively reduce foodborne pathogens *(124)*. To our knowledge, though there are a number of reports on the sanitizing effects of EOW or NECAW on food or food equipment surfaces, most of those studies have used only a very limited number of strains such as one *(117, 341)* or a five-strain mixture of specific pathogens for testing sanitizing effects *(312, 313)*.

Due to possible differing sensitivities among strains and usage of different parameters of ECAW, the antimicrobial activity of ECAW on foodborne pathogens has varied significantly *(60)*. For biofilms formed on utensil surfaces, only studies involving *L. monocytogenes* have been published, and the results varied significantly even under similar conditions from the same research group *(11, 12)*. These reports provide some insight into understanding the properties and effects of ECAW; however, due to the reported inconsistent results, ECAW's effectiveness and broad-spectrum activity against

pathogens remain unclear.

The usage of NECAW as a sanitizer appears to be safe for workers and the environment, and the neutral pH of NECAW minimizes the loss of chlorine due to evaporation and typically maintains antimicrobial activity longer than acidic ECAW *(23, 36, 42, 49)*. Determination of NECAW's effects against different strains of foodborne pathogens would contribute to corroborate its application as a sanitizer for food processing equipment and food itself.

The objective of this study was to determine the effectiveness and broad-spectrum activity of NECAW against different strains of foodborne pathogens including *E. coli* O157:H7, *L. monocytogenes*, and *S. enterica*. The sanitizing efficacy of NECAW against different strains of the pathogens as liquid cultures, dried cells on stainless steel (SS) surfaces, and biofilms on SS was examined.

3.2. Materials and methods

3.2.1. Bacterial strains

Different strains of *E. coli* O157:H7 (11 strains), *S. enterica* (21 strains) and *L. monocytogenes* (8 strains) were included in this study (Table 3.1). All strains have been causal organisms in food outbreaks. For each strain, a loop of glycerol-culture from -60°C storage was inoculated and transferred three consecutive times in tryptic soy broth (TSB) (Neogen Corp,, Lansing, MI) and inoculated at 37ºC at 24 h intervals.

3.2.2. Preparation and analysis of neutral electrochemically activated water (NECAW)

NECAW was freshly produced from softened tap water and saturated NaCl solutions by a generator (Zap Water Technology, Inc., Richfield, MN, USA) at a voltage range of 7 to 9 volts. After a stable voltage reading was reached, NECAW was collected using a sterile glass bottle from the anode side, covered and used within 2 h post generation. FAC of NECAW was determined with a chorine test kit by calculating the drop counts (LaMotte Company, Chestertown, MD). ORP and pH were measured with an ORP meter (ORPTestr 10, Oakton Instruments Inc., Vernon Hills, IL) and a pH meter (pHTestr 10, Oakton Instruments Inc.), respectively.

3.2.3. Liquid culture testing

For each bacterium, 24 h-old cultures (20 ml each) were centrifuged (3, 600 × *g*, 23°C) for 10 min. Pellets were washed using 10 ml of peptone water (PW, Neogen, Inc.), centrifuged and re-suspended in 10 ml of PW. One ml of each suspension was added to 99 ml of liquid sanitizer solution (NECAW or deionized water [DIW] as control) in bottles. After the bottles were shaken by hand for 30 s, 1 ml aliquots were each added to 9 ml neutralizing buffer solutions (5.2 g/l; Becton, Dickinson and Company, Sparks, MD) and shaken for 40 s. The neutralized mixture was serially diluted for plating analysis *(7, 11)*.

3.2.4. Bacteria dried on stainless steel surface

For each strain, 10 ml of 24 h-old cultures were centrifuged. Pellets were washed with 5 ml sterile TSB, spun and re-suspended in 2 ml TSB. Then 25 µl of each bacterial suspension was inoculated on clean, sterile stainless steel coupons (1 cm in diameter and approximately 0.7 mm in thickness) *(9, 98)*. Coupons were dried in a biosafety cabinet for 3 h. Microbial cells on coupons were immersed in sterile petri plates with 100 mg/l FAC NECAW or DIW for 1 min, with or without slight agitation (approx. 10 rpm). After treatment, coupons and solutions were neutralized with neutralizing buffer and the microbial cells were recovered by plating on TSA plates and enrichment test *(332)*.

3.2.5. Biofilm experiments

Individual strains were prepared as described above for liquid culture testing. Suspensions were mixed with 9 ml sterile low nutrient TSB (1:10 dilution of normal TSB solution, LN-TSB) at a dilution of 1:100. Sterilized stainless steel coupons were immersed in the suspensions, mixed well and left in the liquid cultures for 4 h at room temperature to allow bacterial attachment. Suspensions were poured off and the coupons were rinsed gently with a circular motion for 10 s using 1 ml PW in order to remove unattached microbial cells. The PW was poured off. Coupons were incubated at room temperature with 10 ml fresh LN-TSB. After 48 h, LN-TSB was discarded and coupons were transferred to new LN-TSB solutions for another 24 h at room temperature. After this incubation, coupons were rinsed gently with 1 ml PW twice to remove loosely attached microbial cells, and then dried in a biosafety cabinet for 2 h *(12)*.

Coupons with biofilms were placed into glass tubes containing 10 ml NECAW

100 mg/l FAC or DIW as control, slightly mixed at speed 2 using a Mdl G-560 Vortex Genie 2 Mixer (Lehman Scientific, LLC, Wrightsville, PA, USA) for 5 s. After a 25 s pause, coupons were mixed again for 5 s, and then kept still for another 25 s. Coupons were transferred to new glass tubes containing 10 ml neutralizing buffer solution and mixed at speed 2 for 5 s. After 35 s of waiting, coupons were transferred to 50 ml disposable plastic tubes containing 10 ml PW and 3 g sterile glass beads (Sigma-Aldrich Co., St. Louis, MO, USA), and vortexed heavily for 5 min to remove bacteria from the coupons. A series of ten-fold dilutions of the PW containing coupons and neutralizing buffer were conducted, after vortexing for direct plating and enrichment test.

3.2.6. Microbial analyses

The numbers of viable cells in the final diluted PW and the neutralizing buffer were determined by directly plating 0.1 ml of each diluent in duplicate on tryptic soy agar (TSA; Neogen Corp) plates, and further counting the colonies after incubation at 37ºC for 24 h (for *E. coli* O157:H7 and *Salmonella*) or 48 h (for *L. monocytogenes*). The CFU of the recovered bacteria was enumerated and transformed to logarithm base 10 per ml or coupon. For enrichment tests, 5 ml PW recovered from coupons and neutralizing buffer after treatment were transferred to 250 ml Erlenmeyer flasks containing 100 ml sterile TSB and incubated at 37ºC for 48 h *(12, 244)*. Selected colonies from TSA plates were streaked onto selective agar and incubated to confirm the presence of pathogens.

3.2.7. Data analyses

Experiments were independently conducted with at least two separate trials. For each trial, the parallel groups were conducted in duplicate with two plating results for any individual condition. Statistical analyses using analysis of variance (ANOVA) ($P < 0.05$) and Duncan's multiple range test for differences among different treatments (between NWECAW and DIW) were performed using SAS software (Version 9.1.3, SAS, Cary, NC, USA). Comparisons that yielded $P < 0.05$ were considered significant.

3.3. Results

NECAW was very effective in reducing the viable count of *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* in liquid cultures. Table 3.2 shows the survival of *E. coli* O157:H7 microbial cells in liquid pure cultures following exposure to NECAW with 50 mg/l FAC. After 30 s treatment, no survivors of 8 out of 11 *E. coli* O157:H7 strains were detected. Among the three *E. coli* O157:H7 isolates that had surviving counts, strain ATCC 43895 was reduced less than 2 log CFU/ml. NECAW was very effective in killing all *L. monocytogenes* strains to below detection level (Table 3.3). Interestingly, *Salmonella* Typhimurium strains were more sensitive to NECAW treatment than non-Typhimurium strains (Table 3.4). Approximately 82% of Typhimurium strains tested were not detected after NECAW treatment, compared to 50% of non-Typhimurium strains. When the bacterial strains were treated with NECAW containing 100 mg/l FAC no survivors were detected above 2 log CFU/ml after an initial count of approximately 9 log CFU/ml (data not shown).

After testing the effectiveness of NECAW using the standard sanitizer protocol in

liquid cultures, the next step was investigating the effect of NECAW on the same pathogenic strains dried on stainless steel (SS) surfaces. To resemble real sanitizing conditions, the treatments with NECAW were done statically or with slight agitation and only 100 mg/l FAC was tested on cells dried on SS coupon surfaces. For *E. coli* O157:H7 treated statically, 64% of strains had less than 2 log CFU reductions, but agitation resulted in no detactable survivors for almost all strains, even if enrichment was used (Table 3.5). The use of agitation was also effective in enhancing killing of *L. monocytogenes*, in that detectable survivorship decreased from less than 1 log CFU reduction without agitation to undetectable counts with slight agitation (Table 3.7). For *Salmonella*, 48% of strains were inactivated to less than 2 log CFU without agitation (Table 3.6). Two strains, Typhimurium UK-1 and Newport B4442CDC, had survival counts above the detection limit even with slight agitation.

NECAW was applied to treat biofilms of pathogen cells formed on SS coupon surfaces. Since biofilms are generally hard to remove, the protocol of Ayebah and others (2005) *(11)* was modified to include two cycles of 5 s disturbance following 25 s still incubation. However, in this current study, this modification did not markedly improve NECAW's effectiveness. More than half of *E. coli* O157:H7 strains had less than 2 log CFU/coupon reductions, while this number was 38% for *L. monocytogenes* and 29% for *Salmonella* strains as biofilms (Tables 3.8 to 3.10).

To better illustrate NECAW's sanitizing effect, the survival count of microbial cells rinsed into washing solutions and neutralizing buffer was also determined (Tables 3.11 to 3.13). Interestingly, no survivors of washing solution and neutralizing buffer from

NECAW groups were detected by direct counting on TSA or enrichment tests, with or without agitation, while significant amounts of surviving microbial cells were found in all the solutions in DIW control groups (5 to 7 log CFU/coupon). The same results were also found in liquid fractions from biofilms treated with NECAW and DIW (Tables 3.14 to 3.16).

Figure 3.1 summarizes all the sanitizing results of liquid cultures, cultures dried on SS coupon surface and biofilms. Overall, NECAW treatment (50 mg/l FAC for 30 s) on most strains yielded more than 5 log CFU/ml reductions, greater than 3 log CFU/coupon reductions for pathogens dried on SS coupon surfaces and less than 2 log reductions for biofilms formed (100 mg/l FAC for 1 min). Among all the strains, *S.* Newport B4442CDC was the most resistant to NECAW on surfaces while *E. coli* O157:H7 ATCC 43895 was the most resistant strain in biofilms and liquid cultures. Compared to *E. coli* O157:H7 and *Salmonella*, *L. monocytogenes* biofilms were more resistant to NECAW treatment.

3.4. Discussion

The three critical parameters affecting the sanitizing effects of ECAW are FAC, ORP and pH. In the current study, we used NECAW with pH near neutrality, which minimized the influence of pH. A FAC of 63 mg/l was determined to be the critical concentration that resulted in more than 5 log CFU/ml reductions for liquid cultures according to European Standard UNE-EN 1276 *(68, 69)*, however, recent research suggested that such generalizations are limited by the influence of other factors. Several

studies have indicated that it is still unclear if FAC or ORP is more important for NECEW *(36, 45, 46)*. Most of those publications agreed, however, that pH was effective, but not very critical, in maintaining the sanitizing effects of NECAW. As supported by our results and some others *(114, 117)*, the neutral pH ECAW was very effective for killing foodborne pathogens.

To our knowledge, published data onantimicrobial activity of ECAW on foodborne pathogens or other microorganisms varied significantly, although in general ECAW has been more effective on liquid cultures than in dried forms or in biofilms. For example, treatment of *E. coli* K 12 (NBRC 3301) for 30 s with ECAW (21.2 mg/l, pH 5.8, ORP 948 mV) achieved 3.85 log CFU/ml reductions and a higher concentration of FAC and ORP (45.3 mg/l, pH 2.6, ORP 1140 mV) resulted in 5.27 log CFU/ml reductions *(130)*. Extending the treatment from 30 s, which was used in AOAC official method 960.09 *(8)*, to 5 min with ECAW (89 mg/l, pH 8.0, ORP 760 mV) achieved greater than 6 log CFU reductions for *E. coli* O157, *Salmonella* Enteritidis, and *L. monocytogenes (66)*. The varied sanitizing effects among different strains could be due to application of different parameters of ECAW and strains' different penetration barriers, thus different sensitivity to NECAW treatment *(59)*.

Large variety in ECAW's sanitizing effects on surfaces is evident in published literature. Neutral EOW with 63 mg/l FAC was reported as effective as NaClO solution but was safer and easier to use *(67)*. Acidic ECAW (40 mg/l, pH 2.65, ORP 1155 mV) treatment resulted in 1.91 log CFU/coupon reductions of *L. monocytogenes* dried on SS coupon surfaces, but these results could have been influenced by the presence of food

residues and usage of tap water controls, resulting in 1 log CFU reductions *(169)*. In contrast, EOW (90 mg/l, pH 2.5, ORP 1160) 10 min caused 4 log CFU per 100 cm^2 reductions of *L. monocytogenes* dried on a cutting board surface *(313)*, and reduced the viable count of *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* by 3 log CFU on tomato surfaces (30.3 mg/l, pH 2.6, ORP 1140 mV) *(16, 17)*. In the current report, NECAW's antimicrobial effects were markedly diminished when bacteria were dried on surfaces compared to liquid suspensions, possibly due to a limited penetration ability of NECAW into microbial cells because of biofilm structure *(6)*.

Biofilms on materials associated with food production is a serious food safety issue. Biofilms can develop when microbial cells are in a disadvantageous environment. It was widely accepted that biofilms are a community of microbial cells embedded in a matrix of extracellular polymeric substances (EPS), being more resistant to sanitizers than viable cells mainly due to the EPS and quorum sensing *(7, 59, 194, 236)*. In addition, the antimicrobial effects of sanitizer on biofilms have been quite variable even with similar treatment conditions *(11, 12)*. For instance, ECAW treatment of *L. monocytogenes* biofilms for 1 min (85 mg/l, pH 2.29, ORP 1163 mV) led to 3.69 to 4.81 log CFU reductions *(11)*. Conversely, less FAC concentrations (47 mg/l, pH 2.40, ORP 1163 mV) resulted in equivalent or greater reductions (4.65 log CFU) *(12)*. However, in the work described here, NECAW treatment on *L. monocytogenes* biofilms resulted in an average of 2.2 log CFU/coupon reductions. The difference was in some degree due to the control group. Our control group (DIW treatment) yielded around 0.8 log CFU/coupon more reductions than controls used by Ayebah *et al. (11)*. For this reason, we applied a

higher concentration of microbial cells to dry on the SS coupon surface for biofilms to develop since 1 to 2 log CFU/coupon could be washed away.

Results for our control group were similar to that reported by Venkitanarayanan *et al. (313)*. For biofilms, our currently used biofilms were relatively younger (less than 4 days) compared to a more realistic situation (more than 7 days). Thus, it is possible that with more developed biofilms, more than 21 days, for instance, the sanitizing effects of NECAW would be lower *(332)*. Even though our observed reductions of biofilm microbial cells by NECAW may be comparable to that reported in the literature for liquid cultures or surfaces, more concentrated ECAW applied to biofilms needs to be taken into account. Overall, ECAW had a lower sanitizing effect on biofilms possibly due to the strong attachment of microbial cells to the SS surfaces and cells being less accessible to ECAW because of outer EPS of biofilms *(7)*.

ECAW's antimicrobial effects were limited by several factors. Chlorine compounds react with organic compounds to form organochloramines, leading to compromised sanitizing effects, which could be one of the reasons for less sanitizing effects on biofilms than liquid cultures as chlorine compounds could react with the EPS component of the biofilms (Figure 3.1). In addition, FAC was transformed to N-chloro compounds by reacting with food amino acids and proteins, or lipids to form carbon-carbon double bonds *(230)*. Both reactions resulted in reduced ECAW antimicrobial activity *(11, 230)* and limited ECAW ability to penetrate into the protective layer of microbial polymers *(6, 244)*. Evidence supporting this was that without food residue or organic compounds, ECAW (approx. 90 mg l^{-1}, pH 2.5, ORP 1160 mV) treatment for

around 10 min generated 3.5 to 4.0 log CFU reductions compared to DIW. This value decreased when food residue existed *(312, 313)*. Thus, the actual effects of NECAW on practical food or food utensils may be reduced compared to bacteria dried on clean surfaces as used in this report *(169)*. For improvingsanitizing effects, especially on fresh produce surfaces, alkaline electrolyzed water pretreatment could be an option, especially with mild heating *(11, 154)*.

To alleviate the limiting factor (accessible of sanitizers)'s effect on sanitization, agitation or soaking could be applied. Agitation facilitated the penetration of ECAW into microbial cells, and improved contact between chlorine and microbial cells, resulting in significant increase of sanitizing effect *(6, 124, 244)*. Without agitation, between 2.2 and 2.4 log CFU/cm^2 reductions of *Enterobacter aerogenes* ATCC 13048 and 1.7-1.9 log reductions for *Staphylococcus aureus* ATCC 6538 were observed with an initial inoculation of 10^7 CFU/cm^2 on the surface. In contrast, agitation of 50 rpm reduced the population of viable cells on the tested surfaces including glass, stainless steel, glazed and unglazed ceramic tiles, and vitreous china to less than 1 log CFU/cm^2 for both strains *(244)*.

To enhance the sanitizing effect and minimize the nonsanitizing effect of washing away the microbial cells from SS coupon surfaces to the solution, in the current manuscript a slight agitation of 10 rpm was applied. Agitation enhanced sanitization to such a level that most of the strains post-NECAW treatment were killed to below detection levels. Soaking also enhances the sanitizing effect. Soaking of cutting boards in EOW at a high temperature decreased the duration needed to achieve the same reduction

of bacterial counts obtained at lower temperatures *(124)*. We did not detect the effect of soaking in the current research.

Prevention of cross contamination is critical in order to maintain food safety during processing. In this report, no viable bacterial cells were detected in washing solution and neutralizing buffer, two sources of potential contamination, of NECAW treatment group, while more than 2 log CFU/coupon of viable microbial cells were recovered from control group. The result indicated that NECAW at the conditions we applied could not only sanitize pathogens but also effectively prevent cross contamination from the washing solutions, which was supported by previous reports as well *(244)*.

3.5. Conclusion

NECAW is effective and had a broad-spectrum activity on inactivating foodborne pathogens including *E. coli* O157:H7, *L. monocytogenes* and *Salmonella*. NECAW (100 mg/l FAC) inactivated all the strains of the pathogens with greater than 5 log CFU/ml reductions. Different strains of these pathogens had different sensitivities to NECAW, having different bacterial cell reductions when treated with 50 mg/l FAC NECAW treatment in liquid cultures. Of the 40 strains, 28 strains in liquid culture had more than 5 log CFU reductions by the treatment of NECAW. NECAW (100 mg/l FAC) resulted in > 3 log CFU/coupon reductions for 92.5% of the strains dried on SS surface and 27.5% of the biofilms. *E. coli* O157:H7 ATCC 43895 was the most resistant strain to NECAW in biofilms and liquid pure cultures while *S*. Newport B4442CDC was the most resistant

strain on surfaces.

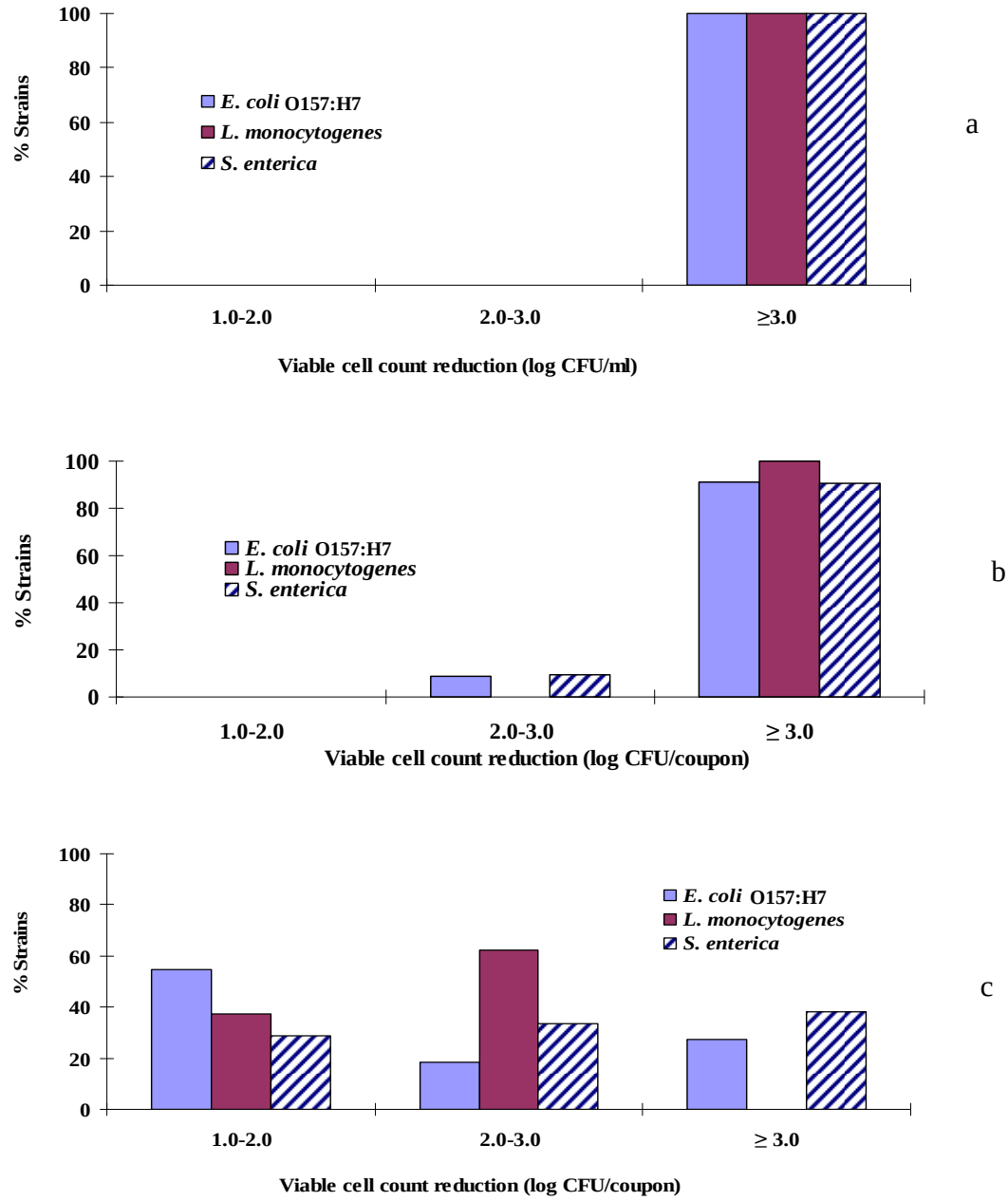


Figure 3.1. Proportion of strains of *Escherichia. coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* killed by neutral electrochemically activated water (NECAW) according to the extent of count reduction. (a) liquid cultures, (b) cultures dried on stainless steel surface with agitation, and (c) biofilms.

Table 3.1. Bacterial strains used in this study to determine efficacy of NECAW.

Bacteria species/serovar	Strain	Source or reference
Escherichia coli O157:H7	ATCC 43890	ATCC
	ATCC 43895	ATCC
	2028, 2029, 2257, 4719, 86-24	ARS
	6058	Dr. Todd Callaway, USDA/ARS
	EK1 TWO8609	MSU
	EK27 TWO 8635	MSU
	E32511 TWO2383	MSU
Listeria monocytogenes	ATCC 19115	ATCC
	U MN-VM	UMNVM
	DUP-1030A	ILSI
	DUP-1038	ILSI
	DUP 1044A	ILSI
	2349, 2422, 3528	FSML
Salmonella enterica		
Serovar Typhimurium	ATCC 700408, ATCC 14028	ATCC
	E2009005811	MDH
	UK-1, I503, I526, I534, I535, I536, I758	FSML
Serovar Newport	AMO 7073, AMO 7076, AMO 5313, B4442CDC	CDC
Serovar Enteritidis	2009595	FSML
	95657613	CDC
Serovar Tennessee	E2007000302	MDH
Serovar Montevideo	95573473	MDH
Serovar Agona		FDA
Serovar Saintpaul	E2008001236	MDH

Abbreviations: ARS, Agricultural Research Service's Southern Plains Agricultural Research Center; ATCC, American Type Culture Collection; CDC, Centers for Disease Control and Prevention; FSML, Food Safety Microbiology Laboratory, University of Minnesota; MDH, Minnesota Department of Health; MSU, Microbial Evolution Laboratory, Michigan State University. ILSI, International Life Sciences Institute, Cornell University; UMNVM, University of Minnesota-Veterinary Medicine. NECAW, neutral electrochemically activated water.

Table 3.2. Survival of *Escherichia coli* O157:H7 cells (log CFU/ml) in liquid cultures after exposure to neutral electrochemically activated water (NECAW). Distilled ionized water (DIW) was used as control.

Strain	Initial	Treatment		
		DIW	NECAW	Reduction
ATCC 43890	9.10±0.04	6.98±0.03	2.19±0.16	4.79
ATCC 43895	9.22±0.03	7.14±0.01	5.25±0.10	1.89
2028	9.22±0.03	7.16±0.01	<2.00	≥5.16
2029	9.27±0.03	7.19±0.03	<2.00	≥5.19
2257	9.18±0.06	7.08±0.02	<2.00	≥5.08
4719	9.12±0.10	7.00±0.09	3.16±2.11	3.84
6058	9.12±0.10	7.15±0.07	<2.00	≥5.15
86-24	9.21±0.04	7.13±0.02	<2.00	≥5.13
EK1 TWO8609	9.18±0.05	7.13±0.04	<2.00	≥5.13
EK27 TWO 8635	8.75±0.04	6.69±0.07	<2.00	≥4.69
E32511 TWO2383	9.03±0.12	6.96±0.18	<2.00	≥4.96

Note: the free available chlorine of the NECAW was 50 mg/l, treatment time was 30 s.

Detection limit: 2 log CFU/ml.

Table 3.3. Survival of *Listeria monocytogenes* microbial cells (log CFU/ml) in liquid cultures after exposure to neutral electrochemically activated water (NECAW). Distilled ionized water (DIW) was used as control.

Strain	Initial	Treatment		
		DIW	NECAW	Reduction
ATCC 19115	9.30±0.05	7.24±0.03	<2.00	≥5.24
UMN-VM	9.27±0.05	7.19±0.04	<2.00	≥5.19
DUP-1030A	9.31±0.04	7.22±0.02	<2.00	≥5.22
DUP-1038	9.50±0.10	7.53±0.05	<2.00	≥5.53
DUP 1044A	9.09±0.08	7.02±0.03	<2.00	≥5.02
2349	9.20±0.03	7.18±0.03	<2.00	≥5.18
2422	8.89±0.09	6.66±0.09	<2.00	≥4.66
3528	9.22±0.03	7.24±0.01	<2.00	≥5.24

Note: the free available chlorine of the NECAW was 50 mg/l, treatment time was 30 s.

Detection limit: 2 log CFU/ml.

Table 3.4. Survival of *Salmonella spp.* microbial cells (log CFU/ml) in liquid cultures after exposure to neutral electrochemically activated water (NECAW). Distilled ionized water (DIW) was used as control.

Serovar	Strain	Initial	Treatment		
			DIW	NECAW	Reduction
Typhimurium	700408	8.95±0.10	6.78±0.04	3.00±0.68	3.78
	ATCC 14028	9.23±0.03	7.10±0.03	3.56±0.14	3.54
	E2009005811	9.29±0.05	7.03±0.07	<2.00	≥5.03
	UK-1	9.22±0.07	7.11±0.02	<2.00	≥5.11
	I503	9.21±0.06	7.13±0.07	<2.00	≥5.13
	I526	9.26±0.07	7.19±0.06	<2.00	≥5.19
	I534	9.06±0.10	6.97±0.07	<2.00	≥4.97
	I535	9.21±0.05	7.12±0.03	<2.00	≥5.12
	I536	9.12±0.14	7.16±0.02	<2.00	≥5.16
	I740	9.19±0.07	7.03±0.02	<2.00	≥5.03
	I758	9.38±0.05	7.32±0.01	<2.00	≥5.32
Newport	AMO 7073	9.03±0.09	6.84±0.01	<2.00	≥4.84
	AMO 7076	9.27±0.07	7.07±0.04	2.65±1.04	4.42
	AMO 5313	9.02±0.12	6.95±0.06	4.05±0.26	2.90
	B4442CDC	9.20±0.04	7.13±0.03	<2.00	≥5.13
Enteritidis	2009595	9.31±0.06	7.25±0.04	<2.00	≥5.25

	95657613	9.22±0.05	7.03±0.10	<2.00	≥5.03
Tennessee	E2007000302	9.34±0.11	7.38±0.03	3.10±0.35	4.28
Montevideo	95573473	9.39±0.05	7.18±0.05	<2.00	≥5.18
Agona	FDA	9.25±0.06	7.04±0.09	2.49±0.76	4.55
Saintpaul	E2008001236	9.20±0.09	7.09±0.02	3.73±0.75	3.36

Note: the free available chlorine of the NECAW was 50 mg/l, treatment time was 30 s.

Detection limit: 2 log CFU/ml.

Table 3.5. Efficacy of neutral electrochemically activated water (NECAW) on the viability of liquid cultures of *Escherichia coli* O157:H7 dried on stainless steel surfaces (log CFU/coupon). Distilled ionized water (DIW) was used as control.

Strain	Initial	Static			With agitation		
		DIW	NECAW	Reduction	DIW	NECAW	Reduction
ATCC 43890	6.99±0.04	4.11±0.28	<2.00	≥2.11	4.33±0.41	N.D.	≥3.33
ATCC 43895	7.58±0.05	5.87±0.27	4.50±0.47	1.36±0.44	4.85±0.46	<2.00	≥2.85
2028	7.07±0.50	5.84±0.61	4.51±0.62	1.33±0.59	4.89±0.37	N.D.	≥3.89
2029	6.43±0.35	4.59±0.51	4.16±0.50	0.43±0.49	4.00±0.48	N.D.	≥3.00
2257	7.24±0.22	5.46±0.29	4.60±0.57	0.86±0.72	4.38±0.21	N.D.	≥3.38
4719	8.03±0.02	5.50±0.32	<2.00	≥3.50	4.35±0.21	N.D.	≥3.35
86-24	7.01±0.13	4.10±0.32	<2.00	≥2.10	4.17±0.27	N.D.	≥3.17
6058	7.94±0.05	5.47±0.34	<2.00	≥3.47	4.02±0.02	N.D.	≥3.02
EK1 TWO8609	7.36±0.34	5.55±0.46	3.88±0.71	1.66±0.92	4.09±0.33	N.D.	≥3.09
EK27 TWO 8635	7.30±0.34	5.60±0.45	4.01±0.66	1.59±0.92	4.18±0.28	N.D.	≥3.18
E32511 TWO2383	7.18±0.55	6.13±0.34	4.84±0.20	1.28±0.22	4.08±0.49	N.D.	≥3.08

Note: N.D.: Not detectable by TSA plate count and negative on enrichment. Free available chlorine of the NECAW was 100 mg/l.

Table 3.6. Efficacy of NECAW on the viability of liquid cultures of *Listeria monocytogenes* dried on stainless steel surfaces (log CFU/coupon). Distilled ionized water (DIW) was used as control.

Strain	Initial	Static			With agitation		
		DIW	NECAW	Reduction	DIW	NECAW	Reduction
ATCC 19115	6.81±0.05	5.70±0.19	5.35±0.28	0.34±0.09	4.26±0.54	N.D.	≥3.26
U MN-VM	7.39±0.48	6.53±0.33	5.58±0.43	0.95±0.11	4.26±0.28	N.D.	≥3.26
DUP-1030A	7.16±0.50	5.95±0.09	5.81±0.16	0.14±0.10	4.24±0.35	N.D.	≥3.24
DUP-1038	7.32±0.50	6.60±0.24	6.29±0.52	0.31±0.29	5.92±0.43	N.D.	≥4.92
DUP 1044A	7.29±0.41	6.26±0.57	6.20±0.58	0.06±0.05	5.61±0.07	<2.00	≥3.61
2349	7.75±0.05	6.07±0.30	5.88±0.72	0.19±0.54	4.03±0.11	N.D.	≥3.03
2422	6.84±0.18	5.79±0.15	5.01±0.71	0.78±0.63	4.32±0.42	N.D.	≥3.32
3528	7.25±0.22	6.04±0.32	5.57±0.53	0.47±0.35	4.07±0.09	N.D.	≥3.07

Note: N.D.: Not detectable by TSA plate count and negative on enrichment. Free available chlorine of the NECAW was 100 mg/l.

Table 3.7. Efficacy of NECAW on the viability of liquid cultures of *Salmonella spp.* dried on stainless steel surfaces (log CFU/coupon). Distilled ionized water (DIW) was used as control.

Serovar	Strain	Initial	Without agitation			Slight agitation		
			DIW	NECAW	Reduction	DIW	NECAW	Reduction
Typhimurium	700408	7.60±0.13	6.13±0.38	5.61±0.43	0.52±0.51	5.45±0.28	<2.00	≥3.45
	ATCC 14028	7.82±0.02	6.26±0.14	5.21±0.29	1.05±0.39	4.73±0.40	<2.00	≥2.73
	E2009005811	7.60±0.08	5.27±0.39	<2.00	≥3.27	4.04±0.27	N.D.	≥3.04
	UK-1	8.04±0.05	5.81±0.17	3.69±1.38	2.11±1.41	5.26±0.08	2.60±0.30	2.66±0.35
	I503	7.75±0.30	6.64±0.11	6.35±0.10	0.29±0.11	5.66±0.19	<2.00	≥3.66
	I526	7.65±0.10	5.62±0.13	<2.00	≥3.62	4.46±0.53	N.D.	≥3.46
	I534	7.91±0.08	5.43±0.15	2.82±0.45	2.61±0.36	4.42±0.85	N.D.	≥3.42
	I535	7.46±0.02	5.68±0.23	N.D.	≥4.68	4.05±0.39	N.D.	≥3.05
	I536	7.01±0.08	4.90±0.17	3.39±0.18	1.51±0.08	4.14±0.49	N.D.	≥3.14
	I740	7.96±0.09	5.47±0.15	2.87±0.46	2.60±0.40	4.43±0.89	N.D.	≥3.43
	I758	7.77±0.11	5.84±0.36	<2.00	≥3.84	4.36±0.74	N.D.	≥3.36

Newport	AMO 7073	7.36±0.10	6.54±0.45	5.50±0.63	1.04±0.20	5.55±0.84	N.D.	≥4.55
	AMO 7076	7.55±0.20	5.87±0.16	3.03±2.13	2.84±2.29	4.29±0.35	N.D.	≥3.29
	AMO 5313	7.86±0.06	6.87±0.07	5.52±0.67	1.35±0.63	5.74±0.76	<2.00	≥3.74
	B4442CDC	7.58±0.14	6.01±0.38	5.60±0.51	0.41±0.20	5.75±0.24	3.23±2.25	2.52±2.48
Enteritidis	2009595	7.32±0.24	6.25±0.55	5.68±0.19	0.57±0.38	5.64±0.48	N.D.	≥4.64
	95657613	7.69±0.15	5.74±0.23	<2.00	3.74	4.04±0.07	N.D.	≥3.04
Tennessee	E2007000302	8.11±0.10	6.01±0.25	<2.00	≥4.01	4.34±0.75	N.D.	≥3.34
Montevideo	95573473	7.54±0.21	6.08±0.20	5.47±0.42	0.61±0.58	5.06±0.29	<2.00	≥3.06
Agona	FDA	7.46±0.34	6.04±0.22	3.01±2.29	3.03±2.09	4.74±0.65	N.D.	≥3.74
Saintpaul	E2008001236	7.80±0.09	5.61±0.15	4.12±0.35	1.49±0.49	4.50±0.23	N.D.	≥3.50

Note: N.D.: Not detectable by TSA plate count and negative on enrichment. FAC of NECAW was 100 mg/l.

Table 3.8. Recovery of *Escherichia coli* O157:H7 cells from biofilms grown on coupons (log CFU/coupon). Distilled ionized water (DIW) was used as control.

Strain	Initial	Treatment		
		DIW	NECAW	Reduction
ATCC 43890	7.54±0.10	6.19±0.24	4.99±0.98	1.20±0.75
ATCC 43895	7.18±0.07	6.29±0.15	5.12±0.67	1.16±0.74
2028	7.74±0.33	6.93±0.60	2.71±0.42	4.21±0.98
2029	7.98±0.46	6.75±0.48	4.01±0.58	2.73±0.52
2257	7.42±0.28	4.99±0.32	3.40±0.48	1.58±0.55
4719	7.33±0.16	5.82±0.27	4.49±0.54	1.33±0.26
6058	7.42±0.23	5.20±0.37	<2.00	≥3.20
86-24	7.94±0.33	7.39±0.47	4.98±0.57	2.41±0.63
EK1 TWO8609	7.25±0.09	4.82±0.16	3.05±0.33	1.76±0.22
EK27 TWO 8635	7.47±0.15	5.62±0.51	4.27±0.40	1.35±0.65
E32511 TWO2383	7.43±0.24	6.39±0.33	2.91±0.28	3.48±0.33

Note: Free available chlorine (FAC) of NECAW was 100 mg/l.

Table 3.9. Recovery of *Listeria monocytogenes* cells from biofilms grown on coupon (log CFU/coupon) after treatment with neutral electrochemically activated water (NECAW). Distilled ionized water (DIW) was used as control.

Strain	**Initial**	**Treatment**		
		DIW	**NECAW**	**Reduction**
ATCC 19115	7.21±0.20	6.01±0.42	3.48±0.87	2.53±0.64
U MN-VM	6.98±0.30	5.88±0.24	4.55±0.43	1.33±0.42
DUP-1030A	6.89±0.12	5.71±0.19	4.08±0.68	1.63±0.81
DUP-1038	7.90±0.17	6.19±0.81	3.53±0.44	2.66±0.67
DUP 1044A	7.47±0.23	6.11±0.31	3.50±0.53	2.61±0.53
2349	7.00±0.16	6.03±0.33	3.11±0.40	2.92±0.13
2422	6.55±0.14	5.89±0.22	4.68±0.50	1.21±0.37
3528	6.91±0.13	5.72±0.21	2.97±0.38	2.74±0.49

Note: Free available chlorine (FAC) of NECAW was 100 mg/l.

Table 3.10. Recovery of *Salmonella spp.* cells from biofilms grown on coupon (log CFU/coupon) after treatment with neutral electrochemically activated water (NECAW). Distilled ionized water (DIW) was used as control.

Serovar	Strain	Initial	Treatment		
			DIW	NECAW	Reduction
Typhimurium	700408	7.83±0.16	7.31±0.32	3.64±1.00	3.67±0.74
	ATCC 14028	7.59±0.12	6.68±0.20	4.78±0.49	1.90±0.39
	E2009005811	7.28±0.26	6.84±0.40	3.11±2.27	3.73±1.93
	UK-1	7.83±0.34	6.95±0.30	3.91±0.48	3.03±0.18
	I503	7.15±0.49	6.55±0.82	4.82±1.34	1.73±0.59
	I526	7.55±0.35	7.10±0.35	3.88±0.31	3.22±0.03
	I534	7.33±0.13	6.40±0.40	4.34±0.44	2.06±0.18
	I535	7.02±0.14	6.01±0.48	4.24±0.43	1.76±0.43
	I536	7.18±0.26	6.39±0.19	3.22±0.99	3.17±0.91
	I740	7.79±0.13	7.48±0.12	4.90±0.23	2.58±0.20
	I758	8.39±0.08	7.85±0.12	6.07±0.37	1.78±0.42

Newport	AMO 7073	7.57±0.15	7.23±0.18	3.81±1.66	3.43±1.74
	AMO 7076	7.62±0.23	7.20±0.29	3.15±1.51	4.05±1.22
	AMO 5313	7.46±0.11	6.46±0.75	3.97±0.83	2.49±0.16
	B4442CDC	7.22±0.42	6.44±0.62	5.13±0.74	1.32±0.16
Enteritidis	2009595	7.34±0.30	6.81±0.52	4.35±0.93	2.45±0.43
	95657613	7.65±0.16	7.07±0.20	3.75±1.10	3.32±0.97
Tennessee	E2007000302	8.12±0.15	7.62±0.20	6.13±0.29	1.49±0.17
Montevideo	95573473	7.71±0.21	6.43±0.10	4.09±0.56	2.34±0.59
Agona	FDA	7.32±0.46	6.06±0.80	3.64±0.86	2.41±0.19
Saintpaul	E2008001236	7.55±0.44	6.12±0.83	4.09±0.82	2.03±0.06

Note: Free available chlorine (FAC) of NECAW was 100 mg/l.

Table 3.11. *Escherichia coli* O157:H7 population survival (log CFU/coupon) from washing solutions of cultures dried on stainless steel surface.

Strain	Static				With agitation			
	Washing water		Neutralizing buffer		Washing water		Neutralizing buffer	
	DIW	NECAW	DIW	NECAW	DIW	NECAW	DIW	NECAW
ATCC 43890	6.51±0.05	N.D.	6.20±0.10	N.D.	6.84±0.10	N.D.	5.90±0.15	N.D.
ATCC 43895	7.09±0.08	N.D.	6.73±0.16	N.D.	7.37±0.09	N.D.	6.10±0.20	N.D.
2028	6.59±0.39	N.D.	6.14±0.13	N.D.	6.86±0.32	N.D.	5.75±0.11	N.D.
2029	5.95±0.43	N.D.	5.58±0.40	N.D.	6.30±0.23	N.D.	5.13±0.28	N.D.
2257	6.35±0.29	N.D.	6.03±0.19	N.D.	6.72±0.28	N.D.	5.64±0.09	N.D.
4719	7.38±0.05	N.D.	7.01±0.17	N.D.	7.72±0.03	N.D.	6.04±0.34	N.D.
86-24	6.59±0.13	N.D.	6.29±0.06	N.D.	6.87±0.09	N.D.	5.97±0.04	N.D.
6058	7.44±0.03	N.D.	7.11±0.13	N.D.	7.75±0.02	N.D.	6.11±0.30	N.D.
EK1 TWO8609	6.26±0.09	N.D.	5.89±0.13	N.D.	6.56±0.12	N.D.	5.57±0.07	N.D.
EK27 TWO8635	6.69±0.40	N.D.	6.42±0.42	N.D.	6.99±0.30	N.D.	6.33±0.21	N.D.
E32511 TWO2883	6.63±0.38	N.D.	6.17±0.11	N.D.	7.00±0.13	N.D.	5.96±0.11	N.D.

Note: N.D.: Not detectable by TSA plate count and negative on enrichment. FAC of NECAW was 100 mg/l.

Table 3.12. *Listeria monocytogenes* population survival (log CFU/coupon) from washing solutions of cultures dried on stainless steel surface.

Strain	Static				With agitation			
	Washing solution		Neutralizing buffer		Washing solution		Neutralizing buffer	
	DIW	NECAW	DIW	NECAW	DIW	NECAW	DIW	NECAW
ATCC 19115	6.11±0.23	N.D.	5.80±0.24	N.D.	6.41±0.02	N.D.	5.35±0.48	N.D.
U MN-VM	6.98±0.49	N.D.	6.33±0.35	N.D.	7.09±0.46	N.D.	5.70±0.14	N.D.
DUP-1030A	6.76±0.56	N.D.	6.34±0.53	N.D.	6.94±0.53	N.D.	5.33±0.38	N.D.
DUP-1038	7.09±0.63	N.D.	6.73±0.70	N.D.	7.24±0.59	N.D.	6.13±0.51	N.D.
DUP 1044A	6.54±0.41	N.D.	6.34±0.10	N.D.	6.85±0.29	N.D.	5.83±0.30	N.D.
2349	6.74±0.34	N.D.	6.17±0.11	N.D.	7.00±0.13	N.D.	5.96±0.11	N.D.
2422	6.09±0.23	N.D.	5.87±0.21	N.D.	6.29±0.26	N.D.	5.03±0.62	N.D.
3528	6.84±0.25	N.D.	6.36±0.30	N.D.	7.00±0.35	N.D.	5.85±0.29	N.D.

Note: N.D.: Not detectable by TSA plate count and negative on enrichment. FAC of NECAW was 100 mg/l.

Table 3.13. *Salmonella spp*. population survival (log CFU/coupon) from washing solutions of cultures dried on stainless steel surface.

Serovar	Strain	Without agitation				Slight agitation			
		Washing solution		Neutralizing buffer		Washing solution		Neutralizing buffer	
		DIW	NECAW	DIW	NECAW	DIW	NECAW	DIW	NECAW
Typhimurium	700408	7.22±0.11	N.D.	6.95±0.08	N.D.	7.42±0.06	N.D.	6.20±0.12	N.D.
	ATCC 14028	7.36±0.04	N.D.	6.96±0.07	N.D.	7.60±0.08	N.D.	6.16±0.12	N.D.
	E2009005811	7.22±0.08	N.D.	6.98±0.03	N.D.	7.44±0.06	N.D.	6.19±0.11	N.D.
	UK-1	7.40±0.02	N.D.	7.00±0.07	N.D.	7.64±0.07	N.D.	6.21±0.11	N.D.
	I503	6.99±0.34	N.D.	6.63±0.53	N.D.	7.48±0.21	N.D.	5.97±0.50	N.D.
	I526	7.26±0.09	N.D.	6.99±0.05	N.D.	7.60±0.11	N.D.	6.17±0.15	N.D.
	I534	7.43±0.05	N.D.	7.21±0.02	N.D.	7.75±0.04	N.D.	6.32±0.14	N.D.
	I535	7.02±0.04	N.D.	6.74±0.08	N.D.	7.33±0.10	N.D.	6.29±0.14	N.D.
	I536	6.52±0.13	N.D.	6.17±0.12	N.D.	6.80±0.10	N.D.	5.58±0.06	N.D.
	I740	7.41±0.06	N.D.	7.16±0.02	N.D.	7.73±0.05	N.D.	6.29±0.14	N.D.
	I758	7.36±0.13	N.D.	7.06±0.12	N.D.	7.65±0.13	N.D.	6.23±0.12	N.D.

Newport	AMO 7073	6.47±0.65	N.D.	5.93±0.55	N.D.	7.07±0.35	N.D.	5.29±0.32	N.D.
	AMO 7076	7.10±0.12	N.D.	6.75±0.12	N.D.	7.38±0.12	N.D.	6.10±0.16	N.D.
	AMO 5313	7.37±0.07	N.D.	7.01±0.14	N.D.	7.61±0.10	N.D.	6.26±0.07	N.D.
	B4442CDC	7.12±0.14	N.D.	6.58±0.20	N.D.	7.33±0.10	N.D.	5.78±0.09	N.D.
Enteritidis	2009595	6.94±0.30	N.D.	6.49±0.36	N.D.	7.08±0.18	N.D.	5.95±0.30	N.D.
	95657613	7.37±0.10	N.D.	7.05±0.08	N.D.	7.60±0.13	N.D.	6.20±0.13	N.D.
Tennessee	E2007000302	7.47±0.05	N.D.	7.14±0.04	N.D.	7.93±0.13	N.D.	6.21±0.12	N.D.
Montevideo	95573473	7.14±0.22	N.D.	6.87±0.19	N.D.	7.29±0.16	N.D.	6.11±0.15	N.D.
Agona	FDA	7.10±0.25	N.D.	6.72±0.30	N.D.	7.35±0.20	N.D.	6.07±0.15	N.D.
Saintpaul	E2008001236	7.31±0.12	N.D.	7.03±0.15	N.D.	7.47±0.12	N.D.	5.97±0.43	N.D.

Note: N.D.: Not detectable by TSA plate count and negative on enrichment. FAC of NECAW was 100 mg/l.

Table 3.14. *Escherichia coli* O157:H7 population survival (log CFU/coupon) from washing solutions of biofilms grown on stainless steel surface.

Strain	Washing solution		Neutralizing buffer	
	DIW	**NECAW**	**DIW**	**NECAW**
ATCC 43890	7.03±0.20	N.D.	5.68±0.39	N.D.
ATCC 43895	6.98±0.11	N.D.	5.19±0.18	N.D.
2028	7.27±0.16	N.D.	5.72±0.34	N.D.
2029	7.54±0.42	N.D.	6.18±0.36	N.D.
2257	7.15±0.23	N.D.	6.09±0.24	N.D.
4719	7.07±0.23	N.D.	5.26±0.26	N.D.
6058	7.07±0.26	N.D.	6.10±0.31	N.D.
86-24	7.22±0.19	N.D.	6.10±0.26	N.D.
EK1 TWO8609	6.90±0.10	N.D.	5.90±0.14	N.D.
EK27 TWO 8635	7.09±0.11	N.D.	5.11±0.44	N.D.
E32511 TWO2383	7.13±0.14	N.D.	5.99±0.44	N.D.

Note: N.D.: Not detectable by TSA plate count and negative on enrichment. FAC of NECAW was 100 mg/l.

Table 3.15. *Listeria monocytogenes* population survival (log CFU/coupon) from washing solutions of biofilms grown on stainless steel surface.

Strain	Washing solution		Neutralizing buffer	
	DIW	NECAW	DIW	NECAW
ATCC 19115	7.07±0.22	N.D.	5.55±0.29	N.D.
U MN-VM	6.78±0.36	N.D.	5.57±0.44	N.D.
DUP-1030A	6.75±0.14	N.D.	5.27±0.25	N.D.
DUP-1038	7.36±0.58	N.D.	5.76±0.57	N.D.
DUP 1044A	7.29±0.20	N.D.	5.87±0.35	N.D.
2349	6.83±0.22	N.D.	5.56±0.23	N.D.
2422	6.16±0.36	N.D.	5.10±0.45	N.D.
3528	6.78±0.16	N.D.	5.32±0.22	N.D.

Note: N.D.: Not detectable by TSA plate count and negative on enrichment. FAC of NECAW was 100 mg/l.

Table 3.16. *Salmonella spp.* population survival (log CFU/coupon) from washing solutions of biofilms grown on stainless steel surface.

Serovar	Strain	Washing solution		Neutralizing buffer	
		DIW	NECAW	DIW	NECAW
Typhimurium	700408	7.12±0.19	N.D.	6.22±0.21	N.D.
	ATCC 14028	6.92±0.07	N.D.	6.15±0.30	N.D.
	E2009005811	6.20±0.18	N.D.	5.84±0.17	N.D.
	UK-1	7.26±0.13	N.D.	6.30±0.15	N.D.
	I503	6.17±0.55	N.D.	5.55±0.37	N.D.
	I526	6.80±0.31	N.D.	5.97±0.29	N.D.
	I534	6.80±0.27	N.D.	5.29±0.16	N.D.
	I535	6.79±0.11	N.D.	6.38±0.51	N.D.
	I536	6.70±0.21	N.D.	5.10±0.17	N.D.
	I740	7.03±0.29	N.D.	6.16±0.19	N.D.
	I758	7.54±0.19	N.D.	6.34±0.17	N.D.
Newport	AMO 7073	6.97±0.15	N.D.	6.06±0.14	N.D.
	AMO 7076	6.99±0.17	N.D.	5.43±0.47	N.D.
	AMO 5313	6.70±0.18	N.D.	5.50±0.50	N.D.
	B4442CDC	6.84±0.28	N.D.	4.98±0.75	N.D.
Enteritidis	2009595	6.74±0.28	N.D.	6.10±0.16	N.D.
	95657613	6.89±0.30	N.D.	6.19±0.21	N.D.

Tennessee	E2007000302	7.29±0.08	N.D.	6.49±0.12	N.D.
Montevideo	95573473	7.28±0.12	N.D.	6.24±0.22	N.D.
Agona	FDA	6.98±0.19	N.D.	6.06±0.13	N.D.
Saintpaul	E2008001236	7.10±0.23	N.D.	5.63±0.44	N.D.

Note: N.D.: Not detectable by TSA plate count and negative on enrichment. FAC of NECAW was 100 mg/l.

CHAPTER IV

SANITIZING EFFECTIVENESS OF COMMERCIAL "ACTIVE WATER" TECHNOLOGIES ON *ESCHERICHIA COLI* O157:H7, *SALMONELLA ENTERICA* AND *LISTERIA MONOCYTOGENES*

Electrochemically activated water (ECAW), also known as electrolyzed water, and ozonized water, are typically effective in inactivating bacteria, but their generation typically uses high current and voltage. A few simpler antimicrobial technologies that are also based on the application of a mild electrical current have been recently marketed to food retail and service customers claiming to have sanitizing properties for controlling bacteria. The objective of this study was to determine the sanitizing effect of some of these commercial technologies (Ionator™ and Lotus™) on *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica*, compared to sterile water, ECAW generated with a pilot size electrolyzing unit, and salt solutions sprayed using Ionator™ sprays. A concentration of 100 mg/L ECAW had sanitizing effects of at least 5 log CFU/ml reductions on liquid culture and more than 4 log CFU/coupon reductions for *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* dried on stainless steel surfaces, respectively. No bacterial cells were detected by direct plate counting post-ECAW treatment. In contrast, treatment of liquid cultures with any of the commercial

technologies resulted in non-significant bacterial cell reductions greater than 0.5 log CFU/ml. Similarly, when cells had been dried on metal surfaces and treated with water generated with those technologies, no reductions were observed. In the case of Ionator^{TM}, when the manufacturer's instructions were followed, the reduction of cells on surfaces was largely due to physical removal by cloth-wiping after water fraction application. These results indicate that treatment with Ionator^{TM} or Lotus^{TM} technologies had no noticeable antimicrobial activity. These results would be helpful for guiding consumers when choosing the right sanitization to ensure food safety.

4.1. Introduction

Escherichia coli O157:H7, *Salmonella spp.* and *Listeria monocytogenes* are three of the most important infectious bacteria targeted for reduction by the Centers for Disease Control and Prevention (CDC) *(192)*. *Salmonella* is the bacterial pathogen that causes most foodborne outbreaks and *L. monocytogenes* is one of the most deadly pathogens transmitted by food *(51, 305)*. The detection of these bacteria lead to most of the food recalls within the category of foodborne pathogen contamination *(3, 4)*. Several foodborne disease outbreaks have been due to the contamination of industrially produced foods, but there could be a range of raw foods that could also be contaminated in the domestic environment.

The possibility of transmission of these pathogens via unsanitary conditions during food preparation is quite high. Microbial surveys of domestic kitchens have found significant contamination with a variety of bacterial microorganisms, including fecal

coliforms, *E. coli,* and *Salmonella (258).* The source of contamination of kitchen surfaces can be multiple, but raw foods such as poultry and meats have been documented to spread some of these pathogenic bacteria. Proper cleaning and sanitizing of kitchen sites and food equipment is critical for preventing the spread of microorganisms and minimizing cross-contamination to ready-to-eat food via food preparation surfaces.

There is a variety of chemical sanitizers currently approved as direct-contact disinfectants for food preparation surfaces. However, the use of chemical compounds presents some issues related to disposal and worker safety. Electrolyzed water and ozone are two alternative sanitizing technologies that generate the active oxidizing component on site and do not use toxic chemical substances. Electrochemically activated water (ECAW) is an electrolyzed water sanitizer used for food and food equipment, which uses electrolysis of dilute sodium chloride solutions generating two distinct fractions, catholyte and anolyte. The anolyte is the sanitizing fraction and contains different forms of chlorine including hypochlorous acid *(124).* ECAW's sanitizing effects depend on free available chlorine (FAC), oxidation-reduction potential (ORP) and pH.

The use of different types of electrolyzed water has been reported to kill various foodborne pathogens including *E. coli* O157:H7, *Salmonella* and *L. monocytogenes (12, 33).* It has many advantages including the usage of safe source materials, safety for handling and distribution and being more environmentally-friendly compared to traditional chlorine sanitizers *(66, 67, 69, 142)*. Its effectiveness is the result of a combination of different forms of chlorine with hypochlorous acid which contributes a great extent of sanitation *(124)*.

Ozone (O_3) is a potent oxidant, formed from oxygen (O_2) by a high energy input. Commercially, ozone can be generated using different types of energy that include photochemical (i.e. ultraviolet radiation), electrical discharge (i.e. coronal discharge) chemical, thermal, chemonuclear, and electrolytic methods *(85, 138, 222)*. Ozone can be spontaneously decomposed into a nontoxic product, oxygen *(318)*, leaving no disinfectant residues *(138)*. Treating food surfaces with ozone can be achieved either by adding gaseous ozone continuously or intermittently to the storage atmosphere throughout the storage period or by washing or dipping in ozonated water to prevent the spread of cross-contamination and inactivate microbial cells *(119, 138, 222, 246)*.

Ozone was approved as a disinfectant or sanitizer in food processing by FDA *(85, 138)*. Treating food surfaces with ozone can be achieved either by adding gaseous ozone continuously or intermittently to the storage atmosphere throughout the storage period or by washing or dipping in ozonated water to prevent the spread of cross-contamination and inactivate the microbial cells *(11, 15, 26, 31)*. An ozone concentration of 0.1 mg/L for 6 h was found to be appropriate to inactivate *E. coli* in whole and ground black peppers without alteration of the organoleptic properties *(85)*.

Both ECAW and ozone are effective sanitizers for inactivating foodborne pathogens *(1, 11, 12, 66, 67, 69, 85, 114, 124, 246, 318)*, but the equipment for generating ECAW or ozonation is typically quite large and expensive for household or and small business applications. In addition, the relative short shelf life of the generated sanitizing solutions may also limit their application in small scales. These limitations have led to demand for small-sized and affordable ECAW or ozone generators. To meet

this market need, currently several companies have developed portable water sanitizing equipment advertising effective sanitization. According to informational materials, these types of equipment also use some sort of electrolysis processing for generating sanitizing water. However, to the best of our knowledge, there are no independent studies supporting their sanitizing claims.

This study was undertaken to investigate the sanitizing effects of some of these commercial technologies and provide guidance for consumers when considering sanitizing equipment. The objectives of this study were to evaluate the efficacy of water products made from several commercial technologies on *Escherichia coli* O157:H7, *Salmonella* and *Listeria monocytogenes* inactivation.

4.2. Materials and Methods

4.2.1. Bacterial strains

Strains of *E. coli* O157:H7 (ATCC 43890, ATCC 43895, 2028, 2257, 2029), *S. enterica* (Typhimurium ATCC 14028, Typhimurium E2009005811, Enteritidis 2009595, Tennessee E2007000302, and Saintpaul E2008001236) and *L. monocytogenes* (ATCC 19115, DUP-1030A, DUP-1038, DUP-1044A, and 2422) were included. For each strain, a loop of culture from -60°C storage was inoculated, transferred three consecutive times in tryptic soy broth (TSB) (Neogen, Inc., Lansing, MI) and inoculated at 37ºC at 24 h intervals.

4.2.2. Preparation and assessment of water sanitizers

ECAW was produced from a STEL 80 ECT US generator (Zap Water Technology, Inc, Richfield, MN) using tap water and saturated NaCl solutions at a voltage of 7 to 9 volts. After the machine reached a stable voltage reading, ECAW was collected from the anode side into a sterile glass beaker, covered to prevent the loss of chlorine and used within 2 h post-production. Free available chlorine (FAC), pH, and oxidation-reduction potential (ORP) of ECAW were determined by a chorine test kit using a drop count method (LaMotte Company, Chestertown, MD), a pH meter (Oakton Instruments Inc., Vernon Hills, IL) and an ORP meter (Oakton Instruments Inc., Vernon Hills, IL), respectively. The generated ECAW was diluted to 50 mg/l (ECAW-50) and 100 mg/l (ECAW-100) FAC for liquid culture testing and 100 mg/l for surface testing, respectively.

For other commercial technologies including the control group, sterile tap water was used and prepared using 0.22 μM filters (Falcon, Oxnard, CA). Ionator^{TM} EXP was purchased from Activeion Cleaning Solutions, LLC (Rogers, MN). Ionato^{TM} EXP was operated using tap water and tap water with 0.1% NaCl. Lotus^{TM} sanitizing system (Model LSR 100, Tersano SRL, Buffalo, NY) also was used with filter-sterilized tap water. Sterile tap water and salt-containing tap water were loaded onto the Ionator^{TM} EXP sprayer, produced and delivered by turning the spraying device on. The filtered tap water for Lotus^{TM} was cooled to 4°C before transfer to the machine according to manufacturer's instructions. Then the equipment was turned on and the water within the container circulated until the apparatus indicated that the cycle was complete.

4.2.3. Liquid culture testing

For each bacterial group, 30 ml cultures of 24 h were centrifuged for 10 min (3,600 × g, 4ºC). Pellets were washed using 15 ml peptone water (PW), centrifuged and re-suspended in 15 ml PW. For all the water sanitizers except Ionator^{TM}, 1 ml resuspended bacterial suspensions were added into bottles containing 99 ml of solution generated by different commercial technologies (or filtered tap water as the control). For Ionator^{TM} streams, 20 ml of generated solution were pre-added to bottles, 1 ml culture was added and an additional 79 ml of the solutions were sprayed. Bottles were shaken by hand for 30 s. Volumes of 1 ml of bacteria-solution mixtures were transferred to 9 ml neutralizing buffer solutions and shaken for 40 s (5.2 g/l; Becton Dickinson, Sparks, MD, USA). The neutralized mixture was then serially diluted. Two 0.1 ml aliquots of the diluents were spread on tryptic soy agar (TSA; Neogen, Inc., Lansing, MI) plates which were incubated at 37°C for 24 h for *E. coli* O157:H7 and *Salmonella* or 48 h for *L. monocytogenes*. Recovered bacteria were enumerated by counting colony forming units (CFU) *(12)*. Bacterial counts as CFU were calculated per ml and the data were transformed to logarithm base 10.

4.2.4. Bacteria dried on stainless steel surfaces

For each strain, approximately 10 ml of 24-h cultures were centrifuged as above. Pellets were washed with 5 ml sterile TSB, centrifuged and re-suspended in 2 ml TSB. Volumes of 25 µl of bacterial suspensions were inoculated on clean, sterile stainless steel

coupons in Petri dishes. The Petri dishes and coupons were dried in a biosafety cabinet for 3 h. Different solutions from each of the control and treatments were sprayed on inoculated coupons for 6 s at a distance of 7 to 10 cm. The coupon surface was wiped dry with a clean sterile cloth (around 3 cm × 3 cm). The coupon and the cloth, respectively, were placed in clean Petri dishes containing 10 ml neutralizing buffer for 40 s, and transferred to 50 ml sterile plastic tubes, to which were added 10 ml PW and 15-20 glass beads (3 mm) using sterile forceps. Tubes were then vortexed with full velocity for 2 min. Sprayed solutions were kept in the Petri dishes for an additional 54 s, then 0.1 ml of the sprayed solutions was spread on TSA plates *(332)*. Bacterial counts as CFU were calculated per stainless steel coupon and the data were transformed to logarithm base 10.

4.2.5. Data analyses

For each strain, at least two separate trials were independently conducted. For each trial, parallel groups were conducted in duplicate with two serials of plating results for any individual condition. Statistical analyses using analysis of variance (ANOVA) ($P < 0.05$) and Tukey Test for differences among different treatments were performed using SAS software (Version 9.1.3, SAS, Cary, NC, USA). Comparisons that yielded $P < 0.05$ were considered significant.

4.3. Results

The detection limits for the recovery of *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* from liquid culture and on stainless steel coupon surfaces were 2 log

CFU/ml and 2 log CFU/coupon, respectively, due to the neutralization step and 0.1 ml of the maximum plating volume of coupon/buffer mixture or liquid culture/buffer mixture. All the results were presented with the assumption that the survival of foodborne pathogens at levels below the detection limits could not be quantified. Thus, when no colony was found on the plates, the result was assigned a value of 2 log CFU/ml or 2 log CFU/coupon level.

Table 4.1 shows the effect of different water fractions on the recovery of 5 individual *E. coli* O157:H7 strains in liquid culture. None of the treatments with Ionator^{TM}, salt Ionator^{TM} or Lotus^{TM} water fractions reduced the cell count of liquid cultures compared with controls. Treatment of liquid bacterial cultures with ECAW (50 mg/l FAC) caused at least 5 log CFU/ml viable cell count reductions ($P < 0.05$) in all strains with the exception of strain ATCC 43895 which was only killed by 2 log CFU/ml. When ECAW fractions with concentrations of 100 mg/l FAC were tested, no survivors were detected.

Similar results were obtained with *Salmonella* strains in liquid cultures (Table 4.2) as only ECAW treatments yielded significant viable count reductions. Exposure to ECAW (50 mg/l FAC) resulted in more than 3 log CFU/ml reductions for all *Salmonella* strains and no detectable levels were found with 100 mg/l FAC. Liquid cultures of *L. monocytogenes* were also only susceptible to ECEW among all water treatments (Table 4.3). Both levels of FAC caused more than 5 log CFU/ml decreases in cell viability to all the strains tested for this Gram positive organism.

E. coli O157:H7, *Salmonella* and *L. monocytogenes* cells dried on stainless steel coupon surface exposed to the different water treatments were determined in each of the following fractions: coupons, wiping cloths, and the rinsing solution after treatment (Tables 4.4 to 4.6). For control, Lotus™, Ionator™, and salt Ionator treatments, from an initial inoculation of approximately 7 log CFU/coupon, more than 90% of the count was consistently recoveried in the water treatment originally sprayed on the coupon (rinse). In all of those treatments, the count of *E. coli* O157:H7 strains remaining on coupons ranged from 2.2 to 3.0 log CFU and transferred to wiping cloth from 3.4 to 4.1 log CFU (Table 4.4). The recovery of *Salmonella* serovars on coupons was slightly higher than for *E. coli* O157:H7 and for *L. monocytogenes*, but it never reached more than 4.0 log CFU. When any of the pathogenic bacterial strains were sprayed with ECAW (100 mg/l FAC) no survivors were detected above the detection level in any coupon, cloth and rinse.

Survival of *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* in buffers after treatment with antimicrobial water treatments were also determined: NB (neutralizing buffer) for coupon and NB for cloth (Tables 4.7 to 4.9). Microbial cells recovered from the two fractions of ECAW-100 (100 mg/l FAC) group were below the detection limit, while other groups had detectable microbial cells, and Ionator™, Salt Ionator and Lotus™ groups did not have significantly different results from the control group

4.4. Discussion

Sanitization plays a very important role in improving food safety. Chemical sanitizers are part of routine utilization in food service and are often recommended to

customers for household use. However, traditional chemical sanitizers such as chlorine, iodine, and hydrogen peroxide among others are often replaced with alternatives that do not employ concentrated potentially toxic compounds. It has long been recognized that electrochemically activated water solutions with antimicrobial activity can be generated from diluted salt solutions by passage through a specially designed electrode. Recently, a few technologies that offer some electrical treatment of plain water have been marketed to the food service industry as a convenient and chemical-free alternative. In this study, two of those commercially available sanitizing water technologies were tested and compared with ECAW. In one of them, the water was supplemented with salt to determine if it would increase antimicrobial activity. The results indicated that with the exception of ECAW, all water sanitizers tested were not effective in inactivating *E. coli* O157:H7, *Salmonella* and *L. monocytogenes*, three representative foodborne pathogens, either in liquid culture or dried on surfaces.

The effectiveness of electrolyzed water and ozonated water has been widely documented in the literature *(2, 6-13, 16, 18-20, 22-24, 29, 33, 38)*. These waters are generated by relatively large equipment with high power, and had been electrolyzed sufficiently or had enough ozone content. The different sanitizing waters can be based on electrolysis or on electrolysis and ozonation. The Lotus™ water used ozone as its sanitizing component, according to its instructions. Ozone has typical ORP values of 2.07 V, which is higher than chlorine-containing sanitizers *(138)*. It is effective both in the aqueous and gaseous phase *(318)*. At sufficient concentration, ozone can effectively and rapidly inactivate foodborne pathogens *(246)*.

Ozonized water has been determined to have almost no effect on food quality properties *(119)*. However, efficiency of ozone is affected by "ozone demand of the medium's residual ozone", which means that ozone remaining on food product or equipment after its application is also needed *(138)*. One possible reason why Lotus™ water was not effective may be that the equipment used relatively low-power treatments, i.e., the current and voltage were not high enough, so not enough ozone was generated to exert sanitizing effects. Ozone is a versatile antimicrobial agent that is relatively stable in air but highly unstable in water, decomposing in a very short time. Due to this property, another possible reason why Lotus™ water did not work could be that the ozone generated by Lotus™ is in a much more unstable form than that generated by large-scale machines. Considering that the Lotus™ water was applied immediately after its preparation, the second reason is highly unlikely. To test any of these hypotheses, further work measuring generated ozone concentrations would need to be conducted.

The action modes of different sanitizers might be different *(1, 159)*. When pH of ECAW is near neutral and within a limited range, only two factors, chlorine (HOCl, OCl^- and Cl_2) and ORP, determine its antimicrobial effects. Active chlorine exerts its effect by destroying the membranes of microorganisms or by reacting with biochemical molecules (amino acids, nucleic acids, or enzymes) as proposed by some researchers *(124, 147, 152, 181, 182)*. High ORP corresponds to strong oxidizing strength. It damages cell membranes, oxidizes cell surfaces, and disrupts cell metabolism, thus inactivating microbial cells *(166)*. Though there is no agreement about which of the two factors, FAC

or ORP, is more important for sanitization, chlorine surely is a necessary component for exerting sanitizing effects by ECAW.

The user manual of Ionator^{TM} EXP indicates that tap water without chlorine-containing salt is enough for generating sanitizers that have sanitizing effects of at least 3 log reductions (http://www.activeion.com/EXP.aspx#frame4). The manufacturer even provided a cartoon showing the electrolysis process with ion exchange and electrically charged nanobubbles. However, our results indicate that its effectiveness did not match the manufacturer's claims. The lack of effect of this technology could be due to the lack of an active chemical component as tap water was the only component. In the case of ECAW, if sodium chloride is not present before electrolysis, the resulting fraction is largely non-effective (data not shown). Tap water itself cannot be electrolyzed to generate high ORP either because limited current and voltage can be applied for electrolysis due to the absence of an electrolyte. The commercial technologies tested here were clearly not effective sanitizers, supporting the importance of electrolytes during electrolysis.

For better understanding of electrolysis and the reasons why the two waters did not work, 0.1% NaCl solution was applied to Ionator^{TM} EXP (salt Ionator). Even this salt Ionator can not generate effective sanitizing components. One possible reason could be that the electrical power delivered by Ionator^{TM} was not sufficient to cause electrolysis. The results of no sanitizing effects by 0.1% NaCl indicated that this portable equipment used to generate sanitizing components might not be sufficiently powerful (Tables 4.1 to 4.6).

Previous results about the antimicrobial activity of ECAW on foodborne pathogens varied significantly *(124, 126, 243)*. Many researchers have demonstrated that ECAW can generate 2 to 6 log CFU reductions of some bacteria such as *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*. A recent report indicated that treatment of *E. coli* NBRC 3301 for 0.5 min with ECAW (FAC 21.2 mg/l, pH 5.8, ORP 948) achieved 3.85 log CFU reductions. Higher concentrations of FAC and ORP (FAC 45.3 mg/l, pH 2.6, ORP 1140) resulted in 5.27 log CFU reductions *(130)*. Another report provided evidence that 5 min treatment of ECAW (FAC 89 mg/l, pH 8.0, ORP 760) achieved greater than 6 log CFU reductions for *E. coli* O157, *Salmonella* Enteritidis, and *L. monocytogenes (66)*. Our results showed ECAW resulted in 3 to more than 5 log CFU reductions for *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* in liquid culture, which was comparable to published results.

In general, ECAW for bacteria dried on surfaces is less effective and more variable than it is for liquid suspensions. For *L. monocytogenes* dried on surfaces, acidic ECAW (40 mg/l, pH 2.65, ORP 1155) only resulted in 1.91 log CFU reductions per chip dirty stainless steel, having 0.88 log CFU more reduction than tap water *(169)*. In the present report, only ECAW fractions containing 100 mg/l FAC were applied for surface treatment. The survival of microbial cells from all five fractions collected was below detection limits at 100 mg/l FAC, indicating that ECAW at this concentration can effectively stop cross contamination during food processing. Several factors may affect the antimicrobial effects of ECAW on surfaces and increase variability *(169, 170)*. Organochloramine and organochlorine are formed when chlorine compounds react with

organic compounds on surfaces *(11, 230, 242)*, resulting in reduced ability to penetrate into the protective layer of microbial polymers and reduced sanitizing effects *(6, 244)*. This may explain why ECAW is less effective on surfaces than in liquid culture and why with similar parameters, sanitizing effects of ECAW on foods and surfaces varied more as compared with liquid culture bacteria *(114, 333)*.

The microbial cells recovered from neutralizing buffers for coupon and for cloth (Tables 4.7-4.9) in groups of Ionator^{TM}, Salt Ionator and Lotus^{TM} alerted that cross contamination could happen.

In conclusion, this study investigated the sanitizing effects of two commercial technologies, which are available and recommended by their manufacturers to consumers, on *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* in liquid or dried on a stainless steel surface. All the tested water sanitizers were not effective in sanitizing any of the above foodborne pathogens except ECAW. The reasons why they did not have sanitizing effects were explained. The result is helpful for guiding food service operators and consumers to choose effective sanitizers for ensuring food safety.

Table 4.1. Effect of water fractions previously treated with different electrolysis technology on the viability *of Escherichia coli* O157:H7 liquid cultures. [a]

Strain	Survival count after treatment (log CFU/ml)					
	Control	**Ionator^{TM}**	**Salt Ionator[b]**	**Lotus^{TM}**	**ECAW-50[c]**	**ECAW-100[d]**
ATCC 43890	7.04 ±0.03 A	7.02 ±0.04 A	7.05 ±0.06 A	7.03 ±0.09 A	2.07 ±0.30 B	<2.00[e] C
ATCC 43895	7.16 ±0.02 A	7.15 ±0.07 A	7.14 ±0.06 A	7.14 ±0.16 A	4.90 ±0.65 B	<2.00 C
2028	7.17 ±0.05 A	7.14 ±0.05 A	7.12 ±0.03 A	7.09 ±0.08 A	<2.00 B	<2.00 B
2257	7.19 ±0.04 A	7.17 ±0.03 A	7.17 ±0.05 A	7.08 ±0.09 A	<2.00 B	<2.00 B
2029	7.10 ±0.04 A	7.10 ±0.05 A	7.07 ±0.07 A	7.04 ±0.08 A	<2.00 B	<2.00 B

[a] Within each row, means with different capital letters are significantly different ($P < 0.05$).

[b] Salt Ionator means Ionator^{TM} using 0.1% NaCl (w/v) solution.

[c] Concentration: FAC 50 mg/l.

[d] Concentration: FAC 100 mg/l.

[e] Detection limit: 2.00 log CFU/ml.

Table 4.2. Effect of water fractions previously treated with different electrolysis technology on the viability of *Salmonella spp.* liquid cultures. [a]

Serovar and strain	**Survival count after treatment (log CFU/ml)**					
	Control	**Ionator**[TM]	**Salt Ionator**[b]	**Lotus**[TM]	**ECAW-50**[c]	**ECAW-100**[d]
Typhimurium ATCC 14028	7.10 ±0.04 A	7.05 ±0.04 A	7.12 ±0.05 A	7.0 3±0.06 A	3.47 ±0.26 B	<2.00[e] C
Typhimurium E2009005811	7.04 ±0.07 A	7.00 ±0.08 A	7.03 ±0.06 A	7.02 ±0.06 A	<2.00 B	<2.00 B
Enteritidis 2009595	7.26 ±0.04 A	7.24 ±0.10 A	7.25 ±0.09 A	7.23 ±0.05 A	<2.00 B	<2.00 B
Tennessee E2007000302	7.34 0.05 A	7.30 ±0.08 A	7.33 ±0.07 A	7.26 ±0.16 A	3.05 ±0.40 B	<2.00 C
Saintpaul E2008001236	7.10 ±0.06 A	7.08 ±0.07 A	7.07 ±0.06 A	7.05 ±0.12 A	3.69 ±0.60 B	<2.00 C

[a] Within each row, means with different capital letters are significantly different ($P < 0.05$).

[b] Salt Ionator means Ionator[TM] using 0.1% NaCl (w/v) solution.

[c] Concentration: FAC 50 mg/l.

[d] Concentration: FAC 100 mg/l.

[e] Detection limit: 2.00 log CFU/ml.

Table 4.3. Effect of water fractions previously treated with different electrolysis technology on the viability of *Listeria monocytogenes* liquid cultures. [a]

Strain	**Survival count after treatment (log CFU/ml)**					
	Control	**Ionator**^{TM}	**Salt Ionator**[b]	**Lotus**^{TM}	**ECAW-50**[c]	**ECAW-100**[d]
ATCC 19115	7.24 ±0.03 A	7.17 ±0.09 A	7.19 ±0.04 A	7.21 ±0.04 A	<2.00[e] B	<2.00 B
DUP-1030A	7.23 ±0.03 A	7.19 ±0.04 A	7.19 ±0.01 A	7.20 ±0.03 A	<2.00 B	<2.00 B
DUP-1038	7.53 ±0.05 A	7.50 ±0.04 A	7.51 ±0.04 A	7.51 ±0.04 A	<2.00 B	<2.00 B
DUP-1044A	7.08 ±0.07 A	7.10 ±0.07 A	7.10 ±0.04 A	7.04 ±0.05 A	<2.00 B	<2.00 B
2422	6.58 ±0.05 A	6.54 ±0.12 A	6.61 ±0.05 A	6.52 ±0.11 A	<2.00 B	<2.00 B

[a] Within each row, means with different capital letters are significantly different ($P < 0.05$).

[b] Salt Ionator means Ionator^{TM} using 0.1% NaCl (w/v) solution.

[c] Concentration: FAC 50 mg/l.

[d] Concentration: FAC 100 mg/l.

[e] Detection limit: 2.00 log CFU/ml.

Table 4.4. Survival of *Escherichia coli* O157:H7 dried on coupons after treatment with antimicrobial water treatments (log CFU/coupon). [a]

Strain	Testing fraction	Survival count after treatment				
		Control	Ionator™	Salt Ionator[b]	Lotus™	ECAW-100[c]
ATCC 43890	Coupon	2.38±0.20 A	2.31±0.12 A	2.42±0.13 A	2.35±0.06 A	<2.00[d] B
	Cloth	3.50±0.06 A	3.48±0.11 A	3.48±0.09 A	3.46±0.11 A	<2.00 B
	Rinse	6.60±0.18 A	6.64±0.10 A	6.65±0.12 A	6.63±0.12 A	<2.00 B
ATCC 43895	Coupon	3.01±0.21 A	2.93±0.09 A	3.00±0.12 A	2.94±0.08 A	<2.00 B
	Cloth	3.44±0.11 A	3.44±0.01 A	3.40±0.02 A	3.39±0.04 A	<2.00 B
	Rinse	6.70±0.15 A	6.71±0.14 A	6.70±0.14 A	6.72±0.12 A	<2.00 B
2028	Coupon	2.98±0.19 A	2.68±0.23 A	2.80±0.33 A	2.61±0.17 A	<2.00 B
	Cloth	4.10±0.04 A	4.06±0.05 A	4.07±0.10 A	3.99±0.05 A	<2.00 B
	Rinse	6.54±0.16 A	6.52±0.21 A	6.51±0.20 A	6.50±0.21 A	<2.00 B
2257	Coupon	2.72±0.67 A	2.16±0.38 A	2.28±0.28 A	2.23±0.29 A	<2.00 B
	Cloth	3.85±0.37 A	3.78±0.34 A	3.83±0.28 A	3.73±0.38 A	<2.00 B
	Rinse	6.45±0.04 A	6.50±0.08 A	6.51±0.05 A	6.47±0.08 A	<2.00 B
2029	Coupon	2.57±0.66 A	2.24±0.26 A	2.30±0.20 A	2.11±0.29 A	<2.00 B
	Cloth	3.47±0.09 A	3.45±0.07 A	3.43±0.10 A	3.38±0.13 A	<2.00 B
	Rinse	6.37±0.42 A	6.47±0.37 A	6.44±0.36 A	6.47±0.38 A	<2.00 B

[a] Initial number of microbial cells was 6.94±0.03, 7.02±0.18, 7.10±0.15, 6.50±0.08, 6.96±0.33 for strains 43890, 43895, 2028, 2257, and 2029, respectively. Within each row, means with different capital letters are significantly different ($P < 0.05$).

[b] Salt Ionator means Ionator™ using 0.1% NaCl (w/v) solution.

[c] Concentration: FAC 100 mg/l.

[d] Detection limit: 2.00 log CFU/coupon.

Table 4.5. Survival of *Salmonella spp.* dried on coupons after treatment with antimicrobial water treatments (log CFU/coupon). [a]

Strain	Testing fraction	Survival count after treatment				
		Control	Ionator™	Salt Ionator[b]	Lotus™	ECAW-100[c]
Typhimurium ATCC 14028	Coupon	3.29±0.26 A	3.23±0.29 A	3.26±0.24 A	3.19±0.20 A	<2.00[d] B
	Cloth	3.54±0.30 A	3.47±0.32 A	3.46±0.32 A	3.46±0.36 A	<2.00 B
	Rinse	6.95±0.26 A	6.98±0.23 A	6.99±0.22 A	6.93±0.25 A	<2.00 B
Typhimurium E2009005811	Coupon	3.77±0.08 A	3.73±0.09 A	3.74±0.09 A	3.67±0.17 A	<2.00 B
	Cloth	3.96±0.03 A	3.93±0.02 A	3.92±0.03 A	3.78±0.20 A	<2.00 B
	Rinse	6.92±0.30 A	6.95±0.28 A	6.95±0.29 A	6.89±0.30 A	<2.00 B
Enteritidis 2009595	Coupon	3.62±0.42 A	3.49±0.16 A	3.51±0.17 A	3.47±0.54 A	<2.00 B
	Cloth	3.81±0.25 A	3.70±0.14 A	3.71±0.15 A	3.55±0.40 A	<2.00 B
	Rinse	6.88±0.25 A	6.81±0.23 A	6.79±0.25 A	6.83±0.18 A	<2.00 B
Tennessee E2007000302	Coupon	3.30±0.16 A	3.19±0.24 A	3.31±0.21 A	3.16±0.30 A	<2.00 B
	Cloth	3.99±0.14 A	3.95±0.16 A	3.99±0.17 A	3.88±0.20 A	<2.00 B
	Rinse	7.14±0.22 A	7.16±0.20 A	7.16±0.22 A	7.09±0.27 A	<2.00 B
Saintpaul E2008001236	Coupon	3.87±0.23 A	3.72±0.14 A	3.77±0.25 A	3.75±0.32 A	<2.00 B
	Cloth	3.29±0.26 A	3.23±0.29 A	3.26±0.24 A	3.19±0.20 A	<2.00 B
	Rinse	7.11±0.18 A	7.10±0.20 A	7.09±0.21 A	7.03±0.27 A	<2.00 B

[a] Initial number of microbial cells was 7.22±0.20, 7.36±0.29, 7.31±0.24, 7.97±0.21, 7.71±0.43 for Typhimurium ATCC 14028, Typhimurium E2009005811, Enteritidis 2009595, Tennessee E2007000302, and Saintpaul E2008001236, respectively. Within each row, means with different capital letters are significantly different ($P < 0.05$).

[b] Salt Ionator means Ionator™ using 0.1% NaCl (w/v) solution.

[c] Concentration: FAC 100 mg/l.

[d] Detection limit: 2.00 log CFU/coupon.

Table 4.6. Survival of *Listeria monocytogenes* dried on coupons after treatment with antimicrobial water treatments (log CFU/coupon). [a]

Strain	Testing fraction	Survival count after treatment				
		Control	Ionator™	Salt Ionator[b]	Lotus™	ECAW-100[c]
ATCC 19115	Coupon	3.28±0.2 A	3.15±0.3 A	3.14±0.2 A	3.20±0.2 A	<2.00[d] B
	Cloth	3.19±0.2 A	3.12±0.2 A	3.17±0.2 A	3.20±0.3 A	<2.00 B
	Rinse	6.2±0.1 A	5.89±0.6A	5.91±0.6 A	5.91±0.6 A	<2.00 B
DUP-1030A	Coupon	3.26±0.3 A	3.2±0.25 A	3.13±0.3 A	3.32±0.3 A	<2.00 B
	Cloth	3.35±0.2A	3.1±0.26 A	3.2±0.28 A	3.44±0.2 A	<2.00 B
	Rinse	6.73±0.5 A	6.58±0.6 A	6.57±0.6 A	6.64±0.6 A	<2.00 B
DUP-1038	Coupon	3.3±0.36 A	3.1±0.28 A	3.19±0.4 A	3.1±0.24 A	<2.00 B
	Cloth	3.4±0.34 A	3.13±0.2 A	3.22±0.2 A	3.2±0.18 A	<2.00 B
	Rinse	6.8±0.95 A	6.4±0.74 A	6.38±0.7 A	6.45±0.8 A	<2.00 B
DUP-1044A	Coupon	3.30±0.3 A	3.06±0.2 A	3.2±0.25 A	3.0±0.37 A	<2.00 B
	Cloth	3.44±0.3 A	3.13±0.2 A	3.2±0.15 A	3.11±0.2 A	<2.00 B
	Rinse	6.61±0.6 A	6.24±0.4 A	6.25±0.5 A	6.31±0.4 A	<2.00 B
2422	Coupon	3.19±0.2 A	3.1±0.18 A	3.12±0.2 A	3.24±0.1 A	<2.00 B
	Cloth	3.3±0.26 A	3.2±0.24 A	3.06±0.2 A	3.3±0.25 A	<2.00 B
	Rinse	6.4±0.46 A	6.0±0.65 A	6.03±0.6 A	6.35±0.4 A	<2.00 B

[a] Initial number of microbial cells was 6.80±0.11, 7.15±0.52, 7.31±0.50, 7.29±0.42, 6.78±0.19 for ATCC 19115, DUP-1030A, DUP-1038, DUP-1044A, and 2422, respectively. Within each row, means with different capital letters are significantly different ($P < 0.05$).

[b] Salt Ionator means Ionator™ using 0.1% NaCl (w/v) solution.

[c] Concentration: FAC 100 mg/l.

[d] Detection limit: 2.00 log CFU/coupon.

Table 4.7. Survival of *Escherichia coli* O157:H7 in buffers after treatment with antimicrobial water treatments (log CFU/coupon). [a]

Strain	Testing fraction	Survival count after treatment				
		Control	Ionator™	Salt Ionator[b]	Lotus™	ECAW-100[c]
ATCC 43890	NB[d] for coupon	2.62±0.29 A	2.54±0.17 A	2.69±0.21 A	2.45±0.10 A	<2.00[e] B
	NB for cloth	3.26±0.17 A	3.18±0.08 A	3.18±0.09 A	3.20±0.11 A	<2.00 B
ATCC 43895	NB for coupon	3.38±0.10 A	3.38±0.06 A	3.35±0.07 A	3.33±0.10 A	<2.00 B
	NB for cloth	3.13±0.08 A	3.01±0.12 A	3.10±0.11 A	3.03±0.12 A	<2.00 B
2028	NB for coupon	3.56±0.15 A	3.53±0.07 A	3.47±0.16 A	3.45±0.09 A	<2.00 B
	NB for cloth	3.42±0.30 A	3.14±0.13 A	3.14±0.26 A	3.33±0.14 A	<2.00 B
2257	NB for coupon	2.69±0.70 A	2.09±0.27 A	2.28±0.24 A	2.12±0.15 A	<2.00 B
	NB for cloth	3.62±0.21 A	3.41±0.32 A	3.45±0.27 A	3.43±0.28 A	<2.00 B
2029	NB for coupon	2.91±0.50 A	2.49±0.16 A	2.48±0.21 A	2.36±0.27 A	<2.00 B
	NB for cloth	3.02±0.43 A	2.48±0.23 A	2.68±0.20 A	2.61±0.21 A	<2.00 B

[a] Initial number of microbial cells was 6.94±0.03, 7.02±0.18, 7.10±0.15, 6.50±0.08, 6.96±0.33 for strains 43890, 43895, 2028, 2257, and 2029, respectively. Within each row, means with different capital letters are significantly different ($P < 0.05$).

[b] Salt Ionator means Ionator™ using 0.1% NaCl (w/v) solution.

[c] Concentration: FAC 100 mg/l.

[d] NB, neutralizing buffer.

[e] Detection limit: 2.00 log CFU/coupon.

Table 4.8. Survival of *Salmonella spp.* in buffers after treatment with antimicrobial water treatments (log CFU/coupon). [a]

Strain	Testing fraction	Survival count after treatment				
		Control	Ionator™	Salt Ionator[b]	Lotus™	ECAW-100[c]
Typhimurium ATCC 14028	NB[d] for coupon	3.15±0.66 A	3.05±0.07 A	3.08±0.08 A	3.03±0.12 A	<2.00[e] B
	NB for cloth	3.19±0.09 A	3.08±0.07 A	3.05±0.09 A	3.13±0.18 A	<2.00 B
Typhimurium E2009005811	NB for coupon	3.68±0.08 A	3.64±0.09 A	3.64±0.10 A	3.56±0.19 A	<2.00 B
	NB for cloth	3.32±0.12 A	3.13±0.15 A	3.24±0.12 A	3.29±0.27 A	<2.00 B
Enteritidis 2009595	NB for coupon	3.43±0.31 A	3.38±0.18 A	3.39±0.20 A	3.26±0.47 A	<2.00 B
	NB for cloth	3.20±0.35 A	2.95±0.13 A	3.09±0.14 A	3.17±0.44 A	<2.00 B
Tennessee E2007000302	NB for coupon	3.38±0.18 A	3.29±0.20 A	3.36±0.25 A	3.20±0.30 A	<2.00 B
	NB for cloth	3.57±0.08 A	3.41±0.21 A	3.52±0.23 A	3.44±0.18 A	<2.00 B
Saintpaul E2008001236	NB for coupon	3.52±0.43 A	3.32±0.23 A	3.40±0.24 A	3.40±0.53 A	<2.00 B
	NB for cloth	3.59±0.42 A	3.48±0.13 A	3.58±0.18 A	3.45±0.52 A	<2.00 B

[a] Initial number of microbial cells was 7.22±0.20, 7.36±0.29, 7.31±0.24, 7.97±0.21, 7.71±0.43 for Typhimurium ATCC 14028, Typhimurium E2009005811, Enteritidis 2009595, Tennessee E2007000302, and Saintpaul E2008001236, respectively. Within each row, means with different capital letters are significantly different ($P < 0.05$).

[b] Salt Ionator means Ionator™ using 0.1% NaCl (w/v) solution.

[c] Concentration: FAC 100 mg/l.

[d] NB, neutralizing buffer.

[e] Detection limit: 2.00 log CFU/coupon.

Table 4.9. Survival of *Listeria monocytogenes* in buffers after treatment with antimicrobial water treatments (log CFU/coupon). [a]

Strain	Testing fraction	Survival count after treatment				
		Control	Ionator™	Salt Ionator[b]	Lotus™	ECAW-100[c]
ATCC 19115	NB[d] for coupon	3.14±0.29 A	3.02±0.34 A	2.99±0.31 A	3.06±0.20 A	<2.00[e] B
	NB for cloth	3.04±0.09 A	3.03±0.25 A	3.05±0.26 A	2.96±0.31 A	<2.00 B
DUP-1030A	NB for coupon	3.17±0.12 A	2.99±0.21 A	2.91±0.34 A	3.18±0.25 A	<2.00 B
	NB for cloth	3.25±0.09 A	3.12±0.22 A	3.19±0.21 A	3.14±0.36 A	<2.00 B
DUP-1038	NB for coupon	3.10±0.40 A	2.93±0.33 A	2.90±0.44 A	2.94±0.32 A	<2.00 B
	NB for cloth	3.09±0.47 A	2.89±0.29 A	3.04±0.22 A	2.97±0.21 A	<2.00 B
DUP-1044A	NB for coupon	3.11±0.38 A	2.92±0.45 A	3.14±0.40 A	2.79±0.35 A	<2.00 B
	NB for cloth	3.17±0.30 A	2.80±0.15 A	2.97±0.15 A	2.88±0.28 A	<2.00 B
2422	NB for coupon	3.06±0.16 A	2.91±0.17 A	3.01±0.18 A	3.17±0.18 A	<2.00 B
	NB for cloth	3.18±0.25 A	3.11±0.24 A	3.14±0.34 A	3.26±0.31 A	<2.00 B

[a] Initial number of microbial cells was 6.80±0.11, 7.15±0.52, 7.31±0.50, 7.29±0.42, 6.78±0.19 for ATCC 19115, DUP-1030A, DUP-1038, DUP-1044A, and 2422, respectively. Within each row, means with different capital letters are significantly different ($P < 0.05$).

[b] Salt Ionator means Ionator™ using 0.1% NaCl (w/v) solution.

[c] Concentration: FAC 100 mg/l.

[d] NB, neutralizing buffer.

[e] Detection limit: 2.00 log CFU/coupon.

CHAPTER V

MORPHOLOGY OF BIOFILMS OF *ESCHERICHIA COLI* O157:H7, *SALMONELLA ENTERICA* AND *LISTERIA MONOCYTOGENES* VISUALIZED BY ATOMIC FORCE MICROSCOPY

Bacterial biofilms are composed of extracellular polymeric substances (EPS) and microbial cells that confer protection against external stressful conditions. *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* are foodborne pathogens that are capable of forming biofilms. The native morphology of biofilms has been studied using a variety of microscopic techniques. In this current study, atomic force microscopy (AFM), which is considered to have the least effect on native biofilm structure, was used for studying the morphology of pathogen biofilms on stainless steel surfaces. Three morphologies of the biofilms were observed: tree-like structures, individual cells and no characteristic structures of the above two (none). The relative percentages of observations of the tree-like, individual cells, and no structures were about 29.8%, 18.1%, and 52.1%, respectively, for *L. monocytogenes* strain ATCC 19115. *E. coli* O157:H7 strain 6058 and *S. enterica* Typhimurium E2009005811 had significantly low percentages of tree-like

morphology, compared to other *E. coli* O157:H7 strains and *Salmonella spp.* There were no significant differences in biofilm morphological distribution within the four strains of *L. monocytogenes*. Morphological distribution was not affected by growth media or inoculation amounts for *L. monocytogenes* biofilm formation. Neutral electrochemically activated water (NECAW) treatment destroyed microbial cells as well as removed the tree-like structures. AFM appeared to be a suitable technique to study and describe biofilms and may offer a unique perspective.

5.1. Introduction

Listeria monocytogenes is an intracellular pathogenic Gram-positive bacterium that causes listeriosis. *Escherichia coli* O157:H7 is an enterohemorrhagic serotype of *E. coli*, responsible for hemorrhagic diarrhea, and in some cases hemolytic uremic syndrome (HUS), especially in children. *Salmonella* is a rod-shaped, non-spore-forming enterobacterial pathogen that infects more people than any other foodborne bacteria. *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* combined are responsible for the largest number of deaths linked to foods (82%), and are three of the most important infectious bacteria targeted for reductions in the U. S. Centers for Disease Control and Prevention (CDC)'s Healthy People 2010 plan *(192)*. For instance, *L. monocytogenes* accounted for approximately 18.9% of deaths caused by foodborne cases due to 31 pathogens in the U.S. *(274)*. The estimated number of infection incidences caused by *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* in 2008 was not significantly less compared to 2005 *(317)*.

Biofilms are organized communities of bacteria growing and surviving in their natural environment under suitable conditions. Biofilms can be formed by a single bacterial species or mixed species *(194)*. They are composed of microbial cells and extracellular polymeric substances (EPS) that cover the cells. In biofilms, microbial cells form clusters, which are believed to be connected by channels that deliver water and nutrients. All these clusters, channels, and EPS form a 3-dimensional structure. The microbial clusters or microcolonies encased by EPS can be physically separated by interstitial voids. It is thought that planktonic *L. monocytogenes* cells might use a quorum sensing mechanism to coordinate themselves as a collective living system and form biofilms *(22)*.

The biofilms of *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* can cause persistent low-level contamination of foods that come in contact with contaminated equipment surfaces, leading to food safety concerns. In food processing, once biofilms are formed, the microbial community becomes more resistant to antimicrobial agents and more difficult to be completely removed than their planktonic status *(73, 194)*. The inability to efficiently remove biofilms is a major problem, which results in contamination of food products and possible foodborne disease outbreaks *(329)*. This is of particular importance for *L. monocytogenes* and *S. enterica* since they are widely distributed in the environment and food processing plants *(73)*. A recent listeriosis outbreak with cantaloupes tragically illustrates the consequences of biofilm formation in the processing environment *(49)*.

Many methods have been applied to characterize foodborne pathogen biofilms. The microtiter plate assay can estimate the growth of bacteria in situ *(86, 345)*, but it is an indirect method and bacterial morphology cannot be easily observed, since the plates must be optically transparent in order to read spectra results *(86)*. The ruthenium red staining technique was introduced to determine the existence of EPS based on the interaction between the ruthenium red dye and carbohydrates. Although EPS contains carbohydrates, cell surface carbohydrates which are not part of EPS will also bind to the dye, therefore affecting the results *(33)*. Transmission/scanning electron microscopy, epifluorescence microscopy *(58, 110, 236)*, and (confocal) laser scanning microscopy are currently widely used for biofilm characterization. These microscopy techniques are applied mainly for targeting microbial cells. Pretreatment or final staining steps might affect the original status of EPS. Due to this reason these microscopes are not perfect for biofilm EPS visualization since the amount of EPS is thought to be related to strain virulence *(186, 320)* and increased resistance to antimicrobial agents *(304)*. In addition, the pretreatments of staining, fluorescence, or vacuum effects involved in these techniques might interfere with the native status of biofilm surface structures *(36, 307, 336)*.

Atomic force microscopy (AFM) generates images by measuring changes in the interaction between a probe and sample surfaces. This instrument has many advantages, including minimal sample preparation, independence of the substance's light transparency, and the ability to measure sample dimensions *(172, 235, 307, 336)*. One special advantage of AFM for biofilms is that it characterizes EPS almost without

affecting native morphology because no treatment for imaging after biofilm formation is applied *(265)*. AFM has been successfully applied in surface characterization including stainless steel surfaces *(264)*, single microbial cells *(23, 79, 110, 158, 162, 203, 207, 239, 269, 334, 337)*, biofilms *(135, 183, 224, 265, 266)*, hydrated biofilm EPS *(80, 307)*, and corresponding physical properties of EPS *(238, 240)*.

Currently, the morphological organization and pattern of biofilms of *E. coli* O157:H7, *Salmonella*, *L. monocytogenes*, and their changes with sanitization treatment remain largely unknown. Moreover, though the sanitizer neutral electrochemically activated water (NECAW) is effective for many bacteria, its antimicrobial mechanism is not clear *(124)*. In-depth understanding of pathogen biofilms, especially their morphologies, is necessary in order to better assess the risk of pathogen contamination, understand the mechanism of NECAW effects and develop strategies to control foodborne pathogen diseases *(186, 320)*.

The aim of the present study was to characterize *E. coli* O157:H7, *Salmonella enterica* and *L. monocytogenes* biofilms developed on stainless steel coupons, and morphological changes of *L. monocytogenes* biofilms as representative of treatment with washing or NECAW. AFM was performed to image biofilms with multimode images applied to characterize biofilm morphologies and NECAW effects.

5.2. Materials and Methods

5.2.1. Bacterial strains

Strains of *E. coli* O157:H7 (ATCC 43890, ATCC 43895, 6058, EK-1), *S. enterica* (Typhimurium E2009005811, UK-1, B4442 and Saintpaul E2008001236) and *L. monocytogenes* (ATCC 19115, 2349, 3528 and 2422) were used in this study. Source information about these strains is listed in Table 3.1. A loop of -60°C storage culture of each strain was inoculated and transferred three consecutive times in tryptic soy broth (TSB) (Neogen, Inc., Lansing, MI) and inoculated at 37ºC at 24 h intervals.

5.2.2. Preparation and characterization of NECAW

NECAW was freshly generated from softened tap water and saturated NaCl solutions by a generator (Zap Water Technology, Inc., Richfield, MN) at a voltage range of 7 to 9 volts. After a stable voltage reading was reached, NECAW was collected using a sterile glass bottle from the anode side, covered and used within 2 h post generation. FAC of NECAW was determined with a chorine test kit by calculating the drop counts (LaMotte Company, Chestertown, MD). ORP and pH were measured with an ORP meter (ORPTestr 10, Oakton Instruments Inc., Vernon Hills, IL) and a pH meter (pHTestr 10, Oakton Instruments Inc.), respectively.

5.2.3. Biofilm formation

Individual strains were prepared as described above for liquid culture testing. Suspensions were mixed with 9 ml sterile low nutrient TSB (1:10 dilution of normal TSB solution, LN-TSB) with a dilution of 1:100. Sterilized stainless steel coupons were

immersed into the suspensions, mixed well and left in the liquid cultures for 4 h at room temperature to allow bacteria attachment. Suspensions were poured off and the coupons were rinsed gently with a circular motion for 10 s using 1 ml PW in order to remove unattached microbial cells. The PW were poured off. Coupons were added with 10 ml fresh LN-TSB and stayed at room temperature. After 48 h, LN-TSB was discarded and coupons were transferred to a new LN-TSB solution for another 24 h at room temperature. After this incubation, coupons were rinsed gently with 1 ml PW twice to remove loosely attached microbial cells. Then the coupons were dried in a biosafety cabinet for 2 h *(12)*.

5.2.4. Biofilm treatment with NECAW

Coupons with biofilms were placed into glass tubes containing 10 ml NECAW 250 mg l^{-1} FAC or DIW as control, slightly mixed at speed 2 using a Mdl G-560 Vortex Genie 2 Mixer (Lehman Scientific, LLC, Wrightsville, PA) for 5 s. After 25 s of stillness, coupons were mixed again for 5 s, then kept still for 25 s again. Coupons were transferred to new glass tubes containing 10 ml neutralizing buffer solution and mixed at speed 2 for 5 s. After 35 s of waiting, coupons were transferred to 50-ml disposable plastic tubes containing 10 ml PW and 3 g sterile glass beads (Sigma-Aldrich Co., St. Louis, MO), and vortexed heavily for 5 min to remove bacteria from the coupons. A series of ten-fold dilutions of the PW containing coupons and neutralizing buffer were conducted, respectively after vortexing for direct plating and enrichment test.

5.2.5. Microbial analysis

The numbers of viable cells in the final diluted PW and the neutralizing buffer were determined by directly plating 0.1 ml of each diluent in duplicate on tryptic soy agar (TSA; Neogen Corp) plates, and further counting the colonies after incubation at 37ºC for 24 h (for *E. coli* O157:H7 and *Salmonella*) or 48 h (for *L. monocytogenes*). The CFU of the recovered bacteria was enumerated and transformed to logarithm base 10 per ml or coupon. For enrichment test, 5 ml PW recovered from coupons and neutralizing buffer after treatment were transferred to 250 ml Erlenmeyer flasks containing 100 ml sterile TSB and incubated at 37ºC for 48 h *(12, 244)*. Selected colonies from TSA plates were streaked onto selective agar and incubated to confirm the presence of pathogens.

5.2.6. AFM experiment and analysis

AFM was carried out using a model 5500 Molecular Imaging PicoPlus/PicoScan 3000 system (now Agilent Technologies, Santa Clara, CA). Tapping mode AFM was applied with rectangular silicon probes and tip radii of curvature 5-10 nm (Applied Nanostructures Inc., Santa Clara, CA). The spring constant of the cantilever was manufacturer-specified in the range of 30-60 N/m, and the resonant frequency was around 300 kHz. AFM imaging of biofilms on stainless steel coupons was conducted at ambient temperature (22-24°C) in air with scanning rate of 0.5-1 Hz, relative humidity of 15%-30%. Different scan sizes (from 5 μm × 5 μm to 50 μm × 50 μm) were applied in order to image bacteria dried on stainless steel surfaces or biofilms. The measurements of biofilms morphology were conducted independently at least twice. For each time, at least

two parallel coupons were independently included for each condition. Different regions were imaged and recorded for the same coupon as well.

Three different mode images were recorded simultaneously with multimode imaging: topography, amplitude, and phase images. Images of topography and amplitudes are shown in the results. Topography imaging records the height of the samples, while amplitude imaging highlights the edges of the features and phase imaging provides contrast of the features and background from features' physical properties. Amplitude and phase modes provide even higher quality images for better viewing the structure of the objects.

The characteristic morphology of biofilms was divided mainly according to amplitude mode images, and the scales (x, y directions) of the images were also doubled checked with the height mode images. The tree-like structure was defined as pure continuous tree-like morphology with no other characteristic structures on the coupon surface. The category of individual cells was defined as when at least one microbial cell was observed from the AFM images, regardless of presence or absence of a tree-like morphology. The feature for individual cells was smooth and regular and in the range of around 1-3 µm in length and 0.5-1.5 µm in width. Features varying from these dimensions were not considered as individual cells *(334)*. Other places on the coupon surfaces without these two features were calculated as the category 'None'. The relative percentages of different types of morphological characteristics were calculated based on the images obtained.

5.2.7. Data analyses

For microbial cell analysis and morphological analysis of each strain, at least two separate trials were independently conducted. For each trial, parallel groups were conducted in duplicate with two serials of plating results for any individual condition. For morphological analysis, different zones of each coupon were scanned and collected for analysis. More than twenty AFM image results were applied for any specific experimental conditions. Statistical analyses using analysis of variance (ANOVA) ($P < 0.05$) and Tukey test for differences among different strains or treatments were performed using SAS software (Version 9.1.3, SAS, Cary, NC). Comparisons that yielded $P < 0.05$ were considered significant.

5.3. Results

Figure 5.1 shows AFM images of the surfaces of a sterile coupon (a and b) and a control coupon obtained with the same procedure as for biofilm growth except that the bacterial strain was inactivated prior to inoculation (c and d). Cut lines formed during manufacture of the coupons were visible on the coupon surfaces but no other characteristic morphologies were observed.

The biofilms were found to have heterogeneous structures with three categories: Tree-like, individual microbial cells and none (do not have tree-like or individual cells). Figures 5.2 and 5.3 show these different morphological categories for *L. monocytogenes* ATCC 19115 and *E. coli* O157:H7 strain 6058, respectively, as examples. Characteristic tree-like structures or individual cells did not cover the whole stainless steel coupon

surface. Instead, they appeared as non-continuous formations on the coupon surface. Biofilms were such a highly heterogeneous community that they were very visible on some parts of the stainless steel surface as mature biofilms, while in other areas biofilms were in developing status or had not formed at all.

Quantitative analysis of the distribution of the above three morphological categories for *L. monocytogenes* biofilms is shown in Table 5.1. The results indicated that the distributions of different morphologies were not significantly different among the four strains of *L. monocytogenes*. However, some *E. coli* O157:H7 and *S. enterica* strains had significantly less tree-like morphology than other strains. For instance, *E. coli* O157:H7 strain 6058 and *S. enterica* Typhimurium E2009005811 had only 8.0% and 8.4% tree-like morphology, respectively (Table 5.2).

The effect of varied inoculation amounts on the final biofilm morphology distribution is also shown in Table 5.1. The inoculation amount '1' meant normal inoculation amount, while 0.1 and 0.001 denoted 10 and 1000 time dilutions of normal inoculation amounts. The results indicated that the morphologies did not change significantly due to different inoculation amounts. The effect of growth media on biofilm growth was also studied and no difference between BHI and TSB (both were used at 1/10 of normal concentrations) was found. The effects of initial inoculation amounts and alternative media (BHI) on the survival of microbial cells in the biofilms are shown in Table 5.3. The results demonstrated that biofilm morphology and the growth and survival of these pathogens were unaffected.

Figure 5.4 depicts represenrative effects of deionized water treatment (a and b) and NECAW treatment (c and d) on biofilms (*L. monocytogenes* ATCC 19115). Biofilm surface morphology was washed off by DI water; however the integrity of the microbial cells were still maintained, as indicated by the smoothness of the microbial surface. In contrast, after NECAW treatment the surface integrity of the microbial cells was damaged, suggesting that NECAW penetrated into the microbial cells and inactivated them.

EPS of biofilms were removed by DI water or NECAW treatment and inner microbial cells appeared (Figure 5.4), leading to observable distribution of the microbial cells by AFM imaging. The effect of NECAW treatment on survival of microbial cells is shown in Table 5.4. DI water only resulted in approximately 1.2 log CFU/coupon reductions. In contrast, NECAW treatment resulted in more than 4.6 log CFU/coupon reductions, thus 3.5 log CFU/coupon more reductions than DI water treatment.

5.4. Discussion

5.4.1. Variation in biofilm morphology

The morphology of the clean coupon surface was very similar to that of a previous report *(264)*. Cut lines on the coupon surface, formed during coupon processing in the factory, were observed by AFM imaging. Current height variation was also comparable to that found in the literature. Considering difference in the z scale, the variation of surface contour over a 25 μm^2 was approximately 500 nm, which was similar

to numbers reported by other groups *(36)*. However, the height of cut lines was taller than the images from Wang and others (2003) *(320)*. One of the reasons could be that Wang and others (2003) might have used offline 'deflection' processing of the AFM software, which alleviated the variation of substrate and decreased the value of height from their images.

Generally, the use of an AFM probe with a low spring constant is suggested for biological samples to minimize damage of the sample surface. However, an AFM probe with a low spring constant can easily be trapped by the samples. As bacterial EPS can interact strongly with AFM tips, a relatively high spring constant (k = 3 N/m) was used, which should result in alleviation of trapping of the AFM probe by the samples *(265)*.

For the control group inoculated with dead bacterial cultures, height mode images showed that the stainless steel coupon surface was covered with a continuous layer of adsorbed substances, which could be from growth media, TSB or BHI (Figure 5.1). For coupons containing biofilms, protrusions could be seen underneath the continuous surface layer of EPS, and below the protrusions could be microbial cells as aggregates or individuals (Figures 5.2-5.4) *(307)*. AFM images demonstrated that biofilms of the three foodborne pathogens on stainless steel were heterogeneous in structure, containing both microbial cells and EPS, which was in accordance with other reports of biofilms formed by *L. monocytogenes* or other bacteria *(36, 77, 194, 265, 266)*. For instance, the existence of heterogeneous distribution of both EPS and microbial cells within the bacterial CCI#8 biofilms was reported with some microbial cells growing in lines on the coupon surface *(36)*. However, it was also found that *L. monocytogenes* microbial cells on mechanically

polished stainless steel grew along the stainless steel crevices *(265)*. And microbial cells did not colonize the whole stainless steel surface but associated as individual associates or as small microcolonies.

The structure of biofilms depended on various intrinsic and extrinsic parameters *(194)*. Intrinsically, EPS, a major component of biofilms composed of polysaccharides, proteins, nucleic acids, and amphiphilic compounds, are so complex and diverse that it is a major factor influencing the structure of biofilms and makes studying biofilm morphology challenging *(213)*. However, the composition and structure of biofilm EPS always vary due to changing microorganisms, their physiological status, nutrients status, and physical conditions *(194, 291, 292)*. Extrinsically, *L. monocytogenes* biofilm morphology could vary under each particular condition with different replicates, as reported previously *(186, 320)*.

The tree-like morphology was first reported for foodborne pathogen biofilms, which was considered to be from EPS and different from previous reports. Wang and others *(320)* reported that *L. monocytogenes* biofilm EPS was thread-like, connecting among microbial cells, or between a microbial cell and the substance. In contrast, Dubravka and others *(77)* thought, based on SEM results, that *L. monocytogenes* strains were weak at producing EPS due to its removal by pretreatment during SEM imaging with three 10 min rinses in 100% ethanol. However, *L. monocytogenes* biofilms imaged by wide-field fluorescence microscopy (WFM) showed that *L. monocytogenes* biofilms of three strains had an organized net-like pattern named a “honeycomb” structure *(186)*;

although this EPS structure might be affected by draining of culture media and the washing process during sample preparation for WFM.

The current AFM imaging overcame the shortcoming of many microscopes, and thus may provide new findings for biofilms research. The biofilms visualized by AFM were nearly at their natural state since the preparation of samples for AFM was very simple, with a major step of immobilizing microbial cells on a supportive material (stainless steel, for instance) and letting them grow into biofilms *(265)*. Thus, the imaging of biofilms, especially surface morphology by AFM, would extend our understanding of the pathogens and the understudied EPS component of biofilms *(213)*.

Previous SEM results of weak EPS by *L. monocytogenes* biofilms could be due to damage during pretreatment steps *(77, 202)*. Epifluorescence microscopy analyzes images of fluorescence staining or fluorescent objectives but its results might overestimate EPS area on the studied surface since microbial cells stained as well *(32, 73, 194)*. On the other hand, confocal microscopy or epifluorescence microscopy can not be used to visualize unstained (nonfluorescent) materials, such as EPS in which biofilm cells were embedded. Hence, conclusions about whether EPS was present when it was not specifically stained was primarily based on assumptions *(235)*. In general, structures in final images could be affected by imaging artifacts *(326)*.

5.4.2. Effect of inoculation level and growth media during biofilm formation on *L. monocytogenes* biofilms

A higher inoculation level resulted in a larger amount of *L. monocytogenes* transferred and attached to a stainless steel surface compared to low inoculation levels, since efficiency of transfer was similar *(264)*. According to McLandsborough and others *(194)*, bacteria begin to anchor themselves to stainless steel by execrating EPS after initial adhesion. Palmer and others *(232)* believed that initial bacterial cell attachment to a surface (stainless steel surface, for instance) was critical for biofilm formation. However, Djordievic and others *(73)* reported that there was no correlation between biofilm formation and microbial cell number, growth rate, or density. On the other hand, Marsh and others *(187)* thought that the organized, net-like patterns of "honeycomb" *L. monocytogenes* biofilms were formed gradually.

Growth media influenced biofilm growth and formation by affecting the attachment of microbial cells on the substrate. In addition, EPS amounts differ among biofilms developed from high nutrient medium (Luria-Bertain medium, for instance) and minimum nutrient medium (M9, for instance) *(224)*. Some researchers believe that different substrate surfaces and media affect microbial cell attachment through influencing surface charges of the microbial cells *(232)*. Generally, the bacterial cell surface is negatively charged while charges of the substrate vary depending on the kinds of substrates and media *(232)*. However, Djordjevic and others found that both electrostatic and exopolymer interactions were responsible for microbial cell attachment to a hydrophilic stainless steel surface. On the other hand, organic components in the

media might not significantly affect biofilm formation and growth. One example is carbon sources of different media did not significantly affect biofilm formation *(73)*. In the current study, biofilms developed in TSB and BHI were similar since both media were non-selective, complex and relatively similar. The media influence was studied by Wang and others who reported that *L. monocytogenes* biofilm formation was significantly inhibited by Tween 80 in deMan-Rogosa-Sharpe broth *(320)*, and a mixed strain (without *L. monocytogenes*) biofilm grown on diclofop was thicker than those grown on TSB *(330)*.

5.4.3. Effect of processing treatment on *L. monocytogenes* biofilms

The structural integrity of biofilms was thought to be determined by the activities of the bacteria, and the bacteria properties affected the integrity of biofilms *(202)*. After NECAW treatment, the morphology of individual cells was altered (Figure 4). Cells became wrinkled and broken, possibly due to the penetration of NECAW into the microbial cells and initiation of sanitizing effects. However, a few microbial cells of biofilms kept their surface morphology (data not shown), which was supported by plate counting results showing some survival (Table 4). There were two possible reasons that could be responsible for this phenomenon. One was that the resistance of microbial cells in biofilms to sanitization treatment was heterogeneous *(202)*. One example was that some *L. monocytogenes* ATCC 19115 microbial cells were much more resistant to sanitization of peroxyacetic acid, based on SEM results *(202)*. Another possible reason was that the penetration of NECAW was limited in such a short treatment and may be

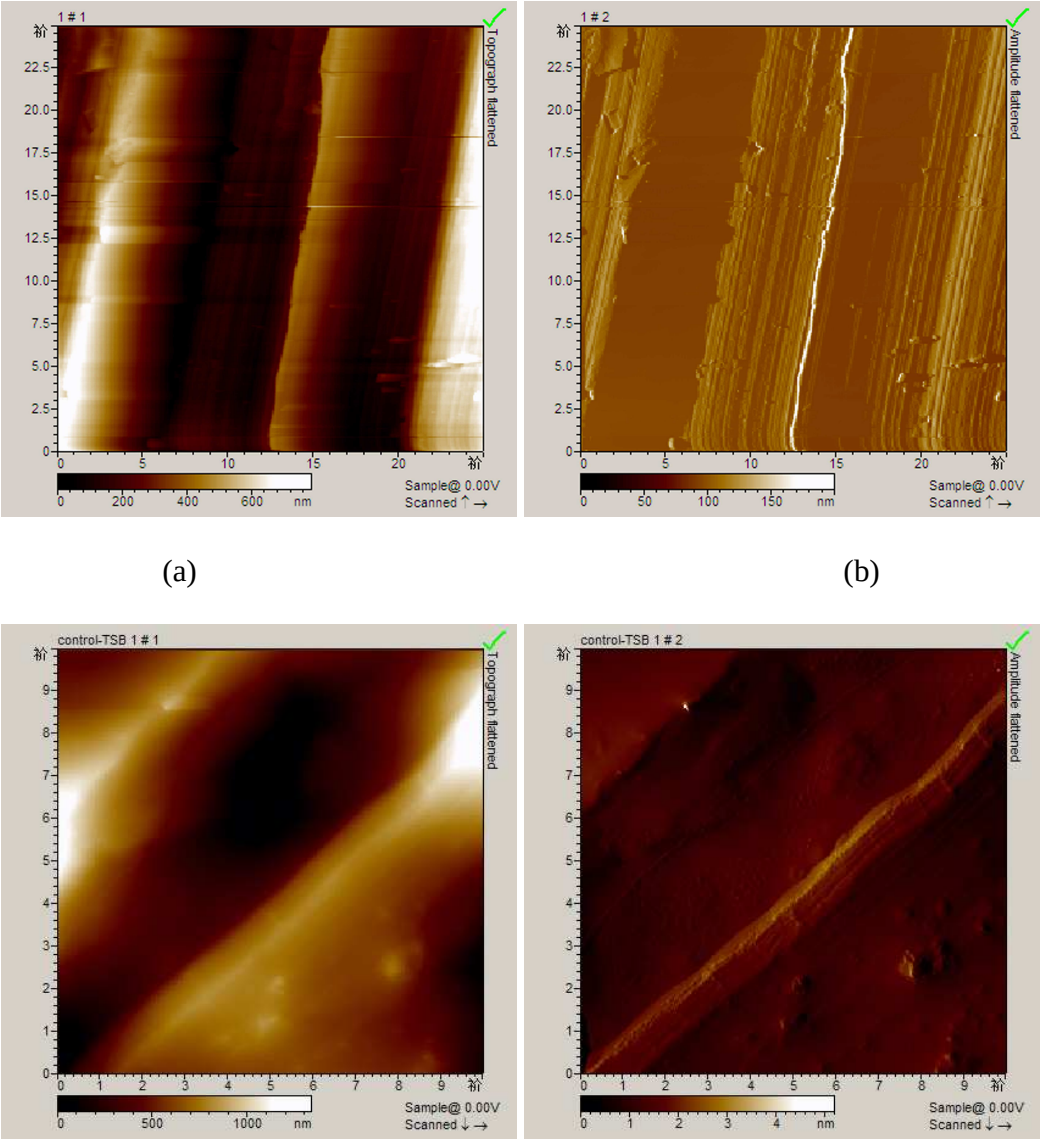
decreased by EPS due to interactions between EPS and sanitizers *(12, 194)*. Therefore, some microbial cells were not penetrated with NECAW. NECAW is an oxidizing sanitizer and can interac with organic matter. This could partially explain why it was hard for NECAW to penetrate into the inner part of biofilms. In the future, study on the sanitizing effects of NECAW on EPS will be necessary to improve NECAW sanitization.

DI water can remove some biofilms from stainless steel surfaces but cannot kill microbial cells. When EPS was removed, its protective effect on bacterial viability disappeared or significantly decreased *(15, 17)*, thus DI water only could cause a low log CFU reduction of bacteria. It should be noted that killing and removal are two different phenomena. Microbial cells removed by DI water did reattach and grow on another surface area or contaminate washing water, while microbial cells killed by NECAW could not reattach and further grow, thus would not contaminate the washing water.

5.5. Conclusion

Bacterial biofilms are composed of EPS and microbial cells. In this reseaerch, AFM was applied to characterize the morphology of foodborne pathogen biofilms on stainless steel surfaces. Three morphologies of the biofilms were tree-like structures, individual cells and no characteristic structures of the above two (none) and the relative percentages of these three morphologies were quantitatively determined. *E. coli* O157:H7 strain 6058 and *S. enterica* Typhimurium E2009005811 had significantly low percentages of the tree-like morphology. Neutral electrochemically activated water (NECAW) treatment destroyed the microbial cells as well as removed the tree-like

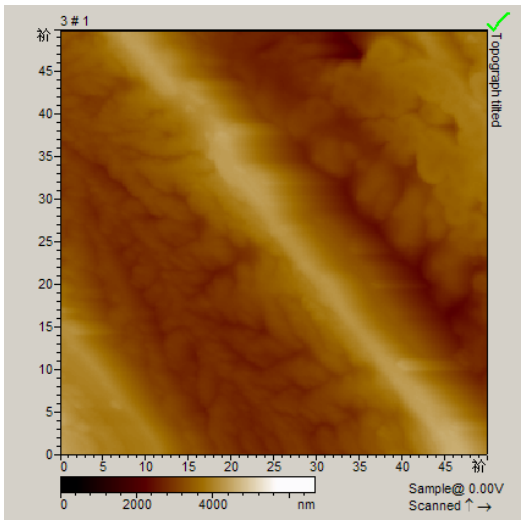
structures. Results from AFM were promising, suggesting that AFM could be a suitable technique for study and characterization of biofilms, and investigating sanitizing effects. More valuable information could be obtained if combined with other microscopic techniques *(91)*.



(a) (b)

(c) (d)

Figure 5.1. AFM images of stainless steel (SS) control coupons. (a) and (b): Height and amplitude mode AFM images of a representative sterile non-treated SS coupon; (c) and (d): height and amplitude mode AFM images of a representative SS coupon incubated with *L. monocytogenes* cells suspensions that had been previously autoclaved.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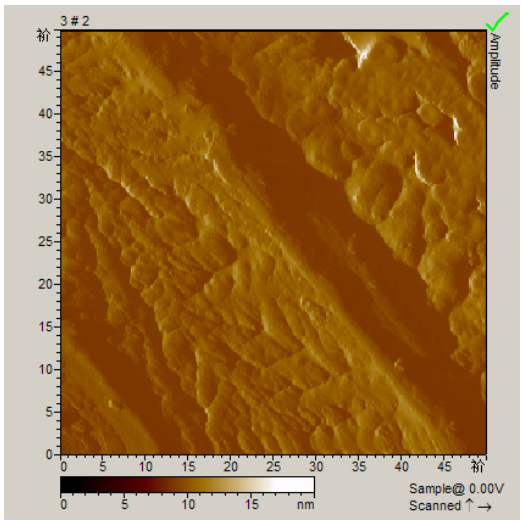
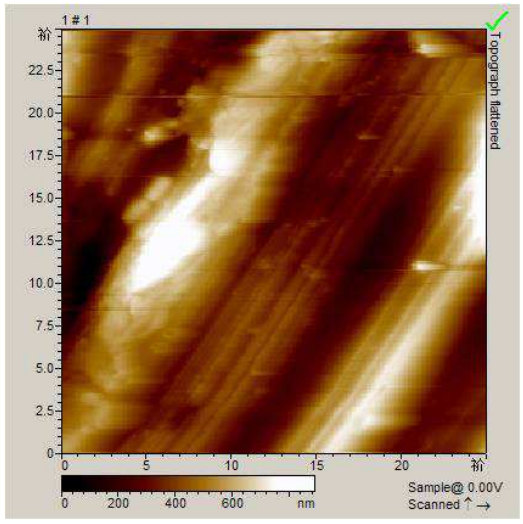
Topograph tilted
Sample@ 0.00V
Scanned

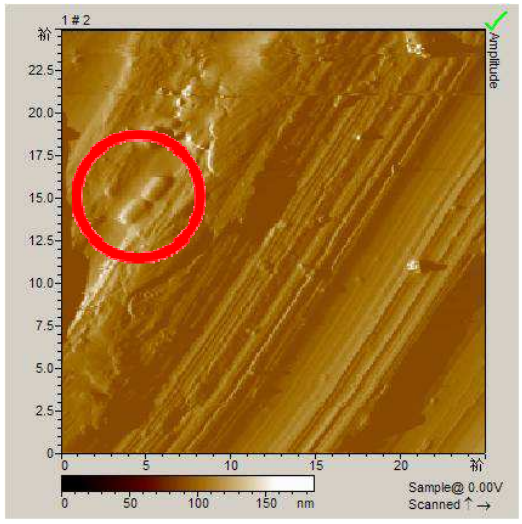
a


3 # 2
Amplitude
Sample@ 0.00V
Scanned

b


1 # 1
Topograph flattened
Sample@ 0.00V
Scanned

c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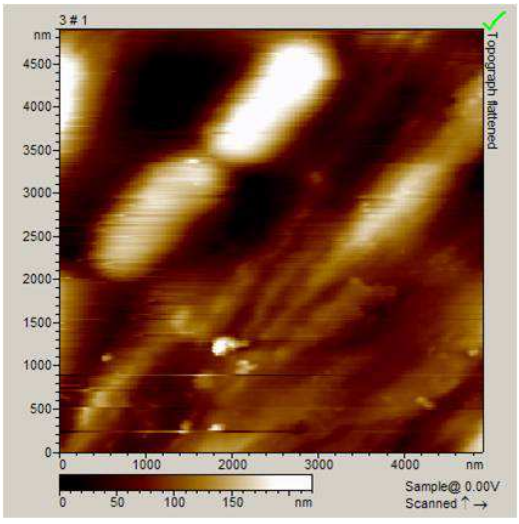
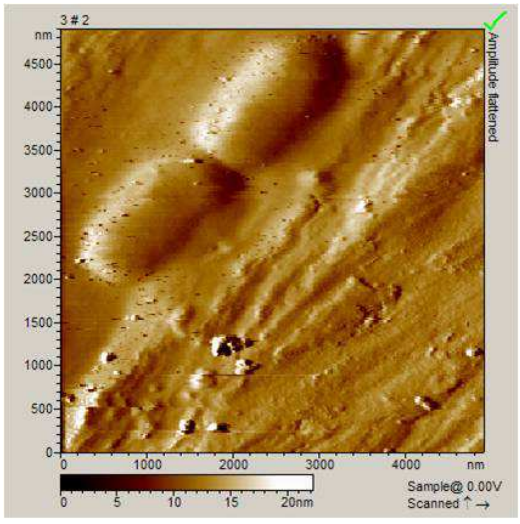
Amplitude
Sample@ 0.00V
Scanned

d

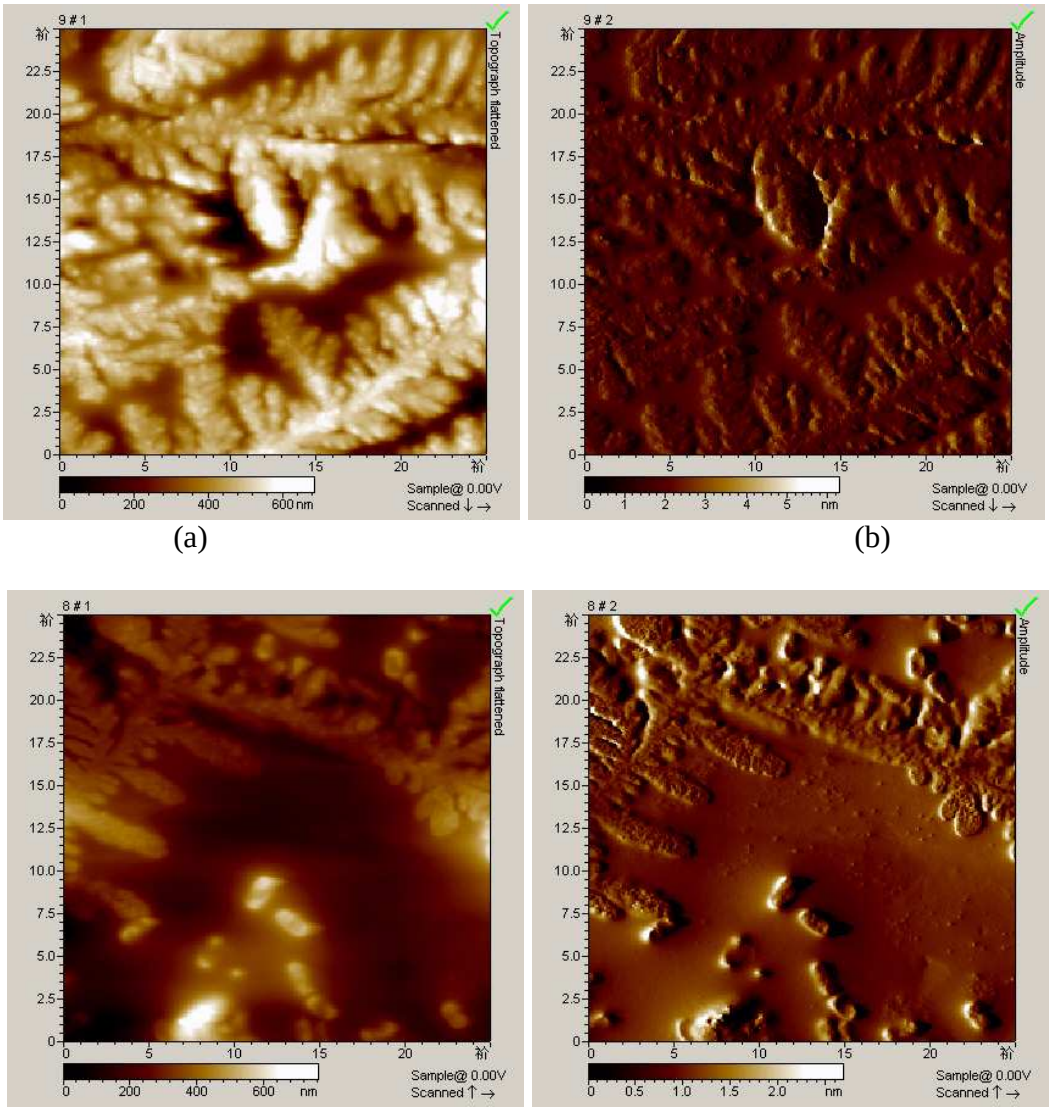

3 # 1
Topograph flattened
Sample@ 0.00V
Scanned

e


3 # 2
Amplitude flattened
Sample@ 0.00V
Scanned

f

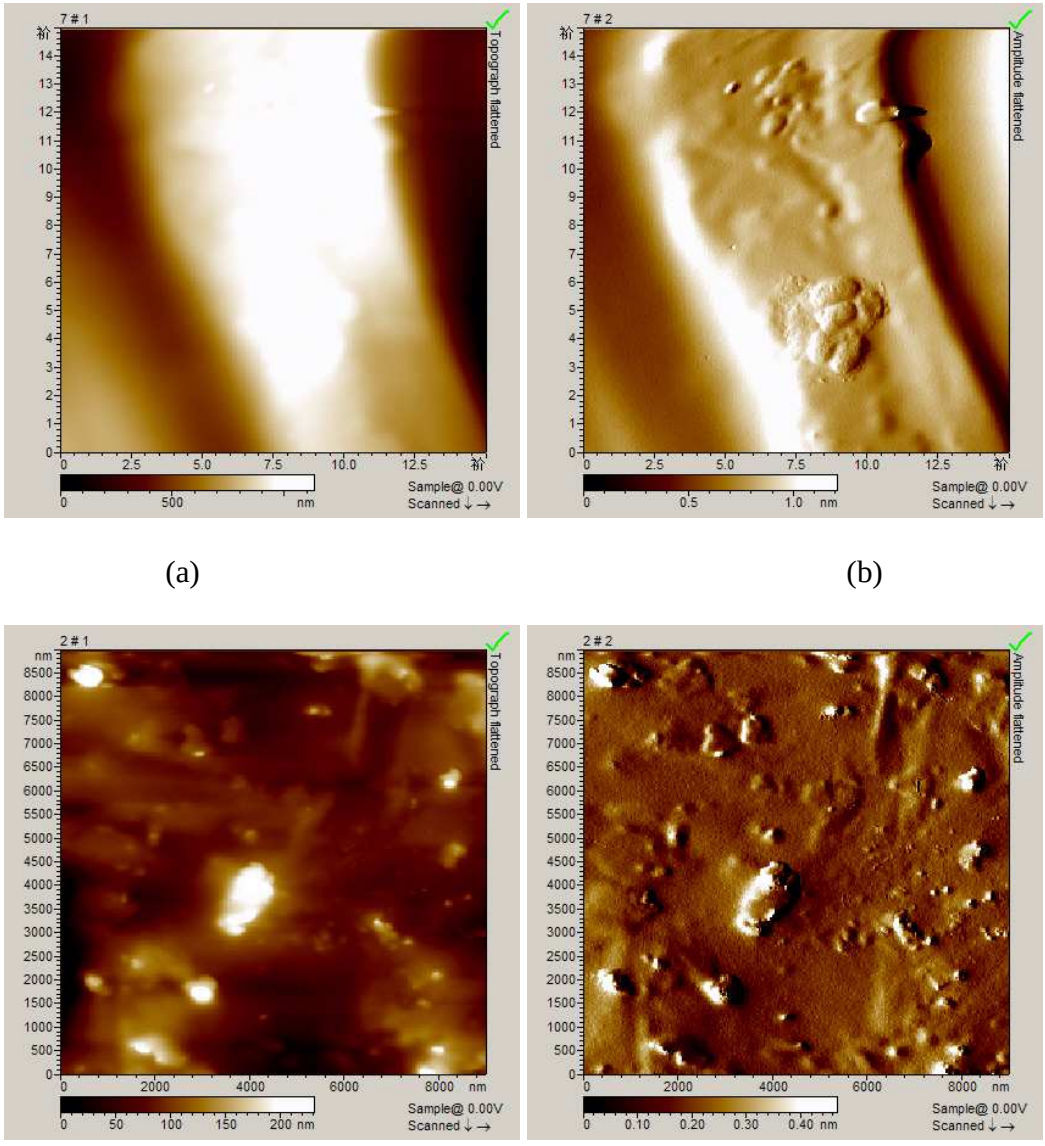
Figure 5.2. Atomic force microscopy (AFM) images of the tree-like structure and individual cells structure of *Listeria monocytogenes* biofilms ATCC 19115 on stainless steel coupons. Height (a) and amplitude (b) mode of tree-like structure biofilms; height (c) and amplitude (d) mode of individual cell structure biofilms; height (e) and amplitude (f) mode of enlarged images of (c) and (d), respectively.



(a) (b)

(c) (d)

Figure 5.3. Atomic force microscopy (AFM) images of the tree-like structure and individual cells structure of *Escherichia coli* O157:H7 biofilms 6058 on stainless steel coupons. Height (a) and amplitude (b) mode of tree-like structure biofilms; height (c) and amplitude (d) mode of individual cell structure biofilms.



(a) (b)

(c) (d)

Figure 5.4. Atomic force microscopy (AFM) images of the morphology of *Listeria monocytogenes* biofilms ATCC 19115 on stainless steel coupons. Height (a) and amplitude (b) mode of biofilms after deionized water (DIW) treatment; height (c) and amplitude (d) mode of biofilms after neutral electrochemically activated water (NECAW) treatment.

Table 5.1. Quantitative analysis of the characteristics of the *Listeria monocytogenes* biofilms (%).

Strain	Inoculation amount	Tree-like morphology	Individual cells	None
2349	1[a]	24.0±8.0 A	14.8±10.8 A	61.2±18.9 A
3528	1	25.2±5.6 A	19.2±10.2 A	55.6±15.1 A
ATCC 19115	1	29.8±10.7 A	18.1±15.3 A	52.1±11.2 A
	0.1	23.1±10.5 A	16.5±9.0 A	60.4±12.5 A
	0.001	24.5±4.9 A	16.4±6.7 A	59.0±6.2 A
	1-BHI[b]	27.4±23.2 A	14.9±11.7 A	57.7±13.5 A
2422	1	19.7±9.3 A	20.6±7.2 A	59.8±7.1 A
	0.1	17.9±13.7 A	27.7±11.8 A	54.5±22.5 A
	0.001	18.7±4.3 A	21.5±8.7 A	59.9±12.5 A

Within each column, means with different capital letters are significantly different ($P < 0.05$).

[a]: '1' is standard amount inoculation amount with 1:100 of bacterial solution to the growth media of LN-TSB, that is, 1/10 of TSB.

[b]: The LN-TSB in the biofilm growth protocol was substituted by LN-BHI.

Table 5.2. Quantitative analysis of the characteristics of the *Escherichia coli* O157:H7 and *Salmonella enterica* biofilms (%).

Pathogen	Strain	Tree-like morphology	Individual cells	None
E. coli O157:H7	43890	26.7±5.6 A	31.3±11.7 A	42.0±10.5 A
	43895	24.1±6.8 AB	34.8±19.0 A	41.1±14.7 A
	6058	8.0±5.6 C	42.7±8.6 A	49.3±5.7 A
	EK-1	10.9±8.3 BC	30.9±13.9 A	58.2±20.8 A
S. enterica	Typhimurium E2009005811	8.4±5.7 C	27.4±14.2 A	64.2±18.9 A
	Typhimurium UK-1	11.3±7.6 BC	37.0±18.7 A	51.7±12.5 A
	Newport B4442	14.1±2.3 ABC	42.9±16.9 A	43.1±15.1 A
	Saintpaul E2008001236	17.6±5.1 ABC	36.5±18.7 A	46.0±14.2 A

Within each column, means with different capital letters are significantly different ($P < 0.05$).

Table 5.3. Effect of initial inoculation amount of *Listeria monocytogenes* on number of viable microbial cells of biofilms (log CFU/coupon) (%).

Relative amount	Strain ATCC 19115	Strain 2422
1[A]	7.06±0.63 A	6.64±0.42 A
0.1	7.12±0.55 A	6.80±0.31 A
0.001	7.04±0.41 A	6.57±0.24 A
1[B]	7.18±0.57 A	-[C]

Note: Within each column, means with different capital letters are significantly different ($P < 0.05$).

[A]: '1' is standard amount inoculation amount with 1:100 of bacterial solution to the growth media of LN-TSB, that is, 1/10 of TSB.

[B]: The LN-TSB in the biofilm growth protocol was substituted by LN-BHI.

[C]: Character '-' means not determined.

Table 5.4. Effect of treatments on number of viable microbial cells of *Listeria monocytogenes* biofilms ATCC 19115 (log CFU/coupon).

Treatment	Strain ATCC 19115
Initial	7.15±0.24 A
DIW treatment	5.96±0.44 B
NB treatment	5.89±0.39 B
NECAW (250 mg/l FAC) treatment	2.46±0.45 C

Note: Within each column, means with different capital letters are significantly different ($P < 0.05$). NB, neutralizing buffer; DIW, deionized water. NECAW, neutral electrochemically activated water; FAC, free available chlorine.

CHAPTER VI

ROLE OF *SIGB* AND *INLA* GENES ON BIOFILM FORMATION AND ANTIMICROBIAL EFFICACY OF NEUTRAL ELECTROCHEMICALLY ACTIVATED WATER ON *LISTERIA MONOCYTOGENES*

Neutral electrochemically activated water (NECAW) is a sanitizer for food and food processing equipment. *sigB* is a global stress regulator and and *inlA* is a virulence gene of *L. monocytogenes*. It has been suggested that these genes play a role in biofilm formation and antimicrobial efficacy of NECAW treatment on *L. monocytogenes*. The objectives of this study were to determine the role of *sigB* and *inlA* gene expression levels in *L. monocytogenes* biofilm formation and antimicrobial efficacy of NECAW treatment. Liquid cultures and biofilms grown on stainless steel coupon surfaces of four *L. monocytogenes* strains (wild type [WT] 10403S, isogenic Δ*inlA*, Δ*inlB*, and Δ*inlA*Δ*sigB* mutants) were treated with NECAW for 10 min. Sanitizing efficacy of NECAW was determined by counting the survivors after treatment using standard plate counting. Gene expression levels were determined using qPCR. Isogenic *inlA* and *sigB* mutants were able

to form biofilms. After NECAW treatment, expression of both genes increased for the WT. While *sigB* gene expression of the Δ*inlA* strain increased at a level comparable to the WT, *inlA* gene expression of the Δ*sigB* strain did not significantly increase. Both genes were expressed more in biofilms than in liquid cultures. The level of *inlA* gene expression in WT increased 4.28 and 5.51-fold with treatment of 4 mg/l NECAW for 10 min in liquid cultures and biofilms, respectively, while the corresponding values were 5.91 and 10.05-fold for the *sigB* gene. Mutant strains were more sensitive to NECAW treatment than the WT strain. For liquid cultures, 10 mg/l NECAW for 10 min resulted in 0.79 and 1.17 more log CFU/ml reductions for Δ*inlA* and Δ*sigB* strains, respectively, than the WT, but there was no significant difference among strains in form of biofilms under the same condition. The *sigB* gene was more important than was *inlA* for survival of NECAW treatment. Surviving *L. monocytogenes* cells post-sublethal NECAW treatment might become resistant to further sanitizer treatment.

6.1. Introduction

Listeria monocytogenes is a ubiquitous, facultative and non-sporeforming intracellular foodborne pathogen that causes a severe invasive disease in humans and animals. *L. monocytogenes* seems to be well adapted to invade human cells as well as to survive in many types of environments. This bacterium can tolerate many harsh conditions including high osmolarity, bile salts and organic acids *(256, 343)*. It can colonize the surface of food processing equipment *(139)*, forming biofilms that can

survive better than planktonic counterparts on food processing equipment under incomplete sanitizing conditions *(308)*.

Biofilms are generally more resistant to antimicrobial agents and sanitizers than individual cells, enhancing the ability of bacterial cells to survive cleaning or sanitizing steps *(308)*. Biofilms are composed of a community of microorganisms and a complex matrix of exopolymeric substances (EPS) *(277)*. Although some reports suggest that some strains of *L. monocytogenes* may not form very resistant biofilms *(249)*, it has long been recognized that this pathogen can survive on food processing environment and food equipment surfaces for a relatively long time *(277)*. Some *L. monocytogenes* strains can even survive for several years in food-processing plants *(139)*.

The mechanisms that explain the increased resistance of biofilms to sanitizers include limited penetration to cells within biofilms, slower growth rate of the microorganism, and induction of resistance responses *(75, 165, 179, 308)*. At the molecular level, the persistence of bacterial survival under stress conditions is thought to be related to transcription redirection via association of alternative sigma factors with core RNA polymerase *(90)*, but the ability to persist was not linked to any specific phenotypic or genetic characteristics *(139)*. Sigma factors are dissociable protein subunits directing the RNA polymerase holoenzyme to accurately recognize a promoter sequence upstream of a gene before transcription initiation. Alternative sigma factors can reprogram specific promoter recognition processes along with the core RNA polymerase when environmental conditions change, thus allowing appropriate expression of specific target genes in response to those changing conditions *(256)*. σ^B, or SigB, a stress-

response alternative sigma factor encoded by *sigB,* was recognized as the global regulator for general stresses in some Gram-positive bacteria with low G+C content including the genera *Bacillus*, *Staphylococcus*, and *Listeria (9, 344)*.

It was originally believed that this stress-response was not associated with specific bacterial strains *(259)*. In *L. monocytogenes*, σ^B helps microbial cells survive under stress conditions including nutrient deficiency, low pH, high or low temperature and oxidative stress *(342)*, and antimicrobial agents *(234, 282)*. *L. monocytogenes* σ^B is activated in order to protect bacterial survival once bacteria are exposed to environmental stress conditions *(256)*. Loss of σ^B (Δ*sigB* strain) reduces the ability of *L. monocytogenes* to invade human intestinal epithelial cells. σ^B was widely viewed as participating in biofilm formation of *L. monocytogenes (308)*. Δ*sigB* strains exhibit significantly decreased biofilm formation compared to wild-type strains *(55, 308)*. However, Schwab and others (2005) view σ^B as unnecessary for initial attachment of *L. monocytogenes* to the surface *(277)*.

σ^B has also been found to be responsible for transcription of several *L. monocytogenes* virulence and stress-response genes, including genes that relate to gastrointestinal infections *(140, 290)*. For example, loss of σ^B results in reduced *inlA* expression, a virulence gene conserved and specific to *L. monocytogenes (253)*, in stationary-phase microbial cells *(143, 144, 193)*. Thus, transcription of *inlA* is at least partially σ^B dependent *(144)*. InlA is a cell-wall anchored protein and a bacterial factor mediating the first step of attachment and internalization of *L. monocytogenes* to human cells as demonstrated by the study of *L. monocytogenes's* entry into the Caco-2 human

colon adenocarcinoma cell line *(144, 220)*. The expression of *inlA* of *L. monocytogenes* changes under different environments *(257)*, which may help confer *L. monocytogenes* ability to adapt for long-term survival while maintaining bacterial virulence *(40)*.

L. monocytogenes can be controlled if proper santization conditions were applied in food processing facilities. It should be noted that the efficiency of sanitizers is compromised by the presence of organic materials including food debris, leading to decreased sanitizing effects. An even worse situation is inappropriate sanitizing treatment in food processing plants, which leads to exposure of pathogens to sublethal concentrations of sanitizers and surviving pathogenic microbial cells after sanitizing. Currently, it is not clear yet whether treatment with a sublethal concentration of sanitizers will affect all pathogen populations or only attack a partial fraction of the population *(139)*.

The objective of this study was to understand the role of *inlA*, *sigB* and their relationship upon sublethal treatment of neutral electrochemically activated water (NECAW) as a sanitizer on *L. monocytogenes*. Isogenic parent Wild type (WT), *inlA* null (Δ*inlA*), *sigB* null (Δ*sigB*) and double null (Δ*inlA*Δ*sigB*) mutant strains in liquid cultures and biofilms were investigated for a comparative study with sublethal concentrations of NECAW. Gene expression levels of *inlA* and *sigB* and the sanitizing effects of NECAW among these strains were quantitatively analyzed.

6.2. Materials and Methods

6.2.1. Bacterial strains

Listeria monocytogenes serotype 1/2a WT strain 10403S and its isogenic *inlA* null mutant (DP-L4405), *sigB* null mutant (FSL A1-254), and double mutant Δ*inlA*Δ*sigB* (FSL B2-042) were kindly provided by Dr. Kathryn J. Boor of Cornell University. Sources for the strains were described in a previous publication *(144)*. Strains were confirmed by one-step RT-PCR for *inlA* and *sigB* gene expression. For each strain, a loop of -60°C storage glycerol-culture was inoculated and transferred three consecutive times in tryptic soy broth (TSB) (Neogen Corp,, Lansing, MI) and inoculated at 37ºC at 24 h intervals.

6.2.2. Preparation and analysis of neutral electrochemically activated water (NECAW)

NECAW was freshly generated from softened tap water and saturated NaCl solutions by a generator (Zap Water Technology, Inc., Richfield, MN, USA) at a voltage range of 7 to 9 volts. After a stable voltage reading was reached, NECAW was collected using a sterile glass bottle from the anode side, covered and used within 2 h post generation. FAC of NECAW was determined with a chorine test kit by calculating the drop counts (LaMotte Company, Chestertown, MD). ORP and pH were measured with an ORP meter (ORPTestr 10, Oakton Instruments Inc., Vernon Hills, IL) and a pH meter (pHTestr 10, Oakton Instruments Inc.), respectively.

6.2.3. Liquid culture testing

For each bacterium, 20 ml of 24-h old cultures were centrifuged (3, 600 × *g*, 23°C) for 10 min. Pellets were washed using 10 ml of peptone water (PW, Neogen, Inc.), centrifuged and re-suspended in 10 ml of PW. One ml of suspensions were added into bottles containing 99 ml of liquid sanitizer solution (NECAW or deionized water [DIW] as control). After the bottles were shaken by hand for 30 s, mixture (1 ml) were transferred to 9 ml neutralizing buffer solutions (5.2 g/l; Becton, Dickinson and Company, Sparks, MD) and shaken for 40 s. The neutralized mixture was serially diluted for plating analysis *(7, 11)*.

6.2.4. Biofilm experiments

Individual strains were prepared as described above for liquid culture testing. Suspensions were mixed with 9 ml sterile low nutrient TSB (1:10 dilution of normal TSB solution, LN-TSB) with a dilution of 1:100. Sterilized stainless steel coupons were immersed into the suspensions, mixed well and left in the liquid cultures for 4 h at room temperature to allow bacteria attachment. Suspensions were poured off and the coupons were rinsed gently with a circular motion for 10 s using 1 ml PW in order to remove unattached microbial cells. The PW were poured off. Coupons were added with 10 ml fresh LN-TSB and stayed at room temperature. After 48 h, LN-TSB was discarded and coupons were transferred to a new LN-TSB solution for another 24 h at room temperature. After this incubation, coupons were rinsed gently with 1 ml PW twice to remove loosely attached microbial cells. Then the coupons were dried in a biosafety cabinet for 2 h *(12)*.

Coupons with biofilms were placed into glass tubes containing 10 ml NECAW 100 mg/l FAC or DIW as control, slightly mixed at speed 2 using a Mdl G-560 Vortex Genie 2 Mixer (Lehman Scientific, LLC, Wrightsville, PA, USA) for 5 s. After 25 s of stillness, coupons were mixed again for 5 s, then kept still for 25 s again. Coupons were transferred to new glass tubes containing 10 ml neutralizing buffer solution and mixed at speed 2 for 5 s. After 35 s of waiting, coupons were transferred to 50-ml disposable plastic tubes containing 10 ml PW and 3 g sterile glass beads (Sigma-Aldrich Co., St. Louis, MO, USA), and vortexed heavily for 5 min to remove bacteria from the coupons. A series of ten-fold dilutions of the PW containing coupons and neutralizing buffer were conducted, respectively after vortexing for direct plating and enrichment test.

6.2.5. Microbial analysis

WT *L. monocytogenes* and isogenic mutants Δ*inlA*, Δ*sigB*, Δ*inlA*Δ*sigB* cultures were used for a series of stress survival assays with 0-50 mg/l NECAW for 0 to 10 min in liquid cultures, and 0-100 mg/l ECAW for 0-10 min for biofilms. The experiment was performed using stationary phase microbial cells, which were the same phase used for RNA experiments. Prior to and after NECAW treatment, survival of *L monocytogenes* was enumerated by plating appropriate serial dilutions in peptone water on tryptic soy agar (TSA) plate after incubation at 37ºC for 48 h. Selected colonies from TSA plates were streaked onto selective agar and incubated to confirm the presence of pathogens.

6.2.6. RNA extraction

RNA was extracted using a TRIzol® Max™ bacterial RNA isolation kit (Life Technologies Corporation, Grand Island, NY, USA). Bacterial cultures grown overnight or biofilm cells (recovered by votexing with glass beads) (1.5 ml) were transferred to pre-chilled microcentrifuge tubes, which were centrifuged at 6,000 × *g* for 5 min at 4ºC. After centrifugation, the supernatant was decanted and the cell pellet was re-suspended in a 200 µl preheated (95ºC) max bacterial enhancement reagent, mixed and incubated at 95ºC for 4 min. After that, 1 ml TRIzol® reagent was added to the lysate, and the solution was mixed and incubated at room temperature for 5 min. Then 0.2 ml cold chloroform was added, and the solution mixed and incubated at room temperature for 3 min. The samples were centrifuged at 12,000 *g* for 15 min at 4ºC. The colorless upper phase was transferred to a new tube, to which 0.5 ml cold isopropanol was added for precipitating RNA. The solution was mixed and incubated at room temperature for 10 min, then centrifuged at 15,000 × g for 10 min at 4ºC. The supernatant was removed and the pellet was resuspended in 1 ml 75% ethanol, and the suspension mixed and centrifuged at 7,500 × *g* for 5 min at 4ºC. The resulting RNA pellet was air-dried and re-suspended in 50 µl RNase-free water. RNA concentration was determined by abosorbance at 280 nm and the quality was determined by the ratio of asborance at 260 nm to that at 280 nm. RNA quality was examined by running extracts on 1% agarose formaldehyde gels.

6.2.7. cDNA synthesis

cDNA syntheis was performed using a SuperScript ® VILO™ cDNA synthesis kit (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer's protocol, RNA (100 ng), 4 µl 5×VILO™ reaction mix, 10×SuperScript® enzyme mix, and diethylpyrocarbonate (DEPC)-treated water were mixed together to a volume of 20 µl. The mixture was gently mixed and incubated at 25ºC for 10 min, 42ºC for 60 min, and then 85ºC for 5 min. Synthesized cDNA was used immediately or stored at -20ºC until used in PCR.

6.2.8. One-step Reverse Transcriptase-Polymerase Chain Reactions

The primers (5' to 3') used for target genes were: TGTGACTGGCGCTTTAATTG and GCGTCACGGTTCCACTAAAT as forward and reverse primers for *inlA*, respectively; CATGAAGATTTAGTTCAAGTTGGAAA and CACATGCACACTCCATGTTTT as forward and reverse primers for *sigB*, respectively; CCCTTATGACCTGGGCTACA and CCTACCGACTTCGGGTGTTA as forward and reverse primers for 16S rRNA, respectively; GGTCACCAGTGTAAGCGTGA and CAGCAACTGGCGATATGAAA as forward and reverse primers for *gapdh*, respectively.

SuperScript™ III one-step Reverse Transcriptase-Polymerase Chain Reactions (RT-PCR) system with Platinum® Taq High Fidelity (Invitrogen Corp., Carlsbad, CA, USA) was used to study *inlA* and *sigB* gene expression in bacterial strains according to the manufacturer's instruction. In a 0.2 ml PCR tube on ice, 25 µl 2× reaction mix, 2.5 µl

(100 ng) template RNA, 0.5 µl sense and 0.5 µl anti-sense primers (10 µM each), 0.5 µl SuperScript™ III RT/Platinum® Taq High Fidelity enzyme superScript mix were added and mixed with water to 25 µl. Then cDNA synthesis was performed with 1 cycle of 55ºC for 30 min, and 94ºC for 2 min; followed by 40 cycles of PCR amplification (94ºC for 15 s, 55ºC for 30 s, and 68ºC for 1 min), and completed with 68ºC for 5 min.

6.2.9. Real time Quantitative PCR (qPCR)

Each reaction mixture was prepared with 7 µl of RNase- and DNase-free water, 1 µl each of sense and anti-sense primer, 10 µl of master mix and 1 µl ofsynthesized cDNA template. qPCR was performed using a LightCycler® 480 instrument (Roche Diagnostics Corporation, Indianapolis, IN, USA).

6.2.10. Statistical analyses

For qPCR data, expression levels of targeted genes were normalized to expression levels of reference genes of the same strain *(251, 311)*. Two reference genes, 16S and *rpoB,* were chosen as two independent genes for data normalization. Relative gene expression was evaluated by analysis of variance among strains, different NECAW concentrations, or NECAW treatment time. Analysis of variance (ANOVA) and Tukey test for differences among different groups were performed by SAS software (version 9.1.3, SAS Inst. Inc., Cary, NC, USA). The quantitative results were represented as means ± standard deviations. Comparisons that yielded $P < 0.05$ were denoted as significant.

6.3. Results

The effects of NECAW (4 mg/l FAC) treatment for as long as 10 min on *inlA* or *sigB* gene expression of *L. monocytogenes* in liquid culture were first investigated (Table 6.1). Deionized water (DIW) treatment did not significantly change gene expression levels for all strains. Expression of *inlA* and *sigB* genes did not significantly increase with NECAW treatment after 30 s. However, after 5 min, *inlA* and *sigB* were significantly up-regulated for all strains. Further treatment (10 min) resulted in even higher gene expression levels. The expression of the *inlA* gene did not increase significantly for strain Δ*sigB*, but *sigB* gene of the Δ*inlA* strain increased similar to that of the WT strain, and was at least 3 times greater than controls after 10 min.

When the NECAW concentration was increased and cells were treated for 5 min, *inlA* expression increased significantly in WT while not significantly in Δ*sigB* (Table 6.2). In contrast, *sigB* expression increased significantly in both WT and Δ*inlA* strains. For the WT strain, *sigB* expression increased at a greater level than *inlA*, for instance a concentration of 6 mg/l FAC caused 6.35- and 2.52-fold increases, respectively.

Tables 6.3 and 6.4 show the effects of time and concentration, respectively, of NECAW treatment on gene expression levels of biofilms. The changes were very similar to that of liquid cultures. Both expression of *inlA* and *sigB* increased in the WT strain with increased concentration and time of NECAW treatment. *sigB* expression of the Δ*inlA* strain increased at similar levels as the WT but *inlA* expression of the Δ*sigB* strain did not change significantly with increased exposure time or FAC concentration.

Compared to liquid cultures, *sigB* gene expression increased almost 2-fold in biofilms after NECAW treatment, in both the WT and the *ΔinlA* strain. For instance, after 10 min of 4 mg/l FAC treatment, *sigB* level in WT was 5.9-fold that of the reference genes in liquid cultures (Table 6.1), while the expression reached 10-fold in biofilms (Table 6.3).

To investigate the role of the genes on microbial survival when cells were exposed to NECAW, survival counts of the four *L. monocytogenes* strains were performed for the same sublethal FAC concentration as well as for increased concentrations. At the maximum FAC and time used for the gene expression studies (6 mg/l, 10 min) the WT strain count of liquid cultures was only reduced by 0.5 log CFU/ml (Table 6.5). At similar sublethal conditions, the viable count of the *ΔinlA* mutant was a little different from that of the WT, but at higher FAC the viability reductions increased more for the *ΔinlA* mutant than the WT. Both the *ΔsigB* and the *ΔsigBΔinlA* mutants were more sensitive than the WT at 6 mg/g FAC after 5 and 10 min, as average reductions of more than 1.2 and 2.2 CFU/ml, respectively, were recorded.

Tables 6.6, 6.7 and 6.8 show the effect of NECAW concentration on *L. monocytogenes* biofilm viable counts for 30 s, 5 min and 10 min, respectively. The viable count of WT biofilms were reduced a maximum of 0.7 CFU/coupon when they were treated for 10 min at 6 mg/l FAC (Table 6.8). Mutants Δ*sigB* and Δ*inlA*Δ*sigB* appeared to be slightly more sensitive to this concentration, but the differences were not statistically significant. At concentrations above 6 mg/l, the killing of WT biofilms reached 3 log CFU/ coupon at 100 mg/l FAC. The Δ*inlA* mutant's sensitivity to NECAW was similar to that of the WT, even at the higher FAC concentrations. The biofilms of Δ*sigB* and

Δ*inlA*Δ*sigB* mutant strains had no detectable survivors (≥ 4.6 log CFU/coupon) at FAC concentrations of 50 or higher for 10 min. However, the reductions of both strains were only 1.3 log CFU/ coupon at 100 mg/l FAC after 1 min.

6.4. Discussion

6.4.1. qPCR analyses and reference genes

qPCR conditions normally need to be optimized to get reliable and reproducible data since many possible factors can affect the outcome of the procedure *(322)*. The first step in a qPCR experiment is typically lysis of microbial cells. The efficiency of this step determines the yield of RNA extracted, thus the sensitivity of qPCR *(322)*. It was reported that appropriate kits for RNA extraction were required depending on the number of microbial cells *(322)*. In this study, the Trizol®Max™ bacterial RNA isolation kit was used, as the whole procedure is relatively convenient and allowed a relatively large volume of bacterial solutions.

The second factor for qPCR analysis is the selection of reference genes. Once reference genes were chosen, qPCR could be used to quantitatively determine expression of the target genes, *sigB* and *inlA*, in the current study. 16S rRNA is a reference gene that has been extensively used for indicating overall cellular mRNA expression levels for microbial cells at a specific physiological status. Gene expression levels of target genes were obtained by dividing by 16S rRNA expression levels according to the formula described by Vandesompele et al. (2002) *(55, 311)*, thus allowing the analysis of relative

target gene expression at a certain physiological status . However, reference gene expression is not always stable, and can be variable under different conditions *(290, 311)*. To minimize the influence from reference gene, a solution was to include additional standard reference genes. For instance, two independent reference genes could be used to calculate relative gene expression of target genes *(55)*. 16S rRNA and *gapdh* were both used as reference genes in our current report *(295, 308)*. The efficiencies of amplification of the *inlA, sigB* and reference genes were found to be in the range of 90%-110%, comparable to that of other reports and satisfactory for qPCR reactions *(96, 225)*.

6.4.2. Stress responses of *sigB* and *inlA* gene with time

While σ^B activation was reported via a single pathway under stress conditions, regulation of the expression of stress-response and virulence genes in the σ^B regulon were hypothesized to require networks involving multiple transcriptional regulators *(55)*. Since σ^B exists in active or inactive states *(24)*, transcriptional levels of σ^B-dependent genes are measured for indirect quantification of protein activity *(55)*, which is an approach taken by many researchers including this research. σ^B expression is related to the growth phase of microorganisms. Cultures approaching stationary phase have the highest σ^B expression *(90)*. However, this expression changes under stress conditions. For instance, static biofilm cells, subjected to a stress condition of nutrient deficiency, exhibit 3-fold increased *sigB* expression compared with planktonic cells *(308)*.

Five minute exposure to stress was reported to be enough for activating stress-induced σ^B-dependent activity in *L. monocytogenes (55, 290)*. Moreover, activation of the

sigB gene was proportional to the level of osmotic stress in *L. monocytogenes* as quick as 5 min in response *(306)*. Our results of the effects of NECAW on activation of *sigB* and *inlA* of *L. monocytogenes* were consistant with these findings.

Gene expression of *inlA* and *sigB* in response to stress might be transient. Under stress conditions, expression levels of *sigB* and *inlA* increased with time within a certain period. For example, *inlA* transcripts significantly accumulates after 5 or 15 min of exposure to osmotic or acid stress conditions compared to the control condition without stress exposure. However, gene expression reverts to its normal level after a relatively longer time. In another report, expression of *sigB* at the mid-exponential growth phase was found to increase with exposure to osmotic stress for 15 min, then gradually decreases, indicating that transcriptional activation is transient in response to osmotic stress *(306)*. In addition, σ^B activity of *L. monocytogenes* increases under exposure to a cold shock for 30 min *(53, 140)*, but its level was relatively stable from the starting point after a relatively longer time (48 h) of exposure *(225)*.

6.4.3. σ^B role in *L. monocytogenes*'s resistance to sanitizers

A functional σ^B protein was found essential for acquiring resistance of *L. monocytogenes* to stress conditions *(90)*. Survival of the Δ*sigB* strain is consistently lower than that of the WT strain in all growth phases under many stress conditions including heating *(225, 286)* Survival is 2- and 4-fold less in mid-log phase and stationary phase, respectively, in Δ*sigB* strain compared to the WT, regardless of a prior acid adaptation *(90)*. σ^B also plays an important role in *L. monocytogenes*'s enhanced

resistance to lethal pH or oxidative treatment *(90)*. Ferreira and others (2001) suggested that *L. monocytogenes* cells in the stationary phase have two mechanisms of acid resistance (AR): σ^B-dependent AR mechanism and σ^B independent AR mechanism *(89, 90)*.

σ^B was found essential for the survival of *L. monocytogenes* at lethal levels of many sanitizers, disinfectants or surfactants *(270)*. A significant 1–2 log decrease in the viability of the Δ*sigB* mutant strain exposed to lethal concentrations of surfactants including benzalkoniumchloride (BC), cetylpyridinium chloride (CPC) and sodium docecyl sulfate (SDS) was observed along with the induction of σ^B by those disinfectants. In addition, planktonic cells of Δ*sigB* mutant strain have a lower survival rate than WT microbial cells as liquid cultures and biofilms *(279, 308)*. However, σ^B does not affect the growth of *L. monocytogenes* when exposed to sublethal levels of those disinfectants *(270)*.

Currently, little information is available for the fundamental mechanisms at the molecular level involved in *L. monocytogenes*'s resistance to detergents or sanitizers *(270)*. For instance, it is not clear why short-term salt stress increases *L. monocytogenes* resistance to H_2O_2 *(26)*. It is highly possible that the σ^B regulon may be critical for this increased resistance *(270)*. Our findings on the increased expression level of virulence gene (*inlA*) and stress-response gene (*sigB*) at sublethal concentration of NECAW sanitizer treatment supports this conclusion. σ^B was found to upregulate genes related to adhesion, acid tolerance, bile tolerance and osmotolerance *(270)*. The evidence of σ^B-regulated mechanisms of detergent resistance provides guidance information for the development of novel sanitizers *(270)*.

6.4.4. Relation between *inlA* and *sigB*

sigB is an autoregulated gene, as expression of the gene regulates several other genes in the σ^B operon and *sigB* itself. The expression of genes in the operon does not significantly change under stress conditions for the Δ*sigB* mutant strain since no σ^B responds to extracellular stress *(306)*, suggesting that when *sigB* is present *sigB*-dependent genes might be influenced by the environmental stress as well. σ^B contributes to the regulation of virulence gene expression in *L. monocytogenes (140)*. σ^B-dependent *inlA* expression was reported previously *(140, 143, 290)*. When exposed to an osmotic stress, *inlA* expression of stationary phase *L. monocytogenes* cultures is up-regulated significantly in the WT strain (9-19-fold) ($P<0.05$), while in the Δ*sigB* strain, expression does not change significantly, demonstrating that σ^B is critical for the up-regulation of *inlA* during stationary phase *(144)*. In the current results, *inlA* gene expression in the Δ*sigB* strain did not change significantly when NECAW concentration and time increased, both for liquid cultures and biofilms (Tables 6.1 to 6.4), revealing that under the NECAW stress condition, the expression of *inlA* was controlled by the *sigB* gene.

Moreover, *inlA* expression in the *L. monocytogenes* isogenic Δ*sigB* null mutant is significantly lower than in the WT strain *(90)*. The current results support this conclusion. However, to make it easier to compare the effects of sanitizing stress on gene expression, the relative fold changes of *inlA* and *sigB* expression in the WT and Δ*sigB* strains were calculated by normalizing to *inlA* and *sigB* levels under DIW treatment, respectively. Recently, *inlA* was further confirmed as transcribed in a σ^B-dependent way by a qRT-

PCR experiment, providing direct evidence that σ^B was critical for regulating *inlA* virulence gene expression during stress conditions within the human intestine *(140, 290)*.

Several other factors could also affect *inlA* expression including oxidative stress *(39)*, therefore it is not clear if σ^B-mediated effects on *inl*A expression were direct or indirect *(144)*. However, it is certain that *prfA* regulates the transcription of the *inlAB* locus, and σ^B contributes to *inlAB* locus transcription indirectly via controlling *prfA* expression *(144)*. Although *inlA* is regulated by σ^B, it should be noted that there are differences between the effects of stress on *inlA* and *sigB* expression. For *inlA*, no significant changes in its transcription levels were found for any of three tested *L. monocytogenes* strains for liver pâtés ($P > 0.05$). While for *sigB*, the relative expression for one of the strains (strain 6896) is significantly increased by 3.2-fold in liver pâté with a 33% reduced NaCl content ($P<0.001$), compared to liver pâté with standard NaCl content ($P > 0.05$) *(225)*. In addition, *L. monocytogenes* grown on food products with changed environments may not increase the expression of virulence genes including *inlA* gene *(15)*. The current results were obtained under one type of stress, NECAW treatment, with varied treatment duration and NECAW concentrations. Changes in the relative expression of *sigB* were greater than that of *inlA*, revealing that *sigB* gene was more important than *inlA* for *L. monocytogenes* survival under NECAW treatment.

6.4.5. Homogeneous sensitivity of *L. monocytogenes* cells to sanitizers

L. monocytogenes biofilms were more resistant than planktonic cells to sanitizers such as NECAW and peracetic acid *(165, 179, 308)*, as indicated by higher surviving

counts and lower maximum inactivation rate of biofilms than their planktonic cells *(308)*. In food production plants, persistent *L. monocytogenes* strains are thought to be more tolerant or resistant to cleaning, especially to sanitizers or disinfectants *(139)*. However, the increased survival of *L. monocytogenes* in food processing plants is not due to an increase in tolerant subpopulation of bacterial cells *(139)*. A study revealed that a population of *L. monocytogenes* microbial cells was homogenous in sensitivity to an acidic disinfectant *(139)*.

Overall, biofilm formation and antimicrobial effects of sanitizers on *L. monocytogenes* is very complicated. One certainty is that the induction of σ^B by sublethal levels of cleaning or sanitizing agents as a stress in the food industry might increase the pathogen's resistance, causing potential safety concerns *(270)*. Future experiments including examination of both RNA and protein levels will be essential for elucidating the roles of *sigB* and *inlA* genes in *L. monocytogenes* resistance. Also, specific agents targeting *sigB* or *inlA* can be developed *(233)*.

6.5. Conclusion

inlA and *sigB* play important roles in the survival and sanitizing resistance of *L. monocytogenes* microbial cells.The current study found that isogenic *inlA* and *sigB* mutants were able to form biofilms, revealing that these two genes were not the decisive factors for biofilm formation. After NECAW treatment, expression of both genes increased in WT. While *sigB* gene expression of the Δ*inlA* strain increased at a level comparable to that of WT, *inlA* gene expression of the Δ*sigB* strain did not change

significantly. Both genes were expressed more in biofilms than in liquid cultures. Mutant strains (Δ*inlA,* Δ*sigB,* Δ*inlA*Δ*sigB)* were more sensitive to NECAW treatment than the WT strain.The *sigB* gene was more important than *inlA* for pathogen survival under NECAW treatment. Surviving *L. monocytogenes* cells post-sublethal NECAW treatment might become resistant to further sanitizer treatment.

Table 6.1. Effect of neutral electrochemically activated water (NECAW, 4 mg/l free available chlorine) treatment time on *inl*A or *sig*B gene expression of liquid cultures of *Listeria monocytogenes* in wild type (WT) and *ΔinlA*, *ΔsigB* strains determined by qPCR. Two housekeeping genes, 16S and *rpoB* were used for reference of expression level.

Treatment		**Relative gene expression level**			
		inlA		***sigB***	
	Time (min)	**WT**	**Δ*sigB***	**WT**	**Δ*inlA***
DIW	0.5	1.00±0.09 Ca	1.00±0.27 Aa	1.00±0.11 Ca	1.00±0.22 Ca
	5	1.01±0.11 Ca	0.98±0.13 Aa	1.06±0.28 Ca	1.01±0.23 Ca
	10	0.99±0.13 Ca	1.16±0.20 Aa	1.14±0.19 Ca	1.16±0.21 Ca
NECAW	0.5	1.53±0.36 BCa	1.42±0.38 Aa	1.82±0.66 BCa	1.95±0.57 BCa
	5	2.26±0.77 Bab	1.49±0.74 Ab	3.72±1.61 ABa	3.57±1.71 ABab
	10	4.28±0.96 Aab	1.68±0.71 Ab	5.91±3.08 Aa	4.79±2.37 Aab

DIW: de-ionized water.

Within each row, means with different lowercase letters are significantly different ($P < 0.05$).

Within each column, means with different capital letters are significantly different ($P < 0.05$).

Table 6.2. Effect of the concentration of free available chlorine (FAC) in neutral electrochemically activated water (NECAW) treatment on the *inl*A or *sig*B gene expression of liquid cultures of *Listeria monocytogenes* wild type (WT) and *ΔinlA*, *ΔsigB* strains after 5 min incubation using qPCR. Two housekeeping genes, 16S and *rpoB* were used for reference of expression level.

FAC (mg/l)	**Relative gene expression level**			
	***inl*A**		***sig*B**	
	WT	**Δ*sigB***	**WT**	**Δ*inlA***
0	1.00±0.07 Ca	1.00±0.14 Aa	1.00±0.09 Ba	1.00±0.16 Ca
2	1.31±0.33 BCa	1.34±0.59 Aa	2.08±0.84 Ba	1.89±0.93 BCa
4	2.11±0.89 ABab	1.59±0.75 Ab	3.97±1.58 ABa	3.81±1.80 ABa
6	2.52±0.41 Ab	1.58±1.12 Ab	6.35±3.44 Aa	4.67±2.14 Aab

Within each row, means with different lowercase letters are significantly different ($P < 0.05$).

Within each column, means with different capital letters are significantly different ($P < 0.05$).

Table 6.3. Effect of neutral electrochemically activated water (NECAW, 4 mg/l free available chlorine) treatment time on *inl*A or *sig*B gene expression of biofilms of *Listeria monocytogenes* in wild type (WT) and *ΔinlA*, *ΔsigB* strains determined by qPCR. Two housekeeping genes, 16S and *rpoB* were used for reference of expression level.

Treatment		**Relative gene expression level**			
		inlA		***sigB***	
	Time (min)	**WT**	**Δ*sigB***	**WT**	**Δ*inlA***
DIW	0.5	1.00±0.11 Ca	1.00±0.16 Aa	1.00±0.20 Ba	1.00±0.16 Ba
	5	1.04±0.30 Ca	1.12±0.24 Aa	1.01±0.30 Ba	0.96±0.21 Ba
	10	1.06±0.24 Ca	1.04±0.23 Aa	1.05±0.23 Ba	1.17±0.52 Ba
NECAW	0.5	1.86±0.26 BCab	1.40±0.49 Ab	2.94±1.10 Ba	2.66±0.60 Ba
	5	4.17±2.26 ABab	1.63±0.87 Ab	8.55±3.28 Aa	7.86±3.64 Aa
	10	5.51±2.75 Aab	1.73±0.88 Ab	10.05±3.60 Aa	9.89±4.55 Aa

DIW: de-ionized water.

Within each row, means with different lowercase letters are significantly different ($P < 0.05$).

Within each column, means with different capital letters are significantly different ($P < 0.05$).

Table 6.4. Effect of the concentration of free available chlorine (FAC) in neutral electrochemically activated water (NECAW) treatment on the *inlA* or *sigB* gene expression of biofilms of *Listeria monocytogenes* wild type (WT) and *ΔinlA*, *ΔsigB* strains after 5 min incubation using qPCR. Two housekeeping genes, 16S and *rpoB* were used for reference of expression level.

FAC (mg/l)	**Relative gene expression level**			
	***inl*A**		***sig*B**	
	WT	**Δ*sigB***	**WT**	**Δ*inlA***
0	1.00±0.16 Ca	1.00±0.21 Aa	1.00±0.20 Ca	1.00±0.17 Ba
2	2.11±0.18 BCab	1.20±0.47 Ab	3.44±1.57 BCa	3.34±0.82 Ba
4	3.61±1.71 ABb	1.39±0.66 Ab	8.50±3.25 ABa	8.70±3.16 Aa
6	5.06±1.95 Ab	1.58±1.15 Ab	14.01±7.46 Aa	12.61±4.94 Aa

Within each row, means with different lowercase letters are significantly different ($P < 0.05$).

Within each column, means with different capital letters are significantly different ($P < 0.05$).

Table 6.5. Effect of the concentration of free available chlorine (FAC) in neutral electrochemically activated water (NECAW) on liquid culture viable count of *Listeria monocytogenes* wild type (WT) and mutant *ΔinlA*, *ΔsigB* strains.

Time (min)	**FAC (mg/l)**	**Viable count reduction[a] (log CFU/ml)**			
		WT	**Δ*inlA***	**Δ*sigB***	**Δ*inlA*Δ*sigB***
0.5	0	0.00±0.00 A	0.00±0.00 A	0.00±0.00 A	0.00±0.00 A
	2	0.04±0.12 A	0.05±0.03 A	0.11±0.05 A	0.20±0.15 A
	4	0.02±0.05 A	0.07±0.03 A	0.44±0.54 A	0.55±0.14 A
	6	0.20±0.21 A	0.62±0.33 A	0.82±0.60 A	0.87±0.27 A
	10	1.27±0.42 B	1.49±0.29 AB	1.86±0.24 AB	2.00±0.26 A
	20	4.43±0.40 A	4.98±0.49 A	≥5.11±0.07	≥5.05±0.04
5.0	0	0.00±0.00 A	0.00±0.00 A	0.00±0.00 A	0.00±0.00 A
	2	0.02±0.11 B	0.05±0.13 B	0.24±0.03 AB	0.41±0.17 A
	4	0.03±0.10 B	0.08±0.13 B	0.97±0.29 A	1.07±0.33 A
	6	0.45±0.30 B	0.79±0.30 AB	1.23±0.39 A	1.32±0.31 A
	10	1.68±0.35 B	1.82±0.42 B	2.34±0.17 AB	2.83±0.33 A
	20	≥5.29±0.05	≥5.23±0.08	≥5.12±0.09	≥5.03±0.08
10.0	0	0.00±0.00 A	0.00±0.00 A	0.00±0.00 A	0.00±0.00 A
	2	0.03±0.11 B	0.11±0.15 B	0.43±0.12 A	0.62±0.06 A
	4	0.11±0.13 C	0.18±0.15 C	1.58±0.36 B	2.17±0.33 A
	6	0.54±0.26 B	0.93±0.30 B	2.23±0.43 A	2.45±0.46 A
	10	1.98±0.27 C	2.77±0.44 B	3.15±0.34 AB	3.63±0.27 A
	20	≥5.28±0.03	≥5.23±0.11	≥5.14±0.10	≥5.05±0.11

[a] Within each row, means with different cap letters are significantly different ($P < 0.05$).

Table 6.6. Effect of the concentration of free available chlorine (FAC) in neutral electrochemically activated water (NECAW) *Listeria monocytogenes* viable count of wild type (WT) and mutant *ΔinlA*, *ΔsigB* strains biofilms on stainless steel coupons treated for one minute.

FAC (mg/l)	**Viable count reduction after 1 min[a] (log CFU/coupon)**			
	WT	**Δ*inlA***	**Δ*sigB***	**Δ*inlA*Δ*sigB***
0	0.00±0.00 A	0.00±0.00 A	0.00±0.00 A	0.00±0.00 A
2	-0.03±0.15 A	0.00±0.15 A	0.01±0.28 A	0.17±0.84 A
4	0.10±0.19 A	0.16±0.26 A	0.21±0.39 A	0.27±0.71 A
6	0.31±0.35 A	0.36±0.31 A	0.42±0.39 A	0.39±0.40 A
10	0.39±0.29 A	0.43±0.25 A	0.50±0.26 A	0.49±0.24 A
20	0.73±0.32 A	1.01±0.83 A	1.03±0.84 A	0.98±0.80 A
50	0.77±0.76 A	0.89±0.87 A	0.98±0.87 A	1.02±1.01 A
100	1.14±0.42 A	1.31±1.01 A	1.34±0.96 A	1.30±0.93 A

[a] Within each row, means with different cap letters are significantly different ($P < 0.05$).

Table 6.7. Effect of the concentration of free available chlorine (FAC) in neutral electrochemically activated water (NECAW) *Listeria monocytogenes* viable count of wild type (WT) and mutant *ΔinlA*, *ΔsigB* strains biofilms on stainless steel coupons treated for five minutes.

FAC (mg/l)	**Viable count reduction after 5 min[a] (log CFU/coupon)**			
	WT	**Δ*inl*A**	**Δ*sig*B**	**Δ*inl*AΔ*sig*B**
0	0.00±0.00 A	0.00±0.00 A	0.00±0.00 A	0.00±0.00 A
2	0.22±0.10 A	0.20±0.14 A	0.32±0.25 A	0.28±0.33 A
4	0.06±0.55 A	0.09±0.47 A	0.55±0.75 A	0.48±0.74 A
6	0.20±0.54 A	0.16±0.44 A	0.45±0.54 A	0.47±0.65 A
10	0.37±0.39 A	0.36±0.36 A	0.75±0.32 A	0.79±0.36 A
20	0.79±0.51 A	0.79±0.43 A	1.12±0.53 A	1.18±0.49 A
35	1.12±0.62 A	1.07±0.51 A	1.43±0.64 A	2.22±1.25 A
50	1.61±0.82 A	1.56±0.78 A	2.01±0.85 A	≥4.62±0.22
75	2.30±0.61 A	2.24±0.62 A	3.15±0.58 A	≥4.62±0.22
100	3.00±0.36 A	3.09±0.43 A	≥4.73±0.12	≥4.62±0.22

[a] Within each row, means with different cap letters are significantly different ($P < 0.05$).

Table 6.8. Effect of the concentration of free available chlorine (FAC) in neutral electrochemically activated water (NECAW) *Listeria monocytogenes* viable count of wild type (WT) and mutant *ΔinlA*, *ΔsigB* strains biofilms on stainless steel coupons treated for 10 minutes.

FAC (mg/l)	Viable count reduction after 10 min[a] (log CFU/coupon)			
	WT	Δ*inl*A	Δ*sig*B	Δ*inl*AΔ*sig*B
0	0.00±0.00 A	0.00±0.00 A	0.00±0.00 A	0.00±0.00 A
2	0.09±0.14 A	0.09±0.17 A	0.13±0.24 A	0.22±0.28 A
4	0.28±0.41 A	0.29±0.44 A	0.77±0.57 A	0.88±0.57 A
6	0.72±0.31 A	0.78±0.31 A	1.00±0.19 A	1.10±0.35 A
10	0.96±0.76 A	1.14±0.81 A	1.45±1.06 A	1.93±1.13 A
20	1.12±0.36 A	1.16±1.08 A	1.49±1.23 A	1.55±1.25 A
35	1.74±0.81 A	1.85±1.33 A	2.97±0.55 A	2.59±1.46 A
50	2.39±0.73 A	2.69±0.76 A	≥4.73±0.12	≥4.62±0.22
75	2.77±0.75 A	3.39±0.19 A	≥4.73±0.12	≥4.62±0.22
100	3.16±0.33 A	3.82±0.52 A	≥4.73±0.12	≥4.62±0.22

[a] Within each row, means with different cap letters are significantly different ($P < 0.05$).

REFERENCES

1. Abadias, M., J. Usall, M. Oliveira, I. Alegre, and I. Vinas. 2008. Efficacy of neutral electrolyzed water (NEW) for reducing microbial contamination on minimally-processed vegetables. *International Journal of Food Microbiology*. 123:151-158.
2. Ackers, M. L., B. E. Mahon, E. Leahy, B. Goode, T. Damrow, P. S. Hayes, W. F. Bibb, D. H. Rice, T. J. Barrett, L. Hutwagner, P. M. Griffin, and L. Slutsker. 1998. An outbreak of *Escherichia coli* O157:H7 infections associated with leaf lettuce consumption. *Journal of Infectious Diseases*. 177:1588-1593.
3. Administration, U. S. F. a. D. Date, 2011, Archive for recalls, market withdrawals & safety alerts. Available at: http://www.fda.gov/Safety/Recalls/ArchiveRecalls/default.htm. Accessed 19 October, 2011.
4. Agriculture, U. S. D. o. Date, 2011, Recall case archive. Available at: http://www.fsis.usda.gov/Fsis_Recalls/Recall_Case_Archive/index.asp. Accessed 19 October, 2011.
5. Ahimou, F., F. A. Denis, A. Touhami, and Y. F. Dufrene. 2002. Probing microbial cell surface charges by atomic force microscopy. *Langmuir*. 18:9937-9941.
6. Al-Haq, M. M., J. Sugiyama, and S. Isobe. 2005. Applications of electrolyzed water in agriculture & food industries. *Food Science and Technology Research*. 11:135-150.
7. Annous, B. A., P. M. Fratamico, and J. L. Smith. 2009. Quorum sensing in biofilms: why bacteria behave the way they do. . *Journal of Food Science*. 74:R24-37.
8. AOAC International. 2000. Germicidal and detergent sanitizing action of disinfectants. *AOAC Official Methods of Analysis*. 6:10-12.
9. Arguedas-Villa, C., R. Stephan, and T. Tasara. 2010. Evaluation of cold growth and related gene transcription responses associated with Listeria monocytogenes strains of different origins. *Food Microbiology*. 27:653-660.
10. Arizona Department of Health Services. Date, 2004, *Escherichia coli* O157:H7-bioterrorism agent profiles for health care workers Available at: http://www.azdhs.gov/phs/edc/edrp/pdf/escherichiacoliset.pdf. Accessed Jul 13, 2012.
11. Ayebah, B., Y. C. Hung, and J. F. Frank. 2005. Enhancing the bactericidal effect of electrolyzed water on *Listeria monocytogenes* biofilms formed on stainless steel. *Journal of Food Protection*. 68:1375-1380.
12. Ayebah, B., Y. C. Hung, C. Kim, and J. F. Frank. 2006. Efficacy of electrolyzed water in the inactivation of planktonic and biofilm *Listeria monocytogenes* in the presence of organic matter. *Journal of Food Protection*. 69:2143-2150.

13. Ayers, L., B. Ferry, S. Craig, D. Nicoll, J. R. Stradling, and M. Kohler. 2009. Circulating cell-derived microparticles in patients with minimally symptomatic obstructive sleep apnoea. *Eur Respir J*. 33:574-80.
14. Badr, H. M. 2005. Elimination of *Escherichia coli* O157:H7 and *Listeria monocytogenes* from raw beef sausage by gamma-irradiation. *Molecular Nutrition & Food Research*. 49:343-349.
15. Bae, D., M. R. Crowley, and C. L. Wang. 2011. Transcriptome Analysis of Listeria monocytogenes Grown on a Ready-to-Eat Meat Matrix. *Journal of Food Protection*. 74:1104-1111.
16. Bari, M. L., Y. Sabina, S. Isobe, T. Uemura, and K. Isshiki. 2003. Effectiveness of electrolyzed acidic water in killing *Escherichia coli* O157:H7, *Salmonella enteritidis*, and *Listeria monocytogenes* on the surfaces of tomatoes. *Journal of Food Protection*. 66:542-548.
17. Bari, M. L., Y. Sabina, S. Isobe, T. Uemura, and K. Isshiki. 2003. Effectiveness of electrolyzed acidic water in killing *Escherichia coli* O157:H7, *Salmonella enteritidis*, and *Listeria monocytogenes* on the surfaces of tomatoes (vol 66, pg 542, 2003). *Journal of Food Protection*. 66:1541-1542.
18. Bartyzel, M. Date, 2008, FDA approves move to zap fresh lettuce and spinach with radiation. Available at: http://www.slashfood.com/2008/08/21/fda-approves-move-to-zap-fresh-lettuce-and-spinach-with-radiatio/. Accessed July 14, 2012.
19. Baumann, A. R., S. E. Martin, and H. Feng. 2009. Removal of *Listeria monocytogenes* biofilms from stainless steel by use of ultrasound and ozone. *Journal of Food Protection*. 72:1306-1309.
20. Bazylinski, D. A., A. J. Garrattreed, A. Abedi, and R. B. Frankel. 1993. Copper association with iron sulfide magnetosomes in a magnetotactic bacterium. *Archives of Microbiology*. 160:35-42.
21. Behravesh, C. B., R. K. Mody, J. Jungk, L. Gaul, J. T. Redd, S. Chen, S. Cosgrove, E. Hedican, D. Sweat, L. Chavez-Hauser, S. L. Snow, H. Hanson, T. A. Nguyen, S. V. Sodha, A. L. Boore, E. Russo, M. Mikoleit, L. Theobald, P. Gerner-Smidt, R. M. Hoekstra, F. J. Angulo, D. L. Swerdlow, R. V. Tauxe, P. M. Griffin, I. T. Williams, and S. S. O. Inve. 2011. 2008 outbreak of *Salmonella Saintpaul* infections associated with raw produce. *New England Journal of Medicine*. 364:918-927.
22. Belval, S. C., L. Gal, S. Margiewes, D. Garmyn, P. Piveteau, and J. Guzzo. 2006. Assessment of the roles of LuxS, S-ribosyl homocysteine, and autoinducer 2 in cell attachment during biofilm formation by Listeria monocytogenes EGD-e. *Applied and Environmental Microbiology*. 72:2644-2650.
23. Ben Abdallah, F., R. Lagha, and A. Bakhrouf. 2008. Resuscitation and morphological alterations of Salmonella bovismorbificans cells under starvation in soil. *World Journal of Microbiology & Biotechnology*. 24:1507-1512.
24. Benson, A. K., and W. G. Haldenwang. 1993. Bacillus-Subtilis Sigma-B Is Regulated by a Binding-Protein (Rsbw) That Blocks Its Association with Core Rna-Polymerase. *Proceedings of the National Academy of Sciences of the United States of America*. 90:2330-2334.

25. Berger, C. N., S. V. Sodha, R. K. Shaw, P. M. Griffin, D. Pink, P. Hand, and G. Frankel. 2010. Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environmental Microbiology*. 12:2385-2397.
26. Bergholz, T. M., B. Bowen, M. Wiedmann, and K. J. Boor. 2012. Listeria monocytogenes Shows Temperature-Dependent and -Independent Responses to Salt Stress, Including Responses That Induce Cross-Protection against Other Stresses. *Applied and Environmental Microbiology*. 78:2602-2612.
27. Beuchat, L. R., C. A. Pettigrew, M. E. Tremblay, B. J. Roselle, and A. J. Scouten. 2005. Lethality of chlorine, chlorine dioxide, and a commercial fruit and vegetable sanitizer to vegetative cells and spores of *Bacillus cereus* and spores of *Bacillus thuringiensis. Journal of Industrial Microbiology & Biotechnology*. 32:301-308.
28. Bialka, K. L., and A. Demirci. 2007. Decontamination of *Escherichia coli* O157:H7 and *Salmonella enterica* on blueberries using ozone and pulsed UV-light. *Journal of Food Science*. 72:M391-M396.
29. Bishop, D. K., and D. J. Hinrichs. 1987. Adoptive transfer of immunity to *Listeria monocytogenes* - the influence of invitro stimulation on lymphocyte subset requirements. *Journal of Immunology*. 139:2005-2009.
30. Bittman, M. Date, 2009, Eating food that's better for you, organic or not. Available at: http://www.nytimes.com/2009/03/22/weekinreview/22bittman.html. Accessed Jul 14, 2012.
31. Bitzan, M. 2009. Treatment options for HUS secondary to *Escherichia coli* O157:H7. *Kidney International*. 75:S62-S66.
32. Blackman, I. C., and J. F. Frank. 1996. Growth of Listeria monocytogenes as a biofilm on various food-processing surfaces. *Journal of Food Protection*. 59:827-831.
33. Borucki, M. K., J. D. Peppin, D. White, F. Loge, and D. R. Call. 2003. Variation in biofilm formation among strains of *Listeria monocytogenes. Applied and Environmental Microbiology*. 69:7336-7342.
34. Brackett, R. E. 1987. Antimicrobial effect of chlorine on *Listeria monocytogenes. Journal of Food Protection*. 50:999-&.
35. Breen, P. J., C. M. Compadre, E. K. Fifer, H. Salari, D. C. Serbus, and D. L. Lattin. 1995. Quaternary ammonium compounds inhibit and reduce the attachment of viable *Salmonella typhimurium* to poultry tissues. *Journal of Food Science*. 60:1191-1196.
36. Bremer, P. J., G. G. Geesey, and B. Drake. 1992. Atomic force microscopy examination of the topography of a hydrated bacterial biofilm on a copper surface. *Current Microbiology*. 24:223-230.
37. Brigati, J. R., S. A. Ripp, C. M. Johnson, P. A. Iakova, P. Jegier, and G. S. Sayler. 2007. Bacteriophage-based bioluminescent bioreporter for the detection of *Escherichia coli* O157:H7. *Journal of Food Protection*. 70:1386-1392.
38. Brooks, J. T., B. T. Matyas, J. Fontana, M. A. DeGroot, L. R. Beuchat, M. Hoekstra, and C. R. Friedman. 2012. An outbreak of *Salmonella* serotype

Typhimurium infections with an unusually long incubation period. *Foodborne Pathogens and Disease*. 9:245-248.
39. Bruck, S., N. Personnic, M. C. Prevost, P. Cossart, and H. Bierne. 2011. Regulated Shift from Helical to Polar Localization of Listeria monocytogenes Cell Wall-Anchored Proteins. *Journal of Bacteriology*. 193:4425-4437.
40. Bruno, J. C., and N. E. Freitag. 2011. Listeria monocytogenes adapts to long-term stationary phase survival without compromising bacterial virulence. *Fems Microbiology Letters*. 323:171-179.
41. Bundrant, B. N., T. Hutchins, H. C. den Bakker, E. Fortes, and M. Wiedmann. 2011. Listeriosis outbreak in dairy cattle caused by an unusual *Listeria monocytogenes* serotype 4b strain. *Journal of Veterinary Diagnostic Investigation*. 23:155-158.
42. Caillet, G., and S. B. Batiot. 2003. 2D modelling of hydrocarbon migration along and across growth faults: an example from Nigeria. *Petroleum Geoscience*. 9:113-124.
43. Camire, K. 2009. Less than 20% of consumers trust food they buy is safe and healthy, IBM survey reveals. *In*.
44. Camps, N., A. Dominguez, M. Company, M. Perez, J. Pardos, T. Llobet, M. A. Usera, L. Salleras, and W. G. I. Outbreak. 2005. A foodborne outbreak of *Salmonella* infection due to overproduction of egg-containing foods for a festival. *Epidemiology and Infection*. 133:817-822.
45. Carlton, R. M., W. H. Noordman, B. Biswas, E. D. de Meester, and M. J. Loessner. 2005. Bacteriophage P100 for control of *Listeria monocytogenes* in foods: Genome sequence, bioinformatic analyses, oral toxicity study, and application. *Regulatory Toxicology and Pharmacology*. 43:301-312.
46. Castillo, A., K. S. McKenzie, L. M. Lucia, and G. R. Acuff. 2003. Ozone treatment for reduction of *Escherichia coli* O157:H7 and *Salmonella* serotype typhimurium on beef carcass surfaces. *Journal of Food Protection*. 66:775-779.
47. Cattlemen's Beef Board & National Cattlemen's Beef Association. Date, 2012, Total beef category breakdown. Available at: http://beefretail.cylosoftncba.com/TotalBeefCategoryBreakdown.aspx. Accessed Jul 14, 2012.
48. CDC. 2006. Ongoing multistate outbreak of *Escherichia coli* serotype O157:H7 infections associated with consumption of fresh spinach - United States, September 2006 (Reprinted from MMWR, vol 55, pg 1045-1046, 2006). *Jama-Journal of the American Medical Association*. 296:2195-2196.
49. CDC. Date, 2011, Multistate outbreak of listeriosis linked to whole cantaloupes from Jensen farms, Colorado. Available at: http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/index.html. Accessed Jul 12, 2012.
50. Center for Infectious Disease Research & Policy. Date, 2012, Food irradiation. Available at: http://www.cidrap.umn.edu/cidrap/content/fs/irradiation/biofacts/irrad-bkgd.html. Accessed.

51. Centers for Disease Control and Prevention (CDC). 2011. Surveillance for foodborne disease outbreaks --- United States, 2008. *Morbidity and Mortality Weekly Report*. 60:1197-1202.
52. Cetin, M. S., C. M. Zhang, R. W. Hutkins, and A. K. Benson. 2004. Regulation of transcription of compatible solute transporters by the general stress sigma factor, sigma(B), in *Listeria monocytogenes*. *Journal of Bacteriology*. 186:794-802.
53. Chan, Y. C., K. J. Boor, and M. Wiedmann. 2007. sigma(B)-Dependent and sigma(B)-Independent mechanisms contribute to transcription of listeria monocytogenes cold stress genes during cold shock and cold growth. *Applied and Environmental Microbiology*. 73:6019-6029.
54. Chancey, B. Date, 2008, Consumers and food safety. Available at: http://www.qsrmagazine.com/news/consumers-and-food-safety. Accessed Jul 13, 2012.
55. Chaturongakul, S., and K. J. Boor. 2006. sigma(B) activation under environmental and energy stress conditions in *Listeria monocytogenes*. *Applied and Environmental Microbiology*. 72:5197-5203.
56. Chen, B. Y., T. J. Kim, Y. S. Jung, and J. L. Silva. 2008. Attachment strength of *Listeria monocytogenes* and its internalin-negative mutants. *Food Biophysics*. 3:329-332.
57. Chen, B. Y., T. J. Kim, J. L. Silva, and Y. S. Jung. 2009. Positive Correlation Between the Expression of inlA and inlB Genes of Listeria monocytogenes and Its Attachment Strength on Glass Surface. *Food Biophysics*. 4:304-311.
58. Chia, T. W. R., R. M. Goulter, T. McMeekin, G. A. Dykes, and N. Fegan. 2009. Attachment of different Salmonella serovars to materials commonly used in a poultry processing plant. *Food Microbiology*. 26:853-859.
59. Cloete, T. E. 2003. Biofouling control in industrial water systems: What we know and what we need to know. *Materials and Corrosion-Werkstoffe Und Korrosion*. 54:520-526.
60. Cloete, T. E., M. S. Thantsha, M. R. Maluleke, and R. Kirkpatrick. 2009. The antimicrobial mechanism of electrochemically activated water against *Pseudomonas aeruginosa* and *Escherichia coli* as determined by SDS-PAGE analysis. *Journal of Applied Microbiology*. 107:379-384.
61. Coffey, B., S. Mills, A. Coffey, O. McAuliffe, and R. P. Ross. 2010. Phage and their lysins as biocontrol agents for food safety applications. *Annual Review of Food Science and Technology, Vol 1*. 1:449-468.
62. Corn, D. Date, 2000, Irradiated food: safe or scandalous? Available at: http://www.alternet.org/module/printversion/9907. Accessed Jul 14, 2012.
63. Cross, S. E., J. Kreth, L. Zhu, F. X. Qi, A. E. Pelling, W. Y. Shi, and J. K. Gimzewski. 2006. Atomic force microscopy study of the structure-function relationships of the biofilm-forming bacterium *Streptococcus* mutans. *Nanotechnology*. 17:S1-S7.
64. Dalton, C. B., C. C. Austin, J. Sobel, P. S. Hayes, W. F. Bibb, L. M. Graves, B. Swaminathan, M. E. Proctor, and P. M. Griffin. 1997. An outbreak of

gastroenteritis and fever due to *Listeria monocytogenes* in milk. *New England Journal of Medicine*. 336:100-105.
65. Denyer, S. P., and G. S. A. B. Stewart. 1998. Mechanisms of action of disinfectants. *International Biodeterioration & Biodegradation*. 41:261-268.
66. Deza, M. A., M. Araujo, and M. J. Garrido. 2003. Inactivation of *Escherichia coli* O157:H7, *Salmonella enteritidis* and *Listeria monocytogenes* on the surface of tomatoes by neutral electrolyzed water. *Letters in Applied Microbiology*. 37:482-487.
67. Deza, M. A., M. Araujo, and M. J. Garrido. 2005. Inactivation of *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* on stainless steel and glass surfaces by neutral electrolysed water. *Letters in Applied Microbiology*. 40:341-346.
68. Deza, M. A., M. Araujo, and M. J. Garrido. 2007. Efficacy of neutral electrolyzed water to inactivate *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* on plastic and wooden kitchen cutting boards. *Journal of Food Protection*. 70:102-108.
69. Deza, M. A., M. Araujo, and M. J. Garrido. 2007. Efficacy of neutral electrolyzed water to inactivate *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* on plastic and wooden kitchen cutting boards (vol 70, pg 102, 2007). *Journal of Food Protection*. 70:1070-1070.
70. Dieguez-Uribeondo, J., H. Forster, and J. E. Adaskaveg. 2008. Visualization of localized pathogen-induced pH modulation in almond tissues infected by *Colletotrichum acutatum* using confocal scanning laser microscopy. *Phytopathology*. 98:1171-1178.
71. Diehl, J. F. 2002. Food irradiation - past, present and future. *Radiation Physics and Chemistry*. 63:211-215.
72. Diez-Gonzalez, F. a. A. M. 2009. Produce safety in organic vs. conventional crops. *In* X. Fan, B. A. Niemira, C. J. Doona, F. E. Feeherry, and R. B. Gravani (ed.), Microbial safety of fresh produce The IFT Press, Ames, Iowa, USA.
73. Djordjevic, D., M. Wiedmann, and L. A. McLandsborough. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology*. 68:2950-2958.
74. Dohnalkova, A. C., B. W. Arey, K. H. Williams, and M. J. Marshall. 2009. Strategies for visualization of extracellular polymeric substances (ExPS) in biofilms by electron microscopy. *Microscopy and Microanalysis*. 15:66-67.
75. Donlan, R. M., and J. W. Costerton. 2002. Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*. 15:167-+.
76. Drevets, D. A., and M. S. Bronze. 2008. *Listeria monocytogenes*: epidemiology, human disease, and mechanisms of brain invasion. *Fems Immunology and Medical Microbiology*. 53:151-165.
77. Dubravka, M., A. Ruzica, V. Branka, D. Krnjaic, P. Jelena, and S. Sara. 2009. Scanning Electron Microscopy of Listeria Monocytogenes Biofilms on Stainless Steel Surfaces. *Acta Veterinaria-Beograd*. 59:423-434.

78. Dufrene, Y. F. 2001. Application of atomic force microscopy to microbial surfaces: from reconstituted cell surface layers to living cells. *Micron*. 32:153-165.
79. Dufrene, Y. F. 2002. Atomic force microscopy, a powerful tool in microbiology. *Journal of Bacteriology*. 184:5205-5213.
80. Dufrene, Y. F. 2003. Recent progress in the application of atomic force microscopy imaging and force spectroscopy to microbiology. *Current Opinion in Microbiology*. 6:317-323.
81. Duong, D. Q., P. G. Crandall, F. W. Pohlman, C. A. O'Bryan, C. W. Balentine, and A. Castillo. 2008. Improving ground beef safety and stabilizing color during irradiation using antioxidants, reductants or TSP. *Meat Science*. 78:359-368.
82. Dykes, G. A. 2006. Laboratory-based simulation of freezing profiles of beef trim for Escherichia coli O157 survival determinations. *Journal of Microbiological Methods*. 64:266-274.
83. *E. coli* Lawyer. Date, 2007, Minnesota *E. coli* O157:H7 cases linked to frozen ground beef patties purchased at Sam's Club stores in August and September. Available at: http://www.marlerblog.com/legal-cases/minnesota-e-coli-o157h7-cases-linked-to-frozen-ground-beef-patties-purchased-at-sams-club-stores-in/. Accessed Jul 14, 2012.
84. Elkest, S. E., and E. H. Marth. 1988. Temperature, pH, and strain of pathogen as factors affecting inactivation of *Listeria monocytogenes* by chlorine. *Journal of Food Protection*. 51:622-625.
85. Emer, Z., M. Y. Akbas, and M. Ozdemir. 2008. Bactericidal activity of ozone against *Escherichia coli* in whole and ground black-peppers. *Journal of Food Protection*. 71:914-917.
86. Erriu, M., G. Genta, E. Tuveri, G. Orru, G. Barbato, and R. Levi. 2012. Microtiter spectrophotometric biofilm production assay analyzed with metrological methods and uncertainty evaluation. *Measurement*. 45:1083-1088.
87. Fabrizio, K. A., and C. N. Cutter. 2005. Application of electrolyzed oxidizing water to reduce *Listeria monocytogenes* on ready-to-eat meats. *Meat Science*. 71:327-333.
88. Fassel, T. A., and C. E. Edmiston. 1999. Bacterial biofilms: Strategies for preparing glycocalyx for electron microscopy. *Biofilms*. 310:194-203.
89. Ferreira, A., C. P. O'Byrne, and K. J. Boor. 2001. Role of sigma(B) in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in *Listeria monocytogenes. Applied and Environmental Microbiology*. 67:4454-4457.
90. Ferreira, A., D. Sue, C. P. O'Byrne, and K. J. Boor. 2003. Role of Listeria monocytogenes sigma(B) in survival of lethal acidic conditions and in the acquired acid tolerance response. *Applied and Environmental Microbiology*. 69:2692-2698.
91. Florian, B., N. Noel, and W. Sand. 2010. Visualization of initial attachment of bioleaching bacteria using combined atomic force and epifluorescence microscopy. *Minerals Engineering*. 23:532-535.
92. Fraser, K. R., D. Sue, M. Wiedmann, K. Boor, and C. P. O'Byrne. 2003. Role of sigma(B) in regulating the compatible solute uptake systems of *Listeria*

monocytogenes: Osmotic induction of *opuC* is sigma(B) dependent. *Applied and Environmental Microbiology*. 69:2015-2022.

93. Fu, X. Y., D. M. Mueller, and J. W. Heinecke. 2002. Generation of intramolecular and intermolecular sulfenamides, sulfinamides, and sulfonamides by hypochlorous acid: A potential pathway for oxidative cross-linking of low-density lipoprotein by myeloperoxidase. *Biochemistry*. 41:1293-1301.
94. Gandhi, M., and M. L. Chikindas. 2007. *Listeria*: A foodborne pathogen that knows how to survive. *International Journal of Food Microbiology*. 113:1-15.
95. Garcia, P., B. Martinez, L. Rodriguez, and A. Rodriguez. 2011. Bacteriophages and phage-encoded proteins: Prospects in food quality and safety. *Natural Antimicrobials in Food Safety and Quality*:10-26.
96. Gasparic, M. B., K. Cankar, J. Zel, and K. Gruden. 2008. Comparison of different real-time PCR chemistries and their suitability for detection and quantification of genetically modified organisms. *Bmc Biotechnology*. 8.
97. Gehring, A. G., D. M. Albin, P. L. Irwin, S. A. Reed, and S. I. Tu. 2006. Comparison of enzyme-linked immunomagnetic chemiluminescence with US Food and Drug Administration's bacteriological analytical manual method for the detection of *Escherichia coli* O157:H7. *Journal of Microbiological Methods*. 67:527-533.
98. Ghebremichael, K., E. Muchelemba, B. Petrusevski, and G. Amy. 2011. Electrochemically activated water as an alternative to chlorine for decentralized disinfection. *Journal of Water Supply Research and Technology-Aqua*. 60:210-218.
99. Ghorbani, R., A. Koocheki, K. Brandt, S. Wilcockson, and C. Leifert. 2010. Organic agriculture and food production: Ecological, environmental, food safety and nutritional quality issues. *Sociology, Organic Farming, Climate Change and Soil Science*. 3:77-107.
100. Gilmour, M. W., M. Graham, G. Van Domselaar, S. Tyler, H. Kent, K. M. Trout-Yakel, O. Larios, V. Allen, B. Lee, and C. Nadon. 2010. High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. *Bmc Genomics*. 11.
101. Giotis, E. S., I. S. Blair, and D. A. McDowell. 2007. Morphological changes in *Listeria monocytogenes* subjected to sublethal alkaline stress. *International Journal of Food Microbiology*. 120:250-258.
102. Giotis, E. S., M. Julotok, B. J. Wilkinson, I. S. Blair, and D. A. McDowell. 2008. Role of sigma B factor in the alkaline tolerance response of *Listeria monocytogenes* 10403S and cross-protection against subsequent ethanol and osmotic stress. *Journal of Food Protection*. 71:1481-1485.
103. Giotis, E. S., D. A. McDowell, I. S. Blair, and B. J. Wilkinson. 2007. Role of branched-chain fatty acids in pH stress tolerance in *Listeria monocytogenes*. *Applied and Environmental Microbiology*. 73:997-1001.
104. Giotis, E. S., A. Muthaiyan, I. S. Blair, B. J. Wilkinson, and D. A. McDowell. 2008. Genomic and proteomic analysis of the alkali-tolerance response (AlTR) in *Listeria monocytogenes* 10403S. *Bmc Microbiology*. 8.

105. Giotis, E. S., A. Muthaiyan, S. Natesan, B. J. Wilkinson, I. S. Blair, and D. A. McDowell. 2010. Transcriptome analysis of alkali shock and alkali adaptation in *Listeria monocytogenes* 10403S. *Foodborne Pathogens and Disease*. 7:1147-1157.
106. Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit, F. Chetouani, E. Couve, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K. D. Entian, H. Fsihi, F. Garcia-Del Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapkat, E. Madueno, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, N. Simoes, A. Tierrez, J. A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart. 2001. Comparative genomics of *Listeria* species. *Science*. 294:849-852.
107. Gomez-Govea, M., L. Solis-Soto, N. Heredia, S. Garcia, G. Moreno, O. Tovar, and G. Isunza. 2012. Analysis of microbial contamination levels of fruits and vegetables at retail in Monterrey, Mexico. *Journal of Food Agriculture & Environment*. 10:152-156.
108. Gossner, C. M., D. van Cauteren, S. Le Hello, F. X. Weill, E. Terrien, S. Tessier, C. Janin, A. Brisabois, V. Dusch, V. Vaillant, and N. Jourdan-da Silva. 2012. Nationwide outbreak of *Salmonella enterica* serotype 4,[5],12:i:- infection associated with consumption of dried pork sausage, France, November to December 2011. *Eurosurveillance*. 17:19-22.
109. Gould, L. H., L. Demma, T. F. Jones, S. Hurd, D. J. Vugia, K. Smith, B. Shiferaw, S. Segler, A. Palmer, S. Zansky, P. M. Griffin, and E. I. Program. 2009. Hemolytic Uremic Syndrome and Death in Persons with Escherichia coli O157:H7 Infection, Foodborne Diseases Active Surveillance Network Sites, 2000-2006. *Clinical Infectious Diseases*. 49:1480-1485.
110. Goulter-Thorsen, R. M., E. Taran, I. R. Gentle, K. S. Gobius, and G. A. Dykes. 2011. Surface roughness of stainless steel influences attachment and detachment of *Escherichia coli* O157. *Journal of Food Protection*. 74:1359-1363.
111. Government of the District of Columbia. Date, 2008, Final investigation report on norovirus outbreak at Georgetown University. Available at: http://newsroom.dc.gov/show.aspx?agency=doh&section=2&release=15261&year=2008&file=file.aspx%2frelease%2f15261%2f20081001gtown_univ_final_report.pdf. Accessed Jul 13, 2012.
112. Gravesen, A., C. Lekkas, and S. Knochel. 2005. Surface attachment of *Listeria monocytogenes* is induced by sublethal concentrations of alcohol at low temperatures. *Applied and Environmental Microbiology*. 71:5601-5603.
113. Gregory, S. H., A. J. Sagnimeni, and E. J. Wing. 1996. Expression of the *inlAB* operon by *Listeria monocytogenes* is not required for entry into hepatic cells in vivo. *Infection and Immunity*. 64:3983-3986.

114. Guentzel, J. L., K. L. Lam, M. A. Callan, S. A. Emmons, and V. L. Dunham. 2008. Reduction of bacteria on spinach, lettuce, and surfaces in food service areas using neutral electrolyzed oxidizing water. *Food Microbiology*. 25:36-41.
115. Gulabivala, K., C. J. R. Stock, J. D. Lewsey, S. Ghori, Y. L. Ng, and D. A. Spratt. 2004. Effectiveness of electrochemically activated water as an irrigant in an infected tooth model. *International Endodontic Journal*. 37:624-631.
116. Hall-Stoodley, L., J. W. Costerton, and P. Stoodley. 2004. Bacterial biofilms: From the natural environment to infectious diseases. *Nature Reviews Microbiology*. 2:95-108.
117. Handojo, A., J. Lee, J. Hipp, and M. A. Pascall. 2009. Efficacy of electrolyzed water and an acidic formulation compared with regularly used chemical sanitizers for tableware sanitization during mechanical and manual ware-washing protocols. *Journal of Food Protection*. 72:1315-1320.
118. Harricane, M. C., E. Caron, F. Porte, and J. P. Liautard. 1996. Distribution of annexin I during non-pathogen or pathogen phagocytosis by confocal imaging and immunogold electron microscopy. *Cell Biology International*. 20:193-203.
119. Hassenberg, K., C. Idler, E. Molloy, M. Geyer, M. Plochl, and J. Barnes. 2007. Use of ozone in a lettuce-washing process: an industrial trial. *Journal of the Science of Food and Agriculture*. 87:914-919.
120. He, S. Y., G. P. Feng, H. S. Yang, Y. Wu, and Y. F. Li. 2004. Effects of pressure reduction rate on quality and ultrastructure of iceberg lettuce after vacuum cooling and storage. *Postharvest Biology and Technology*. 33:263-273.
121. Heckman, J. 2006. A history of organic farming: Transitions from Sir Albert Howard's war in the soil to USDA national organic program. *Renewable Agriculture and Food Systems*. 21:143-150.
122. Hedican, E., C. Hooker, T. Jenkins, C. Medus, S. Jawahir, F. Leano, and K. Smith. 2009. Restaurant *Salmonella enteritidis* outbreak associated with an asymptomatic infected food worker. *Journal of Food Protection*. 72:2332-2336.
123. Hilborn, E. D., J. H. Mermin, P. A. Mshar, J. L. Hadler, A. Voetsch, C. Wojtkunski, M. Swartz, R. Mshar, M. A. Lambert-Fair, J. A. Farrar, M. K. Glynn, and L. Slutsker. 1999. A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. *Archives of Internal Medicine*. 159:1758-1764.
124. Hricova, D., R. Stephan, and C. Zweifel. 2008. Electrolyzed water and its application in the food industry. *Journal of Food Protection*. 71:1934-1947.
125. Huang, Y. R., H. S. Hsieh, S. Y. Lin, S. J. Lin, Y. C. Hung, and D. F. Hwang. 2006. Application of electrolyzed oxidizing water on the reduction of bacterial contamination for seafood. *Food Control*. 17:987-993.
126. Huang, Y. R., Y. C. Hung, S. Y. Hsu, Y. W. Huang, and D. F. Hwang. 2008. Application of electrolyzed water in the food industry. *Food Control*. 19:329-345.
127. Huang, Y. X., M. Ye, and H. Q. Chen. 2012. Efficacy of washing with hydrogen peroxide followed by aerosolized antimicrobials as a novel sanitizing process to inactivate *Escherichia coli* O157:H7 on baby spinach. *International Journal of Food Microbiology*. 153:306-313.

128. Hunter, D. M., and D. V. Lim. 2010. Rapid detection and identification of bacterial pathogens by using an ATP bioluminescence Immunoassay. *Journal of Food Protection*. 73:739-746.
129. Hunter, R. Date, 2006, Food irradiation-questions and answers. Available at: http://www.fipa.us/q%26a.pdf. Accessed July 13, 2012.
130. Issa-Zacharia, A., Y. Kamitani, K. Morita, and K. Iwasaki. 2010. Sanitization potency of slightly acidic electrolyzed water against pure cultures of *Escherichia coli* and *Staphylococcus aureus*, in comparison with that of other food sanitizers. *Food Control*. 21:740-745.
131. Issa-Zacharia, A., Y. Kamitani, A. Tiisekwa, K. Morita, and K. Iwasaki. 2010. In vitro inactivation of *Escherichia coli, Staphylococcus aureus* and *Salmonella spp.* using slightly acidic electrolyzed water. *Journal of Bioscience and Bioengineering*. 110:308-313.
132. Izumi, H. 2001. Effects of electrolyzed neutral water on the bacterial populations in a flower vase and in stems of cut roses. *Journal of the Japanese Society for Horticultural Science*. 70:599-601.
133. Jackson, K. A., M. Biggerstaff, M. Tobin-D'Angelo, D. Sweat, R. Klos, J. Nosari, O. Garrison, E. Boothe, L. Saathoff-Huber, L. Hainstock, and R. P. Fagan. 2011. Multistate outbreak of *Listeria monocytogenes* associated with Mexican-style cheese made from pasteurized milk among pregnant, Hispanic women. *Journal of Food Protection*. 74:949-953.
134. Jiang, L. L., J. S. Chen, J. J. Xu, X. F. Zhang, S. Wang, H. C. Zhao, K. Vongxay, and W. H. Fang. 2008. Virulence characterization and genotypic analyses of *Listeria monocytogenes* isolates from food and processing environments in eastern China. *International Journal of Food Microbiology*. 121:53-59.
135. Jonas, K., H. Tomenius, A. Kader, S. Normark, U. Romling, L. M. Belova, and O. Melefors. 2007. Roles of curli, cellulose and BapA in *Salmonella* biofilm morphology studied by atomic force microscopy. *Bmc Microbiology*. 7.
136. Julian, A., A. Jimenez, M. de Gorgolas, R. Fernandez, and M. L. Fernandez. 2001. *Listeria monocytogenes* infections in the adult clinical and microbiological issues of a changing disease. *Enfermedades Infecciosas Y Microbiologia Clinica*. 19:297-303.
137. Kalmokoff, M. L., J. W. Austin, X. D. Wan, G. Sanders, S. Banerjee, and J. M. Farber. 2001. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. *Journal of Applied Microbiology*. 91:725-734.
138. Karaca, H., and Y. S. Velioglu. 2007. Ozone applications in fruit and vegetable processing. *Food Reviews International*. 23:91-106.
139. Kastbjerg, V. G., D. S. Nielsen, N. Arneborg, and L. Gram. 2009. Response of *Listeria monocytogenes* to disinfection stress at the single-cell and population levels as monitored by intracellular pH measurements and viable-cell counts. *Applied and Environmental Microbiology*. 75:4550-4556.

140. Kazmierczak, M. J., S. C. Mithoe, K. J. Boor, and M. Wiedmann. 2003. *Listeria monocytogenes* sigma(B) regulates stress response and virulence functions. *Journal of Bacteriology*. 185:5722-5734.
141. Kim, C., Y. C. Hung, R. E. Brackett, and J. F. Frank. 2001. Inactivation of *Listeria monocytogenes* biofilms by electrolyzed oxidizing water. *Journal of Food Processing and Preservation*. 25:91-100.
142. Kim, C., Y. C. Hung, R. E. Brackett, and C. S. Lin. 2003. Efficacy of electrolyzed oxidizing water in inactivating *Salmonella* on alfalfa seeds and sprouts. *Journal of Food Protection*. 66:208-214.
143. Kim, H., K. J. Boor, and H. Marquis. 2004. *Listeria monocytogenes* sigma(B) contributes to invasion of human intestinal epithelial cells. *Infection and Immunity*. 72:7374-7378.
144. Kim, H., H. Marquis, and K. J. Boor. 2005. sigma(B) contributes to *Listeria monocytogenes* invasion by controlling expression of *inlA* and *inlB*. *Microbiology-Sgm*. 151:3215-3222.
145. Kim, I. J., J. H. Ha, Y. S. Kim, H. I. Kim, H. C. Choi, D. H. Jeon, Y. J. Lee, A. J. Kim, D. H. Bae, K. S. Kim, C. Lee, and S. D. Ha. 2009. Evaluation for efficacies of commercial sanitizers and disinfectants against *Bacillus cereus* strains. *Food Science and Biotechnology*. 18:537-540.
146. Kimura, A. C., M. S. Palumbo, H. Meyer, S. Abbott, R. Rodriguez, and S. B. Werner. 2005. A multi-state outbreak of *Salmonella serotype* Thompson infection from commercially distributed bread contaminated by an ill food handler. *Epidemiology and Infection*. 133:823-828.
147. Kiura, H., K. Sano, S. Morimatsu, T. Nakano, C. Morita, M. Yamaguchi, T. Maeda, and Y. Katsuoka. 2002. Bactericidal activity of electrolyzed acid water from solution containing sodium chloride at low concentration, in comparison with that at high concentration. *Journal of Microbiological Methods*. 49:285-293.
148. Klumpp, J., J. Dorscht, R. Lurz, R. Bielmann, M. Wieland, M. Zimmer, R. Calendar, and M. J. Loessner. 2008. The terminally redundant, nonpermuted genome of *Listeria* bacteriophage A511: a model for the SPO1-like myoviruses of gram-positive bacteria. *Journal of Bacteriology*. 190:5753-5765.
149. Knight, C., C. Hofacre, G. Mathis, M. Quiroz, and J. Dibner. 2006. Organic acid water treatment reduced *Salmonella* horizontal transmission in broiler chickens. *Poultry Science*. 85:132-133.
150. Knobloch, J. K. M., K. Bartscht, A. Sabottke, H. Rohde, H. H. Feucht, and D. Mack. 2001. Biofilm formation by *Staphylococcus* epidermidis depends on functional RsbU, an activator of the *sigB* operon: Differential activation mechanisms due to ethanol and salt stress. *Journal of Bacteriology*. 183:2624-2633.
151. Koide, S., J. Takeda, J. Shi, H. Shono, and G. G. Atungulu. 2009. Disinfection efficacy of slightly acidic electrolyzed water on fresh cut cabbage. *Food Control*. 20:294-297.
152. Koseki, S., and K. Itoh. 2000. The effect of available chlorine concentration on the disinfecting potential of acidic electrolyzed water for shredded vegetables.

Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi. 47:888-898.
153. Koseki, S., K. Yoshida, S. Isobe, and K. Itoh. 2004. Efficacy of acidic electrolyzed water for microbial decontamination of cucumbers and strawberries. *Journal of Food Protection*. 67:1247-1251.
154. Koseki, S., K. Yoshida, Y. Kamitani, S. Isobe, and K. Itoh. 2004. Effect of mild heat pre-treatment with alkaline electrolyzed water on the efficacy of acidic electrolyzed water against *Escherichia coli* O157:H7 and *Salmonella* on lettuce. *Food Microbiology*. 21:559-566.
155. Kovacevic, J., L. F. McIntyre, S. B. Henderson, and T. Kosatsky. 2012. Occurrence and Distribution of Listeria Species in Facilities Producing Ready-to-Eat Foods in British Columbia, Canada. *Journal of Food Protection*. 75:216-224.
156. Kovacevic, J., L. R. Mesak, and K. J. Allen. 2012. Occurrence and characterization of Listeria spp. in ready-to-eat retail foods from Vancouver, British Columbia. *Food Microbiology*. 30:372-378.
157. Kreske, A. C., K. Bjornsdottir, F. Breidt, and H. Hassan. 2008. Effects of pH, dissolved oxygen, and ionic strength on the survival of *Escherichia coli* O157:H7 in organic acid solutions. *Journal of Food Protection*. 71:2404-2409.
158. Kuda, T., T. Iwase, C. Yuphakhun, H. Takahashi, T. Koyanagi, and B. Kimura. 2011. Surfactant-disinfectant resistance of Salmonella and Staphylococcus adhered and dried on surfaces with egg compounds. *Food Microbiology*. 28:920-925.
159. Kumar, M., R. Hora, M. Kostrzynska, and K. Warriner. 2007. Mode of *Salmonella* and *Escherichia coli* O157:H7 inactivation by a stabilized oxychloro-based sanitizer. *Journal of Applied Microbiology*. 102:1427-1436.
160. Lacroix, M., and B. Ouattara. 2000. Combined industrial processes with irradiation to assure innocuity and preservation of food products - a review. *Food Research International*. 33:719-724.
161. Latorre, A. A., J. S. Van Kessel, J. S. Karns, M. J. Zurakowski, A. K. Pradhan, K. J. Boor, B. M. Jayarao, B. A. Houser, C. S. Daugherty, and Y. H. Schukken. 2010. Biofilm in milking equipment on a dairy farm as a potential source of bulk tank milk contamination with *Listeria monocytogenes. Journal of Dairy Science*. 93:2792-2802.
162. Lee, K. G., S. R. Pillai, S. R. Singh, and G. A. Willing. 2008. The investigation of protein a and Salmonella antibody adsorption onto biosensor surfaces by atomic force microscopy. *Biotechnology and Bioengineering*. 99:949-959.
163. Leifert, C. 2007. Handbook of organic food safety and quality Introduction. *Handbook of Organic Food Safety and Quality*:1-6.
164. Len, S. V., Y. C. Hung, D. Chung, J. L. Anderson, M. C. Erickson, and K. Morita. 2002. Effects of storage conditions and pH on chlorine loss in electrolyzed oxidizing (EO) water. *Journal of Agricultural and Food Chemistry*. 50:209-212.
165. Lewis, K. 2001. Riddle of biofilm resistance. *Antimicrobial Agents and Chemotherapy*. 45:999-1007.

166. Liao, L. B., W. M. Chen, and X. M. Xiao. 2007. The generation and inactivation mechanism of oxidation-reduction potential of electrolyzed oxidizing water. *Journal of Food Engineering*. 78:1326-1332.
167. Licciardello, J. J., J. T. Nickerson, and S. A. Goldblith. . 1970. Inactivation of *Salmonella* in poultry with gamma radiation. *Poultry Science*. 49:663-675.
168. Lingnau, A., E. Domann, M. Hudel, M. Bock, T. Nichterlein, J. Wehland, and T. Chakraborty. 1995. Expression of the *Listeria monocytogenes* EGD *inlA* and *inlB* genes, whose products mediate bacterial entry into tissue-culture cell-lines, by PrfA-dependent and PrfA-independent mechanisms. *Infection and Immunity*. 63:3896-3903.
169. Liu, C. C., J. Y. Duan, and Y. C. Su. 2006. Effects of electrolyzed oxidizing water on reducing *Listeria monocytogenes* contamination on seafood processing surfaces. *International Journal of Food Microbiology*. 106:248-253.
170. Liu, C. C., and Y. C. Su. 2006. Efficiency of electrolyzed oxidizing water on reducing *Listeria monocytogenes* contamination on seafood processing gloves. *International Journal of Food Microbiology*. 110:149-154.
171. Liu, D. Y., M. L. Lawrence, A. J. Ainsworth, and F. W. Austin. 2007. Toward an improved laboratory definition of *Listeria monocytogenes* virulence. *International Journal of Food Microbiology*. 118:101-115.
172. Liu, S. Y., and Y. F. Wang. 2010. Application of AFM in Microbiology: A Review. *Scanning*. 32:61-73.
173. Looper, M. L., T. S. Edrington, R. Flores, C. F. Rosenkrans, and G. E. Aiken. 2006. *Escherichia coli* O157:H7 and *Salmonella* in water and soil from tall fescue paddocks. *Foodborne Pathogens and Disease*. 3:203-208.
174. Lopez-Gonzalez, V., P. S. Murano, R. E. Brennan, and E. A. Murano. 1999. Influence of various commercial packaging conditions on survival of *Escherichia coli* O157:H7 to irradiation by electron beam versus gamma rays. *Journal of Food Protection*. 62:10-15.
175. Lu, H. G., and V. J. Higgins. 1999. The effect of hydrogen peroxide on the viability of tomato cells and of the fungal pathogen *Cladosporium fulvum*. *Physiological and Molecular Plant Pathology*. 54:131-143.
176. Lyytikainen, O., T. Autio, R. Maijala, P. Ruutu, T. Honkanen-Buzalski, M. Miettinen, M. Hatakka, J. Mikkola, V. J. Anttila, T. Johansson, L. Rantala, T. Aalto, H. Korkeala, and A. Siitonen. 2000. An outbreak of *Listeria monocytogenes* serotype 3a infections from butter in Finland. *Journal of Infectious Diseases*. 181:1838-1841.
177. MacDonald, P. D., R. E. Whitwam, J. D. Boggs, J. W. Reardon, R. R. Saah, M. Beatty, J. Sobel, L. M. Graves, S. B. Hunter, and J. N. MacCormack. 2001. Outbreak of *Listeria*-associated birth complications linked with homemade Mexican-style cheese, North Carolina, 2000. *Clinical Infectious Diseases*. 33:1236-1236.
178. Magkos, F., F. Arvaniti, and A. Zampelas. 2006. Organic food: Buying more safety or just peace of mind? A critical review of the literature. *Critical Reviews in Food Science and Nutrition*. 46:23-56.

179. Mah, T. F. C., and G. A. O'Toole. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*. 9:34-39.
180. Mahapatra, A. K., K. Muthukumarappan, and J. L. Julson. 2005. Applications of ozone, bacteriocins and irradiation in food processing: A review. *Critical Reviews in Food Science and Nutrition*. 45:447-461.
181. Mahmoud, B. S. M. 2007. Electrolyzed water: A new technology for food decontamination - A review. *Deutsche Lebensmittel-Rundschau*. 103:212-221.
182. Mahmoud, B. S. M., K. Yamazaki, K. Miyashita, S. Il-Shik, C. Dong-Suk, and T. Suzuki. 2004. Decontamination effect of electrolysed NaCl solutions on carp. *Letters in Applied Microbiology*. 39:169-173.
183. Mangalappalli-Illathu, A. K., J. R. Lawrence, and D. R. Korber. 2009. Cells in shearable and nonshearable regions of *Salmonella enterica* serovar Enteritidis biofilms are morphologically and physiologically distinct. *Canadian Journal of Microbiology*. 55:955-966.
184. Mansfield, M. E., K. J. Vining, M. A. Townley, and T. M. Davis. 2010. Using confocal microscopy to study the infection of *Mentha longifolia* by a GFP strain of the verticillium wilt pathogen. *Phytopathology*. 100:S77-S77.
185. Marais, J. T., and W. P. Williams. 2001. Antimicrobial effectiveness of electro-chemically activated water as an endodontic irrigation solution. *International Endodontic Journal*. 34:237-243.
186. Marsh, E. J., H. L. Luo, and H. Wang. 2003. A three-tiered approach to differentiate *Listeria monocytogenes* biofilm-forming abilities. *Fems Microbiology Letters*. 228:203-210.
187. Marsh, P. N., D. Burns, and J. M. Girkin. 2003. Practical implementation of adaptive optics in multiphoton microscopy. *Optics Express*. 11:1123-1130.
188. Marth, E. H. 1988. Disease characteristics of *Listeria monocytogenes*. *Food Technology*. 42:165-168.
189. Martinez, M. B., M. Flickinger, L. Higgins, T. Krick, and G. L. Nelsestuen. 2001. Reduced outer membrane permeability of *Escherichia coli* O157:H7: Suggested role of modified outer membrane porins and theoretical function in resistance to antimicrobial agents. *Biochemistry*. 40:11965-11974.
190. Masip, L., K. Veeravalli, and G. Georgioui. 2006. The many faces of glutathione in bacteria. *Antioxidants & Redox Signaling*. 8:753-762.
191. Matic, S., V. Mihokovic, B. Katusinrazem, and D. Razem. 1990. The eradication of *Salmonella* in egg powder by gamma-irradiation. *Journal of Food Protection*. 53:111-114.
192. Matyas, B., A. Cronquist, M. Cartter, M. Tobin-D'Angelo, D. Blythe, K. Smith, S. Lathrop, D. Morse, P. Cieslak, J. Dunn, K. G. Holt, O. L. Henao, K. E. Fullerton, B. E. Mahon, R. M. Hoekstra, P. M. Griffin, R. V. Tauxe, and A. Bhattarai. 2010. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food-10 states, 2009 (Reprinted from MMWR, vol 59, pg 418-422, 2010). *Jama-Journal of the American Medical Association*. 303:2130-2132.

193. McGann, P., M. Wiedmann, and K. J. Boor. 2007. The alternative sigma factor sigma(B) and the virulence gene regulator PrfA both regulate transcription of Listeria monocytogenes internalins. *Applied and Environmental Microbiology*. 73:2919-2930.
194. McLandsborough, L., A. Rodriguez, D. Perez-Conesa, and J. Weiss. 2006. Biofilms: At the interface between biophysics and microbiology. *Food Biophysics*. 1:94-114.
195. Medus, C., S. Meyer, K. Smith, S. Jawahir, B. Miller, K. Viger, M. Forstner, E. Brandt, S. Nowicki, E. Salehi, Q. Phan, A. Kinney, M. Cartter, J. Flint, J. Bancroft, J. Adams, E. Hyytia-Trees, L. Theobald, D. Talkington, M. Humphrys, C. Bopp, P. Gerner-Smidt, C. B. Behravesh, I. T. Williams, S. Sodha, A. Langer, C. Schwensohn, F. Angulo, R. Tauxe, K. Date, E. Cavallaro, and C. Kim. 2009. Multistate outbreak of *Salmonella* infections associated with peanut butter and peanut butter-containing products-United States, 2008-2009 (Reprinted from MMWR, vol 58, pg 85-90, 2009). *Jama-Journal of the American Medical Association*. 301:1119-1122.
196. Medus, C., K. E. Smith, J. B. Bender, J. M. Besser, and C. W. Hedberg. 2006. *Salmonella* outbreaks in restaurants in Minnesota, 1995 through 2003: Evaluation of the role of infected foodworkers. *Journal of Food Protection*. 69:1870-1878.
197. Medus, C., K. E. Smith, J. B. Bender, F. Leano, and C. W. Hedberg. 2010. *Salmonella* infections in food workers identified through routine public health surveillance in Minnesota: Impact on outbreak recognition. *Journal of Food Protection*. 73:2053-2058.
198. Mendonca, A., A. Daraba, D. Manu, and F. Woods. 2011. Inactivation of Salmonella enterica on whole tomatoes and serrano peppers immersed in pre-san, a biodegradable vegetable sanitizer. *Journal of Environmental Protection and Ecology*. 12:769-780.
199. Mereghetti, L., R. Quentin, N. Marquet-Van der Mee, and A. Audurier. 2000. Low sensitivity of *Listeria monocytogenes* to quaternary ammonium compounds. *Applied and Environmental Microbiology*. 66:5083-+.
200. Miettinen, M. K., A. Siitonen, P. Heiskanen, H. Haajanen, K. J. Bjorkroth, and H. J. Korkeala. 1999. Molecular epidemiology of an outbreak of febrile gastroenteritis caused by *Listeria monocytogenes* in cold-smoked rainbow trout. *Journal of Clinical Microbiology*. 37:2358-2360.
201. Milillo, S. R., and S. C. Ricke. 2010. Synergistic reduction of *Salmonella* in a model raw chicken media using a combined thermal and acidified organic acid salt intervention treatment. *Journal of Food Science*. 75:M121-M125.
202. Minei, C. C., B. C. Gomes, R. P. Ratti, C. E. M. D'Angelis, and E. C. P. De Martinis. 2008. Influence of peroxyacetic acid and nisin and coculture with Enterococcus faecium on Listeria monocytogenes biofilm formation. *Journal of Food Protection*. 71:634-638.
203. Moldovan, C., C. Mihailescu, D. Stan, L. Ruta, R. Iosub, R. Gavrila, M. Purica, and S. Vasilica. 2009. Characterization of self-assembled monolayers (SAMs) on

silicon substrate comparative with polymer substrate for Escherichia coli O157:H7 detection. *Applied Surface Science*. 255:8953-8959.
204. Molins, R. A., Y. Motarjemi, and F. K. Kaferstein. 2001. Irradiation: a critical control point in ensuring the microbiological safety of raw foods. *Food Control*. 12:347-356.
205. Montanez-Izquierdo, V. Y., D. I. Salas-Vazquez, and J. J. Rodriguez-Jerez. 2012. Use of epifluorescence microscopy to assess the effectiveness of phage P100 in controlling *Listeria monocytogenes* biofilms on stainless steel surfaces. *Food Control*. 23:470-477.
206. Morris, V. J., A. R. Kirby, and A. P. Gunning. 1999. Atomic force microscopy for biologists. Imperial College Press, London.
207. Mostowy, S., S. Janel, C. Forestier, C. Roduit, S. Kasas, J. Pizarro-Cerda, P. Cossart, and F. Lafont. 2011. A Role for Septins in the Interaction between the Listeria monocytogenes Invasion Protein InlB and the Met Receptor. *Biophysical Journal*. 100:1949-1959.
208. Mukherjee, A., D. Speh, E. Dyck, and F. Diez-Gonzalez. 2004. Preharvest evaluation of coliforms, *Escherichia coli*, *Salmonella*, and *Escherichia coli* O157:H7 in organic and conventional produce grown by Minnesota farmers. *Journal of Food Protection*. 67:894-900.
209. Muller, D. J., and Y. F. Dufrene. 2011. Atomic force microscopy: a nanoscopic window on the cell surface. *Trends in Cell Biology*. 21:461-469.
210. Munoz, A. I., M. Vargas, L. Otero, G. Diaz, and V. Guzman. 2011. Presence of *Listeria monocytogenes* in ready-to-eat foods available in open markets, delicatessens and supermarkets, Bogota, 2002-2008. *Biomedica*. 31:428-439.
211. Nelson, K. E., D. E. Fouts, E. F. Mongodin, J. Ravel, R. T. DeBoy, J. F. Kolonay, D. A. Rasko, S. V. Angiuoli, S. R. Gill, I. T. Paulsen, J. Peterson, O. White, W. C. Nelson, W. Nierman, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, R. J. Dodson, A. S. Durkin, R. Madupu, D. H. Haft, J. Selengut, S. Van Aken, H. Khouri, N. Fedorova, H. Forberger, B. Tran, S. Kathariou, L. D. Wonderling, G. A. Uhlich, D. O. Bayles, J. B. Luchansky, and C. M. Fraser. 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. *Nucleic Acids Research*. 32:2386-2395.
212. Neu, T. R., F. Dijk, G. J. Verkerke, H. C. Vandermei, and H. J. Busscher. 1992. Scanning electron-microscopy study of biofilms on silicone voice prostheses. *Cells and Materials*. 2:261-269.
213. Neu, T. R., B. Manz, F. Volke, J. J. Dynes, A. P. Hitchcock, and J. R. Lawrence. 2010. Advanced imaging techniques for assessment of structure, composition and function in biofilm systems. *Fems Microbiology Ecology*. 72:1-21.
214. Ngadi, M., J. P. Smith, and B. Cayouette. 2003. Kinetics of ultraviolet light inactivation of *Escherichia coli* O157:H7 in liquid foods. *Journal of the Science of Food and Agriculture*. 83:1551-1555.

215. Nguyenthe, C., and F. Carlin. 1994. The microbiology of minimally processed fresh fruits and vegetables. *Critical Reviews in Food Science and Nutrition*. 34:371-401.
216. Niggli, U. 2007. History and concepts of food quality and safety in organic food production and processing. *Handbook of Organic Food Safety and Quality*:9-24.
217. Nightingale, K. K., R. A. Ivy, A. J. Ho, E. D. Fortes, B. L. Njaa, R. M. Peters, and M. Wiedmann. 2008. *inlA* premature stop codons are common among *Listeria monocytogenes* isolates from foods and yield virulence-attenuated strains that confer protection against fully virulent strains. *Applied and Environmental Microbiology*. 74:6570-6583.
218. Nightingale, K. K., K. Windham, K. E. Martin, M. Yeung, and M. Wiedmann. 2005. Select *Listeria monocytogenes* subtypes commonly found in foods carry distinct nonsense mutations in *inlA*, leading to expression of truncated and secreted internalin A, and are associated with a reduced invasion phenotype for human intestinal epithelial cells. *Applied and Environmental Microbiology*. 71:8764-8772.
219. Nightingale, K. K., K. Windham, and M. Wiedmann. 2005. Evolution and molecular phylogeny of *Listeria monocytogenes* isolated from human and animal listeriosis cases and foods. *Journal of Bacteriology*. 187:5537-5551.
220. Nikitas, G., C. Deschamps, O. Disson, T. Niault, P. Cossart, and M. Lecuit. 2011. Transcytosis of Listeria monocytogenes across the intestinal barrier upon specific targeting of goblet cell accessible E-cadherin. *Journal of Experimental Medicine*. 208:2263-2277.
221. Nou, X. W., and Y. G. Luo. 2010. Whole-Leaf Wash Improves Chlorine Efficacy for Microbial Reduction and Prevents Pathogen Cross-Contamination during Fresh-Cut Lettuce Processing. *Journal of Food Science*. 75:M283-M290.
222. Novak, J., A. Demirci, and Y. Han. 2008. Novel chemical processes: Ozone, supercritical CO_2, electrolyzed oxidizing water, and chlorine dioxide gas. *Food Science and Technology International*. 14:437-441.
223. Oh, Y. J., W. Jo, Y. Yang, and S. Park. 2007. Biofilm formation and local electrostatic force characteristics of *Escherichia coli* O157:H7 observed by electrostatic force microscopy. *Applied Physics Letters*. 90.
224. Oh, Y. J., W. Jo, Y. Yang, and S. Park. 2007. Influence of culture conditions on *Escherichia coli* O157:H7 biofilm formation by atomic force microscopy. *Ultramicroscopy*. 107:869-874.
225. Olesen, I., L. Thorsen, and L. Jespersen. 2010. Relative transcription of *Listeria monocytogenes* virulence genes in liver pates with varying NaCl content. *International Journal of Food Microbiology*. 141:S60-S68.
226. Oliveira, M., J. Usall, I. Vinas, M. Anguera, F. Gatius, and M. Abadias. 2010. Microbiological quality of fresh lettuce from organic and conventional production. *Food Microbiology*. 27:679-684.
227. Oliveira, M., I. Vinas, J. Usall, M. Anguera, and M. Abadias. 2012. Presence and survival of *Escherichia coli* O157:H7 on lettuce leaves and in soil treated with

contaminated compost and irrigation water. *International Journal of Food Microbiology*. 156:133-140.

228. Olsen, S. J., M. C. Evans, S. Hunter, V. Reddy, L. Kornstein, W. R. Mackenzie, K. Lane, S. A. Bidol, G. Stoltman, D. M. Frye, I. Lee, S. Hurd, D. Gerber, T. F. Jones, T. N. Laporte, W. Dewitt, L. M. Graves, M. Wiedmann, D. J. Schoonmaker-Bopp, A. J. Huang, C. Vincent, A. Bugenhagen, J. Corby, E. R. Carloni, M. Holcomb, R. Woron, S. M. Zansky, G. Dowdle, F. Smith, S. Ahrabi-Fard, A. R. Ong, N. Tucker, N. A. Hynes, and P. Mead. 2001. Multistate outbreak of *Listeria monocytogenes* infections linked to deli turkey meat. *Clinical Infectious Diseases*. 33:1237-1237.
229. Olsen, S. J., M. Patrick, S. B. Hunter, V. Reddy, L. Kornstein, W. R. MacKenzie, K. Lane, S. Bidol, G. A. Stoltman, D. M. Frye, I. Lee, S. Hurd, T. F. Jones, T. N. LaPorte, W. Dewitt, L. Graves, M. Wiedmann, D. J. Schoonmaker-Bopp, A. J. Huang, C. Vincent, A. Bugenhagen, J. Corby, E. R. Carloni, M. E. Holcomb, R. F. Woron, S. M. Zansky, G. Dowdle, F. Smith, S. Ahrabi-Fard, A. R. Ong, N. Tucker, N. A. Hynes, and P. Mead. 2005. Multistate outbreak of *Listeria monocytogenes* infection linked to delicatessen turkey meat. *Clinical Infectious Diseases*. 40:962-967.
230. Oomori, T., T. Oka, T. Inuta, and Y. Arata. 2000. The efficiency of disinfection of acidic electrolyzed water in the presence of organic materials. *Analytical Sciences*. 16:365-369.
231. Ouattara, B., M. Giroux, R. Yefsah, W. Smoragiewicz, L. Saucier, J. Borsa, and M. Lacroix. 2002. Microbiological and biochemical characteristics of ground beef as affected by gamma irradiation, food additives and edible coating film. *Radiation Physics and Chemistry*. 63:299-304.
232. Palmer, J., S. Flint, and J. Brooks. 2007. Bacterial cell attachment, the beginning of a biofilm. *Journal of Industrial Microbiology & Biotechnology*. 34:577-588.
233. Palmer, M. E., S. Chaturongakul, M. Wiedmann, and K. J. Boor. 2011. The Listeria monocytogenes sigma(B) Regulon and Its Virulence-Associated Functions Are Inhibited by a Small Molecule. *Mbio*. 2.
234. Palmer, M. E., M. Wiedmann, and K. J. Boor. 2009. sigma(B) and sigma(L) Contribute to Listeria monocytogenes 10403S Response to the Antimicrobial Peptides SdpC and Nisin. *Foodborne Pathogens and Disease*. 6:1057-1065.
235. Palmer, R. J., and C. Sternberg. 1999. Modern microscopy in biofilm research: confocal microscopy and other approaches. *Current Opinion in Biotechnology*. 10:263-268.
236. Pan, Y., F. Breidt, and S. Kathariou. 2006. Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. *Applied and Environmental Microbiology*. 72:7711-7717.
237. Pangloli, P., Y. C. Hung, L. R. Beuchat, C. H. King, and Z. H. Zha. 2009. Reduction of *Escherichia coli* O157:H7 on produce by use of electrolyzed water under simulated food service operation conditions. *Journal of Food Protection*. 72:1854-1861.

238. Park, B., and N. I. Abu-Lail. 2010. Variations in the nanomechanical properties of virulent and avirulent Listeria monocytogenes. *Soft Matter*. 6:3898-3909.
239. Park, B. J., and N. I. Abu-Lail. 2011. The role of the pH conditions of growth on the bioadhesion of individual and lawns of pathogenic Listeria monocytogenes cells. *Journal of Colloid and Interface Science*. 358:611-620.
240. Park, B. J., T. Haines, and N. I. Abu-Lail. 2009. A correlation between the virulence and the adhesion of Listeria monocytogenes to silicon nitride: An atomic force microscopy study. *Colloids and Surfaces B-Biointerfaces*. 73:237-243.
241. Park, C. M., Y. C. Hung, M. P. Doyle, G. O. I. Ezeike, and C. Kim. 2001. Pathogen reduction and quality of lettuce treated with electrolyzed oxidizing and acidified chlorinated water. *Journal of Food Science*. 66:1368-1372.
242. Park, E. J., E. Alexander, G. A. Taylor, R. Costa, and D. H. Kang. 2008. Effects of organic matter on acidic electrolysed water for reduction of foodborne pathogens on lettuce and spinach. *Journal of Applied Microbiology*. 105:1802-1809.
243. Park, H., Y. C. Hung, and D. Chung. 2004. Effects of chlorine and pH on efficacy of electrolyzed water for inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *International Journal of Food Microbiology*. 91:13-18.
244. Park, H., Y. C. Hung, and C. Kim. 2002. Effectiveness of electrolyzed water as a sanitizer for treating different surfaces. *Journal of Food Protection*. 65:1276-1280.
245. Parsek, M. R., and E. P. Greenberg. 2005. Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends in Microbiology*. 13:27-33.
246. Pascual, A., I. Llorca, and A. Canut. 2007. Use of ozone in food industries for reducing the environmental impact of cleaning and disinfection activities. *Trends in Food Science & Technology*. 18:S29-S35.
247. Pasko, O. A., and V. M. Ketskalo. 1991. Effect of electrochemically activated water on seed-germination. *Soviet Plant Physiology*. 38:284-288.
248. Patil, S., V. P. Valdramidis, P. J. Cullen, J. M. Frias, and P. Bourke. 2010. Ozone inactivation of acid stressed *Listeria monocytogenes* and *Listeria innocua* in orange juice using a bubble column. *Food Control*. 21:1723-1730.
249. Perez-Conesa, D., J. Cao, L. Chen, L. McLandsborough, and J. Weiss. 2011. Inactivation of Listeria monocytogenes and Escherichia coli O157:H7 Biofilms by Micelle-Encapsulated Eugenol and Carvacrol. *Journal of Food Protection*. 74:55-62.
250. Pexara, A., D. Angelidis, and A. Govaris. 2012. Shiga toxin-producing *Escherichia coli* (STEC) food-borne outbreaks. *Journal of the Hellenic Veterinary Medical Society*. 63:45-53.
251. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*. 29.
252. Powell, D. Date, 2008, Georgetown outbreak: emergency so backed up there was 'vomiting in the waiting room'. Available at: http://barfblog.foodsafety.ksu.edu/blog/139838/08/10/02/georgetown-outbreak-emergency-so-backed-there-was-vomiting-waiting-room. Accessed Jul 13, 2012.

253. Poyart, C., P. TrieuCuot, and P. Berche. 1996. The *inlA* gene required for cell invasion is conserved and specific to *Listeria monocytogenes. Microbiology-Sgm*. 142:173-180.
254. Prakash, A. 2002. Pathogen control and shelf-life extension of fresh-cut produce using irradiation. *Abstracts of Papers of the American Chemical Society*. 224:U73-U74.
255. Prasadarao, N. V., C. A. Wass, and K. S. Kim. 1996. Endothelial cell GlcNAc beta 1-4GlcNAc epitopes for outer membrane protein a enhance traversal of *Escherichia coli* across the blood-brain barrier. *Infection and Immunity*. 64:154-160.
256. Raengpradub, S., M. Wiedmann, and K. J. Boor. 2008. Comparative analysis of the sigma(B)-dependent stress responses in *Listeria monocytogenes* and *Listeria innocua* strains exposed to selected stress conditions. *Applied and Environmental Microbiology*. 74:158-171.
257. Rantsiou, K., M. Mataragas, V. Alessandria, and L. Cocolin. 2012. Expression of Virulence Genes of Listeria Monocytogenes in Food. *Journal of Food Safety*. 32:161-168.
258. Redmond, E. C., and C. J. Griffith. 2003. Consumer food handling in the home: A review of food safety studies. *Journal of Food Protection*. 66:130-161.
259. Ringus, D. L., R. A. Ivy, M. Wiedmann, and K. J. Boor. 2012. Salt Stress-Induced Transcription of sigma(B)- and CtsR-Regulated Genes in Persistent and Non-persistent Listeria monocytogenes Strains from Food Processing Plants. *Foodborne Pathogens and Disease*. 9:198-206.
260. Ripp, S., P. Jegier, C. M. Johnson, J. R. Brigati, and G. S. Sayler. 2008. Bacteriophage-amplified bioluminescent sensing of *Escherichia coli* O157:H7. *Analytical and Bioanalytical Chemistry*. 391:507-514.
261. Roberts, A. J., S. K. Williams, M. Wiedmann, and K. K. Nightingale. 2009. Some *Listeria monocytogenes* outbreak strains demonstrate significantly reduced invasion, *inlA* transcript levels, and swarming motility in vitro. *Applied and Environmental Microbiology*. 75:5647-5658.
262. Roberts, R. G., and S. T. Reymond. 1994. Chlorine dioxide for reduction of postharvest pathogen inoculum during handling of tree fruits. *Applied and Environmental Microbiology*. 60:2864-2868.
263. Roche, S. M., O. Grepinet, Y. Corde, A. P. Teixeira, A. Kerouanton, S. Temoin, L. Mereghetti, A. Brisabois, and P. Velge. 2009. A *Listeria monocytogenes* strain is still virulent despite nonfunctional major virulence genes. *Journal of Infectious Diseases*. 200:1944-1948.
264. Rodriguez, A., W. R. Autio, and L. A. McLandsborough. 2007. Effects of inoculation level, material hydration, and stainless steel surface roughness on the transfer of Listeria monocytogenes from inoculated bologna to stainless steel and high-density polyethylene. *Journal of Food Protection*. 70:1423-1428.
265. Rodriguez, A., W. R. Autio, and L. A. Mclandsborough. 2008. Effect of surface roughness and stainless steel finish on *Listeria monocytogenes* attachment and biofilm formation. *Journal of Food Protection*. 71:170-175.

266. Rodriguez, A., W. R. Autio, and L. A. McLandsborough. 2008. Effects of contact time, pressure, percent relative humidity (%RH), and material type on *Listeria* biofilm adhesive strength at a cellular level using atomic force microscopy (AFM). *Food Biophysics*. 3:305-311.
267. Rogers, J. V., G. R. Ducatte, Y. W. Choi, and P. C. Early. 2006. A preliminary assessment of *Bacillus anthracis* spore inactivation using an electrochemically activated solution (ECASOL (TM)). *Letters in Applied Microbiology*. 43:482-488.
268. Romanova, N. A., P. V. Gawande, L. Y. Brovko, and M. W. Griffiths. 2007. Rapid methods to assess sanitizing efficacy of benzalkonium chloride to *Listeria monocytogenes* biofilms. *Journal of Microbiological Methods*. 71:231-237.
269. Ruan, C. M., L. J. Yang, and Y. B. Li. 2002. Immunobiosensor chips for detection of Escherichia coli O157 : H7 using electrochemical impedance spectroscopy. *Analytical Chemistry*. 74:4814-4820.
270. Ryan, E. M., C. G. M. Gahan, and C. Hill. 2008. A significant role for Sigma B in the detergent stress response of *Listeria monocytogenes. Letters in Applied Microbiology*. 46:148-154.
271. Saour, G., and H. Makee. 2002. Effects of gamma irradiation used to inhibit potato sprouting on potato tuber moth eggs *Phthorimaea operculella* Zeller (Lep., Gelechiidae). *Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie*. 126:315-319.
272. Saunders, J., M. Murray, and A. Alleman. 2011. Biofilms in chronic suppurative otitis media and cholesteatoma: scanning electron microscopy findings. *American Journal of Otolaryngology*. 32:32-37.
273. Scallan, E., P. M. Griffin, F. J. Angulo, R. V. Tauxe, and R. M. Hoekstra. 2011. Foodborne iIlness acquired in the United States-unspecified agents. *Emerging Infectious Diseases*. 17:16-22.
274. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States-major pathogens. *Emerging Infectious Diseases*. 17:7-15.
275. Schellekens, M. M., J. Wouters, S. Hagens, and J. Hugenholtz. 2007. Bacteriophage P100 application to control *Listeria monocytogenes* on smeared cheese. *Milchwissenschaft-Milk Science International*. 62:284-287.
276. Schmit, J. Date, 2007, Meat recalls point to possibility threat is growing. Available at: http://www.usatoday.com/money/industries/food/2007-10-01-meat-recall_N.htm?csp=34. Accessed Jul 14, 2012.
277. Schwab, U., Y. Hu, M. Wiedmann, and K. J. Boor. 2005. Alternative sigma factor sigma(B) is not essential for *Listeria monocytogenes* surface attachment. *Journal of Food Protection*. 68:311-317.
278. Scott, B. G. Date, 2012, Keeping our food safe. Available at: http://www.worldandi.com/newhome/public/2003/September/ns2pub.asp. Accessed.
279. Shannon, E. M., S. R. Milillo, M. G. Johnson, and S. C. Ricke. 2011. Inhibition of Listeria monocytogenes by Exposure to a Combination of Nisin and Cold-Pressed Terpeneless Valencia Oil. *Journal of Food Science*. 76:M600-M604.

280. Sheth, A. N., M. Hoekstra, N. Patel, G. Ewald, C. Lord, C. Clarke, E. Villamil, K. Niksich, C. Bopp, T. A. Nguyen, D. Zink, and M. Lynch. 2011. A national outbreak of *Salmonella* serotype Tennessee infections from contaminated peanut butter: A new food vehicle for salmonellosis in the United States. *Clinical Infectious Diseases*. 53:356-362.
281. Shi, X., A. Namvar, M. Kostrzynska, R. Hora, and K. Warriner. 2007. Persistence and growth of different *Salmonella* serovars on pre- and postharvest tomatoes. *Journal of Food Protection*. 70:2725-2731.
282. Shin, J. H., J. Kim, S. M. Kim, S. Kim, J. C. Lee, J. M. Ahn, and J. Y. Cho. 2010. Sigma B-dependent protein induction in Listeria monocytogenes during vancomycin stress. *Fems Microbiology Letters*. 308:94-100.
283. Smith, J. S., and S. Pillai. 2004. Irradiation and food safety. *Food Technology*. 58:48-55.
284. Solanki, R. B., M. Prasad, A. U. Sonawane, and S. K. Gupta. 2012. Probabilistic safety assessment for food irradiation facility. *Annals of Nuclear Energy*. 43:123-130.
285. Solovyeva, A. M., and P. M. H. Dummer. 2000. Cleaning effectiveness of root canal irrigation with electrochemically activated anolyte and catholyte solutions: a pilot study. *International Endodontic Journal*. 33:494-504.
286. Somolinos, M., L. Espina, R. Pagan, and D. Garcia. 2010. sigB absence decreased Listeria monocytogenes EGD-e heat resistance but not its Pulsed Electric Fields resistance. *International Journal of Food Microbiology*. 141:32-38.
287. Soni, K. A., M. Desai, A. Oladunjoye, F. Skrobot, and R. Nannapaneni. 2012. Reduction of *Listeria monocytogenes* in queso fresco cheese by a combination of listericidal and listeriostatic GRAS antimicrobials. *International Journal of Food Microbiology*. 155:82-88.
288. Soni, K. A., and R. Nannapaneni. 2010. Removal of *Listeria monocytogenes* biofilms with bacteriophage P100. *Journal of Food Protection*. 73:1519-1524.
289. Soni, K. A., R. Nannapaneni, and S. Hagens. 2010. Reduction of *Listeria monocytogenes* on the surface of fresh channel catfish fillets by bacteriophage Listex P100. *Foodborne Pathogens and Disease*. 7:427-434.
290. Sue, D., D. Fink, M. Wiedmann, and K. J. Boor. 2004. sigma(B)-dependent gene induction and expression in Listeria monocytogenes during osmotic and acid stress conditions simulating the intestinal environment. *Microbiology-Sgm*. 150:3843-3855.
291. Sutherland, I. W. 2001. Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology-Uk*. 147:3-9.
292. Sutherland, I. W. 2001. The biofilm matrix - an immobilized but dynamic microbial environment. *Trends in Microbiology*. 9:222-227.
293. Tang, W. W., X. P. Zeng, Y. S. Zhao, G. Q. Ye, W. C. Gui, and Y. M. Ni. 2011. Disinfection effect and its mechanism of electrolyzed oxidizing water on spores of *Bacillus subtilis* var. niger. *Food Science and Biotechnology*. 20:889-895.
294. Tarkowski, J. A., S. C. C. Stoffer, R. R. Beumer, and E. H. Kampelmacher. 1984. Low-dose gamma-irradiation of raw meat .1. Bacteriological and sensory quality

effects in artificially contaminated samples. *International Journal of Food Microbiology*. 1:13-23.
295. Tasara, T., and R. Stephan. 2007. Evaluation of housekeeping genes in Listeria monocytogenes as potential internal control references for normalizing mRNA expression levels in stress adaptation models using real-time PCR. *Fems Microbiology Letters*. 269:265-272.
296. Thayer, D. W., G. Boyd, J. B. Fox, L. Lakritz, and J. W. Hampson. 1995. Variations in radiation sensitivity of foodborne pathogens associated with the suspending meat. *Journal of Food Science*. 60:63-67.
297. Thomas, P., A. N. Srirangarajan, and S. P. Limaye. 1975. Studies on sprout inhibition of onions by gamma-irradiation .1. Influence of time interval between harvest and irradiation, radiation-dose and environmental conditions on sprouting. *Radiation Botany*. 15:215-&.
298. Trepka, M. J., J. R. Archer, S. F. Altekruse, M. E. Proctor, and J. P. Davis. 1999. An increase in sporadic and outbreak-associated *Salmonella* enteritidis infections in Wisconsin: The role of eggs. *Journal of Infectious Diseases*. 180:1214-1219.
299. Trinetta, V., N. Vaidya, R. Linton, and M. Morgan. 2011. Evaluation of chlorine dioxide gas residues on selected food produce. *Journal of Food Science*. 76:T11-T15.
300. Tschape, H., R. Prager, W. Streckel, A. Fruth, E. Tietze, and G. Bohme. 1995. Verotoxinogenic citrobacter-freundii associated with severe gastroenteritis and cases of hemolytic-uremic syndrome in a nursery-school - green butter as the infection source. *Epidemiology and Infection*. 114:441-450.
301. Tserenpuntsag, B., H. G. Chang, P. F. Smith, and D. L. Morse. 2005. Hemolytic uremic syndrome risk and *Escherichia coli* O157:H7. *Emerging Infectious Diseases*. 11:1955-1957.
302. U.S. Food and Drug Administration. Date, 2008, FDA announces final rule amending the food additive regulations to allow for the irradiation of fresh iceberg lettuce and fresh spinach. Available at: http://www.fda.gov/Food/NewsEvents/ConstituentUpdates/ucm047176.htm. Accessed July 14, 2012.
303. U.S. Food and Drug Administration. Date, 2008, Irradiation: a safe measure for safer iceberg lettuce and spinach. Available at: http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm093651.htm. Accessed Jul 14, 2012.
304. Uhlinger, D. J., and D. C. White. 1983. Relationship between Physiological Status and Formation of Extracellular Polysaccharide Glycocalyx in Pseudomonas-Atlantica. *Applied and Environmental Microbiology*. 45:64-70.
305. University, P. Date, 2011, Purdue News. Available at: http://www.purdue.edu/uns/html4ever/0801.Bhunia.listeria.html. Accessed 19 October, 2011.
306. Utratna, M., I. Shaw, E. Starr, and C. P. O'Byrne. 2011. Rapid, transient, and proportional activation of sigma(B) in response to osmotic stress in *Listeria monocytogenes*. *Applied and Environmental Microbiology*. 77:7841-7845.

307. van der Aa, B. C., and Y. F. Dufrene. 2002. In situ characterization of bacterial extracellular polymeric substances by AFM. *Colloids and Surfaces B-Biointerfaces*. 23:173-182.
308. van der Veen, S., and T. Abee. 2010. Importance of SigB for *Listeria monocytogenes* static and continuous-flow biofilm formation and disinfectant resistance. *Applied and Environmental Microbiology*. 76:7854-7860.
309. van Schaik, W., and T. Abee. 2005. The role of sigma(B) in the stress response of Gram-positive bacteria - targets for food preservation and safety. *Current Opinion in Biotechnology*. 16:218-224.
310. Vandekinderen, I., F. Devlieghere, J. Van Camp, B. Kerkaert, T. Cucu, P. Ragaert, J. De Bruyne, and B. De Meulenaer. 2009. Effects of food composition on the inactivation of foodborne microorganisms by chlorine dioxide. *International Journal of Food Microbiology*. 131:138-144.
311. Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*. 3.
312. Venkitanarayanan, K. S., G. O. Ezeike, Y. C. Hung, and M. P. Doyle. 1999. Efficacy of electrolyzed oxidizing water for inactivating *Escherichia coli* O157:H7, *Salmonella* enteritidis, and *Listeria monocytogenes. Applied and Environmental Microbiology*. 65:4276-4279.
313. Venkitanarayanan, K. S., G. O. I. Ezeike, Y. C. Hung, and M. P. Doyle. 1999. Inactivation of *Escherichia coli* O157:H7 and *Listeria monocytogenes* on plastic kitchen cutting boards by electrolyzed oxidizing water. *Journal of Food Protection*. 62:857-860.
314. Viazis, S., M. Akhtar, J. Feirtag, A. D. Brabban, and F. Diez-Gonzalez. 2011. Isolation and characterization of lytic bacteriophages against enterohaemorrhagic *Escherichia coli*. *Journal of Applied Microbiology*. 110:1323-1331.
315. Viazis, S., M. Akhtar, J. Feirtag, and F. Diez-Gonzalez. 2011. Reduction of *Escherichia coil* O157:H7 viability on hard surfaces by treatment with a bacteriophage mixture. *International Journal of Food Microbiology*. 145:37-42.
316. Viazis, S., M. Akhtar, J. Feirtag, and F. Diez-Gonzalez. 2011. Reduction of *Escherichia coli* O157:H7 viability on leafy green vegetables by treatment with a bacteriophage mixture and trans-cinnamaldehyde. *Food Microbiology*. 28:149-157.
317. Vugia, D., A. Cronquist, M. Cartter, M. Tobin-D'Angelo, D. Blythe, K. Smith, S. Lathrop, D. Morse, P. Cieslak, J. Dunn, K. G. Holt, O. L. Henao, R. M. Hoekstra, F. J. Angulo, P. M. Griffin, R. V. Tauxe, and K. K. Trivedi. 2009. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food-10 states, 2008 (Reprinted from MMWR, vol 58, pg 333-337, 2009). *Jama-Journal of the American Medical Association*. 301:2088-2090.
318. Vurma, M., R. B. Pandit, S. K. Sastry, and A. E. Yousef. 2009. Inactivation of *Escherichia coli* O157:H7 and natural microbiota on spinach leaves using gaseous

ozone during vacuum cooling and simulated transportation. *Journal of Food Protection*. 72:1538-1546.
319. Wade, W. N., A. J. Scouten, K. H. McWatters, R. L. Wick, A. Demirci, W. F. Fett, and L. R. Beuchat. 2003. Efficacy of ozone in killing *Listeria monocytogenes* on alfalfa seeds and sprouts and effects on sensory quality of sprouts. *Journal of Food Protection*. 66:44-51.
320. Wang, Y., E. B. Somers, S. Manolache, F. S. Denes, and A. C. L. Wong. 2003. Cold plasma synthesis of poly(ethylene glycol)-like layers on stainless-steel surfaces to reduce attachment and biofilm formation by *Listeria monocytogenes. Journal of Food Science*. 68:2772-2779.
321. Wei, C. I., D. L. Cook, and J. R. Kirk. 1985. Use of chlorine compounds in the food-industry. *Food Technology*. 39:107-115.
322. Werbrouck, H., N. Botteldoorn, M. Uyttendaele, L. Herman, and E. Van Coillie. 2007. Quantification of gene expression of Listeria monocytogenes by real-time reverse transcription PCR: Optimization, evaluation and pitfalls. *Journal of Microbiological Methods*. 69:306-314.
323. Wiedmann, M., T. J. Arvik, R. J. Hurley, and K. J. Boor. 1998. General stress transcription factor sigma(B) and its role in acid tolerance and virulence of *Listeria monocytogenes. Journal of Bacteriology*. 180:3650-3656.
324. Williams, R. C., S. S. Sumner, and D. A. Golden. 2005. Inactivation of *Escherichia coli* O157:H7 and *Salmonella* in apple cider and orange juice treated with combinations of ozone, dimethyl dicarbonate, and hydrogen peroxide. *Journal of Food Science*. 70:M197-M201.
325. Wimpenny, J. 1995. Biofilms: Structure and organisation. *Microbial Ecology in Health and Disease*. 8:305-308.
326. Wimpenny, J., W. Manz, and U. Szewzyk. 2000. Heterogeneity in biofilms. *Fems Microbiology Reviews*. 24:661-671.
327. Wimpenny, J. W. T., and R. Colasanti. 1997. A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models. *Fems Microbiology Ecology*. 22:1-16.
328. Winder, C. L., I. S. I. Al-Adham, S. M. A. A. Malek, T. E. J. Buultjens, A. J. Horrocks, and P. J. Collier. 2000. Outer membrane protein shifts in biocide-resistant *Pseudomonas aeruginosa* PAO1. *Journal of Applied Microbiology*. 89:289-295.
329. Wirtanen, G., M. Aalto, P. Harkonen, P. Gilbert, and T. Mattila-Sandholm. 2001. Efficacy testing of commercial disinfectants against foodborne pathogenic and spoilage microbes in biofilm-constructs. *European Food Research and Technology*. 213:409-414.
330. Wolfaardt, G. M., J. R. Lawrence, R. D. Robarts, and D. E. Caldwell. 1998. In situ characterization of biofilm exopolymers involved in the accumulation of chlorinated organics. *Microbial Ecology*. 35:213-223.
331. Wong, C. S., J. C. Mooney, J. R. Brandt, A. O. Staples, S. Jelacic, D. R. Boster, S. L. Watkins, and P. I. Tarr. 2012. Risk factors for the hemolytic uremic syndrome

in children infected with *Escherichia coli* O157:H7: A multivariable analysis. *Clinical Infectious Diseases*. 55:33-41.
332. Yang, H., P. A. Kendall, L. C. Medeiros, and J. N. Sofos. 2009. Efficacy of sanitizing agents against *Listeria monocytogenes* biofilms on high-density polyethylene cutting board surfaces. *Journal of Food Protection*. 72:990-998.
333. Yang, H., B. L. Swem, and Y. Li. 2003. The effect of pH on inactivation of pathogenic bacteria on fresh-cut lettuce by dipping treatment with electrolyzed water. *Journal of Food Science*. 68:1013-1017.
334. Yang, H., and Y. Wang. 2008. Application of atomic force microscopy on rapid determination of microorganisms for food safety. *Journal of Food Science*. 73:N44-N50.
335. Yang, H., Y. Wang, J. M. Regenstein, and D. B. Rouse. 2007. Nanostructura characteization of catfish skin gelatin using atomic force microscopy. *Journal of Food Science*. 72:C430-C440.
336. Yang, H. S., Y. F. Wang, S. J. Lai, H. J. An, Y. F. Li, and F. S. Chen. 2007. Application of atomic force microscopy as a nanotechnology tool in food science. *Journal of Food Science*. 72:R65-R75.
337. Yang, L. J., and Y. B. Li. 2005. AFM and impedance spectroscopy characterization of the immobilization of antibodies on indium-tin oxide electrode through self-assembled monolayer of epoxysilane and their capture of Escherichia coli O157 : H7. *Biosensors & Bioelectronics*. 20:1407-1416.
338. Yang, Y., Y. G. Luo, P. Millner, E. Turner, and H. Feng. 2012. Assessment of *Escherichia coli* O157:H7 transference from soil to iceberg lettuce via a contaminated field coring harvesting knife. *International Journal of Food Microbiology*. 153:345-350.
339. Yong, D. M., C. Liu, D. B. Yu, and S. J. Dong. 2011. A sensitive, rapid and inexpensive way to assay pesticide toxicity based on electrochemical biosensor. *Talanta*. 84:7-12.
340. Yuk, H. G., M. Y. Yoo, J. W. Yoon, K. D. Moon, D. L. Marshall, and D. H. Oh. 2006. Effect of combined ozone and organic acid treatment for control of *Escherichia coli* O157:H7 and *Listeria monocytogenes* on lettuce. *Journal of Food Science*. 71:M83-M87.
341. Zeng, X. P., W. W. Tang, G. Q. Ye, T. Ouyang, L. Tian, Y. M. Ni, and P. Li. 2010. Studies on disinfection mechanism of electrolyzed oxidizing water on *E. coli* and *Staphylococcus aureus. Journal of Food Science*. 75:M253-M260.
342. Zhang, D. L., T. Ross, and J. P. Bowman. 2010. Physiological aspects of Listeria monocytogenes during inactivation accelerated by mild temperatures and otherwise non-growth permissive acidic and hyperosmotic conditions. *International Journal of Food Microbiology*. 141:177-185.
343. Zhang, Q. A., Y. Y. Feng, L. F. Deng, F. F. Feng, L. Wang, Q. C. Zhou, and Q. Luo. 2011. SigB plays a major role in Listeria monocytogenes tolerance to bile stress. *International Journal of Food Microbiology*. 145:238-243.

344. Zhou, Q. C., L. Wang, X. J. Yin, X. Q. Feng, J. L. Shang, and Q. Luo. 2012. SigB-Dependent Tolerance to Protein Synthesis-Inhibiting Antibiotics in Listeria monocytogenes EGDe. *Current Microbiology*. 64:234-241.
345. Zmantar, T., B. Kouidhi, H. Miladi, K. Mandouani, and A. Bakhrouf. 2010. A Microtiter plate assay for Staphylococcus aureus biofilm quantification at various pH levels and hydrogen peroxide supplementation. *New Microbiologica*. 33:137-145.

Appendices

A1. Survival of *Escherichia coli* O157:H7 cells (log CFU ml^{-1}) in liquid cultures after exposure to neutral electrochemically activated water (NECAW). Distilled ionized water (DIW) was used as control.

Trial	Strain	Initial	DIW	NECAW -50
Trial 1	ATCC 43890	9.05	6.98	2.18
		9.09	7.03	2.18
	ATCC 43895	9.22	7.14	5.39
		9.18	7.15	5.23
	2028	9.18	7.14	2.88
		9.26	7.17	2.30
	2029	9.29	7.21	<2.00
		9.23	7.16	<2.00
	2257	9.23	7.08	<2.00
		9.16	7.08	<2.00
	4719	9.19	7.01	4.33
		9.22	7.12	4.21
	6058	9.16	7.19	<2.00
		9.14	7.17	<2.00
	86-24	9.25	7.15	<2.00
		9.17	7.10	<2.00
	EK1 TWO8609	9.15	7.12	<2.00
		9.25	7.19	<2.00
	EK27 TWO 8635	8.69	6.60	<2.00
		8.73	6.67	<2.00
	E32511 TWO2383	9.07	7.05	<2.00
		9.16	7.11	<2.00
Trial 2	ATCC 43890	9.16	6.95	2.00
		9.09	6.98	2.40
	ATCC 43895	9.23	7.15	5.17
		9.26	7.13	5.22
	2028	9.22	7.17	<2.00
		9.24	7.16	<2.00
	2029	9.30	7.22	<2.00
		9.26	7.17	<2.00
	2257	9.10	7.05	<2.00
		9.22	7.11	<2.00
	4719	9.02	6.96	4.10
		9.04	6.93	<2.10

6058	8.98	7.04	<2.00
	9.21	7.18	<2.00
86-24	9.23	7.12	<2.00
	9.17	7.13	<2.00
EK1 TWO8609	9.16	7.10	<2.00
	9.17	7.09	<2.00
EK27 TWO 8635	8.79	6.76	<2.00
	8.77	6.71	<2.00
E32511 TWO2383	9.04	6.98	<2.00
	8.87	6.71	<2.00

Note: the free available chlorine of the NECAW was 50 mg l^{-1}, treatment time was 30 s. Detection limit: 2 log CFU ml^{-1}. All NECAW-100 were below the detection limit (< 2.00).

A2. Survival of *Listeria monocytogenes* cells (log CFU ml^{-1}) in liquid cultures after exposure to neutral electrochemically activated water (NECAW). Distilled ionized water (DIW) was used as control.

Trial	Strain	Initial	DIW	NECAW -50
Trial 1	ATCC 19115	9.32	6.59	<2.00
		9.27	6.79	<2.00
	UMN-VM	9.20	7.19	<2.00
		9.32	7.21	<2.00
	DUP-1030A	9.34	7.22	<2.00
		9.26	7.23	<2.00
	DUP-1038	9.37	7.47	<2.00
		9.48	7.57	<2.00
	DUP 1044A	9.01	7.01	<2.00
		9.21	7.06	<2.00
	2349	9.16	7.16	<2.00
		9.21	7.21	<2.00
	2422	8.82	6.59	<2.00
		9.03	6.79	<2.00
	3528	9.24	7.24	<2.00
		9.21	7.22	<2.00
Trial 2	ATCC 19115	9.25	7.23	<2.00
		9.37	7.27	<2.00
	UMN-VM	9.27	7.14	<2.00
		9.31	7.22	<2.00
	DUP-1030A	9.34	7.20	<2.00
		9.31	7.25	<2.00
	DUP-1038	9.56	7.52	<2.00
		9.61	7.55	<2.00
	DUP 1044A	9.09	7.02	<2.00
		9.06	7.00	<2.00
	2349	9.20	7.15	<2.00
		9.23	7.21	<2.00
	2422	8.85	6.65	<2.00
		8.86	6.59	<2.00
	3528	9.23	7.25	<2.00
		9.18	7.23	<2.00

A3. Survival of *Salmonella enterica* cells (log CFU ml^{-1}) in liquid cultures after exposure to neutral electrochemically activated water (NECAW). Distilled ionized water (DIW) was used as control.

Trial	**Sevovar**	**Strain**	**Initial**	**DIW**	**NECAW -50**
Trial 1	Typhimurium	700408	8.88	6.79	2.54
			9.09	7.13	3.60
		ATCC 14028	9.24	7.07	3.45
			9.27	7.13	3.70
		E2009005811	9.26	7.07	<2.00
			9.35	7.02	<2.00
		UK-1	9.29	7.09	<2.00
			9.18	7.13	<2.00
		I503	9.18	7.11	<2.00
			9.30	7.24	<2.00
		I526	9.21	7.20	<2.00
			9.35	7.26	<2.00
		I534	9.14	7.04	<2.00
			8.98	6.88	<2.00
		I535	7.15	7.15	<2.00
			7.13	7.13	<2.00
		I536	9.15	7.19	<2.00
			8.92	7.16	<2.00
		I740	9.21	7.03	<2.00
			9.24	7.05	<2.00
		I758	9.34	7.31	<2.00
			9.42	7.33	<2.00
	Newport	AMO 7073	9.12	6.83	<2.00
			8.91	6.83	<2.00
		AMO 7076	9.24	7.08	2.00
			9.36	7.13	2.18
		AMO 5313	8.97	6.99	3.95
			9.12	6.99	4.38
		B4442CDC	9.23	7.16	<2.00
			9.17	7.10	<2.00
	Enteritidis	2009595	9.30	7.28	<2.00
			9.38	7.30	<2.00
		95657613	9.23	7.20	<2.00
			9.28	7.24	<2.00
	Tennessee	E2007000302	9.19	7.39	3.40
			9.34	7.41	3.39
	Montevideo	95573473	9.34	7.20	<2.00
			9.37	7.24	<2.00

	Agona	FDA	9.23	7.03	2.00
			9.19	6.94	2.32
	Saintpaul	E2008001236	9.29	7.07	4.33
			9.13	7.10	3.04
Trial 2	Typhimurium	700408	8.98	6.82	2.30
			8.87	6.72	3.56
		ATCC 14028	9.23	7.10	3.42
			9.19	7.11	3.66
		E2009005811	9.31	6.94	<2.00
			9.24	7.09	<2.00
		UK-1	9.14	7.13	<2.00
			9.28	7.11	<2.00
		I503	9.16	7.07	<2.00
			9.20	7.12	<2.00
		I526	9.20	7.12	4.33
			9.27	7.19	4.21
		I534	9.17	7.01	<2.00
			8.98	6.95	<2.00
		I535	9.23	7.14	<2.00
			9.17	7.08	<2.00
		I536	9.23	7.16	<2.00
			9.20	7.13	<2.00
		I740	9.22	7.04	<2.00
			9.10	7.01	<2.00
		I758	9.35	7.34	<2.00
			9.43	7.31	<2.00
	Newport	AMO 7073	9.01	6.85	<2.00
			9.07	6.85	<2.00
		AMO 7076	9.19	7.04	4.13
			9.28	7.04	2.30
		AMO 5313	9.11	6.94	3.76
			8.87	6.87	4.10
		B4442CDC	9.24	7.16	<2.00
			9.15	7.10	<2.00
	Enteritidis	2009595	9.31	7.22	<2.00
			9.22	7.21	<2.00
		95657613	9.15	6.92	<2.00
			9.23	7.00	<2.00
	Tennessee	E2007000302	9.39	7.35	3.76
			9.45	7.38	4.10
	Montevideo	95573473	9.38	7.12	<2.00
			9.46	7.16	<2.00

Agona	FDA	9.34	7.06	2.00
		9.24	7.15	2.62
Saintpaul	E2008001236	9.27	7.11	4.43
		9.13	7.07	3.11

Note: the free available chlorine of the NECAW was 50 mg l^{-1}, treatment time was 30 s. Detection limit: 2 log CFU ml^{-1}. All NECAW-100 were below the detection limit (< 2.00).

B1. Effect of water fractions previously treated with different electrolysis technology on the viability *of Escherichia coli* O157:H7 liquid cultures.

Trial	**Strain**	**Survival count after treatment (log CFU/ml)**					
		Control	**Ionator**	**Salt Ionator**[b]	**Lotus**	**ECAW-50**[c]	**ECAW-100**[d]
Trial 1	ATCC	7.01	7.00	7.10	7.00	2.18	<2.00[e]
	43890	7.04	7.01	7.04	7.06	2.00	<2.00
	ATCC	7.15	7.14	7.14	7.21	5.43	<2.00
	43895	7.17	7.16	7.20	7.29	4.14	<2.00
	2028	7.14	7.08	7.08	6.98	2.00	<2.00
		7.19	7.19	7.15	7.13	2.00	<2.00
	2257	7.10	7.07	7.11	7.06	<2.00	<2.00
		7.07	7.12	7.08	7.09	2.00	<2.00
	2029	7.21	7.14	7.18	7.14	<2.00	<2.00
		7.14	7.17	7.13	7.05	<2.00	<2.00
Trial 2	ATCC	7.01	6.99	6.97	6.92	2.40	<2.00
	43890	7.08	7.08	7.10	7.13	1.70	<2.00
	ATCC	7.19	7.07	7.06	6.93	4.57	<2.00
	43895	7.14	7.24	7.17	7.12	5.44	<2.00
	2028	7.11	7.15	7.15	7.15	2.00	<2.00
		7.23	7.16	7.11	7.11	<2.00	<2.00
	2257	7.23	7.04	6.97	6.93	<2.00	<2.00
		7.19	7.15	7.13	7.10	<2.00	<2.00
	2029	7.07	7.18	7.13	6.98	<2.00	<2.00
		7.16	7.21	7.23	7.17	<2.00	<2.00

[b] Salt Ionator means Ionator using 0.1% NaCl (w/v) solution.
[c] Concentration: FAC 50 mg/l.
[d] Concentration: FAC 100 mg/l.
[e] Detection limit: 2.00 log CFU/ml.

B2. Effect of water fractions previously treated with different electrolysis technology on the viability of *Salmonella spp.* liquid cultures.

Trial	Serovar and Strain	Survival count after treatment (log CFU/ml)					
		Control	Ionator	Salt Ionator[b]	Lotus	ECAW-50[c]	ECAW-100[d]
Trial 1	Typhimurium ATCC 14028	7.04 7.13	7.00 7.08	7.06 7.15	6.95 7.06	3.38 3.69	<2.00[e] <2.00
	Typhimurium E2009005811	7.09 7.05	7.02 7.04	7.08 7.02	7.03 7.00	<2.00 2.30	<2.00 <2.00
	Enteritidis 2009595	7.28 7.31	7.26 7.34	7.30 7.36	7.24 7.29	<2.00 <2.00	<2.00 <2.00
	Tennessee E2007000302	7.36 7.39	7.23 7.24	7.32 7.38	7.23 7.43	3.29 3.35	<2.00 <2.00
	Saintpaul E2008001236	7.10 7.16	7.05 7.17	7.00 7.11	7.07 7.18	3.94 3.24	<2.00 <2.00
Trial 2	Typhimurium ATCC 14028	7.10 7.14	7.07 7.07	7.11 7.16	7.05 7.08	3.15 3.69	<2.00 <2.00
	Typhimurium E2009005811	6.94 7.09	6.89 7.08	6.95 7.07	6.95 7.09	<2.00 <2.00	<2.00 <2.00
	Enteritidis 2009595	7.22 7.23	7.11 7.24	7.18 7.18	7.19 7.20	<2.00 <2.00	<2.00 <2.00
	Tennessee E2007000302	7.27 7.35	7.31 7.40	7.23 7.37	7.05 7.32	2.48 3.10	<2.00 <2.00
	Saintpaul E2008001236	7.01 7.22	7.04 7.09	7.03 7.14	6.89 7.04	4.39 3.16	<2.00 <2.00

[b] Salt Ionator means Ionator using 0.1% NaCl (w/v) solution.
[c] Concentration: FAC 50 mg/l.
[d] Concentration: FAC 100 mg/l.
[e] Detection limit: 2.00 log CFU/ml.

B3. Effect of water fractions previously treated with different electrolysis technology on the viability of *Listeria monocytogenes* liquid cultures.

Trial	Strain	Survival count after treatment (log CFU/ml)					
		Control	Ionator	Salt Ionator[b]	Lotus	ECAW-50[c]	ECAW-100[d]
Trial 1	ATCC 19115	7.23	7.15	7.17	7.16	<2.00[e]	<2.00
		7.26	7.19	7.22	7.23	<2.00	<2.00
	DUP-1030A	7.22	7.19	7.20	7.18	<2.00	<2.00
		7.26	7.23	7.19	7.23	<2.00	<2.00
	DUP-1038	7.48	7.45	7.48	7.46	<2.00	<2.00
		7.58	7.55	7.56	7.53	<2.00	<2.00
	DUP-1044A	7.12	7.12	7.08	6.99	<2.00	<2.00
		7.17	7.19	7.15	7.11	<2.00	<2.00
	2422	6.51	6.37	6.63	6.38	<2.00	<2.00
		6.59	6.56	6.54	6.51	<2.00	<2.00
Trial 2	ATCC 19115	7.20	7.05	7.13	7.21	<2.00	<2.00
		7.28	7.28	7.23	7.26	<2.00	<2.00
	DUP-1030A	7.20	7.14	7.19	7.17	<2.00	<2.00
		7.25	7.20	7.21	7.22	<2.00	<2.00
	DUP-1038	7.51	7.49	7.47	7.50	<2.00	<2.00
		7.56	7.53	7.52	7.56	<2.00	<2.00
	DUP-1044A	7.03	7.04	7.06	7.05	<2.00	<2.00
		7.02	7.06	7.13	7.02	<2.00	<2.00
	2422	6.62	6.60	6.67	6.57	<2.00	<2.00
		6.61	6.63	6.61	6.64	<2.00	<2.00

[b] Salt Ionator means Ionator using 0.1% NaCl (w/v) solution.
[c] Concentration: FAC 50 mg/l.
[d] Concentration: FAC 100 mg/l.
[e] Detection limit: 2.00 log CFU/ml.

B4. Survival of *Escherichia coli* O157:H7 dried on coupons after treatment with antimicrobial water treatments (log CFU/coupon).

Trial	Strain	Testing fraction	Survival count after treatment				
			Control	Ionator	Salt Ionator[b]	Lotus	ECAW-100[c]
Trail 1	ATCC 43890	Coupon	2.40	2.18	2.40	2.30	<2.00[d]
			2.65	2.48	2.30	2.40	<2.00
		Cloth	3.42	3.39	3.45	3.43	<2.00
			3.49	3.45	3.39	3.40	<2.00
		Rinse	6.86	6.77	6.74	6.77	<2.00
			6.57	6.64	6.62	6.65	<2.00
	ATCC 43895	Coupon	3.26	2.93	3.06	3.00	<2.00
			3.06	2.93	2.85	2.98	<2.00
		Cloth	3.31	3.44	3.43	3.45	<2.00
			3.56	3.43	3.37	3.39	<2.00
		Rinse	6.49	6.55	6.75	6.60	<2.00
			6.74	6.77	6.70	6.81	<2.00
	2028	Coupon	2.95	2.60	2.40	2.40	<2.00
			3.26	2.78	3.11	2.74	<2.00
		Cloth	4.07	3.99	3.92	3.95	<2.00
			4.16	4.10	4.16	4.03	<2.00
		Rinse	6.76	6.79	6.81	6.76	<2.00
			6.41	6.30	6.36	6.32	<2.00
	2257	Coupon	2.18	2.54	2.30	2.60	<2.00
			3.54	2.00	2.18	2.00	<2.00
		Cloth	4.16	4.05	4.00	4.08	<2.00
			3.59	3.62	3.77	3.39	<2.00
		Rinse	6.40	6.40	6.45	6.37	<2.00
			6.46	6.59	6.54	6.56	<2.00
	2029	Coupon	2.00	2.18	2.40	2.40	<2.00
			2.00	2.60	2.40	2.18	<2.00
		Cloth	3.56	3.44	3.37	3.53	<2.00
			3.36	3.54	3.58	3.41	<2.00
		Rinse	6.92	6.98	6.94	7.01	<2.00
			6.47	6.49	6.47	6.46	<2.00
Trial 2	ATCC 43890	Coupon	2.18	2.30	2.40	2.30	<2.00
			2.30	2.30	2.60	2.40	<2.00
		Cloth	3.50	3.44	3.48	3.40	<2.00
			3.57	3.65	3.61	3.62	<2.00
		Rinse	6.51	6.62	6.65	6.63	<2.00
			6.46	6.52	6.59	6.48	<2.00

ATCC	Coupon	3.00	3.04	3.13	2.95	<2.00
43895		2.74	2.81	2.98	2.81	<2.00
	Cloth	3.48	3.43	3.41	3.39	<2.00
		3.39	3.46	3.40	3.35	<2.00
	Rinse	6.69	6.64	6.84	6.64	<2.00
		6.86	6.86	6.70	6.84	<2.00
2028	Coupon	2.88	2.40	2.65	2.54	<2.00
		2.85	2.93	3.02	2.74	<2.00
	Cloth	4.07	4.04	4.07	4.03	<2.00
		4.11	4.08	4.11	3.95	<2.00
	Rinse	6.53	6.57	6.50	6.57	<2.00
		6.44	6.51	6.39	6.36	<2.00
2257	Coupon	2.98	2.00	2.65	2.30	<2.00
		2.18	2.00	2.00	2.00	<2.00
	Cloth	4.17	4.07	4.09	4.05	<2.00
		3.48	3.37	3.45	3.41	<2.00
	Rinse	6.49	6.50	6.55	6.49	<2.00
		6.45	6.51	6.51	6.47	<2.00
2029	Coupon	3.06	2.00	2.00	2.18	<2.00
		3.22	2.18	2.40	<2.00	<2.00
	Cloth	3.54	3.44	3.39	3.38	<2.00
		3.42	3.36	3.40	3.20	<2.00
	Rinse	6.09	6.16	6.15	6.16	<2.00
		6.00	6.24	6.44	6.24	<2.00

[b] Salt Ionator means Ionator using 0.1% NaCl (w/v) solution.
[c] Concentration: FAC 100 mg/l.
[d] Detection limit: 2.00 log CFU/coupon.

B5. Survival of *Salmonella spp.*dried on coupons after treatment with antimicrobial water treatments (log CFU/coupon).

Trial	Strain	Testing fraction	Survival count after treatment				
			Control	Ionator	Salt Ionator[b]	Lotus	ECAW-100[c]
Trail 1	Typhimurium ATCC 14028	Coupon	3.53	3.48	3.49	3.44	<2.00[d]
			5.04	2.90	2.98	2.95	
		Cloth	3.27	3.19	3.24	3.15	<2.00
			3.83	3.73	3.74	3.76	
		Rinse	7.16	7.18	7.17	7.13	<2.00
			6.69	6.74	6.74	6.66	
	Typhimurium E2009005811	Coupon	3.66	3.63	3.66	3.45	<2.00
			3.86	3.82	3.84	3.87	
		Cloth	3.93	3.91	3.89	3.53	<2.00
			3.99	3.96	3.94	4.01	
		Rinse	6.67	6.69	6.66	6.62	<2.00
			7.15	7.22	7.22	7.14	
	Enteritidis 2009595	Coupon	3.97	3.67	3.69	3.84	<2.00
			3.98	3.57	3.59	4.01	
		Cloth	3.95	3.87	3.88	3.53	<2.00
			4.04	3.76	3.78	4.11	
		Rinse	7.04	6.98	6.97	6.99	<2.00
			7.03	6.93	6.95	6.95	
	Tennessee E2007000302	Coupon	3.11	2.90	3.10	2.90	<2.00
			3.33	3.19	3.22	3.04	
		Cloth	3.80	3.73	3.74	3.64	<2.00
			4.01	3.96	4.03	3.84	
		Rinse	6.86	6.90	6.90	6.76	<2.00
			7.07	7.09	7.07	7.00	
	Saintpaul E2008001236	Coupon	3.87	3.52	3.54	3.69	<2.00
			3.95	3.74	3.71	3.82	
		Cloth	3.47	3.68	3.77	3.30	<2.00
			4.05	3.86	3.84	3.53	
		Rinse	6.85	6.80	6.78	6.64	<2.00
			7.17	7.21	7.23	7.10	
							<2.00
Trial 2	Typhimurium ATCC 14028	Coupon	3.51	3.48	3.43	3.26	<2.00
			3.10	3.06	3.13	3.13	
		Cloth	3.28	3.19	3.13	3.16	<2.00
			3.76	3.76	3.73	3.78	
		Rinse	7.18	7.17	7.18	7.15	<2.00
			6.75	6.81	6.86	6.77	

Typhimurium	Coupon	3.79	3.67	3.66	3.66	<2.00
E2009005811		3.76	3.78	3.80	3.69	
	Cloth	3.98	3.93	3.92	3.83	<2.00
		3.93	3.93	3.95	3.77	
	Rinse	6.66	6.73	6.75	6.63	<2.00
		7.21	7.18	7.17	7.16	
Enteritidis	Coupon	3.23	3.31	3.31	2.95	<2.00
2009595		3.28	3.40	3.45	3.06	
	Cloth	3.76	3.56	3.54	3.41	<2.00
		3.48	3.62	3.66	3.16	
	Rinse	6.51	6.48	6.44	6.59	<2.00
		6.92	6.84	6.82	6.79	
Tennessee	Coupon	3.26	3.18	3.22	3.10	<2.00
E2007000302		3.50	3.48	3.59	3.60	
	Cloth	4.02	4.00	4.06	3.92	<2.00
		4.12	4.10	4.12	4.12	
	Rinse	7.27	7.29	7.32	7.22	<2.00
		7.36	7.35	7.36	7.38	
Saintpaul	Coupon	4.11	3.77	3.69	4.12	<2.00
E2008001236		3.57	3.86	4.12	3.34	
	Cloth	4.24	3.94	3.87	4.08	<2.00
		3.31	4.00	4.09	2.81	
	Rinse	7.17	7.19	7.17	7.11	<2.00
		7.24	7.21	7.18	7.26	

[b] Salt Ionator means Ionator using 0.1% NaCl (w/v) solution.
[c] Concentration: FAC 100 mg/l.
[d] Detection limit: 2.00 log CFU/coupon.

B6. Survival of *Listeria monocytogenes* dried on coupons after treatment with antimicrobial water treatments (log CFU/coupon).

Trial	Strain	Testing fraction	Survival count after treatment				
			Control	Ionator	Salt Ionator[b]	Lotus	ECAW-100[c]
Trail 1	ATCC 19115	Coupon	3.10	3.13	3.02	3.15	<2.00[d]
			3.06	2.98	2.90	3.06	<2.00
		Cloth	3.06	2.95	2.95	3.02	<2.00
			3.20	3.10	3.10	3.19	<2.00
		Rinse	6.42	6.15	6.12	6.17	<2.00
			6.05	6.22	6.24	6.24	<2.00
	DUP-1030A	Coupon	3.24	3.16	3.22	3.42	<2.00
			3.20	2.88	2.70	3.00	<2.00
		Cloth	3.16	3.31	3.37	3.55	<2.00
			3.40	2.74	2.78	3.27	<2.00
		Rinse	6.23	6.17	6.16	6.24	<2.00
			6.58	6.09	6.07	6.17	<2.00
	DUP-1038	Coupon	3.51	3.34	3.66	3.19	<2.00
			3.63	3.40	3.40	3.29	<2.00
		Cloth	3.52	3.23	3.34	3.38	<2.00
			3.73	3.20	3.45	3.23	<2.00
		Rinse	7.55	6.36	6.41	6.41	<2.00
			7.66	7.42	7.36	7.56	<2.00
	DUP-1044A	Coupon	3.51	3.35	3.43	3.53	<2.00
			3.53	3.08	3.08	2.98	<2.00
		Cloth	3.56	3.37	3.31	3.39	<2.00
			3.64	3.13	3.29	3.08	<2.00
		Rinse	7.31	6.28	6.21	6.35	<2.00
			6.80	6.75	6.86	6.80	<2.00
	2422	Coupon	3.41	2.95	3.06	3.19	<2.00
			3.24	3.37	3.22	3.45	<2.00
		Cloth	3.48	2.98	3.11	3.58	<2.00
			3.32	2.98	3.35	3.35	<2.00
		Rinse	6.43	6.12	6.10	6.18	<2.00
			6.90	6.84	6.86	6.87	<2.00
Trial 2	ATCC 19115	Coupon	3.15	2.93	3.11	3.10	<2.00
			3.63	3.58	3.51	3.56	<2.00
		Cloth	3.02	3.93	3.13	2.95	<2.00
			3.20	3.51	3.48	3.62	<2.00
		Rinse	3.02	4.95	5.03	4.97	<2.00
			3.45	6.24	6.24	6.27	<2.00

DUP-1030A	Coupon	3.65	3.47	3.36	3.66	<2.00
		2.93	3.30	3.26	3.18	<2.00
	Cloth	3.55	3.20	3.29	3.72	<2.00
		3.28	3.23	3.31	3.24	<2.00
	Rinse	6.67	6.63	6.61	6.68	<2.00
		7.44	7.44	7.42	7.47	<2.00
DUP-1038	Coupon	2.85	2.93	2.81	3.13	<2.00
		3.16	2.85	2.88	2.74	<2.00
	Cloth	2.93	2.70	3.06	3.23	<2.00
		3.38	2.60	3.02	2.95	<2.00
	Rinse	5.96	5.85	5.88	5.97	<2.00
		5.97	5.84	5.87	5.88	<2.00
DUP-1044A	Coupon	3.30	2.85	2.95	2.70	<2.00
		2.88	2.95	3.45	2.82	<2.00
	Cloth	3.55	3.00	3.00	2.95	<2.00
		3.02	3.00	3.32	3.00	<2.00
	Rinse	6.43	6.21	6.25	6.30	<2.00
		5.89	5.75	5.70	5.80	<2.00
2422	Coupon	2.95	2.95	2.85	3.20	<2.00
		3.13	3.19	3.23	3.13	<2.00
	Cloth	2.90	2.90	2.90	3.41	<2.00
		3.41	3.36	3.43	2.98	<2.00
	Rinse	5.79	5.79	5.66	5.89	<2.00
		6.56	5.38	5.38	6.47	<2.00

[b] Salt Ionator means Ionator using 0.1% NaCl (w/v) solution.
[c] Concentration: FAC 100 mg/l.
[d] Detection limit: 2.00 log CFU/coupon.

B7. Survival of *Escherichia coli* O157:H7 in buffers after treatment with antimicrobial water treatments (log CFU/coupon).

Trial	Strain	Testing fraction	Survival count after treatment				
			Control	Ionator	Salt Ionator[b]	Lotus	ECAW-100[c]
Trial 1	ATCC 43890	NB[d] for coupon	2.40	2.30	2.40	2.40	<2.00[e]
			2.70	2.54	2.70	2.40	<2.00
		NB for cloth	3.16	3.10	3.13	3.43	<2.00
			3.41	3.13	3.19	3.40	<2.00
	ATCC 43895	NB for coupon	3.45	3.38	3.45	3.40	<2.00
			3.38	3.39	3.31	3.41	<2.00
		NB for cloth	3.06	2.90	3.19	3.15	<2.00
			3.06	2.90	3.11	2.88	<2.00
	2028	NB for coupon	3.69	3.58	3.45	3.45	<2.00
			3.69	3.58	3.68	3.54	<2.00
		NB for cloth	3.42	2.95	2.74	3.13	<2.00
			3.70	3.24	3.27	3.47	<2.00
	2257	NB for coupon	2.18	1.70	2.18	2.00	<2.00
			3.31	2.30	2.40	2.18	<2.00
		NB for cloth	3.84	3.65	3.67	3.61	<2.00
			3.48	3.36	3.31	3.34	<2.00
	2029	NB for coupon	2.18	2.48	2.60	2.65	<2.00
			3.28	2.70	2.48	2.40	<2.00
		NB for cloth	2.54	2.18	2.54	2.65	<2.00
			2.88	2.54	2.81	2.30	<2.00
Trial 2	ATCC 43890	NB for coupon	2.40	2.60	2.78	2.40	<2.00
			3.00	2.70	2.88	2.60	<2.00
		NB for cloth	3.39	3.29	3.31	3.40	<2.00
			3.08	3.19	3.10	3.62	<2.00
	ATCC 43895	NB for coupon	3.45	3.45	3.35	3.19	<2.00
			3.23	3.31	3.29	3.33	<2.00
		NB for cloth	3.22	3.15	2.95	3.10	<2.00
			3.18	3.08	3.16	3.02	<2.00
	2028	NB for coupon	3.40	3.42	3.29	3.48	<2.00
			3.48	3.53	3.45	3.33	<2.00
		NB for cloth	3.55	3.22	3.29	3.33	<2.00
			3.00	3.15	3.25	3.38	<2.00
	2257	NB for coupon	3.27	2.18	2.54	2.00	<2.00
			2.00	2.18	2.00	2.30	<2.00
		NB for cloth	3.75	3.64	3.68	3.69	<2.00
			3.41	2.98	3.13	3.06	<2.00
	2029	NB for coupon	3.06	2.30	2.18	2.00	<2.00
			3.11	2.48	2.65	2.40	<2.00
		NB for	3.57	2.48	2.48	2.78	<2.00

cloth	3.10	2.74	2.88	2.70	<2.00

[b] Salt Ionator means Ionator using 0.1% NaCl (w/v) solution.
[c] Concentration: FAC 100 mg/l.
[d] NB, neutralizing buffer.
[e] Detection limit: 2.00 log CFU/coupon.

B8. Survival of *Salmonella spp.* in buffers after treatment with antimicrobial water treatments (log CFU/coupon).

Trial	Serovar and Strain	Testing fraction	Survival count after treatment				
			Control	Ionator	Salt Ionator[b]	Lotus	ECAW-100[c]
Trial 1	Typhimurium ATCC 14028	NB[d] for coupon	3.06	2.95	3.02	2.88	<2.00[e]
			3.18	3.08	3.10	3.02	<2.00
		NB for cloth	3.08	2.98	3.02	3.00	<2.00
			3.26	3.13	3.18	3.16	<2.00
	Typhimurium E2009005811	NB for coupon	3.59	3.56	3.59	3.37	<2.00
			3.79	3.74	3.72	3.80	<2.00
		NB for cloth	3.18	2.90	3.08	2.98	<2.00
			3.40	3.23	3.27	3.63	<2.00
	Enteritidis 2009595	NB for coupon	3.72	3.55	3.58	3.56	<2.00
			3.69	3.51	3.50	3.75	<2.00
		NB for cloth	3.42	2.95	3.11	3.26	<2.00
			3.57	2.98	3.06	3.76	<2.00
	Tennessee E2007000302	NB for coupon	3.45	3.08	3.02	2.90	<2.00
			3.68	3.30	3.43	3.10	<2.00
		NB for cloth	3.55	3.13	3.18	3.33	<2.00
			3.79	3.40	3.66	3.35	<2.00
	Saintpaul E2008001236	NB for coupon	3.45	2.98	3.19	3.15	<2.00
			3.68	3.38	3.28	3.54	<2.00
		NB for cloth	3.55	3.31	3.53	3.36	<2.00
			3.79	3.46	3.41	3.53	<2.00
Trial 1	Typhimurium ATCC 14028	NB for coupon	3.18	3.08	3.00	3.06	<2.00
			3.18	3.10	3.15	3.18	<2.00
		NB for cloth	3.16	3.10	2.98	2.98	<2.00
			3.27	3.11	3.04	3.36	<2.00
	Typhimurium E2009005811	NB for coupon	3.64	3.56	3.53	3.46	<2.00
			3.71	3.69	3.72	3.61	<2.00
		NB for cloth	3.43	3.19	3.24	3.26	<2.00
			3.28	3.20	3.37	3.31	<2.00
	Enteritidis 2009595	NB for coupon	3.15	3.16	3.13	2.90	<2.00
			3.19	3.11	3.36	2.81	<2.00
		NB for cloth	2.85	2.78	2.93	2.81	<2.00
			2.98	3.08	3.27	2.85	<2.00
	Tennessee E2007000302	NB for coupon	3.99	3.23	3.34	3.18	<2.00
			2.98	3.55	3.63	3.61	<2.00
		NB for cloth	3.99	3.47	3.57	3.37	<2.00
			3.02	3.64	3.67	3.71	<2.00
	Saintpaul E2008001236	NB for coupon	3.99	3.43	3.37	4.08	<2.00
			2.98	3.48	3.74	2.85	<2.00

	NB for cloth	3.99 3.02	3.57 3.60	3.56 3.84	4.08 2.81	<2.00 <2.00

[b] Salt Ionator means Ionator using 0.1% NaCl (w/v) solution.
[c] Concentration: FAC 100 mg/l.
[d] NB, neutralizing buffer.
[e] Detection limit: 2.00 log CFU/coupon.

B9. Survival of *Listeria monocytogenes* in buffers after treatment with antimicrobial water treatments (log CFU/coupon).

Trial	Strain	Testing fraction	Survival count after treatment				
			Control	Ionator	Salt Ionator[b]	Lotus	ECAW-100[c]
Trial 1	ATCC 19115	NB[d] for coupon	3.00	2.90	2.81	3.02	<2.00[e]
			3.02	2.85	2.79	2.95	<2.00
		NB for cloth	2.98	2.88	2.81	2.81	<2.00
			3.02	3.27	3.02	3.15	<2.00
	DUP-1030A	NB for coupon	3.11	3.02	3.15	3.23	<2.00
			3.10	2.70	2.40	2.95	<2.00
		NB for cloth	3.23	3.00	3.23	2.81	<2.00
			3.15	2.88	2.88	3.41	<2.00
	DUP-1038	NB for coupon	3.36	3.23	3.24	3.08	<2.00
			3.48	3.20	3.29	3.10	<2.00
		NB for cloth	3.35	3.04	3.15	3.23	<2.00
			3.54	3.23	3.29	2.81	<2.00
	DUP-1044A	NB for coupon	3.50	3.54	3.52	3.30	<2.00
			3.32	2.95	3.08	2.70	<2.00
		NB for cloth	3.29	2.95	2.85	2.78	<2.00
			3.43	2.85	3.00	3.26	<2.00
	2422	NB for coupon	3.29	2.88	2.93	3.23	<2.00
			3.04	3.27	2.79	3.40	<2.00
		NB for cloth	3.45	3.43	2.81	3.36	<2.00
			3.16	3.08	3.02	3.59	<2.00
Trial 2	ATCC 19115	NB for coupon	2.98	2.81	2.93	2.90	<2.00
			3.57	3.53	3.44	3.35	<2.00
		NB for cloth	2.98	2.88	2.95	2.60	<2.00
			3.18	3.40	3.41	3.28	<2.00
	DUP-1030A	NB for coupon	3.35	3.16	3.08	3.51	<2.00
			3.13	3.10	3.00	3.02	<2.00
		NB for cloth	3.36	3.30	3.30	3.50	<2.00
			3.28	3.32	3.34	2.85	<2.00
	DUP-1038	NB for coupon	2.60	2.70	2.65	3.15	<2.00
			2.95	2.60	2.40	2.48	<2.00
		NB for cloth	2.48	2.60	2.85	3.04	<2.00
			2.98	2.70	2.88	2.78	<2.00
	DUP-1044A	NB for coupon	2.98	2.54	2.60	2.54	<2.00
			2.65	2.65	3.37	2.60	<2.00
		NB for cloth	3.22	2.60	2.88	2.60	<2.00
			2.74	2.81	3.18	2.90	<2.00
	2422	NB for coupon	2.90	2.90	2.65	3.08	<2.00
			3.00	3.00	3.04	2.98	<2.00
		NB for	2.85	2.85	2.70	2.85	<2.00

cloth	3.24	3.10	3.19	3.28	<2.00

[b] Salt Ionator means Ionator using 0.1% NaCl (w/v) solution.
[c] Concentration: FAC 100 mg/l.
[d] NB, neutralizing buffer.
[e] Detection limit: 2.00 log CFU/coupon.

C1. Quantitative analysis of the characteristics of the *Listeria monocytogenes* biofilms.

Trial	Strain	Inoculation amount	Tree-like morphology	Individual cells	None
Trial 1	2349	1[a]	25.0	25.0	50.0
			30.0	20.0	50.0
	3528	1	22.2	11.1	66.7
			25.0	12.5	62.5
	ATCC	1	25.0	37.5	37.5
	19115		18.2	18.2	63.6
		0.1	20.0	30.0	50.0
			37.5	12.5	50.0
		0.001	18.2	18.2	63.6
			25.0	12.5	62.5
		1-BHI[b]	42.9	14.3	42.9
			0.0	28.6	71.4
	2422	1	12.5	25.0	62.5
			25.0	25.0	50.0
		0.1	0.0	25.0	75.0
			28.6	28.6	42.9
		0.001	20.0	20.0	60.0
			22.2	33.3	44.4
Trial 2	2349	1[a]	28.6	14.3	57.1
			12.5	0.0	87.5
	3528	1	33.3	33.3	33.3
			20.0	20.0	60.0
	ATCC	1	42.9	0.0	57.1
	19115		33.3	16.7	50.0
		0.1	22.2	11.1	66.7
			12.5	12.5	75.0
		0.001	30.0	10.0	60.0
			25.0	25.0	50.0
		1-BHI[b]	50.0	0.0	50.0
			16.7	16.7	66.7
	2422	1	30.0	10.0	60.0
			11.1	22.2	66.7
		0.1	28.6	42.9	28.6
			14.3	14.3	71.4
		0.001	12.5	12.5	75.0
			20.0	20.0	60.0

[a]: '1' is standard amount inoculation amount with 1:100 of bacterial solution to the growth media of LN-TSB, that is, 1/10 of TSB.

[b]: The LN-TSB in the biofilm growth protocol was substituted by LN-BHI.

C2. Quantitative analysis of the characteristics of the *Escherichia coli* O157:H7 and *Salmonella enterica* biofilms.

Trial	Pathogen	Strain	Tree-like morphology	Individual cells	None
Trial 1	*E. coli* O157:H7	43890	28.5	14.3	57.1
			33.3	33.3	33.3
		43895	28.5	14.3	57.1
			25.0	25.0	50.0
		6058	8.33	50.0	41.7
			11.11	33.3	55.6
		EK-1	20.0	40.0	40.0
			0.0	14.3	85.7
	S. enterica	Typhimurium	0.0	10.0	90.0
		E2009005811	10.0	30.0	60.0
		Typhimurium	16.7	16.7	66.7
		UK-1	0.0	60.0	40.0
		Newport	14.3	42.9	42.9
		B4442	11.1	66.7	22.2
		Saintpaul	16.7	33.3	50.0
		E2008001236	25.0	12.5	62.5
Trial 2	*E. coli* O157:H7	43890	25.0	37.5	37.5
			20.0	40.0	40.0
		43895	14.3	57.1	28.6
			28.6	42.9	28.6
		6058	12.5	37.5	50.0
			0.0	50.0	50.0
		EK-1	11.1	44.4	44.0
			12.5	25.0	62.5
	S. enterica	Typhimurium	11.1	44.4	44.4
		E2009005811	12.5	25.0	62.5
		Typhimurium	14.3	42.9	42.9
		UK-1	14.3	28.6	57.1
		Newport	16.7	33.3	50.0
		B4442	14.3	28.6	57.1
		Saintpaul	14.3	57.1	28.6
		E2008001236	14.3	42.9	42.6

C3. Effect of initial inoculation amount of *Listeria monocytogenes* on the viable microbial cells of biofilms (log CFU/coupon).

Trial	Relative amount	Strain ATCC 19115	Strain 2422
Trial 1	1[A]	6.65	6.42
		6.40	6.49
	0.1	6.55	6.53
		6.76	6.99
	0.001	6.83	6.88
		6.58	6.65
	1[B]	6.83	-
		6.58	-
Trial 2	1[A]	7.69	7.26
		7.49	6.38
	0.1	7.66	7.14
		7.50	6.55
	0.001	7.38	6.43
		7.39	6.33
	1[B]	7.79	-
		7.51	-

Character '-' means not determined.

C4. Effect of treatments on the viable microbial cells of *Listeria monocytogenes* biofilms ATCC 19115 (log CFU/coupon).

Trial	Treatment	Strain ATCC 19115
Trial 1	Initial	7.32
		7.36
	DIW treatment	6.16
		5.82
	NB treatment	5.83
		5.58
	NECAW (250 mg/l FAC) treatment	3.06
		2.30
Trial 2	Initial	6.83
		7.08
	DIW treatment	5.41
		6.44
	NB treatment	5.69
		6.45
	NECAW (250 mg/l FAC) treatment	2.47
		2.00

Note: NB, neutralizing buffer; DIW, deionized water. NECAW, neutral electrochemically activated water; FAC, free available chlorine.

D1. Effect of neutral electrochemically activated water (NECAW, 4 mg/l free available chlorine) treatment on *inl*A or *sig*B gene expression of liquid cultures of *Listeria monocytogenes* in wild type (WT) and *ΔinlA*, *ΔsigB* strains

Trial	**Treatment**		**Gene expression level**			
		Time (min)	***inlA***		***sigB***	
			WT	**Δ*sigB***	**WT**	**Δ*inlA***
1	DIW	0.5	1.118 0.964 0.918	1.129 0.861 1.010	1.114 1.011 0.874	1.048 1.147 0.805
		5	1.058 1.134 0.871	0.983 0.969 1.105	1.381 0.937 0.880	1.255 1.005 0.716
		10	1.166 1.005 0.884	1.031 0.861 1.333	1.307 1.228 0.887	1.092 0.971 1.290
	NECAW	0.5	1.461 1.995 1.146	0.826 1.429 1.479	2.525 1.954 1.219	2.580 1.345 1.997
		5	1.780 1.615 3.208	0.949 1.711 0.873	5.564 2.782 2.841	4.493 2.246 2.563
		10	4.203 3.710 5.565	0.892 2.289 1.596	8.374 2.542 9.423	8.501 3.405 4.340
2	DIW	0.5	1.122 0.954 0.924	0.572 1.067 1.360	0.898 1.145 0.956	1.090 1.252 0.657
		5	0.905 0.944 1.122	0.929 0.755 1.128	1.440 0.937 0.782	1.270 1.024 0.804
		10	1.092 0.967 0.810	1.278 1.097 1.370	1.362 0.983 1.061	0.867 1.399 1.315
	NECAW	0.5	1.420 1.940 1.194	1.510 1.261 1.992	2.631 1.106 1.460	2.487 1.210 2.077
		5	1.411 2.525 3.045	2.876 1.296 1.226	5.410 4.215 1.532	4.361 6.125 1.607
		10	3.990 2.993 5.228	1.687 0.955 2.684	7.757 2.228 5.154	6.843 2.470 3.170

D2. Effect of the concentration of free available chlorine (FAC) in neutral electrochemically activated water (NECAW) treatment on the *inl*A or *sig*B gene expression of liquid cultures of *Listeria monocytogenes* wild type (WT) and *ΔinlA*, *ΔsigB* strains after 5 min incubation using qPCR.

Trial	**FAC (mg/l)**	**Gene expression level**			
		inlA		***sigB***	
		WT	**Δ*sigB***	**WT**	**Δ*inlA***
1	0	1.035	0.978	1.129	1.128
		1.013	0.851	0.997	1.046
		0.952	1.171	0.874	0.826
	2	1.164	0.945	3.022	3.083
		1.764	0.945	1.511	1.193
		1.049	1.997	1.631	1.448
	4	2.988	1.019	5.372	5.480
		1.205	2.510	2.524	2.150
		1.944	1.179	2.371	2.917
	6	2.866	0.384	9.418	8.307
		2.967	3.405	4.775	4.939
		2.098	2.424	3.746	3.192
2	0	0.994	1.044	1.034	1.223
		0.902	0.822	1.020	0.830
		1.103	1.134	0.945	0.947
	2	1.015	2.184	3.269	3.055
		1.684	0.881	1.318	1.065
		1.207	1.084	1.704	1.475
	4	2.852	0.636	4.560	3.012
		2.755	1.982	6.058	2.678
		0.934	2.199	2.926	6.639
	6	2.717	0.944	11.785	3.485
		2.465	1.385	5.059	5.661
		1.989	0.949	3.315	2.413

D3. Effect of neutral electrochemically activated water (NECAW, 4 mg/l free available chlorine) treatment on *inl*A or *sig*B gene expression of biofilms of *Listeria monocytogenes* in wild type (WT) and *ΔinlA*, *ΔsigB* strains determined by qPCR.

Trial	**Treatment**		**Gene expression level**			
		Time (min)	***inlA***		***sigB***	
			WT	**Δ*sigB***	**WT**	**Δ*inlA***
1	DIW	0.5	1.162 0.957 0.881	1.073 0.847 1.080	1.241 0.825 0.934	1.203 0.827 0.970
		5	0.991 0.957 0.788	1.043 0.940 1.073	1.518 0.780 0.909	1.280 0.766 0.893
		10	1.170 0.899 0.810	0.915 1.182 1.396	1.330 0.830 0.947	1.927 0.845 0.963
	NECAW	0.5	1.577 2.154 1.599	0.859 1.455 1.581	4.175 2.269 2.570	2.244 2.213 3.241
		5	5.452 6.219 1.092	0.764 2.533 1.767	12.310 7.119 5.783	11.602 4.647 9.622
		10	9.427 3.802 4.948	0.987 2.753 1.258	12.833 5.586 13.102	4.396 17.104 9.555
2	DIW	0.5	1.075 1.046 0.879	0.758 1.086 1.156	1.270 0.844 0.886	1.165 0.841 0.994
		5	0.745 1.244 1.542	0.946 1.109 1.579	1.244 0.850 0.782	0.895 1.141 0.774
		10	1.469 0.976 1.017	1.007 0.727 1.042	1.342 0.983 0.856	1.415 0.940 0.953
	NECAW	0.5	2.048 2.077 1.734	1.140 1.125 2.249	4.485 2.021 2.107	2.363 2.298 3.607
		5	5.915 4.805 1.552	1.148 0.801 2.769	13.133 6.941 6.042	11.397 2.810 7.065
		10	8.307 2.386 4.212	2.968 1.301 1.132	9.351 6.042 13.409	13.092 8.115 7.065

D4. Effect of the concentration of free available chlorine (FAC) in neutral electrochemically activated water (NECAW) treatment on the *inlA* or *sigB* gene expression of biofilms of *Listeria monocytogenes* wild type (WT) and *ΔinlA*, *ΔsigB* strains after 5 min incubation using qPCR.

Trial	**FAC (mg/l)**	**Gene expression level**			
		inlA		***sigB***	
		WT	**Δ*sigB***	**WT**	**Δ*inlA***
1	0	1.202	0.943	1.165	1.208
		0.911	0.695	0.946	0.825
		0.886	1.362	0.889	0.967
	2	2.138	0.874	4.757	4.898
		1.900	0.777	2.567	2.755
		2.228	1.619	3.117	2.625
	4	3.151	0.924	11.714	13.016
		5.963	1.700	6.499	5.825
		1.967	0.937	6.917	7.322
	6	3.195	0.425	22.011	13.290
		7.140	3.216	11.714	16.939
		5.227	1.822	6.775	6.927
2	0	1.064	0.956	1.315	1.210
		0.795	1.004	0.793	0.827
		1.141	1.039	0.892	0.963
	2	2.394	1.861	5.918	3.353
		2.000	0.766	1.784	3.284
		1.972	1.289	2.523	3.128
	4	5.539	0.597	13.503	12.427
		2.713	2.213	6.476	6.478
		2.345	1.967	5.877	7.138
	6	4.198	0.844	24.678	15.730
		3.010	2.595	8.908	16.743
		7.567	0.585	9.953	6.002

D5. Effect of the concentration of free available chlorine (FAC) in neutral electrochemically activated water (NECAW) on liquid culture viable count of *Listeria monocytogenes* wild type (WT) and mutant *ΔinlA*, *ΔsigB* strains.

Trial	Time (min)	FAC (mg/l)	Viable count reduction[a] (log CFU/ml)			
			WT	**Δ*inlA***	**Δ*sigB***	**Δ*inlA*Δ*sigB***
Trial 1	0.5	0	0.00	0.00	0.00	0.00
			0.00	0.00	0.00	0.00
		2	0.16	0.10	0.09	0.10
			-0.08	0.03	0.15	0.25
		4	0.07	0.10	0.13	0.54
			-0.04	0.04	1.24	0.64
		6	0.18	0.36	0.14	0.85
			0.50	0.96	0.98	1.16
		10	1.89	1.34	1.72	1.80
			1.23	1.45	1.97	2.15
		20	4.28	5.32	≥5.05	≥5.12
			4.87	5.11	≥5.01	≥5.07
	5.0	0	0.00	0.00	0.00	0.00
			0.00	0.00	0.00	0.00
		2	0.11	0.13	0.02	0.25
			-0.03	-0.03	0.27	0.57
		4	0.11	0.16	0.69	1.05
			-0.03	0.02	1.03	0.88
		6	0.30	0.58	0.76	1.04
			0.90	1.01	1.45	1.67
		10	2.00	2.21	2.38	2.61
			1.42	1.45	2.23	3.01
		20	≥5.29	≥5.26	≥5.10	≥5.04
			≥5.33	≥5.31	≥5.13	≥5.01
	10.0	0	0.00	0.00	0.00	0.00
			0.00	0.00	0.00	0.00
		2	0.08	0.20	0.27	0.56
			-0.06	0.09	0.50	0.63
		4	0.12	0.26	1.39	2.66
			-0.02	0.16	1.44	1.93
		6	0.35	0.77	2.65	3.12
			0.93	1.25	2.20	2.40
		10	2.16	2.98	2.70	3.67
			1.81	3.10	3.16	3.28
		20	≥5.30	≥5.31	≥5.07	≥5.17
			≥5.31	≥5.27	≥5.03	≥5.12

Trial 2	0.5	0	0.00	0.00	0.00	0.00
			0.00	0.00	0.00	0.00
		2	-0.03	0.05	0.04	0.06
			0.13	0.02	0.15	0.38
		4	0.03	0.09	0.12	0.36
			0.03	0.05	0.27	0.68
		6	0.06	0.32	0.58	0.95
			0.06	0.84	1.57	0.52
		10	0.95	1.91	1.60	1.76
			1.08	1.27	2.13	2.30
		20	3.95	5.25	≥5.21	≥5.01
			4.61	4.26	≥5.26	≥4.98
	5.0	0	0.00	0.00	0.00	0.00
			0.00	0.00	0.00	0.00
		2	0.11	0.18	0.24	0.29
			-0.11	-0.10	0.23	0.54
		4	0.12	0.21	0.80	1.53
			-0.09	-0.08	1.35	0.81
		6	0.34	0.48	1.09	1.07
			0.27	1.07	1.63	1.49
		10	1.96	2.15	2.57	2.50
			1.32	1.47	2.20	3.17
		20	≥5.23	≥5.18	≥5.00	≥5.13
			≥5.24	≥5.12	≥5.23	≥4.97
	10.0	0	0.00	0.00	0.00	0.00
			0.00	0.00	0.00	0.00
		2	0.16	0.24	0.54	0.58
			-0.05	-0.10	0.40	0.71
		4	0.27	0.33	1.37	1.99
			0.05	-0.02	2.12	2.10
		6	0.44	0.60	1.65	2.17
			0.45	1.11	2.42	2.10
		10	2.26	2.87	3.50	3.94
			1.71	2.13	3.25	3.64
		20	≥5.26	≥5.12	≥5.21	≥5.01
			≥5.23	≥5.07	≥5.26	≥4.98

D6. Effect of the concentration of free available chlorine (FAC) in neutral electrochemically activated water (NECAW) *Listeria monocytogenes* viable count of wild type (WT) and mutant *ΔinlA*, *ΔsigB* strains biofilms on stainless steel coupons treated for one minute.

Trial	**FAC (mg/l)**	**Viable count reduction after 1 min[a] (log CFU/coupon)**			
		WT	**Δ*inlA***	**Δ*sigB***	**Δ*inlA*Δ*sigB***
Trial 1	0	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00
	2	0.01	0.03	0.07	1.09
		0.13	0.02	-0.03	-0.04
	4	0.05	0.08	0.11	0.08
		-0.03	0.02	0.03	0.62
	6	0.41	0.47	0.78	0.83
		-0.15	-0.11	-0.14	-0.12
	10	0.68	0.70	0.76	0.71
		-0.01	0.11	0.15	0.14
	20	0.95	2.00	2.00	1.90
		0.32	0.07	0.09	0.10
	50	-0.37	-0.32	-0.10	-0.06
		1.08	1.04	1.06	0.96
	100	1.13	1.18	1.27	1.33
		0.74	-0.08	0.00	-0.00
Trial 2	0	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00
	2	-0.03	0.15	0.34	0.51
		-0.23	-0.21	-0.33	-0.88
	4	0.38	0.54	0.78	1.01
		-0.01	-0.02	-0.08	-0.62
	6	0.31	0.51	0.53	0.53
		0.68	0.56	0.49	0.31
	10	0.41	0.42	0.45	0.58
		0.45	0.51	0.63	0.54
	20	1.01	1.31	1.41	1.35
		0.66	0.68	0.63	0.58
	50	1.24	1.76	2.03	2.38
		1.10	1.10	0.94	0.81
	100	1.73	2.05	2.07	2.12
		0.97	2.08	2.01	1.76

D7. Effect of the concentration of free available chlorine (FAC) in neutral electrochemically activated water (NECAW) *Listeria monocytogenes* viable count of wild type (WT) and mutant *ΔinlA*, *ΔsigB* strains biofilms on stainless steel coupons treated for five minutes.

Trial	**FAC (mg/l)**	**Viable count reduction after 1 min[a] (log CFU/coupon)**			
		WT	**Δ*inlA***	**Δ*sigB***	**Δ*inlA*Δ*sigB***
Trial 1	0	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00
	2	0.37	0.39	0.56	0.64
		0.19	0.14	0.23	0.21
	4	0.10	0.13	1.17	1.22
		0.18	0.20	0.49	0.25
	6	0.61	0.64	1.05	1.11
		0.00	-0.02	0.20	0.13
	10	0.63	0.65	1.08	1.18
		0.30	0.27	0.61	0.46
	20	1.18	1.10	1.19	1.51
		0.73	0.74	1.13	1.14
	35	1.39	1.61	1.69	3.54
		1.21	1.39	2.21	2.25
	50	1.67	1.73	2.20	≥4.94
		2.16	2.47	3.07	≥4.57
	75	2.31	2.53	3.48	≥4.94
		2.90	2.96	3.52	≥4.57
	100	3.41	3.50	≥4.66	≥4.94
		2.69	3.41	≥4.91	≥4.57
Trial 2	0	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00
	2	0.15	0.07	0.00	-0.13
		0.16	0.20	0.48	0.42
	4	0.65	0.59	1.03	0.88
		-0.67	-0.54	-0.48	-0.42
	6	0.65	0.40	0.72	0.89
		-0.47	-0.36	-0.18	-0.27
	10	0.70	0.63	0.92	1.00
		-0.15	-0.11	0.37	0.53
	20	1.17	1.11	1.72	1.57
		0.10	0.19	0.43	0.50
	35	1.64	0.55	0.83	2.57
		0.23	0.72	1.00	0.53
	50	2.20	0.60	1.02	≥4.58

	0.43	1.42	1.78	≥4.40
75	2.52	1.63	3.32	≥4.58
	1.46	1.83	2.29	≥4.40
100	3.22	2.82	≥4.68	≥4.58
	2.70	2.64	≥4.68	≥4.40

D8. Effect of the concentration of free available chlorine (FAC) in neutral electrochemically activated water (NECAW) *Listeria monocytogenes* viable count of wild type (WT) and mutant *ΔinlA*, *ΔsigB* strains biofilms on stainless steel coupons treated for ten minutes.

Trial	**FAC (mg/l)**	**Viable count reduction after 1 min[a] (log CFU/coupon)**			
		WT	**Δ*inlA***	**Δ*sigB***	**Δ*inlA*Δ*sigB***
Trial 1	0	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00
	2	0.23	0.25	0.31	0.45
		-0.10	-0.14	-0.16	-0.16
	4	-0.02	0.02	-0.06	0.06
		-0.12	-0.19	0.85	0.94
	6	1.08	1.15	1.20	1.34
		0.39	0.46	0.73	0.77
	10	0.25	0.35	0.86	0.80
		0.54	0.84	0.84	2.59
	20	1.29	1.43	1.91	2.06
		0.58	-0.36	-0.17	-0.21
	35	0.91	0.61	3.04	3.14
		1.88	0.89	2.58	0.42
	50	1.56	1.88	≥4.66	≥4.94
		2.01	2.19	≥4.91	≥4.57
	75	2.18	3.32	≥4.66	≥4.94
		2.05	3.15	≥4.91	≥4.57
	100	2.95	3.18	≥4.66	≥4.94
		3.34	4.18	≥4.91	≥4.57
Trial 2	0	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00
	2	0.14	0.07	0.03	0.18
		0.09	0.19	0.35	0.41
	4	0.65	0.67	1.05	1.17
		0.63	0.66	1.22	1.36
	6	0.87	0.91	1.04	1.44
		0.54	0.61	1.03	0.83
	10	1.07	1.12	1.06	1.17
		1.98	2.26	3.03	3.17
	20	1.23	2.17	2.77	2.71
		1.35	1.40	1.47	1.65
	35	1.36	2.47	2.55	3.26
		2.80	3.43	3.72	3.56
	50	2.83	3.22	≥4.68	≥4.58

	3.14	3.45	≥4.68	≥4.40
75	3.41	3.51	≥4.68	≥4.58
	3.43	3.56	≥4.68	≥4.40
100	2.83	4.28	≥4.68	≥4.58
	3.52	3.60	≥4.68	≥4.40