REDUCTION OF *SALMONELLA, ESCHERICHIA COLI* O157:H7, AND *LISTERIA MONOCYTOGENES* ON THE SURFACE OF LEAFY GREENS AND GREEN ONIONS USING INNOVATIVE HOME WASHING TECHNOLOGIES

by

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(Under the Direction of Joseph F. Frank)

ABSTRACT

Recent disease outbreaks linked to fresh produce attracted consumer's attention regarding interventions that minimize microbiological risk at home. This study compared the efficacy of various washing technologies in reducing pathogens on lettuce, spinach and green onions. Trimmed samples were inoculated with *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* and then subjected to the following procedures: (1) rinse for 15 seconds under running tap water, (2) immersion for 2 minutes in household chlorine bleach, Veggie Wash®, ozonated water and electrolyzed oxidizing (EO) water. Veggie Wash® provided the lowest antimicrobial effect, resulting in < 1 log reduction of the tested pathogens. Ozonated water produced significantly greater pathogen reduction on green onions, but was not able to further reduce pathogens on leafy vegetables as compared to water rinse. Chlorine based technologies (bleach and EO water) produced equal or greater pathogen reduction than other treatments, but exhibited minimal antimicrobial effect when tested on spinach.

INDEX WORDS: Decontamination, Lettuce, Spinach, Green Onion, *Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, Chlorine bleach, Veggie Wash, Ozonated water, Electrolyzed oxidizing water

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CHAPTER 1

INTRODUCTION

Foodborne illnesses associated with the consumption of fresh uncooked produce are on the rise. Fresh vegetables, primarily those of leafy nature, have been implicated as primary vectors for the transmission of foodborne disease worldwide *(59)*. Lettuce and spinach are of primary concerns since they have been linked to several recent multi-state outbreaks *(4, 41, 46, 60, 66, 74, 76, 88, 134-135, 145, 149, 162, 188, 194)*, including the large spinach-*E.coli* O157:H7 outbreak in 2006 that led to at least 276 consumer illnesses and 3 deaths *(41)*. The consumption of green onions has been associated with lower microbiological risks *(121)*, but an increase has been reported, as evidenced by the recent Hepatitis A outbreak in USA associated with imported Mexico green onions *(39)*. The leading pathogens of primary concern are norovirus, *Salmonella*, *Escherichia coli* and *Listeria monocytogenes (72)*.

In light of the increasing disease outbreaks linked to vegetables, consumers need more practical information on interventions to minimize microbial hazard caused by pathogenic bacteria. Various food grade sanitizers including: sodium hypochlorite *(19, 22, 61, 92)*, chlorine dioxide *(79, 99, 111, 116, 118)*, hydrogen peroxide *(115)*, and organic acids *(6, 22)* have been investigated for their antimicrobial potential. Although these antimicrobial methods are capable of reducing or inactivating pathogens on the surfaces of fruits and vegetables, their use at home is limited by the potential adverse effect on sensory and quality characteristics of foods and hazards associated with handling of these chemicals.

Currently, water rinse is the most commonly used method for washing fresh produce at home, but immersing or rinsing with tap water has limited efficacy, typically reducing pathogens on lettuce by less than 1.5 log CFU/g *(95, 109, 159, 182)*. In addition, the resulted water rinse solution was not able to destroy pathogen and therefore become a source of cross-contamination. Commercial produce wash products, such as Veggie Wash®, claim to be able to remove wax, dirt, soil, and pesticide residues, and claim to have greater antimicrobial effect than water. However, Kilonzo-Nthenge *et al. (95)* demonstrated that there is no significant difference between reduction of *Listeria innocua* on lettuce after 15 s rinse in running tap water (1.4 log CFU/g) and 2 min immersion in Veggie Wash® followed by 15 s water rinse (1.7 log CFU/log).

Novel washing technologies, such as aqueous ozone, may be a promising alternative to chemical sanitizers or water wash, and are now commercially available at household level. Ozonated water was capable of killing a broad spectrum of microorganisms including many that are resistant to chlorine *(65)*, and spontaneously decomposes to nontoxic product after washing treatments. Rodgers *et al*. reported that ozone at 3 ppm was more effective than chlorine dioxide (3 to 5 ppm), chlorinated trisodium phosphate (100 to 200 ppm), and peroxyacetic acid (80 ppm) at reducing populations of *E. coli* O157:H7 and *L. monocytogenes (147)*.

More recently, a consumer-size generator for electrolyzed oxidizing (EO) water was introduced as a potent antimicrobial technology for food preparation at home. EO water is produced by electrolysis of a 0.1% sodium chloride solution and has characteristics of low pH (approximately 2.5), high oxidation-reduction potential (> 1,100 mV), and chlorine-based reactants (10 to 100 ppm) *(92)*. The effect of EO water in reducing pathogenic microorganisms on fresh produce has been investigated. Izumi *(81)* reported that mesophilic aerobic microorganisms on fresh-cut produce (carrots, bell peppers, spinach, Japanese radish, and

potatoes) were reduced by 0.6 to 2.6 log CFU/g after treatment with EO water containing 20 ppm available chlorine. When EO water was compared with acidified chlorinated water for treating lettuce *(139),* no significant difference in pathogen reduction (2.41 and 2.65 log CFU per leaf) was found under equivalent pH (2.5), ORP (1,130 mV) and residual chlorine concentration (45 ppm). In a separate study, EO water (pH 2.6, 30 ppm of available chlorine) showed a significantly higher bactericidal effect than did ozonated water (5 ppm ozone, pH 6.6), but was as effective as sodium hypochlorite solution (pH 9.3, 150 ppm of available chlorine) *(105).*

Most of the previous studies evaluating pathogen reduction by EO water were carried out in laboratory or industry scenarios. This study was conducted to evaluate application of washing technologies under simulated home washing conditions. The efficacy of water rinse, household chlorine bleach, Veggie Wash, ozonated water and electrolyzed oxidizing water were determined and compared for their ability to reduce populations of *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* inoculated on the surfaces of lettuce, spinach and green onions. Since the quality of incoming water may affect the efficacy of treatment solution, the performance of aforementioned washing technologies with different incoming water quality (pH and degree of hardness) was also identified prior to the comparative efficacy study.

CHAPTER 2

LITERATURE REVIEW

Consumption and Production of Fresh Produce

It is widely recognized that consumption of fresh produce is critical to a healthy diet. Fresh produce vary considerably in the nutritional content, but generally are good sources of vitamins, dietary minerals, fiber, as well as a great variety of other beneficial phytochemicals *(177)*. Fruits and vegetables also add variety and pleasure to the diet mainly because of their pleasing colors, aromas, textures, and flavors.

Driven by these health benefits, consumer demand for fresh produce with improved quality and safety, increased variety and year-around availability has been on the rise *(90)*. For example, human fruit and vegetable consumption increased by an average of 4.5% per annum between 1990 and 2004 worldwide *(56)*. This increasing consumption provided the fresh produce industry with tremendous business opportunities. In the United Sates, the estimated total sales of fresh produce (fresh-cut and bulk) via retail and foodservice channels surpassed $81 billion in 2002, up from $70.8 billion in 1997 and $34.6 billion in 1987 *(44, 90)*.

Within the total fresh produce market, packaged and fresh-cut produce has become one of the fastest-growing segments over the past ten years *(59)*. This burst is mainly due to the growing demand for convenience in food preparation and consumption. Compared to $3.3 billion in sales in 1994, fresh-cut fruit and vegetable sales have grown to approximately $12 billion per year in the North American foodservice and retail market and account for nearly 15% of all

produce sales within the past decade *(34)*. Fresh-cut produce sales are even higher in Europe, and beginning to develop in Latin America and Asia as well *(44)*. Therefore, it is anticipated that the consumption and thus the production of fresh produce will continue to increase in the future.

Disease Outbreaks Linked to Fresh Produce

Fresh Produce of Concern

As the fresh produce market continues to grow, there are new challenges that require attention, namely the microbiological hazard caused by foodborne pathogenic microorganisms. Fresh vegetables, primarily those of a leafy nature, have been linked to several large outbreaks *(21, 59)*, and the characteristics of which have been described extensively by researchers. Herman *et al*. *(72)* analyzed data from the CDC foodborne disease outbreak surveillance system reported between 1973 and 2006. Of the 10,421 foodborne disease outbreaks reported, 4.8% outbreaks, 6.5% illness, and 4.0% deaths were associated with leafy greens. Higher incidence was documented when number of leafy greens-associated outbreaks was compared with the total fresh produce outbreaks during the period 1998–2005, accounting for 70% of the latter *(59)*.

In consideration of large volumes of cultivation and production, complex and diverse post-harvest handling steps, and the economic impact to fresh produce industry, lettuce is probably the most important leafy vegetables of concern. Sivapalasingam *et al*. (2004) detailed that among 190 produce-associated outbreaks reported by 32 states in the U.S. from 1973 to 1997, 25 of which were associated with lettuce causing 2,078 reported illnesses, 181 hospitalizations, and six deaths *(160)*. Lettuce (shredded, salad, iceberg, romaine) has been implicated in outbreaks of *E. coli* O157:H7 *(4)*, *Salmonella (160)*, *Listeria monocytogenes (75)*, *Campylobacter jejuni (37)*, *Shigella sonnei (18, 47)*, Norovirus *(160)*, Hepatitis A *(36, 117, 149)*

and *Cyclospora (73)*. Spinach has drawn worldwide attention to its potential microbiological hazard through several severe outbreaks, such as the large *E. coli* O157:H7 outbreak in 2006 that led to at least 276 consumer illnesses and 3 deaths *(41)*.

Green onions has been associated with lower risks of foodborne infectious diseases *(78)* and their production is relatively small compared to that of leafy vegetables *(59)*, nevertheless, they are widely used as minor components of a meal and an increase has been observed in the frequency of outbreaks linked to this commodity. During the past decade, the consumption of green onions has been associated with Hepatitis A virus *(39, 178)*, *E. coli* O157:H7 *(27)*, *Shigella flexneri 6A (169)*.

Microbiological safety issues associated with other vegetables and all varieties of fruits have also been recognized, a comprehensive review of which is yet beyond the scope of this review. Further information is directed to the following sources: cabbage *(154, 183)*, watercress *(119)*, parsley *(130)*, tomatoes *(40, 68)*, cantaloupe *(38, 128)*, berries *(59)*, sprouted seeds *(129)*, carrot *(82, 87)*, celery *(58, 186)*.

Foodborne Microorganisms

Foodborne disease outbreaks related to fresh produce include cases of bacteria, viral pathogens, protozoan parasites and a variety of other foodborne pathogens *(53)*. Among the greatest concerns of the bacterial pathogens are *E.coli* O157:H7, *Salmonella* and *Listeria monocytogenes (122)*. In this section, the characteristics of these three bacteria were described and their incidence in fresh produce related outbreaks of foodborne disease was reviewed in greater details.

1. Salmonella spp.

The *Salmonella* genus consists of a large and diverse group of facultatively anaerobic gram-negative rod-shaped bacteria. Through the development of taxonomic systems based on biochemical traits and genomic relatedness, the following nomenclature is now widely accepted by academia: the genus *Salmonella* consists of two species (*S. enterica* and *S. bongori*), each of which includes several serovars *(53)*. Optimum growth occurs at neutral pH and temperatures between 35 and 37^{o}C, while a condition of pH < 3.8 and > 9.0, temperature < 7^{o}C, or water activity < 0.94 results in complete inhibition of growth *(84)*. A population of < 10 cells is sufficient to cause disease symptoms, primarily gastroenteritis followed by abdominal cramps and diarrhea *(52)*.

Although natural reservoirs of *Salmonella* is the intestinal tract of birds, reptiles, amphibians and mammals, it has been identified as the leading cause of bacterial infection associated with fresh produce-related outbreaks, accounting for 48% of these bacterial-related infectious cases from 1973 to 1997 in the USA *(160)*. Cantaloupe and tomatoes are among the most commonly identified produce commodities causing human Salmonellosis *(20)*. Three high-profile outbreaks of *Salmonella* serotype Poona during 2000 – 2002 was traced back to cantaloupe imported from Mexico, resulting in 58 cases of infection *(38)*. More recently, two major tomato-related *Salmonella* outbreaks occurred in the USA, accounting for 23.2% of reported *Salmonella* cases in 2006 *(42)*.

2. *Escherichia coli* O157:H7

E. coli O157:H7, a gram-negative, rod-shaped, facultative anaerobic bacterium, is the most predominant serotype of the Enterohemorrhagic *E. coli* that causes several life-threatening

infections such as hemorrhagic colitis, hemolytic uremic syndrome, and thrombocytopenic purpura at low dose *(53)*. Leafy vegetables, particularly lettuce and spinach have been extensively reported in several large outbreaks of *E. coli* O157:H7 infection in the United States *(54)*. Due to its presence in animal manures and slurries, *E. coli* O157:H7 may contaminate fresh produce via livestock's entry into field, or improperly composted manure applied as fertilizer. For example, *E. coli* O157:H7-related infectious cases in Montana in 1995 were identified on lettuce that was grown downhill from a cattle pasture *(184)*.

3. *Listeria monocytogenes*

Listeria monocytogenes, a gram-positive, rod-shaped bacterium, belongs to one of six species in the genus *Listeria* and consists of 13 serotypes of which more than 90% of human isolates belong to three serotypes: 1/2a, 1/2b, and 4b *(53)*. *L. monocytogenes* is a typical pathogen of concern because it is widely distributed and can persist under diverse stress conditions including low pH, relatively high sodium chloride (NaCl) concentrations and low temperature (2 to 4^{o}C) *(83)*. Success of survival of the pathogen in food and food-related environments presents a major public health concern. Listeriosis is a rare but potentially lethal infection in immunocompromised individuals due to the severity of the disease (abortion, meningitis, septiemia) and a high case fatality rate (approximately 20 – 30%) *(53)*.

Although food commodities from which *L. monocytogenes* has been isolated are predominantly raw and ready-to-eat meat products *(70)*, it has also been identified in outbreaks of fresh produce items such as lettuce *(12, 23)*, cabbage *(71, 143)*, bean sprouts *(12)*. Due to its nature of being a soil bacterium, *L. monocytogenes* was more prevalent on sprouted seeds or other root vegetables than high-grow leafy vegetables *(45)*. An investigation in Malaysia in 1994 revealed that 85% of bean sprouts and 22% of leafy vegetable samples were positive for *L.*

monocytogenes. Similarly, Thunberg et al. (2002) tested a range of fresh produce and only isolated *L. monocytogenes* from potatoes (50%) and field cress (18%) purchased at farmers' markets *(171)*.

Ecology of Pathogens in Fresh Produce

To prevent microbial contamination of fresh produce, we must first be able to answer some fundamental questions: how do human pathogens make their way into fresh produce? What environmental conditions make their survival and multiplication possible? In this section, these questions were addressed and a comprehensive picture of bacterial contamination of produce was offered.

Sources of Contamination

Contamination of raw fruits and vegetables with human pathogens can occur during production, harvest, processing, transport, retail and foodservice, and in the home kitchen (Table 1). When in field, animal feces have shown to be the primary source of contamination *(127, 184)*. In postharvest operations, microbiological risks rise up from concern came from considerable contacts between fresh produce and workers, poorly sanitized tools and equipment surfaces and contaminated water for washing or cooling. After introduction, pathogens are able to infiltrate and well established within produce long before sanitizers are applied.

Poor hygiene practice of plant workers has been proposed to be a significant source of contamination *(48)*. One investigation showed that an outbreak of Hepatitis A virus was traced back to an infected food handler shredding lettuce by hand *(117)*. Poorly cleaned and maintained equipment may also serve as reservoirs of contamination and provide ideal site for biofilm

formation which protects them from being removed or inactivated *(163)*. Water is used widely for harvesting (maintaining hydration), cleaning (produce, equipment and surfaces), transport, cooling and packing. If wash water was contaminated via workers or facility surfaces, it may represent a problem of cross-contamination. Although chlorine and other wash water disinfectants are used by industry to prevent this problem, bacterial pathogens such as *L. monocytogenes*, have been detected in unchlorinated wash water *(143).*

TABLE 1. Routes of contamination on fresh produce [a]

Sources of Contamination	Reference
Pre-Harvest Soil Irrigation water Improperly composted manure used as fertilizer Wild and domestic animals	*(21, 49, 91, 127, 155, 164, 166)*
Harvest & Processing Harvesting equipment transport bins, conveyor belts, crates Sorting, packing, cutting and further-processing equipment Wash and rinse water Cooling Medium (ice or water) Field and factory workers	*(29, 49, 59, 64, 83, 85-86, 122, 165)*
Storage and Distribution Transport vehicles Improper storage environments Improper display conditions Improper consumer handling Cross contamination by other foods in storage, preparation and display areas	*(2, 85, 137)*

[a] Adapted from *(174)*

Survival and Proliferation of Pathogens on Produce

Prior to harvest, bacteria must be able to cope with a range of environmental stresses that are subject to intense fluctuations, including ultraviolet radiation, desiccation, osmotic stress and temperature *(13, 54)*. In response, bacteria localize and aggregate at sites that provide more nutrient availability to support bacterial growth, such as the base of trichromes, substomatal cavities and cracks in veins and cuticles *(125-126)*. But generally, pathogens will not proliferate on the uninjured outer surface of fresh fruits or vegetables, mainly due to lack of nutrients and water which are protected and retained by the plant's natural barriers (cell walls and wax layers).

Postharvest operations differ from in-farm environments in that these postharvest activities would cause high extent of mechanical injuries to produce tissues via cutting, shredding, dicing or peeling. Bacteria seem to attach preferentially at cut surfaces *(114, 156, 168)* or in punctures or cracks *(33)* that release nutrients essential for their proliferation, although attachment to intact surfaces (pores, indentations or other natural irregularities) has also been reported *(156)*. As cells start to grow, newly formed cells produce microcolonies and biofilms embedded in a polysaccharide polymer matrix that protect cells from bactericidal agents and retain water and nutrients for microbial reproduction *(13, 35, 43, 57, 105, 127)*. Once the bacteria have colonized these niches, they are very difficult to kill or remove by washing treatments *(43, 152)*.

Internalization of bacterial cells into the produce tissues during postharvest has been recently recognized *(16-17, 31)*. When the water used for processing is colder than the commodity, the resulting negative temperature differential causes the contraction of the tissue which can draw human pathogens though pores, channels, or damaged/cut surface *(151)*. Besides the improper temperature maintenance, vacuum cooling has also been found to provide a

significant opportunity for pathogen internalization. A mechanism for this phenomenon was proposed, suggesting that the strong pressures of vacuum cooling possibly disturb the structure of the lettuce tissue, such as the stomata, and thus create openings for pathogen internalization *(113)*.

Subsequent growth of human pathogens during storage and distribution depends on several factors, such as temperature, relative humidity, nutrient availability and competition with indigenous microflora *(54)*. Among the most significant concerns is temperature abuse since pathogens with low infectious dose may amplify under refrigeration conditions to a population that was sufficient to trigger infection *(23)*. Growth of *L. monocytogenes* at 3–5°C in refrigerated fresh-cut packaged leafy vegetables has been demonstrated *(131)*.

Home Washing Methods for Fresh Produce

Although fresh produce industry implemented intensive sanitizing interventions to minimize levels of contamination, recent disease outbreaks emphasized the importance of consumer handling of fresh produce at home *(63)*. In this section, several conventional and novel washing technologies for washing fresh produce are discussed, with respect to their mode of action, efficacy against human pathogens, advantages and limitations.

Consumer Attitudes toward Washing Produce

Studies of consumer behavior indicate they may not wash produce adequately because they believe produce is pre-washed thoroughly or because the rind or skin is not consumed *(112)*. Similarly, consumers do not properly clean their hands, food preparation surfaces and knives before and during food preparation *(11, 30)*. Some population groups are more likely to practice

unsafe produce handling practices, namely people over 45 years, women, non-college graduates, lower-income households *(10, 112, 190)*.

Consumers use different washing procedure to reduce the microbiological risk associated with their foods, such as peeling, rubbing with hands, scrubbing with a brush, and washing under running tap water *(112)*. Among the most commonly used methods is rinsing under running water, however the efficacy of which has shown to be limited. Kilonzo-Nthenge *et al*. *(95)* demonstrated that a 1.4 log CFU/g *Listeria innocua* reduction on lettuce was obtained by running tap water for 15 s. Some consumers (approximately 20 percent) immerse produce in water *(112)*, nevertheless, it resulted in a lower microbial reduction than that achieved by running tap water rinse, typically less than 1 log CFU/g *(109, 159, 182)*.

Household Chlorine Bleach

Sodium hypochlorite (NaClO), the active compound in household bleach, is the most common chlorine based derivatives for washing fresh produce, with free chlorine concentration of 50 – 200 ppm frequently being used in industry *(43)*. When hypochlorites are in the aqueous form, it is the resulting hypochlorous acid (HOCl) that has the most biocidal activity *(133)* as shown below. HClO may further dissociate to produce hydrogen ion (H^+) and hypochlorite ion (ClO^-) when pH increases above 8 *(26)*.

$$NaClO + H_2O \rightarrow HOCl + NaOH$$

$$HOCl \rightarrow H^+ + ClO^-$$

Several mechanisms of how HOCl destroys microorganisms have been elucidated. Hypochlorous acid is generally considered to be a highly destructive, nonselective oxidant which reacts with a variety of subcellular compounds and inhibits essential cytoplasmic metabolic

processes *(8-9, 123)*. Inhibition of glucose oxidation has been proposed to be a major factor, as evidenced by the observation that HClO oxidizes the sulfhydryl groups of certain vital enzymes important in carbohydrate metabolism *(101)*. Other mechanisms of bactericidal action of HClO include: post-translational modification of protein *(191)*, oxidative damage to amino acids *(80)*, depletion of adenine nucleotides *(15)*, inhibition of DNA replication *(148)*, and chromosomal aberration *(120)*.

The antimicrobial activity of hypochlorite depends on the amount of hypochlorous acid formed. This, in turn, depends on the pH of the water, the amount of organic material in the water and, to some degree, the temperature of the water *(26)*. The optimum pH of hypochlorite solution used for disinfection was observed at 6.0 – 7.5 *(133)*. With proper pH control, hypochlorite solution is effective in preventing cross-contamination through wash solutions during processing and to retard spoilage.

However, extensive studies showed that chlorine has limited efficacy for killing or removing pathogens that were within the produce, resulting in only 1- to 2-log reduction in bacterial population *(5, 19, 28, 50, 61-62, 109, 153, 187, 195-196)* depending on treatment conditions. Organic material in chlorine solution has the most significant detrimental effect on the capacity of chlorine in reducing pathogens. It was found that damaged tissues of shredded produce may release juices that contain organic matter. Beuchat *et al*. *(22)* reported the highest reductions in free chlorine concentration in solutions used to treat shredded lettuce as compared to treatment of unshredded lettuce pieces. Rodgers *et al*. *(147)* confirmed this phenomenon by the comparison of whole and shredded produce: *L .monocytogenes* and *E. coli* O157:H7 were not detectable on whole apples and lettuce after treatment with 100 ppm chlorine for 5 min, while 1 log CFU/g of these pathogens remained on sliced apples and shredded lettuce.

The efficacy of chlorine treatments was also affected by the type of produce, namely the characteristics of the produce surface *(63)*. Micro-niches (cracks, crevices and cut tissues) that pathogens tend to hide and colonize, as well as hydrophobic nature of the waxy cuticle of much fresh produce prevent contact of chlorine solutions with microorganisms on the produce, which made subsequent proliferation of pathogens during storage possible.

Electrolyzed Oxidizing Water

Electrolyzed oxidizing (EO) water is a special case of chlorination *(81)* in that the antimicrobially active compound is generated directly in the water. Application of EO water in food sanitation is a relatively new concept *(158)* but has received attention from both consumers and industry. Major advantages of using EO Water over sodium hypochlorite are: (1) it has demonstrated a greater effectiveness than most commonly used washing technologies against food borne pathogens on a variety of food commodities *(80)*, (2) there is no need for handling or storage of potentially dangerous sodium hypochlorite in liquid or solid form since it is produced by simple electrolysis using pure water and table salt with no added chemicals *(97)*, (3) it leaves less residual chlorine than does hypochlorite solution and thus may potentially be more environment- and operator-friendly *(7)*.

Electrolyzed oxidizing water, along with electrolyzed reducing (ER) water, is generated by electrochemical disassociation of diluted salt solution (0.1% of NaCl) between anode and cathode electrodes separated by a membrane within an electrolytic chamber (Figure 1). Solution collected from the anode side are EO water, which posses at least three antimicrobial properties, including low pH (approximately 2.5), high oxidation-reduction potential (ORP) (> 1,100 mV), and chlorine-based reactants (10 to 100 ppm) *(139)*.

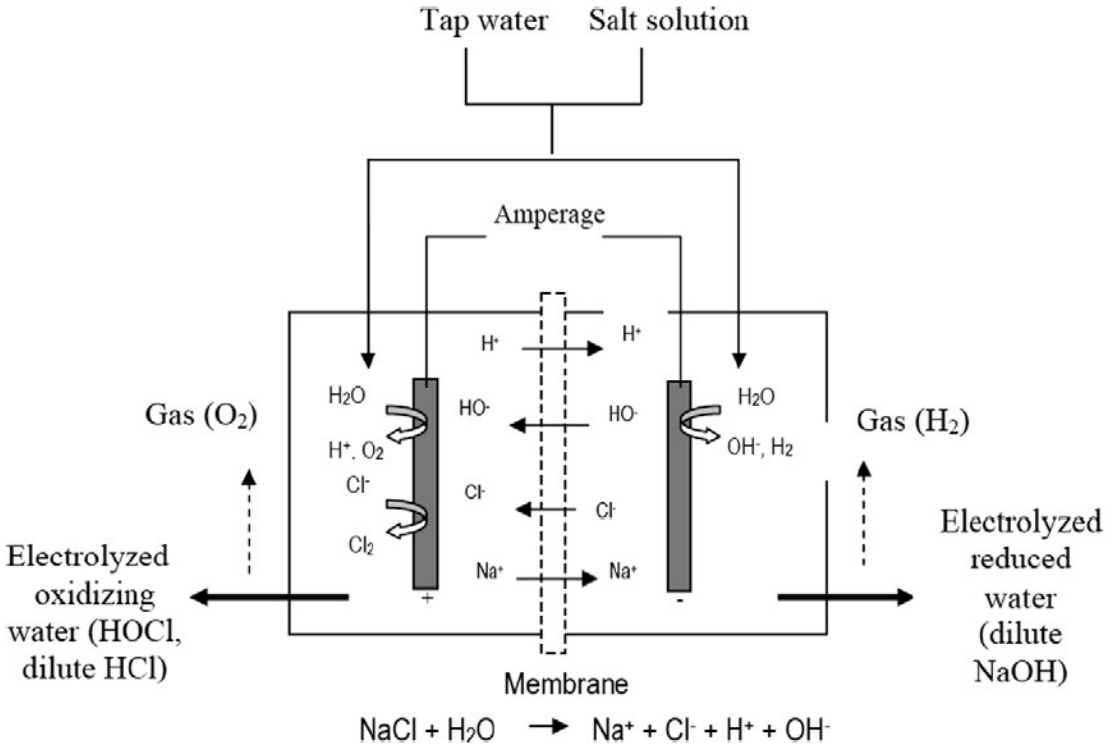


FIGURE 1. Principle of acidic electrolyzed water: process flow of apparatus and chemical reaction during electrolysis (adapted from Huang 2008) *(80)*

Since EO water has the same active antimicrobial compound as that of chlorine bleach, namely hypochlorous acid (HClO), its superior in efficacy over hypochlorite is most likely attributed to low pH and high ORP value. While low pH drives the chlorine equilibrium in water to form more HClO, it also sensitized the outer membrane to the entry of HClO into the intercellular space of bacterial cells *(80)*. The high ORP of EO water interrupts the electron flow in bacterial cells, thus causing disruption of metabolic fluxes and ATP production *(124)*. However, agreement on the role of ORP in killing microorganisms is not made by scientists. Kim *et al*. *(96)* suggested that ORP of EO water might be the primary factor responsible for the

bactericidal effect, while Koseki *et al. (105)* contracts this study by the observation that the higher ORP of ozonated water did not show higher disinfectant effect than lower ORP of EO water.

The efficacy of electrolyzed oxidizing water for reducing or inactivating microorganisms has been investigated on a wide variety of fresh produce commodities including lettuce *(51, 67, 92, 103-105, 107-108, 139-140, 180, 192),* spinach *(67, 81, 140),* tomato *(1, 14),* cucumber *(106),* strawberries *(106),* bell pepper *(81),* cilantro *(185),* carrot *(81),* and Japanese radish *(81).*

The results of electrolyzed water treatments have been mixed. Venkitanarayanan *et al. (181)* conducted pure culture studies to investigate the efficacy of EO water for inactivating *E. coli* O157:H7, *Salmonella enteritidis,* and *L. monocytogenes* incubated at different times and temperatures. They demonstrated that an exposure time of 5 min reduced the populations of all three pathogens in the treatment samples by approximately 7 log CFU/ml at 4^{o}C. Similarly, a reduction of$\geq$ 7 log CFU/ml in the levels of the three pathogens occurred in the treatment samples incubated for 1 min at 45^{o}C or for 2 min at 35^{o}C.

Although EO water provides a great inactivation for pure culture scenario, its efficacy is reduced when EO water is evaluated on food commodities. Izumi *(81)* reported that mesophilic aerobic microorganisms on fresh-cut produce (carrots, bell peppers, spinach, Japanese radish, and potatoes) were reduced by 0.6 to 2.6 log CFU/g after treatment with EO water containing 20 ppm available chlorine. Later, EO water was compared with acidified chlorinated water for treating lettuce *(139).* No significant difference ($P > 0.05$) in pathogen reduction (2.41 and 2.65 log CFU per leaf) was found between EO water and treatment using chlorinated water of equivalent pH (2.5), ORP (1,130 mV) and residual chlorine concentration (45 ppm) for 3 min. Similarly, EO water (pH 2.6, 30 ppm of available chlorine) showed a significantly higher

bactericidal effect than did ozonated water (5 ppm ozone, pH 6.6), but as effective as sodium hypochlorite solution (pH 9.3, 150 ppm of available chlorine) *(105)*. Unfortunately, higher chlorine concentration (300 ppm) would not be able to offer further bacterial reduction *(192)*. Possible explanations for the reduced efficacy of EO water on foods have been previously discussed in the "household bleach" part, including microbial internationalization to microstructures in foods which protected pathogens from sanitation, and the release of organic matters from cut tissues.

Increased biocidal activity can be achieved by using EO water in combination with other antimicrobial technologies, such as electrolyzed reducing (ER) water or ultrasonication. Koseki *et al*. *(106)* showed that treatment of 5 min ER water + 5 min EO water had at least 2 log CFU of aerobic mesophiles per cucumber greater reduction than only immersion in EO water (30 ppm free chlorine), ozonated water (5 ppm ozone) or sodium hypochlorite solution (150 ppm free chlorine) for 10 min. This result is in agreement with their previous findings *(105)*, indicating that ER water provides additional microbicidal effect to EO water treatment alone. Similarly, Kim *et al*. *(97)* found that application of EO water in conjunction with ultrasonication enhanced the bactericidal effectiveness of EO water by 80%.

However, there are several issues limiting the acceptance of EO water by consumers: (1) since electrolyzed water is chlorine-based antimicrobial agent with low pH, future research is required to determine the impact of EO water on food sensory quality, and human health at home situation, (2) the bactericidal activity of EO water is reduced due to chlorine loss over time *(105)*, (3) more importantly, the initial purchase of EO machine may be costly as compared to commonly used washing methods at home, although the operational expenses are minimal *(7)*.

Ozonated Water

Ozonated water is a promising agent to control pathogenic microorganisms on foods *(65, 98)*. Ozone was first introduced as a chemical disinfectant in drinking water and municipal wastewater *(172)*, however, it was not until 2001 that ozone was approved by U.S. Food and Drug Administration (FDA) as antimicrobial agent for direct contact with foods, including raw and minimally processed fruits and vegetables *(173)*.

Ozone is generated by passing a stream of oxygen through a high voltage field called corona discharge, where oxygen is recombined to form the triatomic ozone molecule *(93)*. Ozone is a blue gas at room temperature with pungent odor *(77)*. Ozone in the aqueous form is relatively unstable and spontaneously decomposes to oxygen. The half-life of aqueous ozone ranges from 2-4 min (pH 7.0 and 25^{o}C) *(189)* to 165 min (20^{o}C) *(100)*, but is generally 20 to 30 min *(93)*. Ozone, directly as molecular ozone or indirectly as ozone-derived free radicals ($^{\cdot}OH$, $^{\cdot}O_2^{-}$, and $HO^{\cdot}_3$), is able to oxidize a variety of organic and inorganic substances.

Temperature and pH influence its stability, and in turn, affect the biocidal activity of ozone. Generally, increasing temperature results in greater destruction of microorganisms and on the contrary, less solubility and stability of ozone in aqueous solutions, but these two factors diminish one another within the temperature range of 0 – 30°C *(93)*. Stability of ozone in water was the greatest at pH 5.0, and decreases as pH increases *(93)*.

Ozone is a more powerful oxidant and destroys a broad-spectrum of microorganisms, including many that are resistant to chlorine-based treatment *(65)*. The mode of ozone action has been shown to be a complex process *(93)*. The bacterial cell surface is suggested to be the primary target of ozone activity *(146)*. Ozone attacks components of the bacterial cell wall, including proteins, unsaturated lipids and respiratory enzymes in the outer membrane, and

peptidoglycans in cell envelopes. Damage to the cell wall by oxidative action of ozone would ultimately lead to lysis of the targeted cell. Once ozone has penetrated into the cytoplasm, it oxidizes enzymes and nucleic acids, and proteins in spore coats *(93)*. Several studies have observed that gram-positive bacteria were more resistant than were gram-negative bacteria to ozone treatment *(110, 146, 161)*.

Evaluation of ozone as an effective antimicrobial agent has been carried out targeted at both pure culture and complex systems, such as fresh produce. In a model system, ozone at 3 ppm was more effective than chlorine dioxide (3 to 5 ppm), chlorinated trisodium phosphate (100 to 200 ppm), and peroxyacetic acid (80 ppm) at reducing populations of *E. coli* O157:H7 and *L. monocytogenes (147)*. In a separate study *(102)*, exposure for 5 min to 1 ppm of ozone provided a similar reduction in the population of Cryptosporidium parvum oocysts (90%) as that achieved by 80-ppm chlorine treatment for 90 min.

When tested on food commodities, mixed results were obtained. In a study evaluating the use of aqueous ozone for reduction of *E. coli* O157:H7 on apples, a reduction of 1.5 – 3.7 log CFU/g was obtained, depending on the location of the pathogen on the fruit surface and the degree of attachment *(3)*. But generally, ozonated water reduces bacterial populations in produce by no greater than 3 log CFU/g *(122)*. Koseki *et al*. *(105)* demonstrated that washing with ozonated water (5 ppm ozone) for 10 min reduced levels of aerobic bacteria on the surfaces of lettuce by only 1.5 log. Recent researches *(61)* observed a even lower reduction (0.6 to 0.8 log) in aerobic plate count following a 10 min ozone treatment (2.5, 5.0, and 7.5 ppm) on shredded lettuce. This limited efficacy may be attributed to the release of readily oxidizable organic matters from cut leaf tissue which may react rapidly with ozone. Inactivation studies on cucumbers showed that ozone was less effective than acidic electrolyzed water at reducing levels

of coliforms and aerobic mesophiles *(106)*. Combinations of ozone with other antimicrobial treatments are capable of increasing its efficacy, and a comprehensive review on this topic is directed to: sonication and high-speed stirring *(98)*, pulsed electric field *(136, 176)* and advanced oxidation processes *(93)*.

In conclusion, ozone is a potent chlorine replacement for reducing microbiological safety risk associated with fresh produce. It is effective in reducing a broad spectrum of microorganisms at relatively low concentrations. It also decomposes to oxygen rapidly and therefore leaves no harmful residues, or carcinogenic by-products. However, some serious drawbacks of ozone for home use are obvious: (1) ozonation is more complex than other disinfection technologies, and the cost of treatment is relatively high, being both capital- and power-intensive, (2) the instability of gaseous and aqueous ozone discourages the prior generation and storage of ozone for later application. Elimination of these problems is essential for a broad application of this promising technology.

Commercial Produce Wash Solutions

Consumer demand for more user friendly, less toxic alternative washing technologies has led the industry to develop a number of novel fresh produce wash products. Although none are currently approved by FDA as antimicrobial agents, there are some that have shown to be effective at both removing soil and applied fruit waxes and capable of removing bacteria from the surface of produce. Examples are Fit® Fruit and Vegetable Wash (HealthPro Brands, Inc., Procter & Gamble, Cincinnati, OH), Veggie Wash® (Refill Beaumont Products, Inc., Kennesaw, GA), and SunSmile® Fruit & Vegetable Rinse (Sunrider International, Torrance, CA). Each appears to provide a benefit to eating quality and to reduce food safety concerns. Ingredients are

all generally recognized as safe (GRAS), typically plant extracts that have antimicrobial activity and surfactant-like properties for cleaning.

Fit® Antibacterial Produce Wash is an alkaline (pH 11.1) surfactant solution composed of water, oleic acid, glycerol, ethanol, potassium hydroxide, sodium bicarbonate, citric acid, and distilled grapefruit oil *(25)*. The surfactant ingredients in FIT® (grapefruit oil and oleic acid) act as wetting agents designed to dissolve hydrophobic wax, dirt, and pesticides that may harboring microorganisms and thus remove them from the surface of fresh produce. Microorganisms in the removed dirt are further destroyed by antimicrobial components of FIT® solution, namely citric acid.

Veggie Wash® is a fruit and vegetable wash made with sodium citrate derived from organic citrus fruits that may exert an antibacterial effect and surfactant properties. Similarly, the active ingredients in SunSmile™ Fruit and Vegetable Rinse include: benzoin extract, an antiseptic preservative, and decyl polyglucose, a biodegradable cleansing agent derived from corn and coconut.

Information on the effectiveness of these products against foodborne pathogens is limited. In a study comparing the efficacy of FIT® with chlorine rinse for microbial reduction, FIT® was approximately as effective as 200 and 20,000 ppm chlorine in reducing levels of *Salmonella* and *E. coli* O157:H7 on alfalfa seeds *(25)*. Burnett *et al*. *(32)* investigated the effectiveness of a 0.5% (wt/vol) FIT® solution and reported only 1.51 log CFU per lettuce piece reduction of *L. monocytogenes*. More recently, Park *et al*. *(142)* undertook a study to evaluate liquid and powdered FIT® in a commercial fresh pack potato operation and in laboratory tests to determine its effectiveness against various foodborne pathogens, aerobic plate count, yeasts, and molds. The authors found that FIT® prepared with flume water showed significantly greater reductions (>

6.0 to 6.4 log CFU/g) in populations of all organisms than treatments consisting of water or 9 ppm ClO_2 (0.7 to 1.4 CFU/g).

Kilonzo-Nthenge *et al. (95)* investigated microbial reduction on fresh produce achieved by several home washing methods, including Veggie Wash®. There was no significant difference ($P < 0.05$) among reduction of *Listeria innocua* on lettuce after (1) 15 s rinse in running tap water (1.4 log CFU/g), (2) 2 min immersion in tap water + 15 s rinse (1.8 log CFU/g), (3) 2 min immersion in vinegar + 15 s water rinse (1.9 CFU/g), (4) 2 min immersion in Veggie Wash + 15 s water rinse (1.7 log CFU/log). They also found that Veggie Wash had a significantly greater effect in reducing *L. innocua* in tomatoes, but not in apples, broccoli, and lettuce, partly due to the differences in surface morphology and properties of these produce.

CHAPTER 3

MATERIALS AND METHODS

Test Cultures

Five strains each of *Salmonella*, *E. coli* O157:H7, and *Listeria monocytogenes* were used to create a cocktail inoculum of each target pathogen (Table 2). Bacterial strains were available as frozen stock (- 80^{o}C) using Microbank^{TM} Bacterial and Fungal Preservation System (Product Code PL.160, Pro-Lab Diagnostics Inc., Austin, Texas, USA).

TABLE 2. List of test cultures of *Salmonella*, *E. coli* O157:H7 and *Listeria monocytogenes*

Strain	Reference	Source
S. Baildon		Human feces, tomatoes-associated outbreak
S. Montevideo	G4639	Patient in a tomato-associated outbreak
S. Poona	01A3923	Cantaloupe-associated outbreak
S. Stanley	H1256	Alfalfa sprout-associated outbreak
S. typhimurium	DT104 H3380	Clinical human isolate
E. coli O157:H7	H-1730	Human feces, lettuce-associated outbreak
E. coli O157:H7	F-4546	Human feces, alfalfa sprout-associated outbreak
E. coli O157:H7	#994	Salami isolate
E. coli O157:H7	SEA 13B88	Apple juice
E. coli O157:H7	CDC658	Human feces, cantaloupe-associated outbreak
L. monocytogenes	LCDC 81-861	Cabbage outbreak
L. monocytogenes	G3982	Clinical isolate-Jalisco cheese outbreak
L. monocytogenes	Scott A	Human feces, milk-associated outbreak
L. monocytogenes	LM254	Drain of chicken processing plant
L. monocytogenes	LM311	Raw chicken product

Preliminary Studies

Preparation of Antibiotic-resistant Strains

Antibiotic-adapted strains were used as inoculum to minimize interference of colony development by naturally occurring microorganisms on produce and to facilitate detection of inoculated pathogens on recovery media. Rifampicin is a widely used antibiotic to induce resistant mutants and was obtained as crystalline powder from SIGMA-ALDRICH (Prod. No. R3501-5G). It was prepared as stock solutions by dissolving 75 mg of rifampicin in 1 ml of dimethylsulfoxide (DMSO; SIGMA-ALDRICH, Inc., St. Louis, MO, USA) to obtain a final concentration of 75 mg/ml. Due to its light-sensitivity *(89)*, stock solution of rifampicin was stored in 3.0-ml polypropylene low temperature freezer vials (VWR International, LLC, West Chester, PA, USA), wrapped in aluminum foil and kept at - 20^{o}C for long-term preservation. For a working solution, the rifampicin was thawed completely and added aseptically to the prepared, cooled (50^{o}C) medium prior to use. Rifampicin-containing media were then poured into Petri dishes, held 1 day at 22^{o}C, and then at 7^{o}C for up to 7 days before use.

Rifampicin-resistant strains were prepared by challenging wild-type cultures in 10 ml of Bacto^{TM} Tryptic Soy Broth (TSB; Becton, Dickinson and Company Sparks, MD, USA) supplemented with 100 µg/ml of rifampicin (TSB-R100). Once the cultures were adapted to 100 µg/ml of rifampicin, the overnight adapted cultures were plated for isolation onto Bacto^{TM} Tryptic Soy Agar (TSA; Becton, Dickinson and Company Sparks) supplemented with 100 µg/ml of rifampicin (TSA-R100) and confirmed by streaking onto respective selective medium containing the same concentration of rifampicin. After 24 h incubation, one typical colony from the selective plate was transferred to TSB-R100 for overnight incubation, followed by storing at - 80^{o}C for long term results.

Growth Curve Characteristics

Growth rate study for both mutants and wild-type organisms was performed to confirm that the rifampicin resistant strain had similar growth characteristics as the parent strain. The cells were subjected to two successive loop transfers into glass tubes containing 10 ml of TSB (TSB-R100 for mutants), followed by a final transfer of 0.25 ml overnight culture into 25 ml of TSB. For growth curve determination, growing culture was collected for sampling at certain time points until cells enter stationary phase. At each sampling, 1 ml of culture was transferred to a disposable polystyrene cuvette with capacity of 1.5 ml (Fisher Scientific Inc., USA) and the absorbance was read at 600 nm using a DU530 UV-VIS life science spectrophotometer. A graph of optical density (OD) of cells versus time was prepared to characterize growth curve for each mutated strain.

Antibiotic Dependency

To ensure that the strains had not developed dependence on the antibiotics, 24 h culture of mutant was streaked onto two sets of enumeration agar: (1) rifampicin-free media (TSA and selective medium), (2) rifampicin-containing media (TSA-R and selective medium with rifampicin). The wild-type strain was also streaked on the same agars for control.

Acquisition of Produce

Produce selected for experiments consisted of romaine lettuce (Lactuca sativa var. longifolia, Publix® Romaine Hearts), spinach (Spinacia oleracea, Publix® Fresh & Tender Spinach, triple washed), and green onions (Allium fistulosum). They were purchased at a local grocery store, transported without refrigeration (18 - 20^{o}C) for 30 minutes and then stored

immediately at 4^{o}C for a 1 day before use in experiments. All produce obtained were free from visual defects such as bruises, cuts or abrasions.

For each bag of produce purchased, background microflora was determined by homogenizing 1 piece of cut sample (lettuce: 4.5 × 4.0 cm, spinach: 6.0 × 4.5 cm, green onion: 7.0 cm) with 50 ml sterile 0.1% peptone water (PW) for 2 min. Serial dilutions (1:10) of each homogenized sample were made in the same diluent and surface spread (in duplicate) on Bacto^{TM} Plate Count Agar (PCA; Becton, Dickinson and Company Sparks).

Preparation of Treatment Solutions

Feed Water

Hard water (200 ppm total hardness) was prepared by mixing calcium carbonate ($CaCO_3$; J.T.Baker) and magnesium carbonate ($MgCO_3$; Basic Hydrate, Fisher Scientific) with a 3:1 ratio in deionized water (DW) (150.0 mg $CaCO_3$ + 50.0 mg $MgCO_3$/L DW). Hard water was prepared at least two days before use and covered with aluminum foil in beakers. DW was used to simulate soft water (0 ppm total hardness). The pH of feed solution was adjusted using white distilled vinegar (5% acidity, Publix® White Vinegar) or 0.1 N Sodium Hydroxide (NaOH; Pellets, 98.6%, J.T.Baker). Temperature of the feed water was maintained at approximately 15^{o}C using Traceable® Memory/Waterproof Thermometer (Cat. No. 4373, Control Company, TX, USA).

In order to optimize the performance of each treatment solution, the influence of pH and hardness on the efficacy of each treatment solution was compared on lettuce inoculated with the *Salmonella* cocktail (Table 3). The water pH and hardness combination that provided the greatest microbial reduction was selected for preparing each treatment solution.

TABLE 3. Combinations of pH and water hardness used for evaluating the effect of water properties on the efficacy of various produce washing technologies in reducing *Salmonella*

Washing Technologies	Water Properties
Chlorine bleach	Hardness (0 and 200 ppm) at pH 9.44 ± 0.22
Veggie Wash	Hardness (0 and 200 ppm) at pH 9.56 ± 0.07
Ozonated water	hardness (0 and 200 ppm) and pH (5 and 8)
Electrolyzed oxidizing water	Hardness (0 and 200 ppm) at pH 2.81 ± 0.06

Household Chlorine Bleach

Clorox® household bleach (Clorox Co., Oakland, CA, USA) containing 6.0% Sodium hypochlorite was used as the chlorine solution. Working solutions were prepared by diluting 0.60 ml of household bleach with 499.5 ml of feed water to obtain solution with free chlorine level of about 75 ppm. The pH and oxidation-reduction potential (ORP) value of chlorine solution were measured in duplicate by an Orion 3-Star Plus Benchtop pH/mV Meter (Thermo Scientific, Beverly, MA, USA), using pH and ORP electrodes (Epoxy Sure-Flow Combination Redox/ORP Electrodes), respectively. Free chlorine levels were verified by Iodine-Chlorine Kit #101 (Ecolab Center, St. Paul, MN, USA).

Veggie Wash® Solution

Veggie Wash® (Refill Beaumont Products, Inc., Kennesaw, GA, USA) is a fruit and vegetable wash made with organic citrus and obtained as 32 oz. soaker bottle. Working solutions were prepared by dilution of Veggie Wash with DW (Ratio of Veggie Wash to DW ~ 1:64),

corresponding to a concentration of 1.6% *(95)*. The pH and ORP value were measured in both fresh-prepared Veggie Wash and wash solution (after treatment) as described above.

Ozonated Water

Ozonated water was generated using the Lotus Sanitizing System (Model LSR 100, Tersano Int., Buffalo, NY, USA) with multi-purpose bowl and lid attachments. According to manufacturer's instruction, the multi-purpose bowl was filled with sufficient water to cover the fruits and vegetables, and then processed to complete ozonation cycle indicated by 100% on the display. After the ozonation was completed, the produce items were left in the bowl attachment for an extra 2 min for antimicrobial treatment. For each experiment, several batches (at least three batches) were processed until the pH and ORP of the ozonated water were relatively stable. Ozone level was determined by the Indigo Colorimeter Method using AccuVac Ampuls (Hach Co., Loveland, CO) of high range ozone (0 - 1.5 mg/L ozone) and a Hach Colorimeter (Model DR/890).

Electrolyzed Oxidizing Water

Electrolyzed oxidizing (EO) water was generated using a Bion-Tech generator (BTM-3000, Bion-Tech Co., Ltd. Seoul, South Korea). One measure spoon (approximately 0.85 g) of Sodium Chloride (NaCl; Kroger® table salt) and 2-L DW were added into each chamber. After 20 min generation, a 2-L portion of EO water was collected from the anode outlet and used within 1 h of preparation. Samples of EO water were taken at the beginning and at the end of each experiment to evaluate the pH and ORP value as described above.

Experimental Design

A flow chart of experimental design of this study is provided in the appendix. Each test case (organism × produce × treatment) was replicated three times with three treatment samples, one positive control (inoculated but untreated) and one negative control sample (uninoculated and untreated) per replicate. Bacterial log reductions in infectivity by each solution were determined by subtracting the populations of bacteria in the treatment samples from the population in the positive control samples.

Preparation of Inoculum

Five-strain mixture of each pathogen was used as inocula. Each variant strain was transferred to TSB-R100 using loop inocula at two successive 24-h intervals and then collected by centrifugation (6,500 rpm, 21^{o}C, 15 min; Beckman Coulter Allegra 21R Refrigerated High speed Table Top Centrifuge). The resulting pellet was washed once in 10 ml of 0.1% PW to remove nutrients or metabolites that would react with sanitizers, followed by resuspending in the same volume of PW to achieve a population of ~ 9 log CFU/ml.

During the day of experiment, equal volumes (2 ml each) of each culture suspension of the target pathogens were combined to obtain a 10 ml inoculum containing approximately 9 log CFU/ml and equal populations of each strain. The inoculum was diluted (1:4) in PW, maintained at 22 $\pm$ 2^{o}C and inoculated onto produce within 1 h of preparation as described below. Populations in the individual cultures and the five-strain cocktail were determined by serial dilution in 0.1% PW and plating on TSA-R100. Plates were incubated at 37^{o}C for 24 h before colonies are counted.

Inoculation of Produce

On the day of each experiment, the original package of produce was taken out of the 4°C refrigerator and allowed to equilibrate to room temperature for a period of 30 minutes. Two serving sizes of each produce (170 g of lettuce, 170 g of spinach, 50 g of green onions) were used for washing treatments.

Inoculation of Lettuce

The outer 3 or 4 damaged or green wrapper leaves and core of the lettuce head were aseptically removed and discarded. The remaining inner leaves were weighed and rinsed under running DW for 15 seconds to remove any dirt, and subjected to a ratchet salad spinner (Progressive International® Corp, Kent, WA, USA) for 30 seconds to remove residual DW. Leaves for inoculation and microbiological analysis were trimmed into pieces (ca. 4.5 cm × 4.0 cm) using sterile carbon steel surgical blades (REF 4-121, miltex®, Inc., York, PA, USA), while the rest of leaves were kept intact.

The trimmed leaves were then placed on sterile aluminum foil with the abaxial side facing up in a biosafety hood. Each mixed-strain cocktail prepared as described earlier was inoculated onto the abaxial surface of each leaf by placing 50 µl at 10 locations with a micropipettor *(139).* Each uninoculated control was treated in a similar manner but used sterile 0.1% PW as the inoculum. To allow attachment of bacteria to the leaf surfaces, inoculated samples were air-dried in a class II biosafety hood with a constant laminar flow at 22 $\pm$ 2°C for 1 h before use in washing treatment.

Inoculation of Spinach and Green Onions

The same procedure described above was used for preparation and inoculation of spinach leaves and green onions, with the following modifications: 1) individual intact spinach leaves with good quality (ca. 6.0 cm × 4.5 cm) were picked directly from the package, trimmed to remove stalks, inoculated with test pathogen and marked by a red dye (Testors® 1103 Enamel Paint Red 1/4 oz) to distinguish themselves from the rest of the leaves during treatment. 2) Roots and peels of green onions were removed and the remaining hollow upper green tissues were then trimmed for inoculation (ca. 7.0 cm long).

Treatment of Produce

Two serving sizes of leaves (including inoculated leaves) were treated by washing solutions (ratio of produce to treatment solution ~ 1:12, g/ml) as follows: (1) rinse under running tap water at 2 L/min for 15 seconds, (2) immersion for 2 minutes in chlorine bleach, Veggie Wash, ozonated water and EO water.

On termination of treatment, the leaves were transferred individually into 710 ml Whirl-Pak® filter bags (product No. B01348WA; Nasco, Fort Atkinson, WI) containing 50 ml of neutralizing solution, while their respective residual wash solutions (25 ml) were combined with 50 ml of neutralizing solution for microbiological analysis. The formulation of the neutralizing solution in this study was developed based on three active reducing agents of Dey-Engley Broth, which are sodium thioglycolate (1g/L DW; Sat. T0632, ≥ 96.5%, SIGMA), sodium thiosulfate (6g/L DW; concentration 0.04 M, 98.5%, ACROS ORGANICS) and sodium bisulfite (2.5g/L DW; ACROS ORGANICS).

Microbiological Analysis

In this study, both produce homogenate (untreated and treated produce) and residual wash solution were evaluated for their microbial content. The Whirl-Pak® bags containing lettuce or spinach samples were macerated by hand for 1 min, while green onions were shaken for 1 min to avoid the disruption of cells and the release of natural antimicrobials inherent in green onions. Undiluted homogenates were surface-plated in quadruplicate (0.25 ml) and also serially (1:10) diluted in 0.1% PW and plated in duplicate (0.1 ml) on TSA-R100 using an automated spiral plater (Spiral Biotech Autoplate® 4000, Spiral Biotech, MD, USA).

The Whirl-Pak® bags containing residual wash solutions were shaken for 1 min. Homogenates were serially diluted and plated as described above. Resulting plates were incubated at 37°C for 24 h before counting presumptive colonies. If low numbers of pathogen were anticipated, 1 ml of each mixture solution was inoculated into 20 ml of TSB for enrichment (24 hr at 37°C).

Presumptive colonies of each pathogen were randomly selected (10 to 20 colonies per treatment) and confirmed by streaking onto appropriate selective agars. These selective media include: Xylose Lysine Desoxycholate agar with 100 μg/ml of rifampicin (XLD-R100, pH 7.4 $\pm$ 0.2) for *Salmonella*, BBL™ MacConkey II Agar with Sorbitol supplemented with 100 μg/ml of rifampicin (SMAC-R100, pH 7.1 $\pm$ 0.2) for *E. coli* O157:H7 and DIFCO™ *Listeria* selective agar base with 100 μg/ml of rifampicin (OX-R100, pH 7.2 $\pm$ 0.2) for *Listeria monocytogenes*. All media were incubated at 37°C for 24 hours before enumeration of colony types typical for the respective pathogen.

Statistical Analysis

Bacterial reduction data (CFU/sample) were analyzed after log transformation. Data were pooled from the three replicate experiments to obtain a set of 9 observations for each test case. Values for the mean log and standard deviation of each set of bacterial counts were calculated on the assumption of a lognormal distribution of microorganisms. Significant differences among means were determined by the least-square-means method using SAS Software Release 9.13 (SAS Institute Inc., Cary, NC), and reported at a significant level of $\alpha = 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

This study consisted of three phases of research. The first phase consisted of determination of background microbial populations on vegetables, preparation and characterization of rifampicin resistant strains, and selection of enumeration media for microbiological analysis. The first phase was designed to provide consistent inocula, thereby reducing variability in later studies. The second phase was aimed at determining the influence of physical properties of water (hardness and pH) on the efficacy of treatment technologies, using lettuce and rifampicin resistant *Salmonella* as test model. Water physical property combinations achieving the greatest *Salmonella* reduction were selected for preparing each washing solutions in subsequent experiments. The third phase compared efficacies of various home washing technologies at reducing pathogenic bacteria on the surfaces of lettuce, spinach and green onions.

Preliminary Studies

Indigenous Microbial Flora on Produce

Prior to analysis of microbial reduction for inoculated samples, it is important to determine levels of background microflora present in untreated (uninoculated) products. High numbers of indigenous microflora may interfere with the efficacy of treatment solutions against artificially contaminated pathogens, thus selection of leaf samples with similar microbiological quality is necessary to produce comparable data.

Spinach. As shown in Table 4, the aerobic plate count (APC) of the spinach samples examined was around 6.61 log CFU/g, with a range of 4.31 to 9.85 log CFU/g. These data are consistent with the results of a 2007 survey in which 100 bagged spinach and lettuce mixes were found to have a mean total bacterial count of 7.0 log CFU/g *(179)*.

Lettuce. Aerobic microbial population on the romaine head lettuce was 9.89 log CFU/g, with a range of 6.67 to 18.1 log CFU/g. These data were in general agreement with previous investigation by Ruiz *et al*. *(150),* in which levels of aerobic bacteria were found to range from 2 to > 8 log CFU/g on both field and retail samples of lettuce (enumerated on plate count agar after incubation at 37^{o}C for 48 h).

Green Onion. A population of 7.09 to 12.18 log CFU/g was noted for green onion, with an average aerobic microbial count of 10.28 log CFU/g. This number is found to be significantly higher than that of a previous investigation in which total bacteria on green onions ranged from 5 – 6 log CFU/g *(193)*. The slightly higher APC levels for romaine lettuce and green onions indicate that these two produce are generally retailed with less processing and washing steps as compared to “triple washed” ready-to-eat vegetables such as spinach.

In general, our data are consistent with those of other studies that examined microbial levels on fresh produce items, indicating that the microbial load can be highly variable and may depend on the produce type and whether or not postharvest processing treatments are performed prior to retail. This implication necessitates the development of bacterial strain markers for facilitating differentiation of vegetable-colonizing bacteria from the inoculums applied in experimental studies.

Growth Curve Characteristics

To eliminate interference of the background microflora in the enumeration of the inoculated pathogens, the strains used in this study were marked with resistance to rifampicin. No colonies were observed when uninoculated fresh produce samples were plated onto TSA-R100. However, when uninoculated produce samples were plated onto agar containing 20 μg/ml or 50 μg/ml of rifampicin, colonies were observed, especially when the background populations were high (data not shown). Therefore, bacterial strains resistant to 100 μg/ml of rifampicin were developed and used in all further studies.

Once a marker is obtained, it is critical to assess the impact of marker introduction on growth rate of the corresponding cells. Growth curves were prepared for both wild type and rifampicin resistant variants of five strains each for *Salmonella* (Figures 2-6), *E. coli* O157:H7 (Figures 7-11) and *Listeria monocytogenes* (Figures 12-16). The growth of rifampicin resistant strains of all pathogens was similar to that of the parent strains in TSB-R100 at 37 °C. We concluded that the introduction of the rifampicin-resistance mutation had no significant impact on bacterial growth rate.

Selection of Enumeration Media

The growth characteristics of wild and resistant strains of all test pathogens were compared after 24-h incubation in TSB-R100 by plating onto two sets of media: TSA with and without 100 μg/ml of rifampicin, and the corresponding selective agar with and without 100 μg/ml of rifampicin. Microbial populations of 24-h cultures recovered on these four types of agar are summarized for *Salmonella* (Figures 17-21), *E. coli* O157:H7 (Figures 22-26), and *L. monocytogenes* (Figures 27-31). Wild-type and resistant variants of all microorganisms

developed a similar number of colonies (~ 9 log CFU/ml) ($P > 0.05$) as well as similar cell morphologies and sizes (data not shown) on non-selective agar (TSA) and selective agar. These observations confirmed the aforementioned results that the impact of antibiotic resistance biomarker on cell physiology is minimal in term of growth rate characteristics.

For all three types of test microorganisms, an equal population of rifampicin resistant mutants ($P > 0.05$) was recovered on TSA-R100 and TSA. It is thus concluded that mutated strains had not developed dependence on rifampicin at the targeted concentration.

Colony development on TSA-R100 and Selective-R100 agar was also compared. Overall, TSA-R100 performed better than rifampicin containing selective agar for supporting colony development for all test strains. This phenomenon was particularly evident when comparing the number of *Salmonella* and *E. coli* O157:H7 recovered on TSA-R100 and their relevant selective agar, XLD-R100 and SMAC-R100. These findings are in general agreement with that observed by Beuchat *et al*. *(24)*. This indicates that some of the cells were not able to resuscitate in the presence of selective chemicals in selective agar and that direct plating of these cells onto selective agar may overestimate the microbial reduction. Therefore, TSA-R100 was selected for enumeration of cells in all further studies.

The Influence of Water Physical Property on Microbial Reduction

Although extensive studies have been conducted on the efficacy of various washing treatments, little information is available on the performance of treatment technologies with variable physical properties of water (such as pH and water hardness). Hardness refers to the amount of dissolved calcium, magnesium and other divalent and trivalent metallic elements in the water. Water hardness may be classified as follows: soft (0-60 ppm of calcium carbonate),

moderately hard (60-120 ppm of calcium carbonate), hard (120-180 ppm of calcium carbonate), and very hard (> 180 ppm of calcium carbonate) *(69)*. Hardness of tap water can differ from state to state, and even area to area within the state depending upon the source and type of treatment. Texas, New Mexico, Kansas, Arizona, and southern California have relatively hard water *(175)*. Harper *et al*. *(69)* found that hardness of some Ohio water supplies ranges from 10 – 385 ppm.

Hard water may present a major problem by reducing effectiveness and by forming surface deposits. In this study, the effect of hardness on the efficacy of treatment solution in killing or removing *Salmonella* was illustrated in Table 5. It was observed that hardness had no significant effect on the efficacy of chlorine bleach, ozonated water and EO water ($P > 0.05$). These results confirmed previous observation that chlorine is less affected by water hardness, while pH has a much greater influence on the antimicrobial activity of chlorine-based agents *(26)*. Shere *(157)* evaluated sodium hypochlorite solution (5 ppm available chlorine) at 0 and 400 ppm hardness at 20^{o}C. A complete kill of bacteria was observed at the two examined levels of hardness, indicating that raising the hardness from 0 to 400 ppm did not reduce the bactericidal activity of hypochlorite solution. Water hardness significantly affected Veggie Wash effectiveness against *Salmonella* ($P < 0.05$), but the differences of bacterial reduction associated with the change in hardness were relatively small (0.25 log CFU/inoculated cut leaf). It is likely that sodium citrate, the main microbicidal and surface-active component of Veggie Wash, may react with calcium and magnesium ions by sequestering these ions, thus diminishing the effectiveness of this produce wash product against *Salmonella*.

The effect of pH on the reduction of *Salmonella* by ozonated water was also investigated (Table 5). Treatment with ozonated water at pH 5.0 reduced *Salmonella* by 1.45 (0 ppm of hardness) or 1.49 (200 ppm of hardness) log CFU/inoculated cut leaf, while reductions of 0.83

and 0.99 log CFU/inoculated cut leaf were achieved at pH 8.0 using washing solutions with 0 ppm and 200 ppm of hardness, respectively. This result demonstrated that antimicrobial efficacy of ozonated water is greater at low pH (5.0) than at high pH values (8.0). Similarly, Khadre *et al. (93)* revealed that the stability of ozone in water was the greatest when pH was 5.0, and decreased as pH increased. It has been proposed that the low pH of aqueous ozone solution sensitizes the bacterial outer membrane to the entry of molecular ozone which is the main inactivator of microorganisms at acidic condition *(93)*.

In summary, treatment solution with 0 ppm of hardness seems to provide an equal or greater efficacy than hard water (200 ppm) against artificially inoculated *Salmonella* on lettuce. In the case of ozonated water, low pH (5.0) also contributes to a greater bactericidal activity. Therefore, we concluded that no pre-adjustment of water hardness be used for the washing technologies in future studies, except that the pH of feed water for ozone generation should pre-adjust to level of 5.0 in order to achieve a greater biocidal activity.

Comparative Efficacies of Various Washing Technologies

Physicochemical Properties of Treatment Solutions

The properties (pH, ORP, and free chlorine concentration) of fresh prepared treatment solutions (tap water, chlorine bleach, Veggie Wash, ozonated water and EO water) tested in this study are summarized in Table 6. After treatment, the corresponding residual wash solutions except for tap water and Veggie Wash were also analyzed to identify the changes in pH, ORP and free chlorine level, and are presented in Tables 7 - 15.

In most test cases, the ORP and pH of bleach, ozonated water and EO water after treatment were not significantly different from the initial values measured before test produce (P >

0.05). The exception was washing spinach with ozonated water which resulted a significant decrease in ORP (ORP values decreased from 1040 mV to 969 mV, 847 mV, 537 mV for *Salmonella*, *E. coli* O157:H7 and *Listeria monocytogenes*, respectively) and an increase in pH (pH increased from 5.02 to 5.54, 5.32, 5.51 for *Salmonella*, *E. coli* O157:H7 and *Listeria monocytogenes*, respectively) after treatment ($P < 0.05$). The free chlorine concentrations of chlorine bleach and EO water decreased by 2 – 11 ppm and 1 – 13 ppm from initial levels of 74 ppm (bleach) and 18 ppm (EO water), respectively.

Pathogen Reduction on the Surface of Lettuce

Data on the populations of rifampicin resistant *Salmonella*, *E. coli* O157:H7 and *Listeria monocytogenes* recovered from the surface of the romaine lettuce leaf after applying different washing treatments are presented in Tables 7 - 9. Pathogens were not detected on uninoculated lettuce leaves. The initial populations of bacteria recovered from the surface of inoculated lettuce after 1 h of air drying were 7.09 log CFU/cut leaf of *Salmonella*, 7.03 log CFU/cut leaf of *E. coli* O157:H7 and 7.09 log CFU/cut leaf of *L. monocytogenes*, respectively. Statistical analysis indicated that the initial counts of the inoculum were not significantly different among different types of pathogens ($P > 0.05$).

All washing treatments produced a significant reduction in bacterial counts ($P < 0.05$). Immersing lettuce in Veggie Wash for 2 min provided the lowest antimicrobial effect on lettuce inoculated with the tested pathogens, resulting in reductions of < 1 log CFU/inoculated cut leaf. Little previous research has been done on the efficacy of Veggie Wash in reducing pathogenic bacteria on lettuce. Kilonzo-Nthenge *et al. (95)* reported a reduction of 1.73 log CFU/g achieved by immersion for 2 min in Veggie Wash solution followed by 15 s water rinse. The surfactant-

like property and weak antimicrobial activity of the sodium citrate in Veggie Wash makes it a good candidate for removing soil and dirt rather than killing pathogens.

Treatment with 15 s running tap water had a moderate effect in removing three tested pathogens, resulting in reductions of 1.5 to 1.7 log CFU/inoculated cut leaf. The same rinsing procedure was tested by Kilonzo-Nthenge *(95)*, who demonstrated that a bacterial reduction of 1.41 log CFU/g was obtained by rinsing lettuce leaves under running tap water for 15 s. Immersion into stationary water was also utilized for washing lettuce, but only reduced initial bacterial populations by an average of 1 log CFU or less on fresh produce surface *(81, 140, 167)*.

Treatment with ozonated water was equally effective compared to rinse with running tap water ($P > 0.05$), resulting in bacterial reductions of 1.36 to 1.85 log CFU/inoculated cut leaf. This is in general agreement with previous observations that ozonated water reduces bacterial populations in fresh produce by no greater than 3 log CFU/g *(122)*. Koseki *et al. (105)* demonstrated that washing with ozonated water (5 ppm ozone) for 10 min reduced levels of aerobic bacteria on the surfaces of inoculated lettuce by only 1.5 log CFU unit.

Use of 75 ppm chlorine bleach to wash lettuce for 2 min was more effective in reducing levels of *E. coli* O157:H7 (reduction of 2.34 log CFU/inoculated cut leaf) and *L. monocytogenes* (reduction of 2.16 log CFU/inoculated cut leaf) compared to either a 15 s tap water rinse or immersion in ozonated water for 2 min ($P < 0.05$). These results agree with a previous experiments by Behrsing et al. *(19)*, showing that *E. coli* cells were reduced by approximately 1.9 – 2.8 log CFU/g following immersion of lettuce leaves into hypochlorite solutions (50 pm or greater chlorine) for 30 s or greater. However, there was no significant difference in efficacy between chlorine bleach and water rinse in decreasing *Salmonella* on lettuce ($P > 0.05$). These results confirmed previous studies demonstrating that the effect of chlorine based antimicrobials

for washing fresh produce was minimal *(5, 81)*. In our study, chlorine bleach was not adjusted to acidic condition, and therefore may result in a reduced effectiveness against *Salmonella* on lettuce.

Immersing lettuce in EO water for 2 min produced equal or greater pathogen reduction than chlorine bleach, resulting in reductions of 2.55 to 3.72 log CFU/inoculated cut leaf, and in turn, exhibited the greatest efficacy among all washing treatments tested ($P < 0.05$). In this study, its superior efficacy over chlorine bleach may be mainly because of the highly acidic pH and the presence of other oxidants such as hydrogen peroxide and hydroxyl radical in EO water in addition to hypochlorous acid *(139)*. However, extensive studies have reported that EO water posses similar bactericidal activity to that of chlorinated water especially when chlorine is acidified from pH 9 to 4.5 – 5.0 *(105, 139)*, since the hypochlorous acid is the major contributor to their antimicrobial activity *(167)*. Park and Kim *et al*. *(139)* compared the efficacy of EO water and acidified chlorinated water and found no significant difference in pathogen reduction (2.41 and 2.65 log CFU per leaf) between EO water and treatment using chlorinated water of equivalent pH (2.5), ORP (1,130 mV) and residual chlorine concentration (45 ppm) for 3 min.

Pathogen Reduction on the Surface of Spinach

Results of the efficacy of various washing technologies in removing or killing *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* on spinach are summarized in Tables 10 - 12. The mean populations of *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* recovered on spinach after 1 h of drying were 7.13, 7.26 and 6.94 log CFU/inoculated cut leaf. None of these tested pathogens was recovered in the deionized water and uninoculated samples used in the experiments, which implied that viable pathogens recovered after cleaning was entirely attributed to the inoculation.

All washing treatments produced a significant decrease in pathogen count as compared to the initial population ($P < 0.05$) except that Veggie Wash was unable to produce a significant reduction of *L. monocytogenes* on the surface of spinach. Immersing spinach in Veggie Wash for 2 min exhibited the lowest bacterial reduction of approximately 0.5 log CFU/inoculated cut leaf among all other treatments, and was therefore relatively ineffective at reducing pathogens on spinach leaves ($P < 0.05$).

Immersing spinach leaves in ozonated water for 2 min produced no difference ($P > 0.05$) in pathogen reductions from immersion in Veggie Wash, resulting in 0.3 to 1.0 log CFU/inoculated cut leaf of reduction. In contrast, tests on tomatoes showed much higher reductions of *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* using the same ozone generator, ranging from 3.43 to 5.28 log CFU/tomato *(170)*. This result confirmed the product statement that this model of ozone generator is better suitable for decontaminating whole produce rather than cut items. It is speculated that the efficacy of the ozone depends on the placement of the produce items in the device. When whole produce were placed in the machine, there are spaces between each item so that the active ozone generated at the bottom is able to spread in the solution. In the case of leafy vegetables, however, chopped leaves tend to overlap each other and collect at the bottom of the device so that active ozone is absorbed by the bottom leaves and there is less reactive species left to interact with the inoculated leaves added to the top.

A 15-s rinse under running tap water was more effective in killing three tested pathogens on spinach than both Veggie Wash and ozonated water, exhibiting bacterial reductions of ~ 1 log CFU/inoculated cut leaf ($P < 0.05$); however, the difference between three pathogens was not significant ($P > 0.05$). A ~ 1 log reduction is consistent with reductions demonstrated by other researchers. Park *et al. (140)* observed a < 1.0 log reduction when inoculated spinach leaves were

immersed in deionized water (22 $\pm$ 2^{o}C) for 15s up to 5 min, followed by agitating for < 3 s. The reduction was attributed to physical wash-off of inoculated cells on the spinach surface.

75 ppm chlorine bleach was similarly effective in killing *Salmonella* and *E. coli* O157:H7 as rinsing with running tap water ($P > 0.05$), resulting in 1.1 – 1.6 log CFU/inoculated cut leaf. A greater reduction of *L. monocytogenes* was obtained by chlorine treatment than with water rinse ($P < 0.05$), however, the difference in their efficacy was less than 0.6 log CFU/inoculated cut leaf. Similar bacterial reduction was observed when all three pathogens were challenged to treatment by immersion in EO water for 2 min, in which reductions of 1.0 – 1.6 log CFU/inoculated cut leaf were obtained. These results were consistent with Izumi's research, which showed that acidic electrolyzed water (20 ppm available chlorine) treatment reduced the microbial load by 0.7 to 1.1 logs in trimmed spinach leaves *(81).* Similarly, Rahman *et al*. *(144)* reported that the neutral and acidic electrolyzed water treatment reduced the microbial load in the spinach leaves by 1.93 and 1.94 log cfu/g, respectively.

Pathogen Reduction on the Surface of Green Onion

The initial analysis of the green onions that were not inoculated revealed the absence of rifampicin resistant *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes*. Green onion samples were inoculated with 7.20 log CFU/cut sample of *Salmonella*, 7.09 log CFU/cut sample of *E. coli* O157:H7, and 7.11 log CFU/cut sample of *L. monocytogenes*, respectively. Tables 13 - 15 show surviving cells of *Salmonella, E. coli* O157:H7 and *L. monocytogenes* from inoculated green onions after treatment with various solutions tested in this study.

Generally, we observed a ~ 1 log pathogen reduction when inoculated green onions were treated by rinsing with running tap water, while a slightly higher reduction (1.5 log

CFU/inoculated cut sample) was achieved for *E. coli* O157:H7. These results indicate a higher bacterial reduction than that achieved by Martínez-Téllez *et al*. *(121)* who demonstrated that green onions washed with sterile water only reduced 0.65 log CFU/g of *Salmonella*. Since dip-inoculation method (produce was dipped into a bacterial suspension) was applied in their study, the discrepancy between results of two studies is likely attributed to different degree of bacterial attachment and internationalization. Dip-inoculation allows microorganisms to preferentially attach to inaccessible sites and further penetrate into hydrophobic pockets, folds or cracks on the surface of vegetables *(159)* which protect the cells from being washed off.

Use of Veggie Wash did not provide further significant reduction of three pathogens as compared to water rinse treatment, resulting in 0.7 – 1.1 log CFU/inoculated cut sample. It is likely that Veggie Wash just removed the inoculated cells physically from the surface of green onions since it contains compounds with weak antimicrobial activity, namely citric acid. Both 75 ppm chlorine bleach and ozonated water showed a more effective pathogen removal on the inoculated green onions as compared to treatment by running tap water, nevertheless, no significant difference in efficacy was observed between these two treatments ($P > 0.05$), achieving bacterial reductions ranging from 2.2 – 2.8 log CFU/inoculated cut sample. Martínez-Téllez *et al. (121)* reported less bacterial reduction by chlorine treatment (200 – 250 ppm), resulting in reduction of *Salmonella* by 1.36–1.74 log CFU/g. Again, this may be due to bacterial attachment and infiltration resulting from the dip-inoculation method, as evidenced by a recent study (unpublished data) by Durak et al. *(55)* where chlorine (200 ppm) were able to decrease populations of *E. coli* O157:H7 by 0.9 log CFU/g for dip-inoculated green onions whereas the same treatment resulted in a 4.4 log reduction for spot-inoculated samples.

EO water exhibited the greatest efficacy against three pathogens on inoculated green onions among all washing treatments, reducing levels of *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* by 2.5, 3.1 and 3.6 log CFU/inoculated cut sample, respectively. These results are in agreement with previous research that treating with EO water (pH 2.06, free chlorine 37.5 ppm) reduced levels of *E. coli* O157:H7 on green onions by 4.45 and > 5.82 log CFU/g after 1 min and 3 min, respectively *(141)*. The slightly lower pathogen reduction observed in this study may be due to the higher pH (2.8) and lower free chlorine level (18 ppm) of the EO water tested.

Microbial Populations in Wash Solutions

The microbial populations in the residual wash solutions represent the number removed from the treated produce and thus, potentially available to cross-contaminate other produce or food preparation surfaces in home-use situations. In this study, no *Salmonella*, *E. coli* O157:H7 and *Listeria monocytogenes* was detected by either direct plating (detection limit: 3 CFU/ml) or enrichment (detection limit: 0.3 CFU/ml) in the wash solutions of chlorine bleach, ozonated water and EO water, which demonstrated that these three washing technologies were able to prevent the potential of cross-contamination. An exception was the ozone treatment of spinach, in which 2 out of 6 treatment solution samples were positive for *Salmonella* and *L. monocytogenes* by enrichment, while the presence of *E. coli* O157:H7 was identified in 3 out of 6 enrichment samples. This exception confirmed our previous observation that aqueous ozone was particularly ineffective at reducing pathogens on spinach leaves. Similarly, Veggie Wash was unable to destroy the remaining microorganisms in the solution, as evidenced by the bacterial population of 3.8 – 4.6 log CFU/ml, 3.8 – 4.4 log CFU/ml, 4.8 – 5.4 log CFU/ml in treatment solutions after being used to wash lettuce, spinach and green onions, respectively.

Comparison of Reductions among Produce and Pathogens

In the current study, differences in the level of pathogen reduction were observed among produce types. For example, pathogen levels on lettuce and green onions were significantly reduced by 0.5 - 3.7 log CFU/inoculated cut sample upon exposure to all treatments, whereas only 0.3 – 1.6 log CFU/inoculated cut sample of pathogen reduction was observed on spinach. The efficacy of washing treatments may be influenced by morphological properties of the leaf tissues, which differ among various produce types *(19, 95)*. The smooth surface of lettuce and green onion is protected by a relatively thick waxy cuticle with hydrophobic properties that repels water and possibly loose bacterial adhesion to its surface, while the abaxial side of spinach surface seems to be rougher and may differ in microstructure (such as cuticle thickness), which may allow greater depth of bacterial penetration. However, high-definition microscopy techniques are required to provide direct evidence on bacterial behavior within spinach leaves.

The differences in pathogen reduction among produce types may also be attributed to the amount of organic material in treatment solutions. Organic matter potentially react with free available chlorine and thus may reduce washing effectiveness *(138)*. In the present study, free chlorine concentration decreased significantly after treatment in both chlorine bleach (75 ppm to 63 – 72 ppm) and EO water (18 ppm to 5 – 17 ppm). This phenomenon is more evident in the case of spinach than lettuce and green onions. It is thus hypothesized that spinach released more organic materials including antioxidants (such as phenolic compounds) from the cut surfaces, thus diminishing the bactericidal activity of chlorine-bearing treatments *(94)*.

Previous researches indicate that pathogens differ in their susceptibilities to antimicrobial agents, as well as their interaction with produce items (such as bacterial attachment) *(122, 168)*. Our results showed that *L. monocytogenes* was reduced more readily than were *Salmonella* and

E.coli O157:H7 to most antimicrobial agents tested including chlorine bleach, ozonated water and EO water, except that *L .monocytogenes* on lettuce was more resistant to EO water treatment than were other two gram-negative pathogens. These observations are in agreement with previous findings that gram-negative bacteria seem to be more resistant to biocides *(98, 132)* except for ozone treatment in which gram-positive bacteria were more resistant than were gram-negative ones *(110, 161)*. However, these mixed results must be interpreted with caution, as the present study differs from previous researches in that pathogen reductions were determined on the surface of fresh produce instead of pure culture suspension.

TABLE 4. Aerobic plate counts of romaine lettuce, spinach and green onions stored under 4°C within 24 h of purchase

Product	No. of samples	Weight (g/cut leaf sample)		Length (cm/cut leaf sample)		Width (cm/cut leaf sample)		Aerobic plate counts (log CFU/g)	
		Mean	Range	Mean	Range	Mean	Range	Mean	Range
Lettuce	10	0.47	0.30 – 0.55	4.4	4.1 – 4.7	3.8	3.5 – 4.0	9.89	6.67 – 18.1
Spinach	11	0.71	0.55 – 0.90	5.9	5.2 – 6.5	4.5	4.2 – 4.9	6.61	4.31 – 9.85
Green Onions	11	0.61	0.45 – 0.85	6.8	6.3 – 7.2	1.0	0.8 – 1.3	10.28	7.09 – 12.18

TABLE 5. The influence of water hardness and pH on the efficacy of treatment solutions in killing *Salmonella*

Treatments	Reduction (log CFU/inoculated cut leaf) [a]		pH
	Water hardness (0 ppm)	Water hardness (200 ppm)	
Veggie Wash	0.96 A	0.71 B	9.56 ± 0.07
Bleach	1.78 A	1.86 A	9.44 ± 0.22
EO Water	3.72 A	3.47 A	2.81 ± 0.06
Ozone	1.45 A	1.49 A	5
	0.83 A	0.99 A	8

[a] Mean values within a row followed by different letter are significantly different ($p \leq 0.05$); inoculum levels were ca. 7 log CFU/inoculated cut leaf.

TABLE 6. Physicochemical properties of washing solutions before treatment

Treatment Solutions	ORP (mV)	pH	Free chlorine concentration (ppm)
Tap Water	554 ± 29	7.40 ± 0.19	1 – 2
Chlorine Bleach	639 ± 14	9.44 ± 0.22	74 ± 2
Veggie Wash	NA [a]	9.56 ± 0.07	NA [a]
Ozonated Water	1040 ± 12	5.02 ± 0.07	0.67 ± 0.05 mg O_3/L of water
EO Water	1109 ± 4	2.81 ± 0.06	18 ± 3

[a] NA, not analyzed

Table 7. Population of *Salmonella* recovered from lettuce leaves and wash solutions after treatment

Treatment	Mean *Salmonella* population			Properties of Treatment Solution [e] (After Treatment)		
	On lettuce (log CFU/inoculated cut sample [a])					
	Recovered [b]	Reduction	In solutions (log CFU/ml)	ORP (mV)	pH	Free Chlorine (ppm)
None	7.09 A					
Tap	5.51 CD	1.58	NA [c]	NA	NA	NA
Bleach	5.04 D	2.05	< 0.3 [d]	615 ± 6	9.52 ± 0.01	70 ± 2
Veggie Wash	6.13 B	0.96	3.84	NA	NA	NA
Ozone	5.64 BC	1.45	< 0.3	1040 ± 6	5.13 ± 0.08	NA
EO Water	3.37 E	3.72	< 0.3	1103 ± 3	2.80 ± 0.01	17 ± 1

[a] The average size of inoculated cut sample is 4.4 cm × 3.8 cm
[b] Mean values within a column followed by different letter are significantly different ($p \leq 0.05$).
[b] NA, not analyzed
[d] ND, not detected by direct plating (3 CFU/ml detection limit) or enrichment (0.3 CFU/ml detection limit)
[e] Sample size: n = 3

Table 8. Population of *E. coli* O157:H7 recovered from lettuce leaves and wash solutions after treatment

Treatment	Mean *E. coli* O157:H7 population			Properties of Treatment Solution [e] (After Treatment)		
	On lettuce (log CFU/inoculated cut sample [a])					
	Recovered [b]	Reduction	In solutions (log CFU/ml)	ORP (mV)	pH	Free Chlorine (ppm)
None	7.03 A					
Tap	5.34 C	1.69	NA [c]	NA	NA	NA
Bleach	4.69 D	2.34	< 0.3 [d]	626 ± 7	9.28 ± 0.04	70 ± 1
Veggie Wash	6.15 B	0.88	4.35	NA	NA	NA
Ozone	5.67 BC	1.36	< 0.3	1015 ± 7	5.27 ± 0.02	NA
EO Water	3.60 E	3.43	< 0.3	1098 ± 4	2.8 ± 0.07	13 ± 1

[a] The average size of inoculated cut sample is is 4.4 cm × 3.8 cm by average
[b] Mean values within a column followed by different letter are significantly different ($p \leq 0.05$).
[c] NA, not analyzed
[d] ND, not detected by direct plating (3 CFU/ml detection limit) or enrichment (0.3 CFU/ml detection limit)
[e] Sample size: n = 3

Table 9. Population of *Listeria monocytogenes* recovered from lettuce leaves and wash solutions after treatment

Treatment	Mean *Listeria monocytogenes* population			Properties of Treatment Solution [e] (After Treatment)		
	On lettuce (log CFU/inoculated cut sample [a])					
	Recovered [b]	Reduction	In solutions (log CFU/ml)	ORP (mV)	pH	Free Chlorine (ppm)
None	7.09 A					
Tap	5.60 C	1.49	NA [c]	NA	NA	NA
Bleach	4.93 DE	2.16	< 0.3 [d]	620 ± 4	9.31 ± 0.03	72 ± 1
Veggie Wash	6.57 B	0.52	4.61	NA	NA	NA
Ozone	5.24 CD	1.85	< 0.3	985 ± 10	5.19 ± 0.09	NA
EO Water	4.54 E	2.55	< 0.3	1089 ± 4	2.81 ± 0.03	13 ± 1

[a] The average size of inoculated cut sample is 4.4 cm × 3.8 cm
[b] Mean values within a column followed by different letter are significantly different ($p \leq 0.05$).
[c] NA, not analyzed
[d] ND, not detected by direct plating (3 CFU/ml detection limit) or enrichment (0.3 CFU/ml detection limit)
[e] Sample size: n = 3

Table 10. Population of *Salmonella* recovered from spinach leaves and wash solutions after treatment

Treatment	Mean *Salmonella* population			Properties of Treatment Solution [f] (After Treatment)		
	On spinach (log CFU/inoculated cut sample [a])					
	Recovered [b]	Reduction	In solutions (log CFU/ml)	ORP (mV)	pH	Free Chlorine (ppm)
None	7.13 A					
Tap	6.12 C	1.01	NA [c]	NA	NA	NA
Bleach	6.04 CD	1.09	< 0.3 [d]	616 ± 6	9.29 ± 0.05	70 ± 2
Veggie Wash	6.59 B	0.54	4.27	NA	NA	NA
Ozone	6.80 B	0.33	2/6 [e]	969 ± 11	5.54 ± 0.19	NA
EO Water	5.81 D	1.32	< 0.3	1103 ± 3	2.87 ± 0.03	8 ± 1

[a] The average size of inoculated cut sample is 5.9 cm × 4.5 cm
[b] Mean values within a column followed by different letter are significantly different ($p \leq 0.05$).
[c] NA, not analyzed
[d] ND, not detected by direct plating (3 CFU/ml detection limit) or enrichment (0.3 CFU/ml detection limit)
[e] 2 out of 6 samples were positive for *Salmonella* by enrichment
[f] Sample size: n = 3

Table 11. Population of *E. coli* O157:H7 recovered from spinach leaves and wash solutions after treatment

Treatment	Mean *E. coli* O157:H7 population			Properties of Treatment Solution [f] (After Treatment)		
	On spinach (log CFU/inoculated cut sample [a])					
	Recovered [b]	Reduction	In solutions (log CFU/ml)	ORP (mV)	pH	Free Chlorine (ppm)
None	7.26 A					
Tap	6.18 C	1.08	NA [c]	NA	NA	NA
Bleach	6.12 C	1.14	< 0.3 [d]	641 ± 6	9.08 ± 0.06	66 ± 1
Veggie Wash	6.73 B	0.53	4.44	NA	NA	NA
Ozone	6.66 B	0.60	3/6 [e]	847 ± 125	5.32 ± 0.02	NA
EO Water	6.28 BC	0.98	< 0.3	1066 ± 13	2.86 ± 0.05	6 ± 1

[a] The average size of inoculated cut sample is 5.9 cm × 4.5 cm
[b] Mean values within a column followed by different letter are significantly different ($p \leq 0.05$).
[c] NA, not analyzed
[d] ND, not detected by direct plating (3 CFU/ml detection limit) or enrichment (0.3 CFU/ml detection limit)
[e] 3 out of 6 samples were positive for *E. coli* O157:H7 by enrichment
[f] Sample size: n = 3

Table 12. Population of *Listeria monocytogenes* recovered from spinach leaves and wash solutions after treatment

Treatment	Mean *Listeria monocytogenes* population			Properties of Treatment Solution [f] (After Treatment)		
	On spinach (log CFU/inoculated cut sample [a])					
	Recovered [b]	Reduction	In solutions (log CFU/ml)	ORP (mV)	pH	Free Chlorine (ppm)
None	6.94 A					
Tap	5.92 C	1.02	NA [c]	NA	NA	NA
Bleach	5.36 D	1.58	< 0.3 [d]	611 ± 4	9.23 ± 0.07	63 ± 1
Veggie Wash	6.47 AB	0.47	4.10	NA	NA	NA
Ozone	5.96 BC	0.98	2/6 [e]	569 ± 49	5.51 ± 0.05	NA
EO Water	5.34 D	1.6	< 0.3	1036 ± 9	2.86 ± 0.02	5 ± 1

[a] The average size of inoculated cut sample is 5.9 cm × 4.5 cm
[b] Mean values within a column followed by different letter are significantly different ($p \leq 0.05$).
[c] NA, not analyzed
[d] ND, not detected by direct plating (3 CFU/ml detection limit) or enrichment (0.3 CFU/ml detection limit)
[e] 2 out of 6 samples were positive for *L. monocytogenes* by enrichment
[f] Sample size: n = 3

Table 13. Population of *Salmonella* recovered from green onions and wash solutions after treatment

Treatment	Mean *Salmonella* population			Properties of Treatment Solution [e] (After Treatment)		
	On green onion (log CFU/inoculated cut sample [a])					
	Recovered [b]	Reduction	In solutions (log CFU/ml)	ORP (mV)	pH	Free Chlorine (ppm)
None	7.20 A					
Tap	6.19 B	1.01	NA [c]	NA	NA	NA
Bleach	4.90 C	2.30	< 0.3 [d]	636 ± 17	9.3 ± 0.02	70 ± 1
Veggie Wash	6.12 B	1.08	4.81	NA	NA	NA
Ozone	4.81 C	2.39	< 0.3	1037 ± 3	4.94 ± 0.03	NA
EO Water	4.68 C	2.52	< 0.3	1105 ± 4	2.83 ± 0.01	14 ± 1

[a] The average length of inoculated cut green onion sample is 6.8 cm
[b] Mean values within a column followed by different letter are significantly different ($p \leq 0.05$).
[c] NA, not analyzed
[d] ND, not detected by direct plating (3 CFU/ml detection limit) or enrichment (0.3 CFU/ml detection limit)
[e] Sample size: n = 3

Table 14. Population of *E. coli* O157:H7 recovered from green onions and wash solutions after treatment

Treatment	Mean *E. coli* O157:H7 population			Properties of Treatment Solution [e] (After Treatment)		
	On green onion (log CFU/inoculated cut sample [a])					
	Recovered [b]	Reduction	In solutions (log CFU/ml)	ORP (mV)	pH	Free Chlorine (ppm)
None	7.09 A					
Tap	5.64 B	1.45	NA [b]	NA	NA	NA
Bleach	4.91 C	2.18	< 0.3 [c]	633 ± 1	9.21 ± 0.09	72 ± 0
Veggie Wash	5.92 B	1.17	5.16	NA	NA	NA
Ozone	4.51 C	2.58	< 0.3	1018 ± 6	4.96 ± 0.04	NA
EO Water	3.99 D	3.10	< 0.3	1099 ± 3	2.78 ± 0.03	10 ± 1

[a] The average length of inoculated cut green onion sample is 6.8 cm
[b] Mean values within a column followed by different letter are significantly different ($p \leq 0.05$).
[c] NA, not analyzed
[d] ND, not detected by direct plating (3 CFU/ml detection limit) or enrichment (0.3 CFU/ml detection limit)
[e] Sample size: n = 3

Table 15. Population of *Listeria monocytogenes* recovered from green onions and wash solutions after treatment

Treatment	Mean *Listeria monocytogenes* population			Properties of Treatment Solution [e] (After Treatment)		
	On green onion (log CFU/inoculated cut sample [a])					
	Recovered [b]	Reduction	In solutions (log CFU/ml)	ORP (mV)	pH	Free Chlorine (ppm)
None	7.11 A					
Tap	6.13 B	0.98	NA [b]	NA	NA	NA
Bleach	4.33 C	2.78	< 0.3 [c]	650 ± 5	8.92 ± 0.15	64 ± 1
Veggie Wash	6.39 B	0.72	5.35	NA	NA	NA
Ozone	4.59 C	2.52	< 0.3	997 ± 8	4.89 ± 0.03	NA
EO Water	3.52 D	3.59	< 0.3	1065 ± 5	2.95 ± 0.04	7 ± 1

[a] The average length of inoculated cut green onion sample is 6.8 cm
[b] Mean values within a column followed by different letter are significantly different ($p \leq 0.05$).
[c] NA, not analyzed
[d] ND, not detected by direct plating (3 CFU/ml detection limit) or enrichment (0.3 CFU/ml detection limit)
[e] Sample size: n = 3

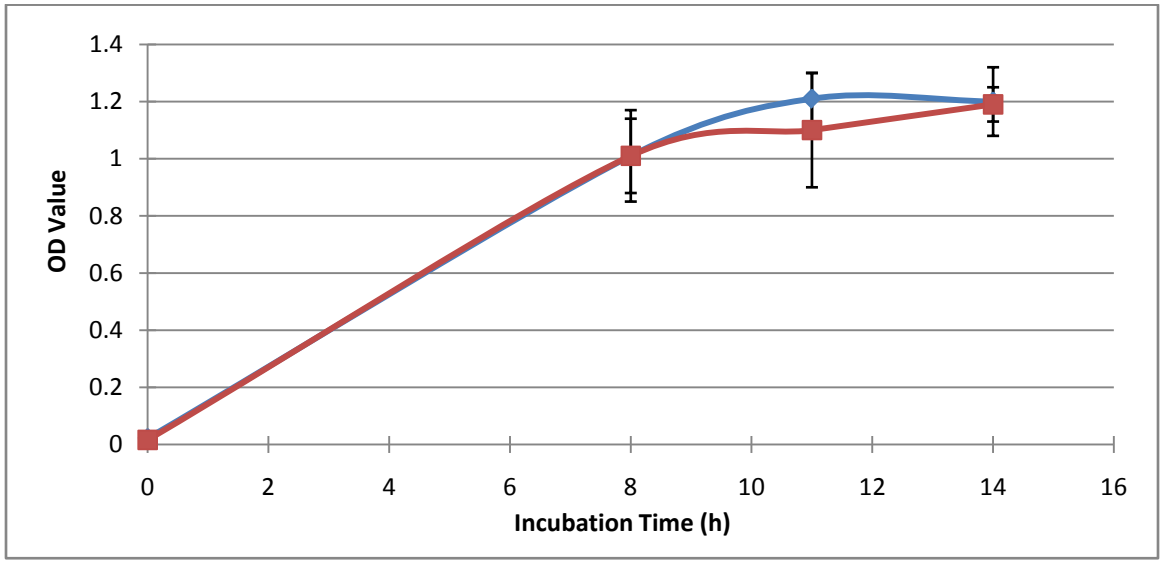


FIGURE 2. Growth curves of the parent strain of *Salmonella Baildon* (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.

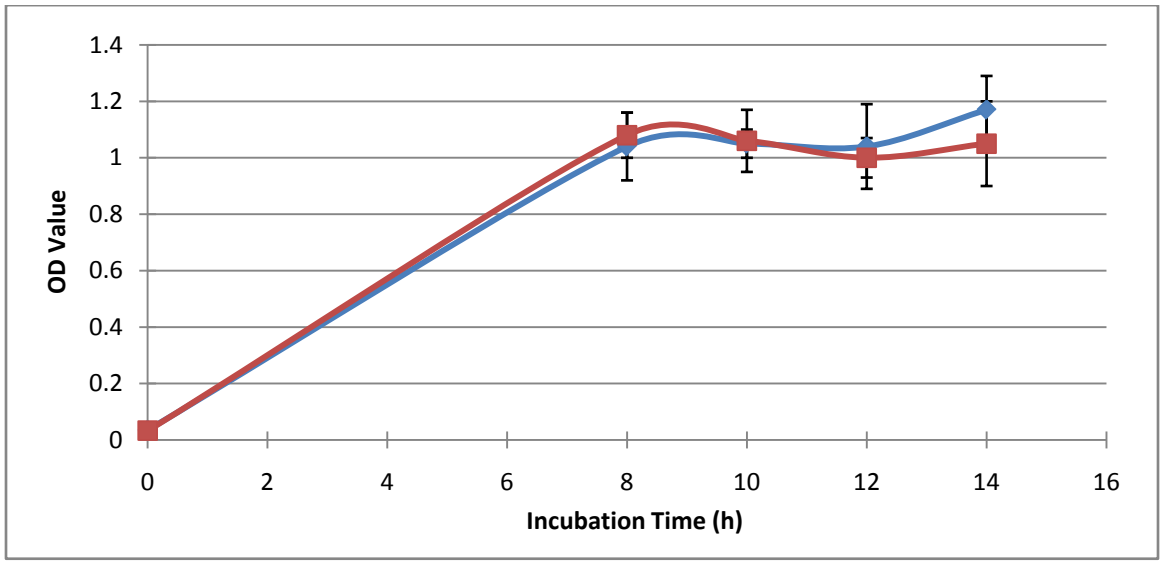


FIGURE 3. Growth curves of the parent strain of *Salmonella Montevideo* G4639 (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.

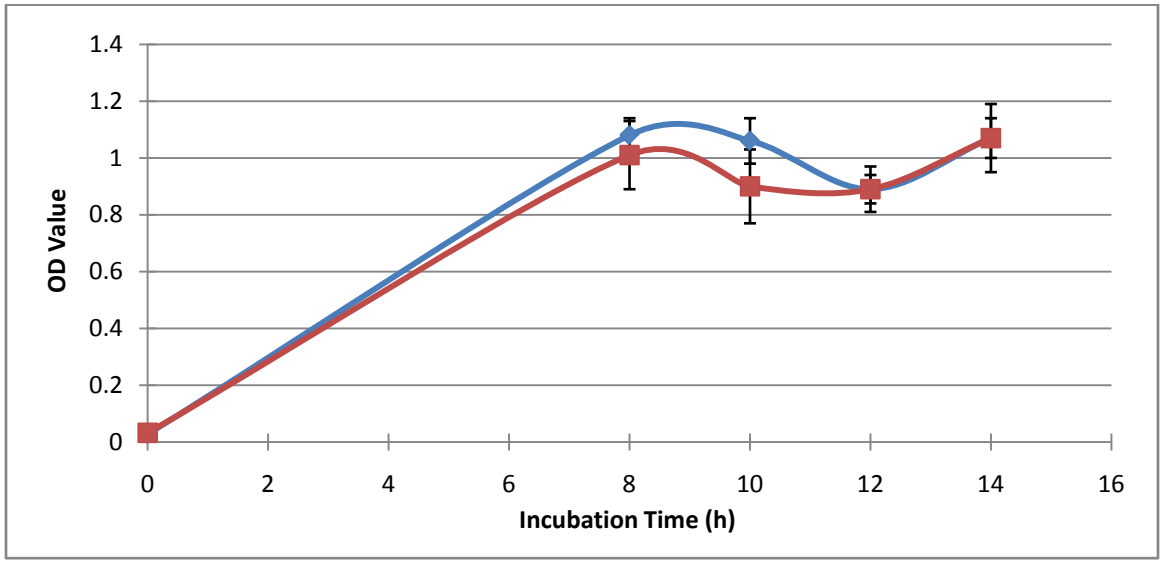


FIGURE 4. Growth curves of the parent strain of *Salmonella Poona* 01A3923 (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.

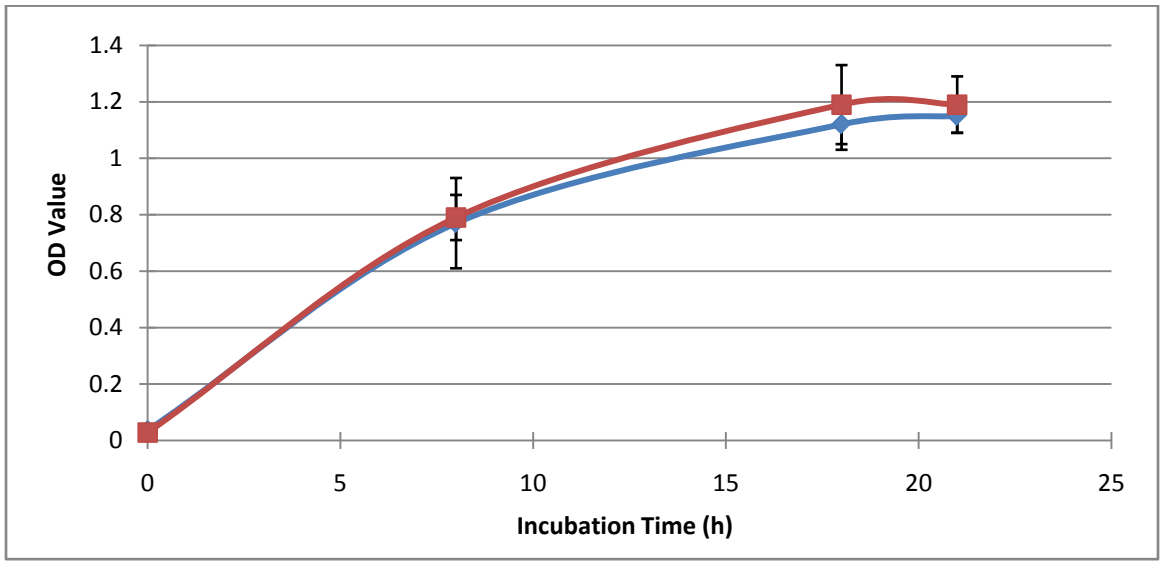


FIGURE 5. Growth curves of the parent strain of *Salmonella Stanley* H1256 (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.

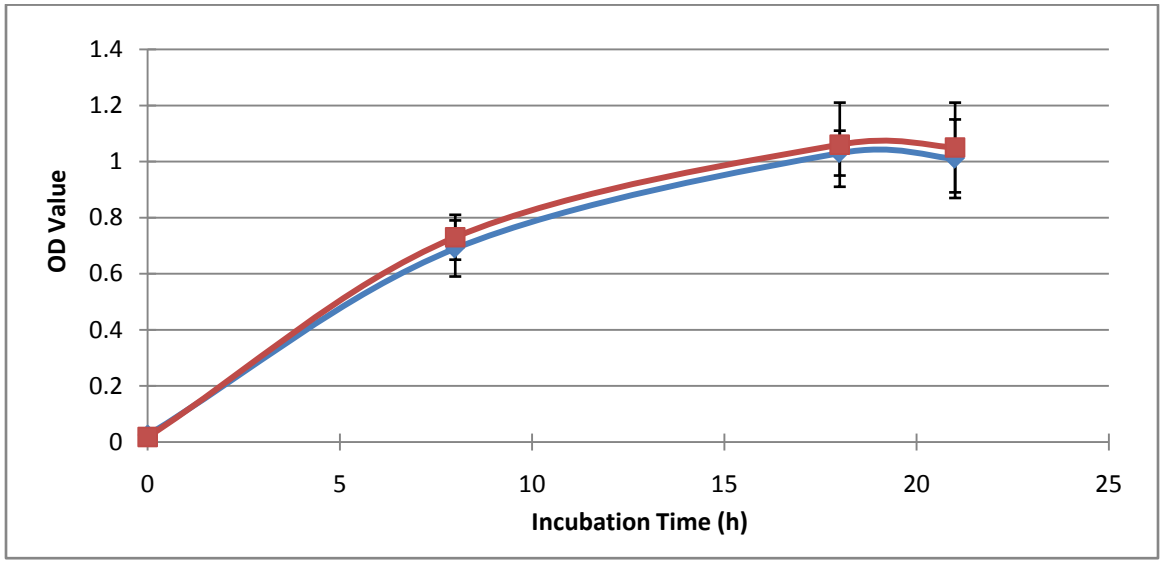


FIGURE 6. Growth curves of the parent strain of *Salmonella Typhimurium* (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.

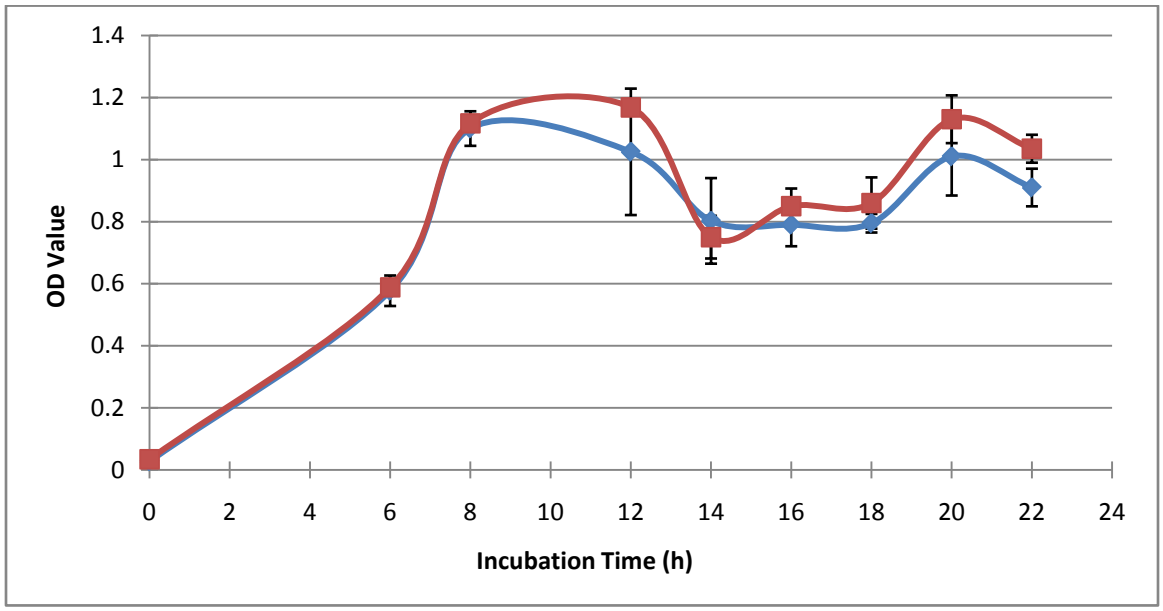


FIGURE 7. Growth curves of the parent strain of *E. coli* O157:H7 H1730 (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.

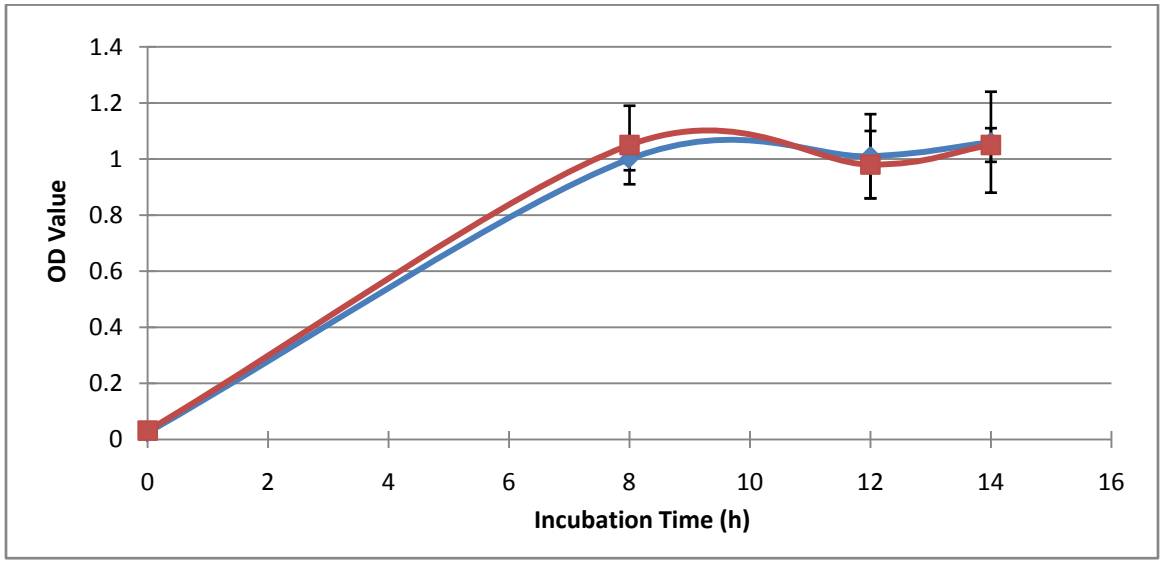


FIGURE 8. Growth curves of the parent strain of *E. coli* O157:H7 F4546 (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.

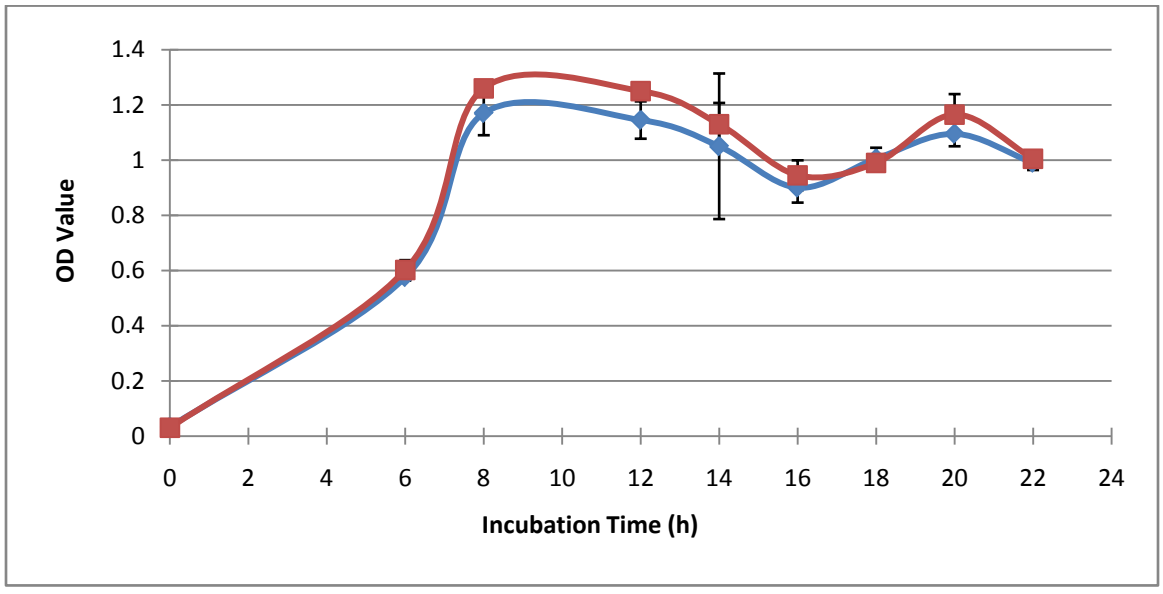


FIGURE 9. Growth curves of the parent strain of *E. coli* O157:H7 #994 (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.

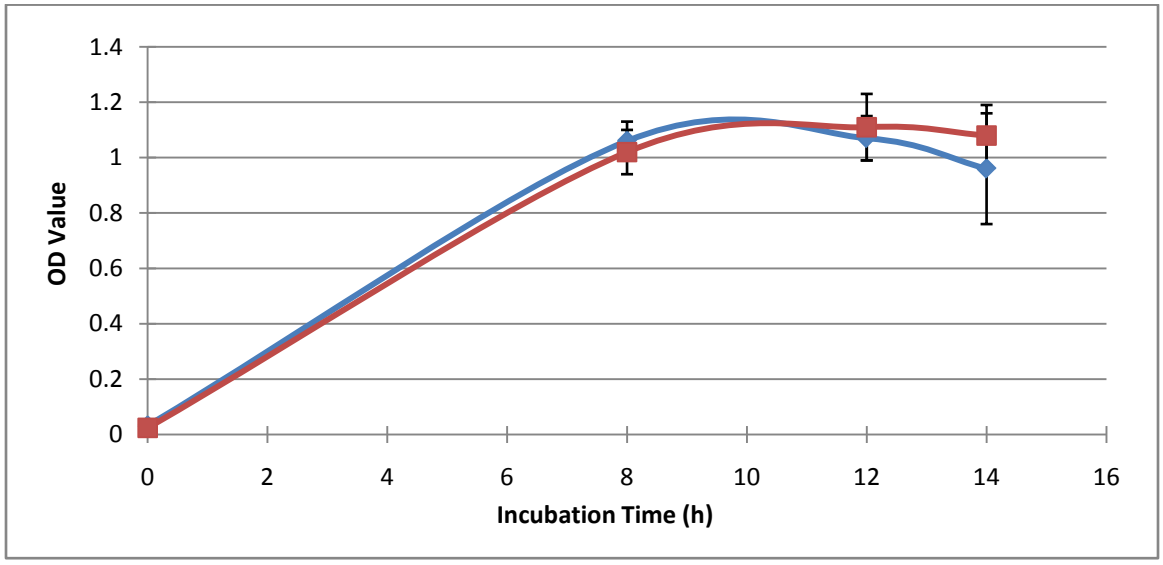


FIGURE 10. Growth curves of the parent strain of *E. coli* O157:H7 SEA 13B88 (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.

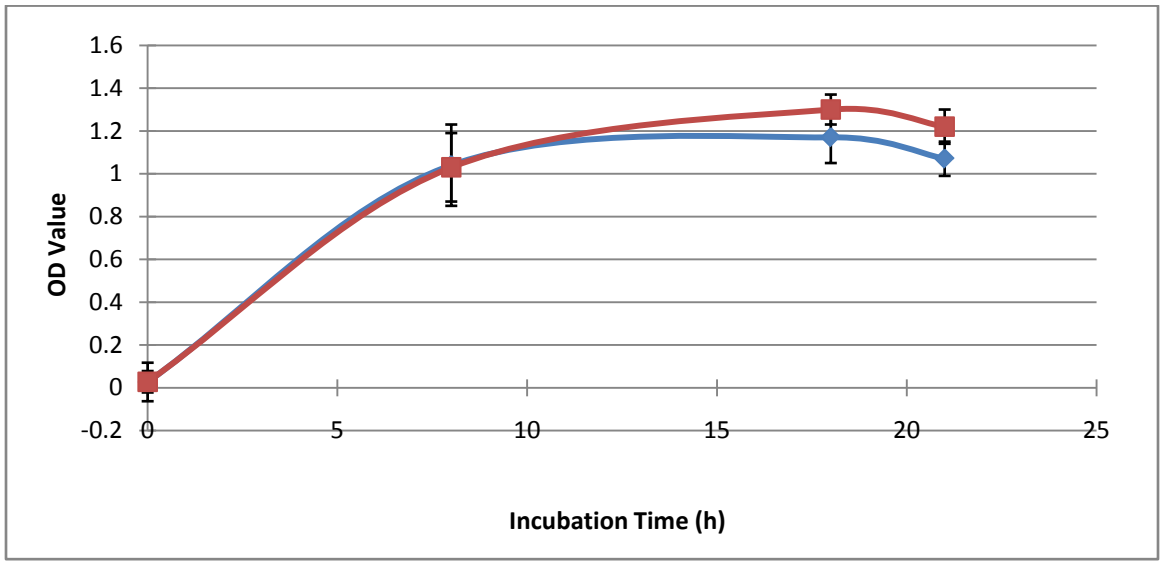


FIGURE 11. Growth curves of the parent strain of *E. coli* O157:H7 CDC658 (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.

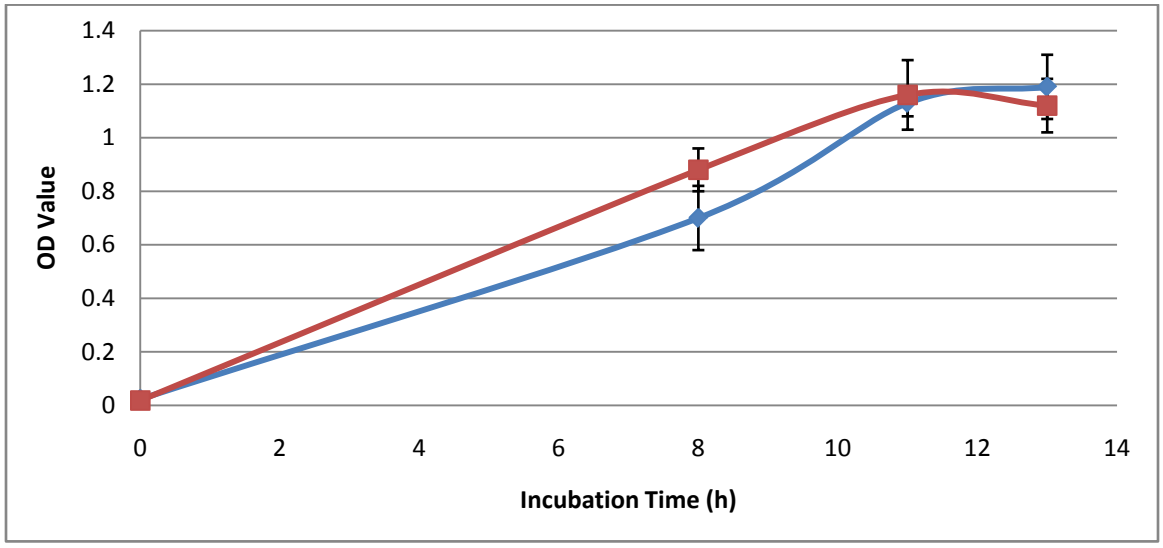


FIGURE 12. Growth curves of the parent strain of *L. monocytogenes* LCDC 81-861 (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.

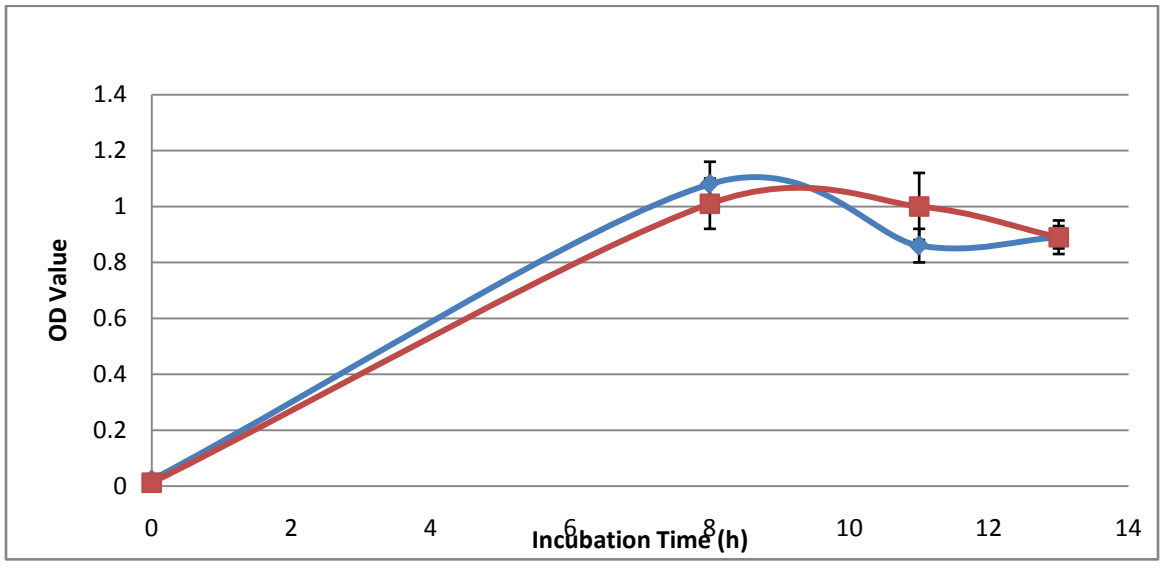


FIGURE 13. Growth curves of the parent strain of *L. monocytogenes* G3982 (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.

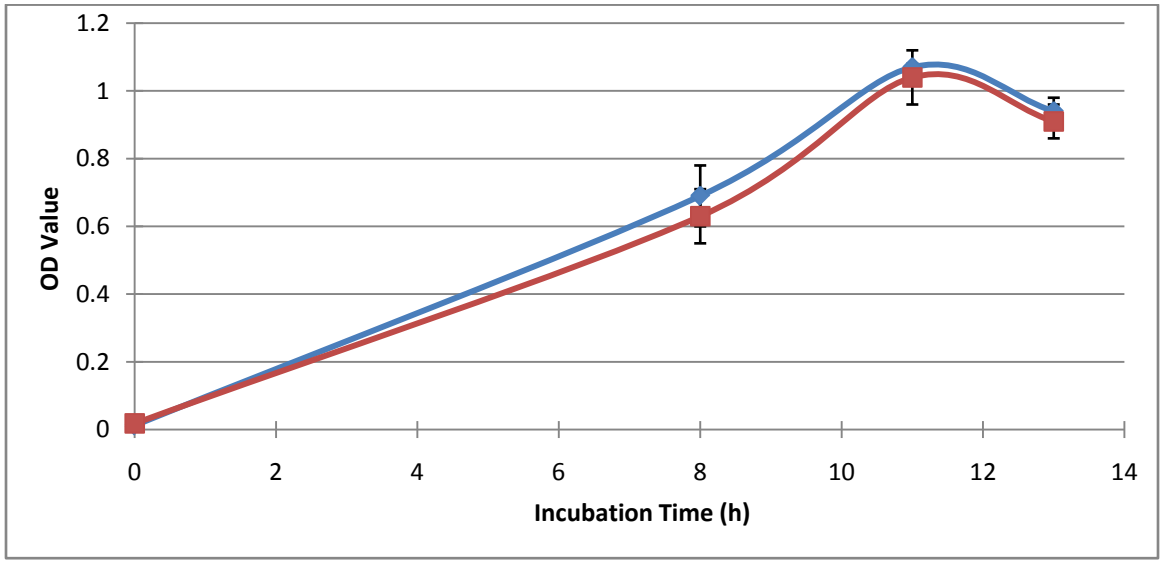


FIGURE 14. Growth curves of the parent strain of *L. monocytogenes* Scott A (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.

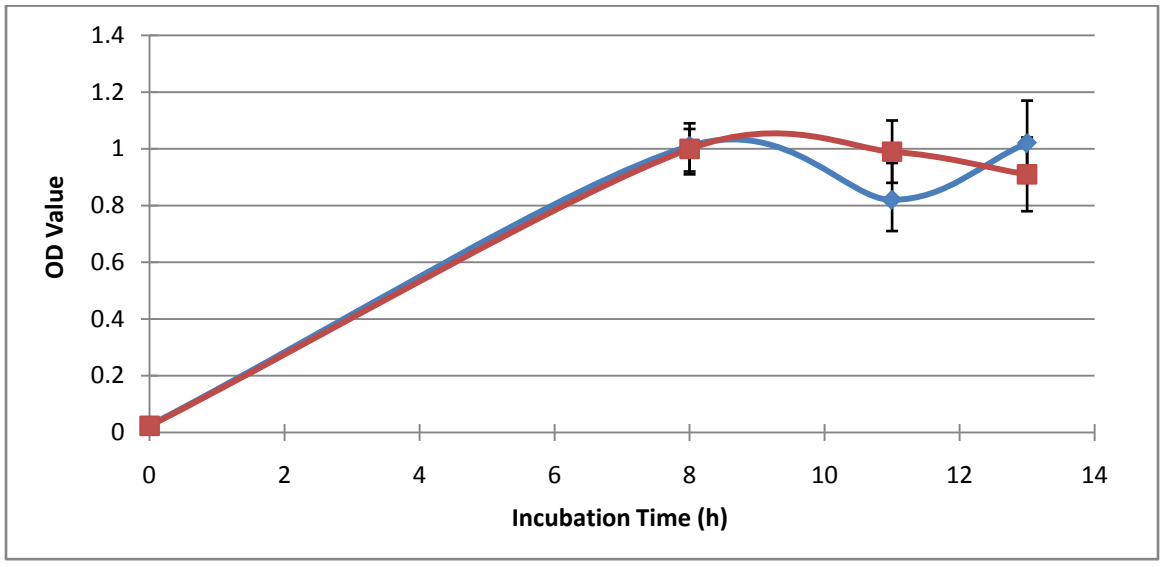


FIGURE 15. Growth curves of the parent strain of *L. monocytogenes* LM 254 (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.

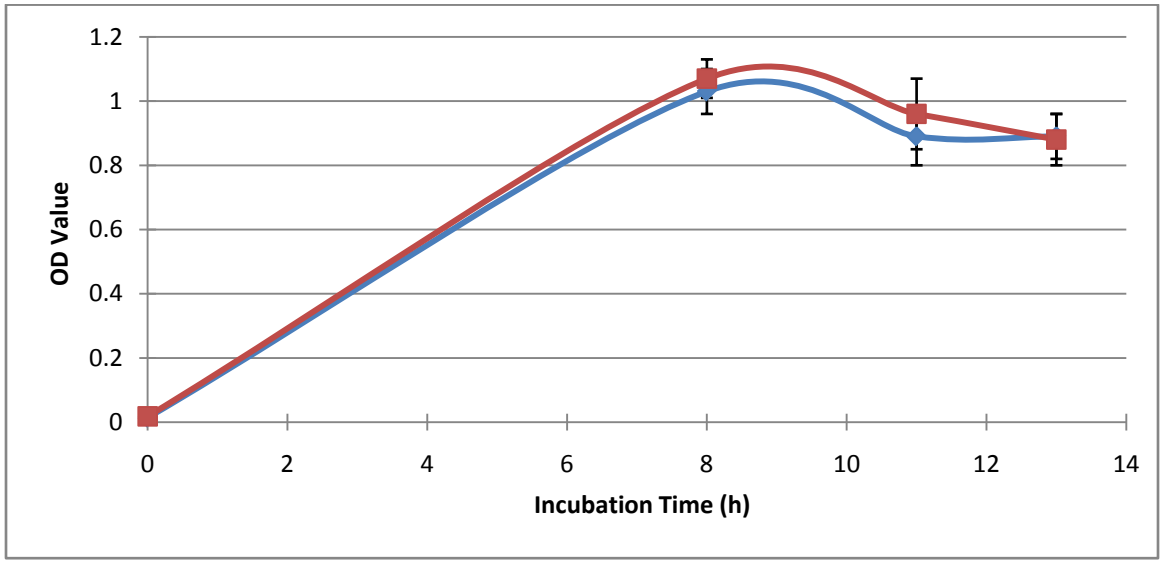
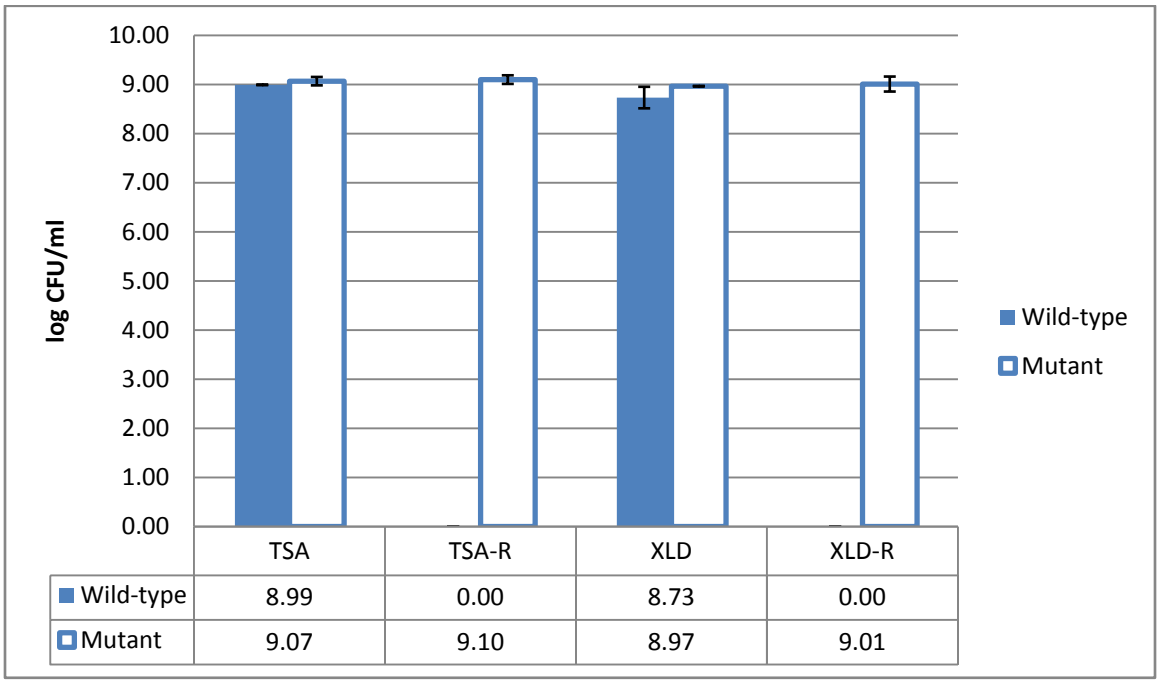
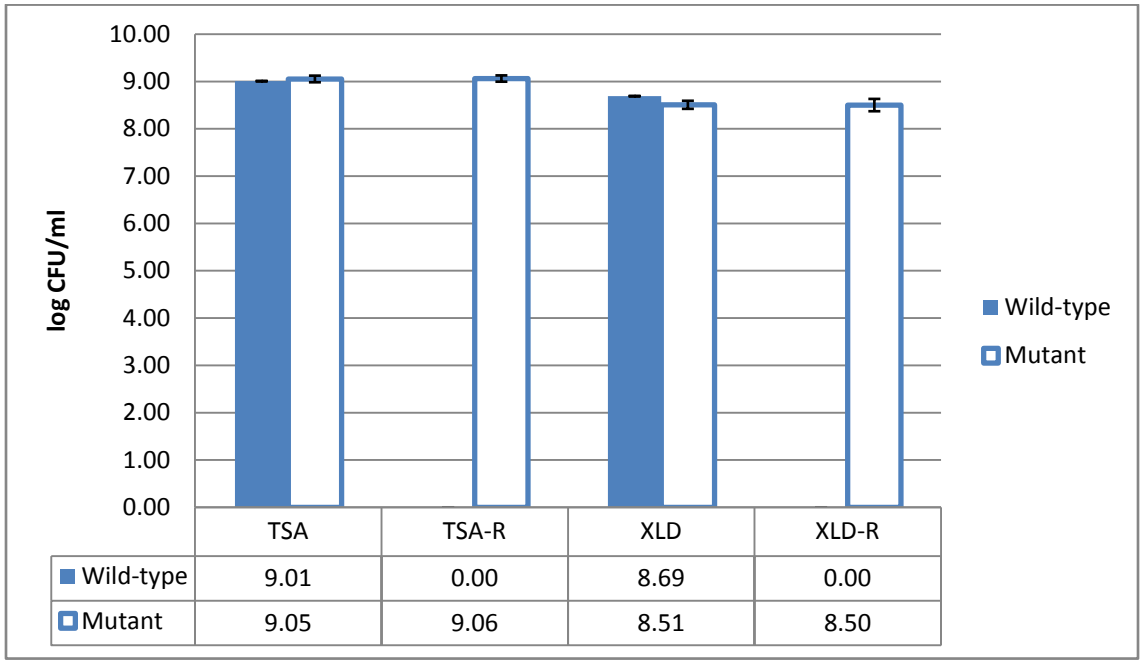


FIGURE 16. Growth curves of the parent strain of *L. monocytogenes* LM 311 (▲) and its rifampicin resistant derivative (■) when incubated at 37 °C in TSB and TSB-R100 respectively.



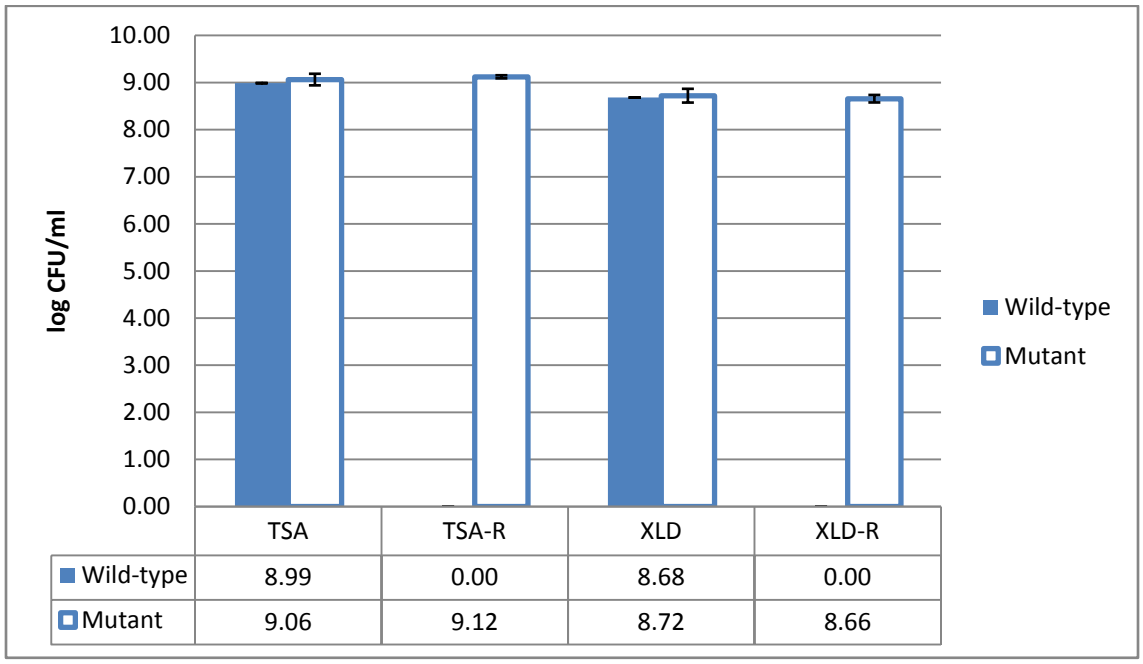
	TSA	TSA-R	XLD	XLD-R
Wild-type	8.99	0.00	8.73	0.00
Mutant	9.07	9.10	8.97	9.01

FIGURE 17. Colony development on TSA, TSA-R, XLD and XLD-R by wild-type (■) and rifampicin resistant strains (□) of *Salmonella Baildon* after 24-h incubation in TSB-R100



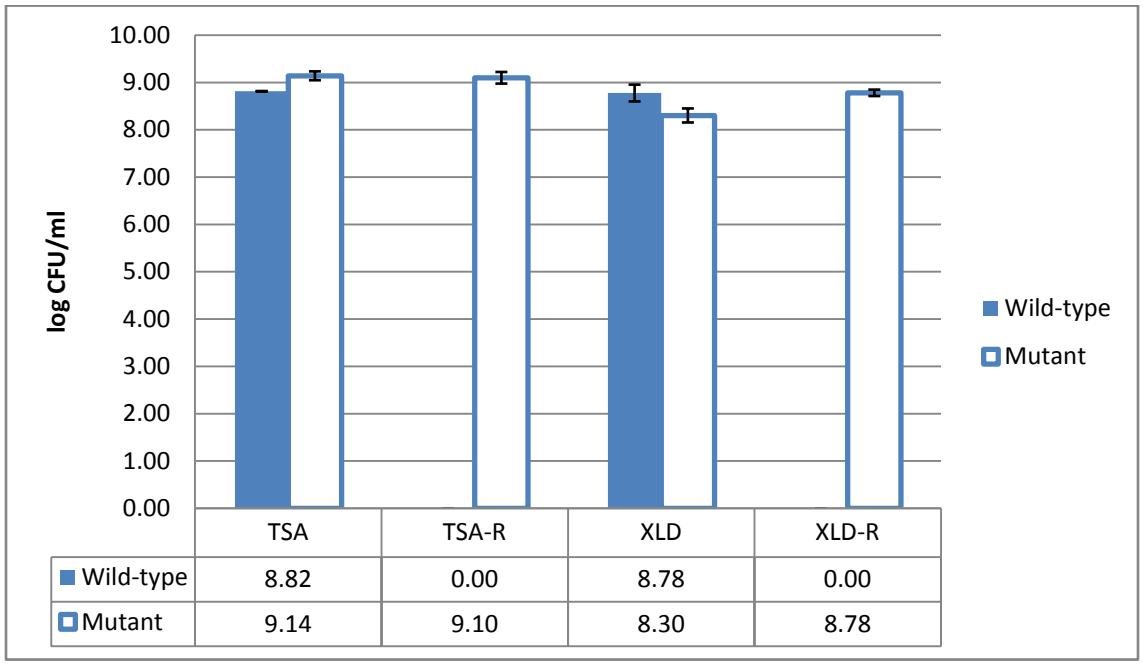
	TSA	TSA-R	XLD	XLD-R
Wild-type	9.01	0.00	8.69	0.00
Mutant	9.05	9.06	8.51	8.50

FIGURE 18. Colony development on TSA, TSA-R, XLD and XLD-R by wild-type (■) and rifampicin resistant strains (□) of *Salmonella Montevideo* after 24-h incubation in TSB-R100



	TSA	TSA-R	XLD	XLD-R
Wild-type	8.99	0.00	8.68	0.00
Mutant	9.06	9.12	8.72	8.66

FIGURE 19. Colony development on TSA, TSA-R, XLD and XLD-R by wild-type (■) and rifampicin resistant strains (□) of *Salmonella Poona* after 24-h incubation in TSB-R100



	TSA	TSA-R	XLD	XLD-R
Wild-type	8.82	0.00	8.78	0.00
Mutant	9.14	9.10	8.30	8.78

FIGURE 20. Colony development on TSA, TSA-R, XLD and XLD-R by wild-type (■) and rifampicin resistant strains (□) of *Salmonella Stanley* after 24-h incubation in TSB-R100

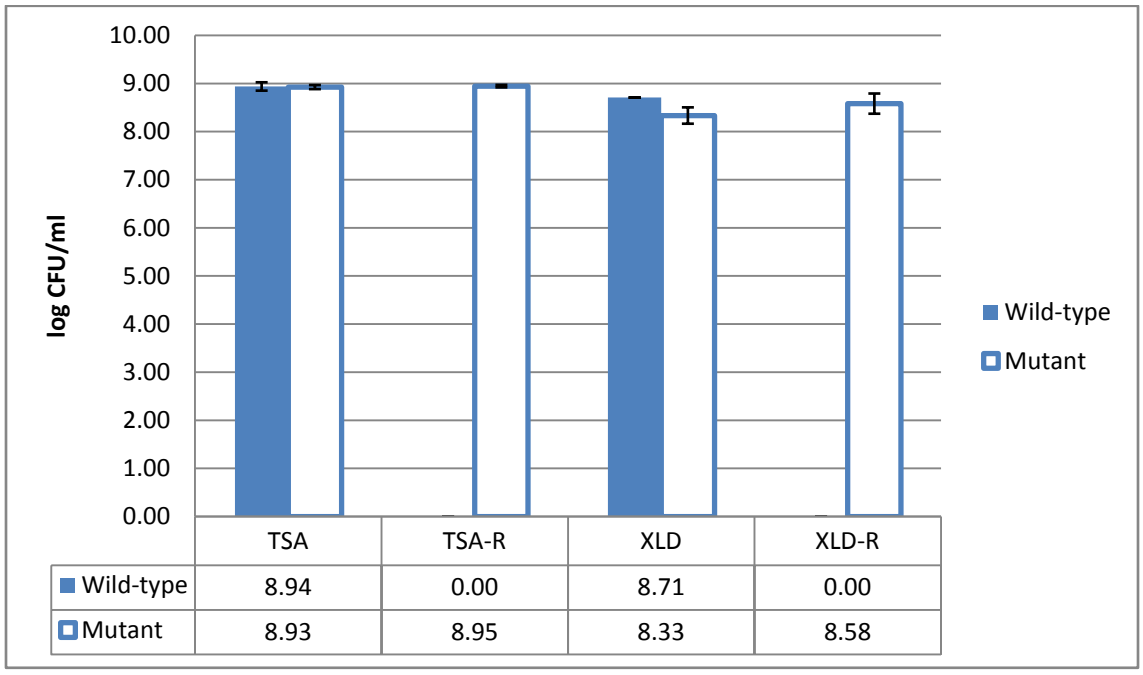


FIGURE 21. Colony development on TSA, TSA-R, XLD and XLD-R by wild-type (■) and rifampicin resistant strains (□) of *Salmonella typhimurium* after 24-h incubation in TSB-R100

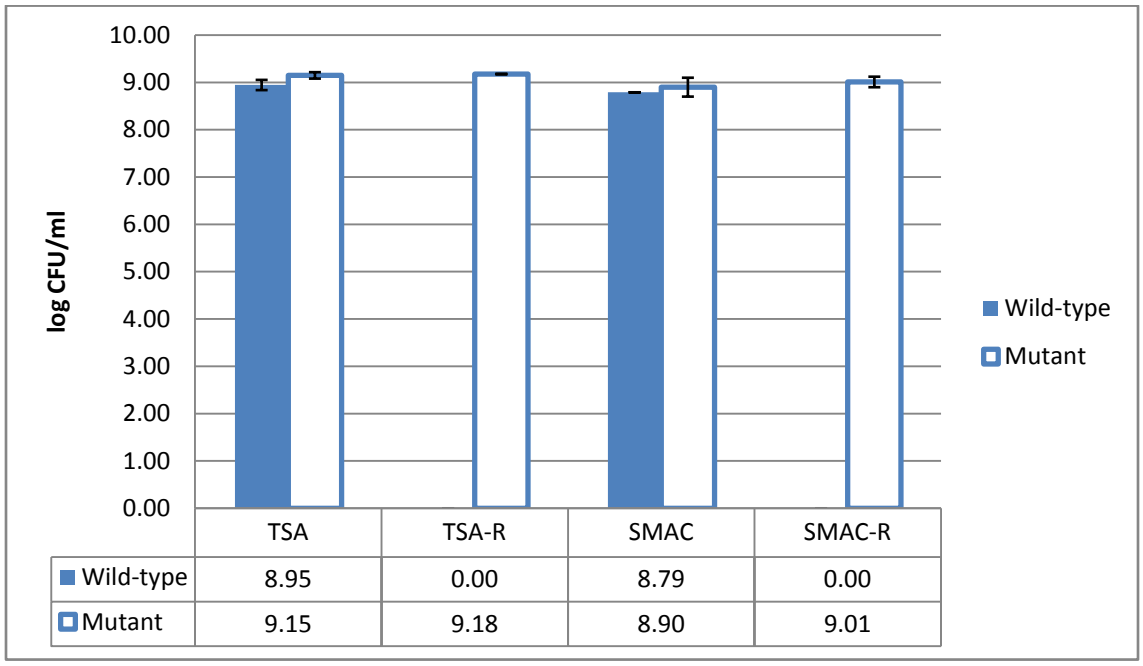
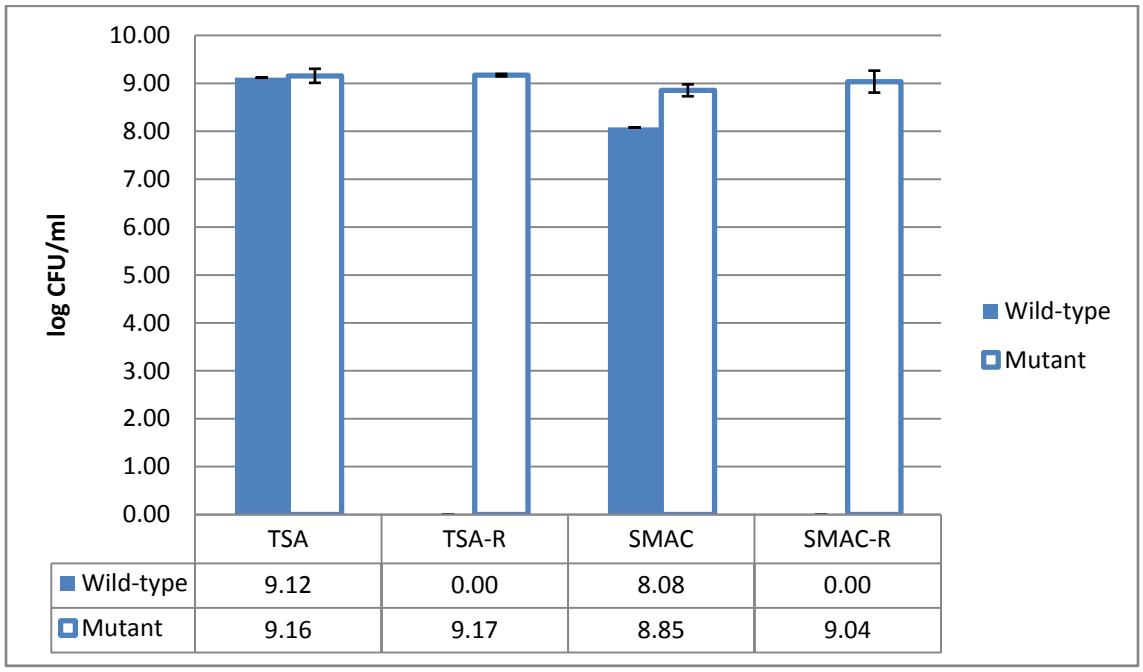
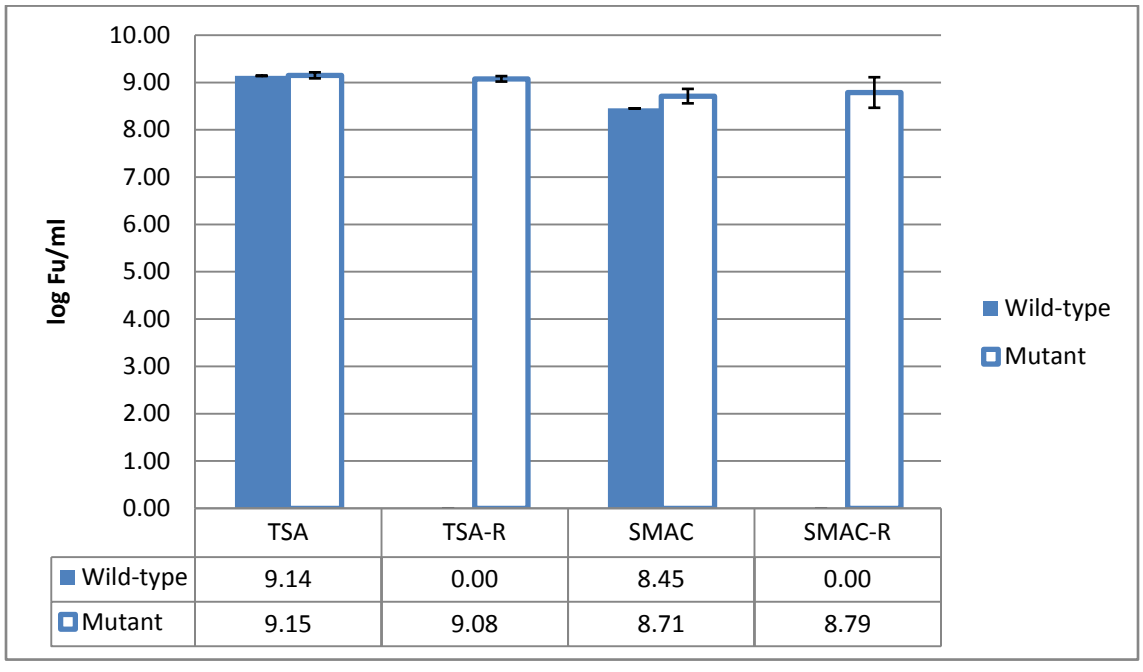


FIGURE 22. Colony development on TSA, TSA-R, SMAC and SMAC-R by wild-type (■) and rifampicin resistant strains (□) of *E. coli* O157:H7 H-1730 after 24-h incubation in TSB-R100



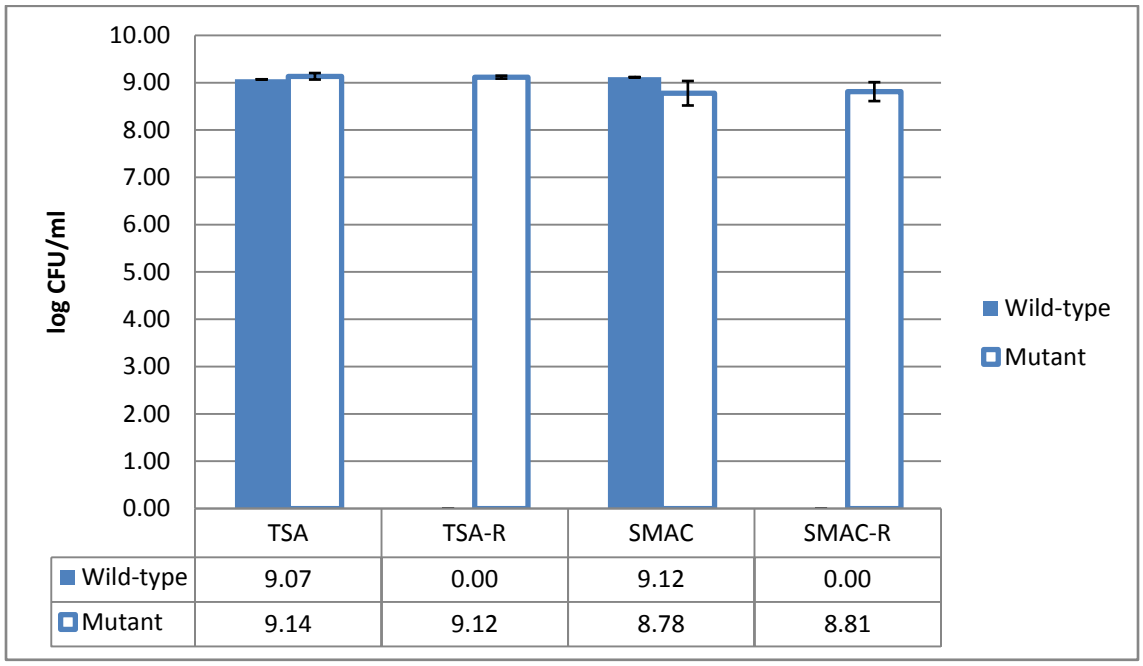
	TSA	TSA-R	SMAC	SMAC-R
Wild-type	9.12	0.00	8.08	0.00
Mutant	9.16	9.17	8.85	9.04

FIGURE 23. Colony development on TSA, TSA-R, SMAC and SMAC-R by wild-type (■) and rifampicin resistant strains (□) of *E. coli* O157:H7 F-4546 after 24-h incubation in TSB-R100



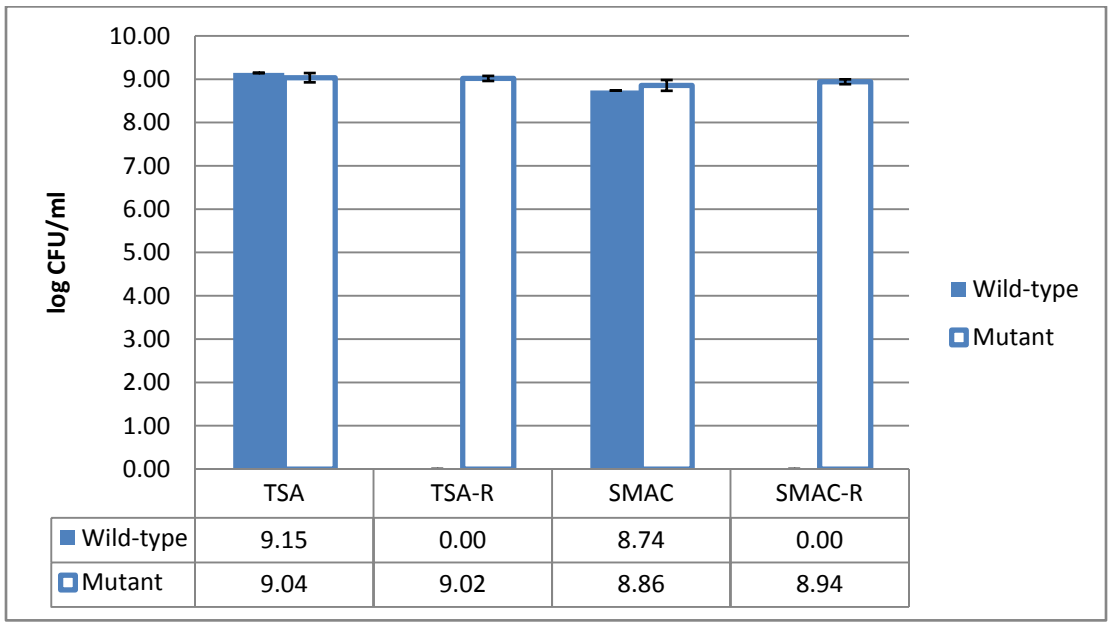
	TSA	TSA-R	SMAC	SMAC-R
Wild-type	9.14	0.00	8.45	0.00
Mutant	9.15	9.08	8.71	8.79

FIGURE 24. Colony development on TSA, TSA-R, SMAC and SMAC-R by wild-type (■) and rifampicin resistant strains (□) of *E. coli* O157:H7 #994 after 24-h incubation in TSB-R100



	TSA	TSA-R	SMAC	SMAC-R
Wild-type	9.07	0.00	9.12	0.00
Mutant	9.14	9.12	8.78	8.81

FIGURE 25. Colony development on TSA, TSA-R, SMAC and SMAC-R by wild-type (■) and rifampicin resistant strains (□) of *E. coli* O157:H7 SEA13B88 after 24-h incubation in TSB-R100



	TSA	TSA-R	SMAC	SMAC-R
Wild-type	9.15	0.00	8.74	0.00
Mutant	9.04	9.02	8.86	8.94

FIGURE 26. Colony development on TSA, TSA-R, SMAC and SMAC-R by wild-type (■) and rifampicin resistant strains (□) of *E. coli* O157:H7 CDC658 after 24-h incubation in TSB-R100

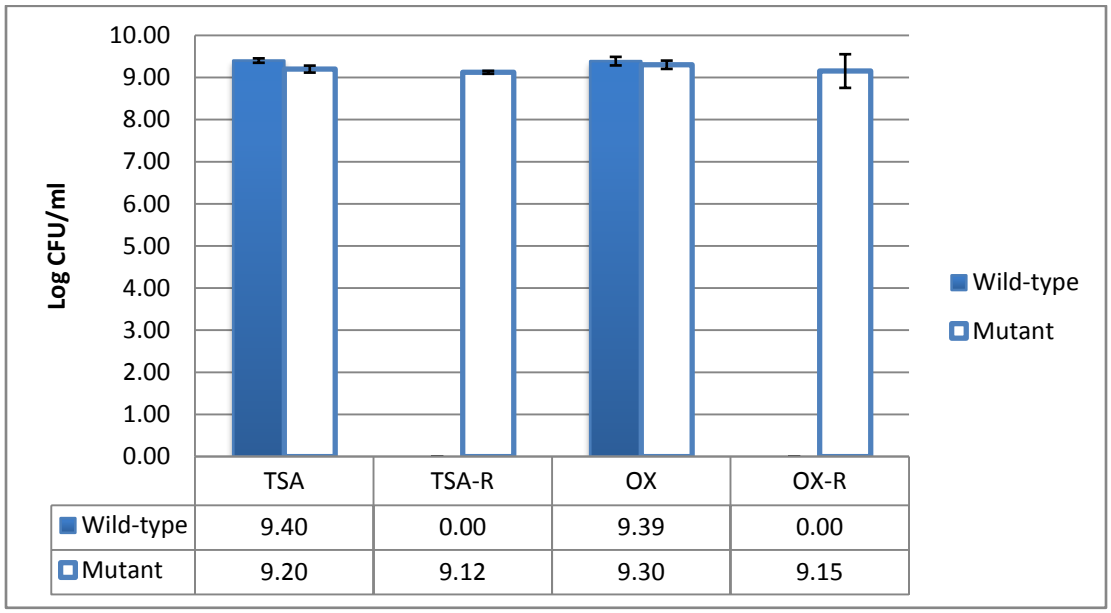


FIGURE 27. Colony development on TSA, TSA-R, OX and OX-R by wild-type (■) and rifampicin resistant strains (□) of *L. monocytogenes* LCDC 81-861 after 24-h incubation in TSB-R100

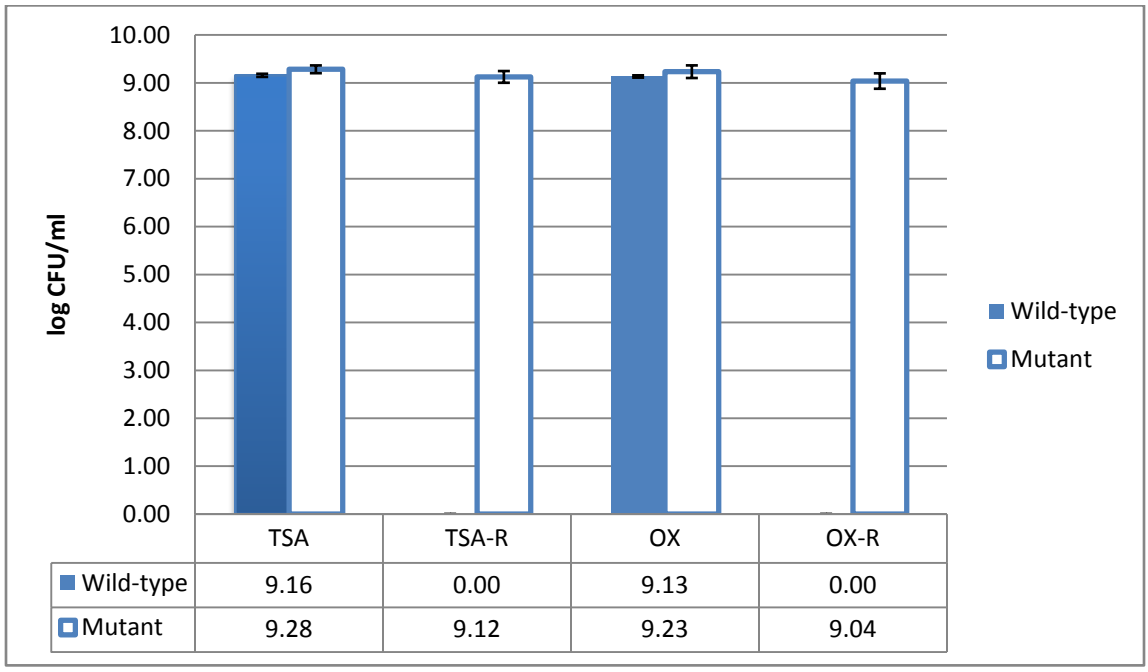
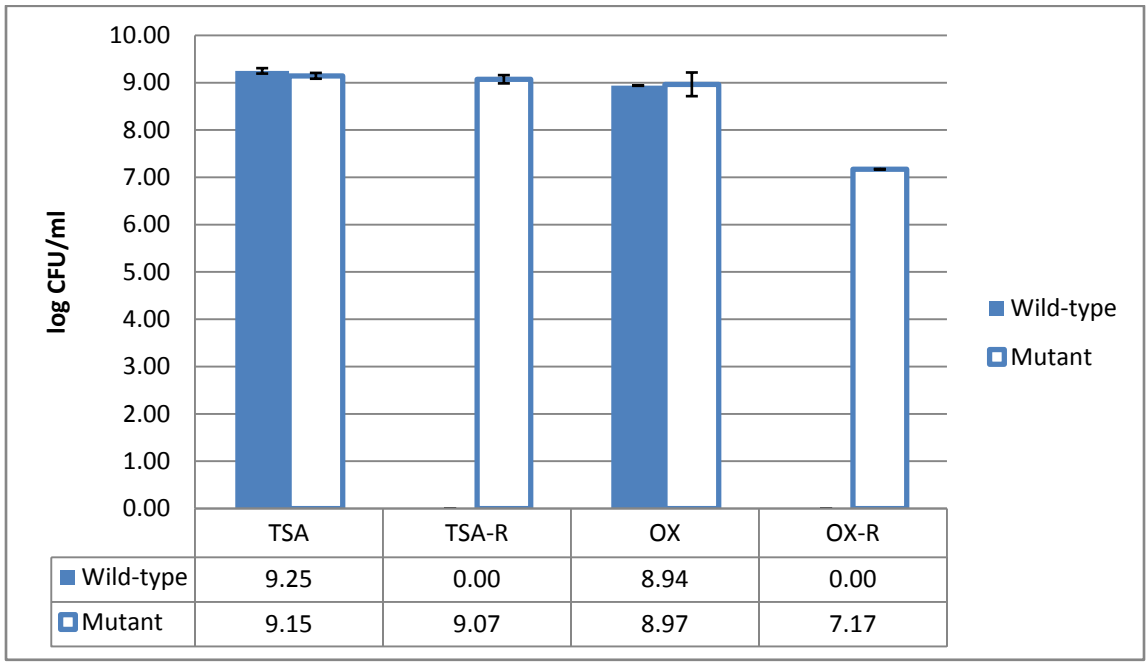
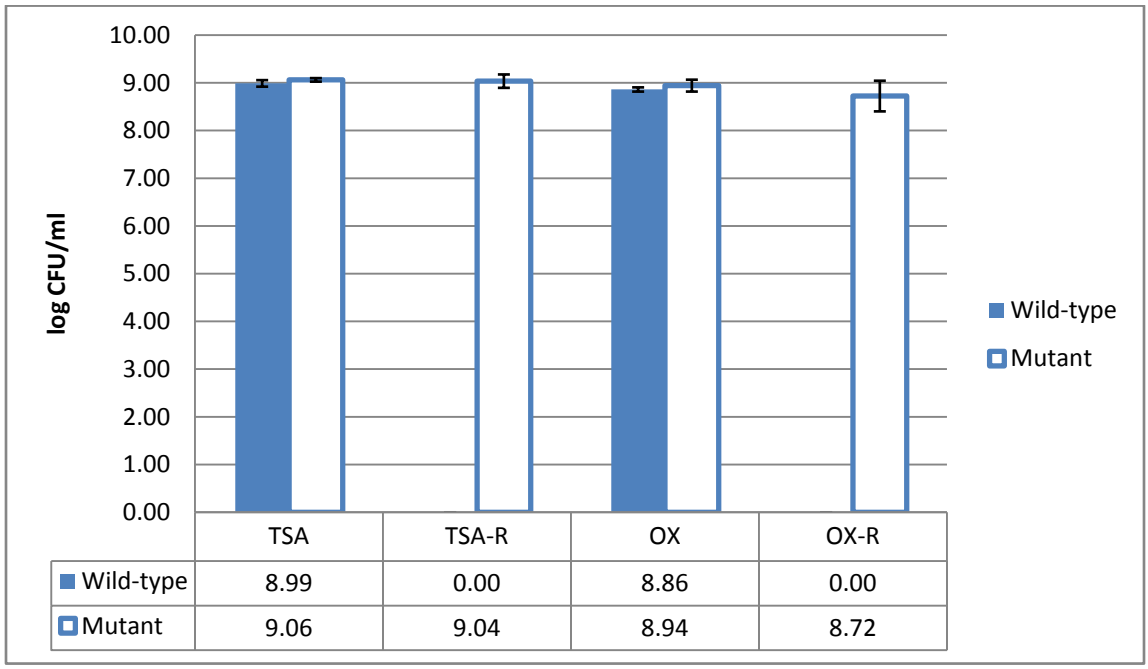


FIGURE 28. Colony development on TSA, TSA-R, OX and OX-R by wild-type (■) and rifampicin resistant strains (□) of *L. monocytogenes* G3982 after 24-h incubation in TSB-R100



	TSA	TSA-R	OX	OX-R
Wild-type	9.25	0.00	8.94	0.00
Mutant	9.15	9.07	8.97	7.17

FIGURE 29. Colony development on TSA, TSA-R, OX and OX-R by wild-type (■) and rifampicin resistant strains (□) of *L. monocytogenes* Scott A after 24-h incubation in TSB-R100



	TSA	TSA-R	OX	OX-R
Wild-type	8.99	0.00	8.86	0.00
Mutant	9.06	9.04	8.94	8.72

FIGURE 30. Colony development on TSA, TSA-R, OX and OX-R by wild-type (■) and rifampicin resistant strains (□) of *L. monocytogenes* LM254 after 24-h incubation in TSB-R100

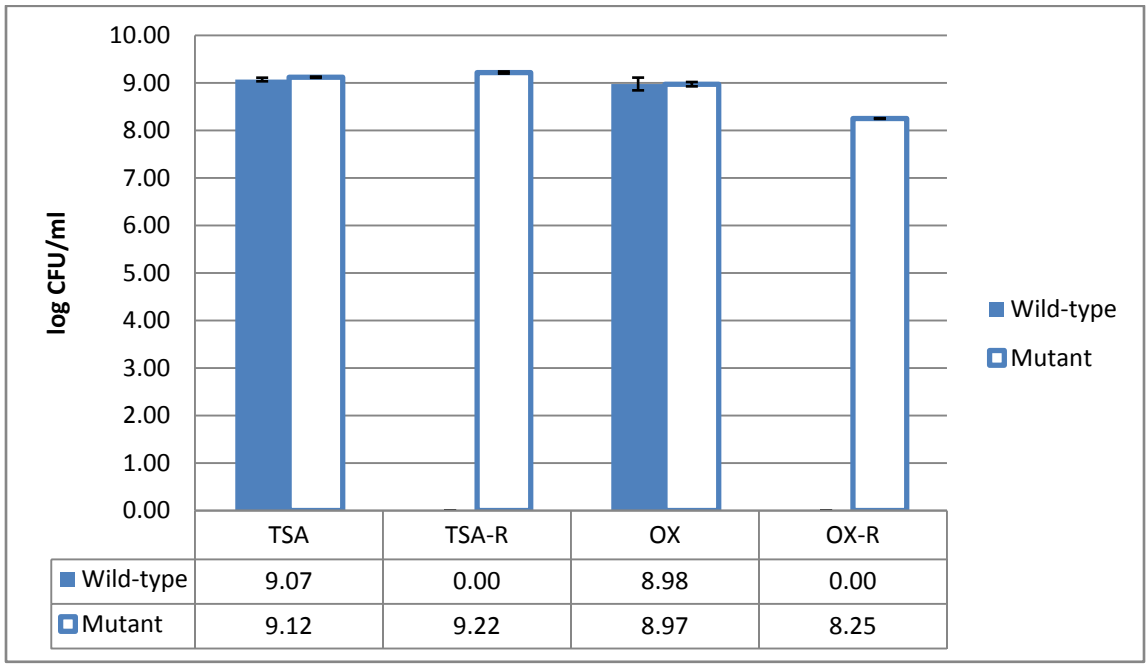


FIGURE 31. Colony development on TSA, TSA-R, OX and OX-R by wild-type (■) and rifampicin resistant strains (□) of *L. monocytogenes* LM311 after 24-h incubation in TSB-R100

CHAPTER 5

CONCLUSIONS AND IMPLICATIONS

Wash solutions prepared with soft water (0 ppm hardness) was equivalent or more effective than those prepared with hard water (200 ppm hardness) in killing *Salmonella* on lettuce. Generally, the effectiveness of washing technologies used in this study decreased in the following order: EO water ≥ chlorine bleach ≥ ozonated water ≥ tap water ≥ Veggie Wash.

Treatment of leafy green vegetables and green onions with either running tap water or Veggie Wash was not effective in reducing populations of pathogenic bacteria from the produce surface, having found to cause a typical reduction of less than 1.5 log.

Household chlorine bleach, at concentrations currently permitted for being used in the fresh produce industry (approximately 75 ppm free chlorine), was able to reduce pathogens by 1.1 – 2.8 log on all three produce tested. However, it is not recommended for home use since consumers may have problems obtaining proper concentrations and handling the concentrated form of chlorine solution.

Ozonated water, a powerful antimicrobial agent, has yet shown to be ineffective for washing leafy green vegetables due to the particularly non-uniform distribution of active ozone in the aqueous solution, resulting in similar pathogen reduction as compared to water rinse. Nevertheless, its superior efficacy for washing green onions and whole fruits, along with its nontoxic nature due to the absence of harmful residue, made it a candidate technology suitable for home use.

In most test cases involving lettuce and green onions, electrolyzed oxidizing water (pH 2.8, free chlorine concentration 18 ppm) was the most effective washing technology, producing pathogen reductions of 2.5 – 3.7 log. EO water can be easily prepared from tap water and table salt without addition of hazardous chemicals, resulting in lower levels of free chlorine than household bleach. From these considerations, it may be a suitable washing technology for use at home. However, its acceptance by consumers would be limited due to the high initial capital cost. More importantly, when tested on fresh-cut produce (such as spinach) it is not able to obtain significantly further microbial reduction than those achieved by chlorine or water rinse ($P > 0.05$), resulting in only 1.0 – 1.6 log reduction of test pathogens.

Overall, this study highlighted the potential application of various home washing technologies to alleviate the risks of bacterial infections associated with consumption of leafy greens and green onions. Results obtained also provided the consumers with scientific evidence to make their own decision regarding the selection of antimicrobial methods for home use.

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APPENDIX

FLOW CHART OF EXPERIMENTAL DESIGN

One Test Case

(1 Organism × 1 Produce × 1 Treatment)