USE OF OREGANO ESSENTIAL OIL AND ACETIC ACID TO REDUCE *SALMONELLA* CONTAMINATION ON ROMAINE LETTUCE

by

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(Under the Direction of Mark A. Harrison)

ABSTRACT

The objective of this study was to evaluate the reduction of *Salmonella enterica* and the prevention of cross-contamination on romaine lettuce leaves in wash water treated with oregano essential oil, acetic acid, or their combination. Whole leaves spot inoculated with *Salmonella* were mixed with uninoculated leaves at ambient temperatures. Surviving levels on the leaves and the amount transferred to uninoculated leaves were determined at 2, 5, and 10 min. The combination treatment was generally more effective in reducing *Salmonella* levels and reducing cross-contamination than its singular components and after 10 min was not significantly different than the chlorine. Additionally, *Salmonella* reduction on inoculated fresh cut lettuce by the combination treatment and other sanitizers was compared. The combination treatment reduction (2.41 cfu/g) was not significantly different from chlorine (1.83 cfu/g). These results suggest the 0.02% oregano essential oil and 1% acetic acid treatment has potential as a small scale natural wash alternative.

INDEX WORDS: Essential oil, Oregano, Acetic acid, Lettuce, Cross-contamination

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CHAPTER 1

INTRODUCTION

Produce related incidents of foodborne illness are well documented *(83)* and raise serious public health concerns. Although washing fresh produce effectively removes soil residues and cools and moves products, improperly managed wash water can become a source for microbial cross-contamination. Antimicrobials are added to wash water to enhance produce safety, most commonly chlorine *(28)*; however, the popularity of "natural" products has the industry seeking alternatives.

Organic acids are natural antimicrobials that are known to inhibit foodborne pathogens *(86, 134, 142)*. Many are recognized as Generally Recognized As Safe (GRAS) substances (21 CFR 184) *(123)*, and treatments are implemented as sprays, dips, and wash additives. Plant essential oils are composed of volatile metabolites that play a major role in plant defense and communication with their environment *(39)*. Most are also GRAS substances (21 CFR 582.20) *(123)* and exhibit antimicrobial activity against a range of yeasts, molds, and bacteria *(51)*. Oregano essential oil exhibits an exceptional antimicrobial effect *(13, 17, 33, 51, 66, 91, 97)* and has shown potential as a chlorine alternative in wash water systems *(47, 71, 102)*. Some studies have suggested a synergistic effect between essential oils and organic acids *(25, 27, 41, 49, 145)*; however, no experiments have been conducted in produce models.

In this study, we evaluated the effectiveness of wash water treated with oregano essential oil, acetic acid, or both in combination at reducing levels of *Salmonella enterica* on inoculated

whole romaine lettuce leaves and preventing cross-contamination. Additionally the antimicrobial effectiveness against other commonly available commercial sanitizers was determined on fresh cut romaine lettuce.

CHAPTER 2

LITERATURE REVIEW

I. Small Farms and "Local Produce"

The United States Department of Agriculture (USDA) national count of farmers markets has consistently been on the rise for the past two decades reaching an estimated 8,000 in 2012 *(132)*. Small farms that practice direct-to-consumer marketing control a minor portion of the U.S. agricultural market yet have more than doubled from 1997 to 2007 *(75)*. The growing number of small farms and farmers markets is a response to the increasing consumer interests in obtaining fresh produce from local farms or "local food".

The "local food" concept refers to relative geographical distance, but it also extends to farming practices and ethics, for example use of fair farm labor practices and reduced use of synthetic chemicals and fertilizers *(75)*. Depending on the product and location, consumers are willing to pay a premium for locally produced foods *(16)*. In fact, almost all states (43 cited in 2006) are participating in state branding programs that market locally grown products *(87)*.

Several surveys cite consumer motivations for purchasing local produce to a perceived higher quality, healthier, and fresher alternative to grocery store produce *(12, 30, 118)*. However, little data has assessed the microbiological safety concerns on small farms relative to large ones. While small scale farms practicing direct-to-consumer sales and producing less than $500,000 in annual sales are partially exempt from the new control plans in the Food Safety Modernization Act (FSMA) *(127)*, the size exclusion has raised a lot of controversy *(54)*. For

this reason, the exclusion is requiring the United States Food and Drug Administration (FDA) to conduct a study determining the impact of size scale on foodborne illness risks.

Regardless of the size risk associations, the fact remains that many small farmers lack resources needed to acquire food safety certifications that large corporations require. Good Agricultural Practice (GAP) guidelines were developed by the USDA in 1999 to help reduce potential microbial contamination and food safety risks. GAP certification is often required by many large retailers and wholesale distributors, but it is an expensive process. Survey and case study estimates suggest that first year modifications can cost up to $150,000 *(6, 36, 52)*. A small-growers specific study in Vermont estimated the average cost to be over $30,000 *(6)*. Because many of the costs associated with GAP compliance are not relative to farm size, like water purification systems, modified bathroom/hand-washing facilities, and third party audit costs, small farmers suffer a greater burden than larger growers *(95, 139)*.

Food Safety Practices on Small Farms

Surveys of small to medium-sized produce growers in southeastern states found 56% of the participants reported amending their soil with manure of which 15.6% did not compost at all *(53)*. The use of animal manure as a crop fertilizer can be effective when properly composted. However, if improperly composted, biosolids can harbor varying amounts of pathogens including bacteria, parasites, and viruses. The survey also found that 15.9% of participating growers used untested water to wash their produce. Similarly, a survey of mostly small produce farmers in New York found that only 16% reported adding chlorine to postharvest wash water suggesting additional training is needed on this topic *(93)*. Our preliminary surveys of small farmers participating in the "local food movement" of North Georgia suggest that many oppose

the use of chemical additives in their wash water and they instead choose to wash their produce in scaled down dump tanks with water only.

II. Produce Contamination

Foodborne illness is a deadly serious concern and is estimated to cause 47.8 million illnesses annually in the United States, so one in six individuals could be affected by foodborne illness each year *(107, 108)*. Through laboratory-based surveillance, the Centers for Disease Control and Prevention (CDC) estimates these illness incidents lead to over 300,000 hospitalizations and over 4,000 deaths annually *(77)*.

Produce related incidents of foodborne illness are on the rise and were linked to 46% of the reported cases between 1998-2008 *(83)*. This number may be an underestimate because produce related incidents of foodborne illness are especially difficult to detect and investigate. Aside from the usual underreporting and tracking issues seen when estimating risks for all foods, produce commodities usually have low levels of contamination and are widely distributed throughout the food chain. This causes widely dispersed outbreaks with low attack rates *(113)*.

As a category, fresh fruits and vegetables refer to many different species and various parts of the plants are consumed at different states of maturity *(14)*. Overall they have a high water content and are exposed to many sources of contamination from farm to table. Sources of contamination include pre-harvest conditions, such as contaminated irrigation water, runoff from nearby pastures, wildlife or insect vectors, and postharvest handling like contact contamination from workers' hands, storage containers, or wash water *(106)*. The microflora among produce is diverse and the likelihood of attachment and/or internalization is also dependent upon the physicality of the produce.

The growing contamination problem is linked to a wide range of infectious agents including foodborne viruses (Hepatitis A and Norwalk virus), parasites (*Cyclspora*, *Giardia*, and *Cryptosporidium*), sporeforming bacteria (*Bacillus cereus* and *Clostridium perfringens*, and *botulinum*), and non-sporeforming bacteria (*Campylobacter jejuni*, *Escheria coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella*, *Shigella*, and *Vibrio* spp.) *(113).*

Salmonella

Salmonella is currently the leading cause of foodborne related deaths and hospitalizations in the U.S. *(5).* Of the 190 produce associated outbreaks from 1973-1997, *Salmonella* was linked to 48% *(113).* A risk based analysis designed to rank the pathogen-food combinations with the greatest burden on U.S. public health, estimated *Salmonella* associated produce incidents were among the top ten and cost $548 million dollars per year *(5).*

Salmonella spp. are a diverse group of *Enterobacteriaceae* that currently include over 2,500 serovars *(89).* The genus is divided into 2 species (*S. enterica* and *S. bongori*) where *S. enterica* contains 6 subspecies –*S. enterica* subsp. *enterica, salamae, arizonae, diarizonae, houtenae,* and *indica. S. enterica* subsp. *enterica* contains over half the designated serovars, of which Typhimurium and Enteritidis are most often implicated in illness outbreaks *(11).* The classification of *Salmonella* serovars is based on an evolving knowledge of biochemical properties, DNA homology, antigenic specificities, and clinical isolation conditions *(67).*

Salmonella spp. optimally grow in an environment with temperatures between 35 and 37°C, pH between 6.5 and 7.5, and with a water activity between 0.96 and 0.99. However, *Salmonella* spp. are resilient organisms than can tolerate a wide range of harsh environments (Table 1) *(23, 125).*

Table 1. Growth conditions for *Salmonella* spp.

Parameter	Minimum	Optimal	Maximum
Temperature (°C)	2	35-37	54
pH	4.2	6.5-7.5	9.5
Water activity (a_W)	0.94	0.96-0.99	0.99

Nontyphoid *Salmonella* enterocolitis is characterized by non-specific symptoms like diarrhea, fever, and/or abdominal cramps that begin 12-72 hours after contamination *(23)*. While many organisms may be killed during passage through the stomach, some survive the harsh, acidic environment, and the infection begins only after the bacteria colonize the distal ileum of the intestines *(90)*. *Salmonella's* released endotoxins then act on the vascular and nervous apparatus eliciting a profound inflammatory response *(31)*. Infections usually cause self-limiting, non-typhoid salmonellosis or gastroenteritis that persists from 4-7 days *(23)*. In serious cases, systemic infections can occur resulting in typhoid salmonellosis or enteric fever. As with many pathogens, infants, the elderly, and immunocompromised individuals are most susceptible. *Salmonella* infections can cause the release of enough blood and fluid from the body to cause hypovolemic shock and perhaps death. It has been estimated that non-typhoidal salmonellosis and typhoid salmonellosis cause 155,000 *(74)* and 200,000 *(21)* respective fatalities annually worldwide.

Salmonella has been linked to leafy greens in several U.S. outbreaks *(113)* and has been more recently implicated in many outbreaks across the European Union. Italian rucola lettuce contaminated with *S.* Thompson caused a multinational outbreak in 2008 *(81)*. In 2004 *S. enterica* adulterated lettuce was the source of contamination in 368 cases of confirmed illness in the United Kingdom and over 100 in Finland in 2008 *(44, 68)*.

Leafy Greens

Leafy green products include butter, green leaf, red leaf, and romaine lettuce, arugla, cabbage, chard, escarole, endive, spring mix, and spinach *(76)*. As a category, they are one of the principal produce commodities associated with foodborne disease, of which lettuce is most often implicated *(106)*. Leafy greens are considered "high-risk" produce items and the classification is partly attributed to consumption patterns. Often, greens are consumed raw, eliminating a kill step for potential pathogens that might be more commonly implemented for other vegetables.

The physical nature of the leaf structure is also an attribute of concern. Leaf surfaces are covered with a generally bacteria impermeable cuticle with less than 1% of the surface concentrated with respiration pores called stomata *(109)*. These natural openings and other artificial ones, like injuries causing cracks to the cuticle or broken trichomes, are entryways for bacterial infection. Other produce such as harder skinned items, for example mangoes and tomatoes, are uniformly better protected against bacterial internalization.

The immersion of fruits in dump tank wash systems causes potential concerns for bacterial internalization. When a warm fruit is placed into cool water, hydrostatic forces draw water into the fruit, bringing along any pathogens present in the wash water. This has been shown in various fruits including the *Salmonella* internalization of tomatoes *(146)* and mangoes *(8)*. To halt this effect, wash water should be maintained at temperatures above the internal temperature of the produce item *(146)*.

Internalization of pathogens into leafy greens has been studied. Takeuchi and Frank *(120)* showed that *Escherichia coli* O157:H7 cells had a greater penetration of lettuce when inoculated and held at 4°C compared to higher temperatures. Gomez et al *(46)* demonstrated

that, unlike fruits, a negative temperature differential was not shown to have an effect on bacterial internalization of baby spinach leaves. However, they did find that preconditioning spinach in a reduced relative humidity environment decreased the uptake of *Salmonella*, perhaps explained by stomata closure in environments with low water availability. A two year study assessing the internalization of *Salmonella* in lettuce, found the summer months had higher incidences of internalization, suggesting another possible connection between reduced moisture and reduced pathogen uptake *(45)*.

Lettuce harvesting is usually done by hand. The heads are trimmed and can be packaged in the field. Throughout the process, contact contamination from workers' hands or knives can occur. In lettuce stems that were cut, similar to field harvesting, and inoculated at the cut site with *E. coli* O157:H7, Brandl *(10)* observed over a 1-$\log_{10}$ increase in bacterial population after just 4 hours, which was notably higher than the 2-fold increase seen on whole lettuce leaves.

Produce processing, specifically the washing and cutting steps, physically destroys the plants' natural defense mechanisms. Bacteria tend to accumulate at cut edges and their internalization into the stomata protects them from wash treatments *(120)*. As one might suspect, shredded, bagged leafy greens are the causative commodity associated with the major produce outbreaks in the United States *(76)*.

Soil is also a potential route for leafy green contamination. Islam et al *(58)* found *E. coli* O157:H7 persisted up to 217 days in inoculated manure amended soils and was detected from lettuce up to 77 days after soil application. In another similar study, *Salmonella* was detected on lettuce 63 days after inoculated manure and irrigation water were applied to the soil *(59)*. Proper manure composting relies on time-temperature principles for the inactivation of pathogens *(55)*, and the FDA's proposed FSMA has specific requirements for proper management.

III. Produce Wash Treatments

Running water can be effective at reducing pathogen levels on contaminated produce, and it has been shown to perform with a similar $\log_{10}$ reduction to other common household sanitizers like diluted chlorine bleach *(40)*. However, most postharvest operations reuse water in dump tanks to conserve resources, energy, and costs. Organic matter, dirt, and microorganisms can collect becoming a route for the spread of human disease or postharvest plant decay *(104)*. For example, if one lettuce leaf is contaminated and then added to a wash tank with other leaves, there is a risk of cross-contamination to the other leaves by direct contact or transmission through the water. Wachtel and Charkowski *(136)* showed that one inoculated leaf is capable of contaminating 100% of all other leaves stored in the same wash water. Several cases of foodborne illness have been associated with unsanitary wash water used during produce handling including a recent salmonellosis multi-state outbreak with fresh whole cantaloupes and another from mangoes in 1993 *(112, 128)*.

Produce Regulations

Based on the demonstrated risks of produce related foodborne illness, several organizations including the FDA, United Fresh Produce Association, and California Leafy Green Products Handler Marketing Agreement, have outlined guidance documents to raise awareness in the industry to facilitate safe produce production using preventative controls. The "Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables" *(124)* compiles general intervention strategies to prevent pathogen spread on the farm and has led to more commodity specific directives like the "Commodity Specific Food Safety Guidelines for the Production and Harvest of Lettuce and Leafy Greens" *(126)*.

Food safety regulations are jointly enforced by local, state and federal agencies *(43)*. The FDA has regulatory authority over large-scale food producers and defines them as those exceeding $500,000 in total agricultural commodity sales *(54)*. Wash water disinfectants, unless they are considered to be GRAS, are regulated as secondary direct food additives and a list of approved ones can be found in part 173 of the Code of Federal Regulations Title 21 (21 CFR173) *(123)*. However, if the product being washed is in a food processing facility, like a ready-to-eat facility, both the FDA and United States Environmental Protection Agency (EPA) have regulatory authority and the disinfectant must also be registered with the EPA *(43)*.

Treatments to Reduce Microorganisms on Produce

Chemical agents, like peroxyacetic acid, quarternary ammonium compounds, electrolyzed water, ozone and most commonly chlorine *(28)*, are added to processing water primarily to reduce the microbial build-up in the water, and as a secondary effect, they may also reduce the microbial load on individually contaminated produce items. Generally large commercial operations have approved chemical treatment strategies in place to maintain wash water quality. Their effectiveness depends on the chemical and physical state of the antimicrobial agent, the treatment conditions (temperature, acidity, and contact time), the resistance of different pathogens, the type of produce, and the water quality *(73)*. However our preliminary surveys suggest that many commercial disinfectants are met with aversions by small scale farmers.

Chlorine Sanitizers. Chlorine based sanitizers are widely used for their effectiveness and relatively low cost. Agricultural chlorine is available in three forms: chlorine gas (Cl_2), calcium hypochlorite ($CaCl_2O_2$), and sodium hypochlorite ($NaCl_2O_2$) and is usually used at a concentration between 50-200 ppm with a contact time of 1-2 minutes *(42)*. Calcium

hypochlorite is the preferred industry formula for wash water disinfection while sodium hypochlorite, the active ingredient in household bleach, is more commonly used in small-scale operations *(119).*

The "available chlorine" or "free chlorine" refers to the more bioactive hypochlorous acid (HOCl) and hypochlorite ion (OCl^-) that are formed when chlorine is added to water. The reaction equilibrium is dependent on the presence of hydrogen ions and acids (commonly citric acid) that are often added to the wash solution to favor the production of HOCl. The desired pH range for greatest efficacy is near neutral, between 6.0 and 7.5. An acidic pH can cause chlorine gas formation while an alkaline pH can be corrosive and disfavors the formation of the active chlorine molecule HOCl *(42)*.

(1) $NaOCl + H_2O \leftrightarrow NaOH + HOCl$

(2) $HOCl \leftrightarrow H^+ + OCl^-$

Continuous monitoring of the wash system is necessary because chlorine is highly reactive. Other factors like, temperature, light, air, metals, and organic matter can also affect the HOCl concentration. Organic matter is a particular problem in fresh cut lettuce operations, as tissue exudates commonly accumulate. Upon contact, free chlorine will combine with the organic matter, vegetable juice, tissue, or soil, thus neutralizing its affectivity. Shen et al *(110)* showed that increasing the organic load in a broth suspension reduced the efficacy of chlorine against *Salmonella* and *E. coli* O157:H7. Zhang et al *(143)* found that chlorine wash water with a 10% organic load, allowed for significantly higher *E. coli* O157:H7 cross-contamination in lettuce compared to treatments without an organic load. Therefore, it is important that wash water is changed regularly.

In addition to pH and turbidity, the oxidation–reduction potential (ORP) is another measure of chlorine efficacy. A device measures the electrical conductivity as an estimate of the chlorine concentration. Readings between 550-650 mV are acceptable; however, accumulated salts from soil or produce can interfere with an accurate measurement *(42)*.

Chlorine has a broad range of activity and is highly effective at inactivating microorganisms in properly maintained wash water. Luo et al *(72)* found that 5 ppm free chlorine was enough to inactivate *E. coli* O157:H7 in a wash solution. However, concentrations 10 ppm or greater were required to stop the cross-contamination from inoculated lettuce leaves to uninoculated leaves. In a similar study, López-Gálvez *(70)* found that 40 ppm chlorine was required to stop *E. coli* cross-contamination in lettuce washing systems. Studies suggest that chlorine wash treatments are not nearly as effective at reducing levels of pathogens inoculated onto produce. Typical chlorine treatments (50-200 ppm) usually show 1-2 $\log_{10}$ reductions *(26, 40, 65, 70, 80)*. However, Rodgers et al *(101)* showed that a 100 ppm chlorine treatment solution reduced *L. monocytogenes* and *E. coli* O157:H7 levels by 4.9 and 5.1 $\log_{10}$ on whole lettuce leaves, respectively.

Even though chlorine is the most common form of disinfection in the produce industry, concerns are raised about its possible negative human health and environmental effects. Disinfection by-products (DBPs) are formed when chlorine reacts with naturally occurring drinking water adulterants (anthropogenic contaminants), and in drinking water, only 11 of the 600+ literature reported DBPs are regulated by the EPA *(98)*. Trihalomethanes (including chloroform), haloketons, and haloacetic acids are just some with known carcinogenic and genotoxic effects *(98)*. This has led some European countries including Belgium, Germany, Switzerland, and the Netherlands, to prohibit chlorine use from ready-to-eat food products

altogether *(4)*. Therefore, alternative, non-harmful, ethical, and accessible wash water disinfectants are in demand.

Residue Free Treatments. Residue free sanitizers are highly desired in the food industry. Ozone, a naturally occurring molecule, is one example with a powerful antimicrobial activity and a strong oxidizing potential. Ozonated water is very effective at inhibiting a wide range of organisms including spoilage organisms and foodborne pathogens *(96)*. The EPA considers it the strongest chemical disinfectant for the inactivation of the parasites *Cryptosporidium* and *Giardia (34)*. However, there are practical working problems, such as the release of toxic ozone off-gas that can occur when ozone is added to wash water because of its partial solubility *(78, 82)*. Thus an appropriate system would need to be designed to prevent worker exposure. There is also a high initial cost tied to the generator equipment making this treatment method impractical for most small farmers *(115)*. Ultraviolet light is another promising residue free antimicrobial, but again, there are issues with the ease of use and accessibility of the technology for small farmers.

Commercial Wash Treatments. The negative consumer perceptions of chemical preservation and demands for more "natural" produce treatments have led the industry to develop several prototypes. Chemstar Liquid Fruit and Vegetable Wash (Chemstar©, Lithia Springs, GA) is a surfactant and citric acid based sanitizer; however, there is currently no literature on its efficacy as a produce wash. FIT Fruit & Vegetable Wash™ (HealthPro Brands Inc.©, Proctor & Gamble, Cincinnati, Ohio) is another commercially available surfactant and citric acid based sanitizer. The liquid solution is made from GRAS substances, water, oleic acid, glycerol, ethanol, potassium hydroxide, sodium bicarbonate, citric acid, and distilled grapefruit oil.

A possible mode of action of these wash treatments relies on the surfactant properties to remove dirt and microorganisms from the produce, which are then acidified in the water by the present citric acid. Similarly, previous studies have shown chlorine efficacy is improved when surfactants are added to the wash water, presumably because microorganisms are removed from otherwise impenetrable locations *(1, 117)*. However, Weissinger and Beuchat *(137)* found that a 2% citric acid + 1% Tween treatment of alfalfa sprouts was not significantly different than citric acid alone.

There is limited information on commercial produce washes. Park et al *(85)* found FIT treated flume water caused no significant effect on inoculated potatoes but did significantly reduce the levels of gram negative and aerobic organisms present in the water. FIT was demonstrated to be more effective than 200 ppm chlorine at reducing levels of *Salmonella* on inoculated alfalfa seeds *(137)* and tomatoes *(7)*. However, when blueberries were inoculated with *E. coli* O157:H7, 100 ppm chlorine showed more reduction (3.9 to 4.4 $\log_{10}$ cfu/g) than the FIT treatment (3.3 to 4.6 $\log_{10}$ cfu/g) *(84)*.

Organic Acid Based Sanitizers. Microorganisms are sensitive to pH and generally cannot grow below 4.5 *(42)*. Organic acids are natural antimicrobials that are known to have antibacterial activity *(28)*. Many are recognized as GRAS substances (21 CFR 184) *(123)* and treatments are useful as sprays, dips, and wash additives throughout the food industry. Organic acids are considered weak acids and do not completely dissociate when added to water.

$$AH + H_2O \leftrightarrow A^- + H_3O^+$$

In the undissociated form, organic acids are able to diffuse through the cellular membrane. Once inside the cell, at a neutral pH, the reaction shifts to favor anion production. The normal cellular physiology is disrupted by the intracellular accumulation of acid anions and

a subsequent pH drop; however, the mechanism of action is not entirely understood *(15)*. Proposed theories suggest a combination of disrupted cellular functions: accumulation of toxic anions, acidification of internal cell membrane components, membrane disruption leading to leakage, and inhibition of essential cellular functions for respiration, protein synthesis, and enzyme activity *(121)*. The acid stress response will synthesize proteins to enhance bacterial survival by mitigating the effects of the reduced pH and thus prolonging the bacterial lag phase. Depending on the acid concentration, acidity constant (pK_a), and the abilities of the microorganisms, the acid stress can be lethal.

Several studies have investigated acid washes for pathogen reduction. Household vinegar contains about 5% acetic acid by volume and reduces pathogens with the same effect *(62)*. These relatively high concentrations of acetic acid are extremely effective at pathogen removal, where up to 7-$\log_{10}$ reductions have been shown in parsley *(62, 140)*. In a broth suspension, undiluted vinegar resulted in a 5-$\log_{10}$ reduction of *Salmonella* levels *(142)*. A 5-$\log_{10}$ reduction was shown when *E. coli* inoculated iceberg lettuce was treated with a diluted vinegar solution (35% vinegar: 1.9% acetic acid), but in this study sensory panelists indicated a wilted, browned appearance in lettuce leaves *(134)*.

Dipping lettuce in acetic acid concentrations above 1% may negatively influence sensory qualities *(2)*. The use of practical amounts of organic acids in wash water usually result in a 1 to 2 $\log_{10}$ reduction of bacterial levels on inoculated produce; however, there is a considerable range in data *(105)*. Wash solutions with 0.5–1% lactic, citric, acetic, or ascorbic acid caused a 1 to 2 $\log_{10}$ reduction of *E. coli* and *Listeria monocytogenes* levels on inoculated cut lettuce *(2)*. Zhang and Farber found only 0.2–0.5 $\log_{10}$ reduction in *Listeria monocytogenes* when cut lettuce and cabbage were treated with 1% lactic or acetic acid *(144)*. When *Y. enterocolitica* inoculated

shredded lettuce was treated with 0.5% acetic acid, Escudero et al *(35)* observed a 3 $\log_{10}$ reduction.

IV. Plant Derived Antimicrobials

Plant derived substances are another type of natural antimicrobial. They have been used for health purposes since ancient history and modern scientific methods recognize their efficacy *(37)*. In recent years, there has been a resurgence in the use of alternative medicine *(32, 64)*, and plant-derived drugs exceed a $18 billion global market share *(94)*.

Many essential oils are GRAS substances (21 CFR 582.20) *(123)*. Essential oils (EO) are lipophilic, volatile plant compounds, primarily composed of monoterpenes and sesquiterpenes *(69)*. Terpenoids are widely distributed throughout the plant kingdom. They are classified by the number of isoprene units (C_5) and differ in linkages, functional groups, and degrees of unsaturation *(100, 103)*. Monoterpenoids (C_{10}), built by two isoprene units, are present in some lower plants and fungi but are most commonly associated with seed plants *(100)*. Examples include thymol, the characteristic aroma of thyme, (+)-carvone, the characteristic aroma of caraway, and carvacrol, the characteristic aroma of oregano. Sesquiterpenes (C_{15}) are derived from three isoprene units and also contribute to the aroma character of fruits and flowers *(100)*.

These volatile metabolites play a major role in plant defense and communication with their environment. They act as pollinator attractants and deterrents to animals, competitor plants, and pathogens *(39)*. Many EOs exhibit antimicrobial activity against a range of yeasts, molds, and bacteria *(51)*. In this study, we chose to specifically look at the aromatic oregano plant, *Origanum vulgare* because its distillate produces a highly active antimicrobial.

Oregano

Oregano is highly valued for its chemical constituents and organoleptic properties. The common name oregano refers to over 60 aroma plants belonging mostly to the Lamiaceae and Verbenaceae families. The most widely cultivated plants are of the *Origanum* genus (Lamiaceae family) in which there is some debate and complexity of taxa and species. *Origanum vulgare* subsp. *hirtum*, of Greek origin, is the most commercially well known variety because it is rich in aromatics and is generally accepted to yield the highest quality oil *(114)*.

Naturally, the composition of the essential oil is dependent on growth and harvest conditions of the plants. Higher phenolic contents from Greek oregano have been associated with plants grown in low altitudes and relatively high temperatures *(135)*. Seasonal variations will also affect the EO yield and composition. Generally, dry and warm periods cause plants to produce more oil than wet and cool ones *(114)*, and herbs harvested during or immediately following flowering show the strongest antimicrobial activity *(13)*.

Post-harvest treatments applied to plants can also change the EO composition. Often oregano leaves are dried before extraction. Exposure to hot air during this process reduces the volatile compounds so methods like vacuum microwave or freeze drying are preferred *(38)*. The method of extraction also affects the final essential oil composition. Many studies have focused on optimizing analytical methods to achieve a better extraction of total phenolics and antioxidants from oregano plants, which in turn may increase their antimicrobial properties *(56, 92)*. For example, *Origanum majorana* extracts were found to exhibit stronger antimicrobial properties when isolated by supercritical fluid extraction in comparison to the hydrodistillation method *(133)*.

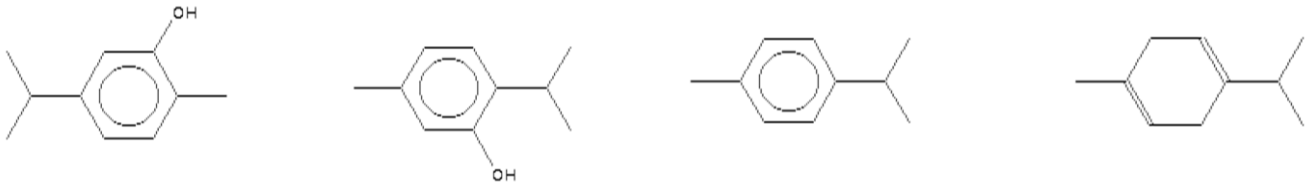
Although cold solvent extraction, supercritical fluid extraction and solid-phase microextraction have all been shown to reduce the transformation of plant components and are better at maintaining the aroma active compounds, steam distillation is still the most common commercial form of extraction for its ease of use and cost *(99)*. During steam distillation the plant material is added to a still that is heated to a boiling point. The oil will vaporize with the steam and enter a condenser where it is cooled and separated *(60)*.

Antimicrobial Activity of Oregano

Oregano EO is well established in the literature as an antimicrobial and possesses biological activity against a range of microorganisms *(51, 122)*. Using broth dilutions, it has been shown to inhibit the growth of foodborne pathogens and spoilage microorganisms at concentrations between 100-800 mg/L *(13, 17, 33, 66, 91, 97)*.

Generally EOs with the strongest antimicrobial activities are rich in phenolic compounds *(13)*. While the composition varies for reasons stated above, the phenolic monoterpenoids carvacrol, thymol, *ρ*-cymene and γ-terpinene are the main components of oregano spp. *(17-19, 22, 114)*.

carvacrol thymol *ρ*-cymene γ-terpinene



Oregano Essential Oil Mechanism of Action

While the antimicrobial activities of various essential oils are well documented, the mechanisms of action are not well understood. Most hypotheses suggest cellular membrane disruption. The two primary functions of the cytoplasmic membrane of bacteria are to: (1) form

barriers for ion gradients and energy transduction that drive various cellular processes and (2) physically hold membrane bound proteins (such as ATP-synthase) to control exchanges *(130)*. The hydrophobicity of the solute determines their permeability into the cellular membrane *(66)*. Because oregano EO is a lipophilic compound, it accumulates within the membrane *(13, 130)*.

In an extensive study of carvacrol's mechanism of action, Ultée et al *(129)* found that the hydroxyl group attached to the phenolic ring in carvacrol was found to be an essential component for antimicrobial activity against *B. cereus*. However, it was not required for accumulation into a simulated liposome membrane. Also, by measuring liposomal expansion, they found that carvacrol caused swelling of the membrane whereby the physical distortion may result in membrane destabilization and allow for cellular leakage. Carvacrol permeation changed the cell's membrane potential, depleted the ATP pools, decreased intracellular pH, and increased the membrane permeability for protons and potassium ions *(130)*. Using confocal scanning laser microscopy images and a fluorescent nuclear stain, Lambert et al *(66)* also found that membrane permeability was affected by oregano essential oil, carvacrol, or thymol and that the pH gradient was destroyed.

Possible consequences of membrane fluidity could be impairment of energy synthesis mechanisms, as suggested by Conner et al *(20)* when oregano EO was found to inhibit ethanol production, respiration and sporulation of yeasts. Ultimately the dissipation of the pH gradient (proton motive force) and electrical potential will impair cellular processes and can lead to cell death.

Oregano Essential Oil in the Food Industry

Based on the demonstrated antimicrobial properties, oregano EO and its constituents have been tested for antimicrobial applications in the food industry. Pérez-Alfonso et al *(88)* found

that when lemons inoculated with *Penicillium digitatum* (a common spoilage microorganism that causes lemon green rot) were dipped in wax containing carvacrol and/or thymol, they showed significant reduction in percentages of infected surfaces, reduced rate of respiration, and acid loss (arguably the lemons most important quality attribute).

Essential oils have also been tested as additives to wash water. Thyme EO significantly reduced *E. coli* O157:H7 levels on lettuce, baby carrots, and grape tomatoes *(71, 111)*. Treatment with 200 mg/L thymol or 400 mg/L carvacrol reduced the residual *Salmonella* levels in wash water from inoculated grape tomatoes to undetectable levels *(71)*. Oregano EO has been shown to significantly reduce *Salmonella* populations on inoculated lettuce leaves, spinach, jalapeno peppers, and cilantro *(47, 48, 102)*. In synthetic gray water (wastewater exiting a building exempting toilet water) oregano EO (468 mg/L) was also found to time-dependently decrease coliform concentration *(138)*.

Gündüz et al *(47)* found that *Salmonella* reductions seen on inoculated lettuce were statistically equivalent when treated with 75 mg/L oregano oil or 50 mg/L chlorine. In another study, Ruiz-Cruz et al *(102)* suggest that oregano EO treatments are not influenced by organic matter as the same *Salmonella* reductions were shown in cut lettuce leaves washed with and without an organic load. Chlorine on the other hand showed over a 1-$\log_{10}$ reduction difference between wash water with (2.8 $\log_{10}$ cfu/g) and without an organic load (1.2 $\log_{10}$ cfu/g).

Combinations of Oregano and Acetic Acid

There is some evidence suggesting a potential additive or synergistic effect between oregano essential oil and acetic acid. Gutierrez et al *(49)* found that *L. monocytogenes* was increasingly inhibited in TSB when the pH decreased (HCl adjustment) from 7.0 to 4.0. Zhou et al *(145)* found that thymol or carvacrol combined with acetic acid showed strong synergistic

activity against *Salmonella*. Combinations with 100 mg/L thymol + 0.10% acetic acid showed the same antibacterial activity as 400 mg/L thymol. Similarly 100 µl/L carvacrol + 0.10% acetic acid performed like 400 µl/L carvacrol treatment alone. De Oliveira et al *(25)* also found that combinations of sub-inhibitory concentrations of thymol or carvacrol and acetic acid performed similarly to the inhibitory concentrations of each antimicrobial individually against *Staphylococcus aureus*.

There is little literature focused on the combined mechanism of organic acids and phenolics. Juven et al *(61)* suggest that at low pH, the essential oils become more hydrophobic and are able to better bind to membrane bound proteins and are thus more membrane permeable. Separately, both categories act on the bacterial membrane and sublethal membrane injuries caused by one treatment might increase the bacteria's susceptibility to the next. Regardless of the mechanism, the results from the few combined phenolic and acid studies suggest more exploration is warranted in this area.

CHAPTER 3

MATERIALS AND METHODS

Culture Maintenance and Preparation

Five serovars of *Salmonella enterica* were used in this study: Agonia, Anatum, Cubana, Montevideo, and Poona. All serovars have been associated with previous produce related foodborne illness outbreaks and were obtained from the University of Georgia Department of Food Science and Technology stock collection. Cultures were maintained on cryogenic beads at -80ºC (Microbank™ Bacterial and Fungal Preservation System, Pro-Lab Diagnostics™, Austin, Texas) and were activated by transferring at least twice in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD) in 18-24 h intervals at 35ºC.

Antibiotic-Resistant Strain Maintenance and Preparation. To selectively recover inoculated strains without the native background lettuce microflora, cultures used in wash treatments were adapted to 100 µg/ml rifampicin (Rif; Fisher BioReagents, Fisher Scientific Inc.). Rifampicin stock solutions were prepared by dissolving Rif powder into methanol (Fisher Scientific) in a 1 g: 20 ml ratio and filtered through 0.22 Millipore Express® PLUS Membrane (Millipore Corporation, Billerica, MA). Cultures were transferred twice at 24 h intervals in TSB containing 100 µg/ml Rif at 35ºC. Mutated cultures were streaked onto xylose lysine deoxycholate agar (XLD; Becton Dickinson) and tryptic soy agar (TSA; Becton Dickinson), incubated at 35ºC for 24 h, and then assessed for similar growth patterns to non-adapted strains. Antibiotic resistant isolates were transferred to cryogenic beads and maintained at -80ºC.

Antibiotic resistant isolates were activated by transferring at least twice in TSB+Rif (100 μg/ml) that was incubated for 18-24 h at 35ºC. Equal volumes of each serovar were combined in 50 ml screw cap conical tubes (Sarstedt Ag & Co. Nümbrecht Rommelsdorf, Germany) and collected by centrifugation (Model 5681; Forma Scientific, Inc., Marietta, OH) at a relative centrifugal force of 2,500 x g for 10 min. The resulting pellet was washed with 10 ml Bacto™ peptone (Becton Dickinson). The centrifuge and wash procedure was followed 3 times to achieve a final concentration of 9 $\log_{10}$ CFU/ml.

Oregano Essential Oil

Oregano essential oil was obtained from Frontier Co-op (Norway, Iowa) where plants classified as *Orignaum vulgare* subsp. *hirtum* were grown in Hungary under National Organic Program *(131)* standards and oil was obtained by steam distillation. Between uses, extracts were maintained at 4ºC in original amber glass containers with screw caps and paper tape.

Oregano Antibacterial Activity Determination. The oregano EO was tested for antibacterial activity using a modified NCCLS M7 broth dilution method *(79)*. A 1% (8,600 mg/L) concentration was serially diluted to 0.02% (172 mg/L) in TSB broth. Solutions were mixed thoroughly by vortexing for 1 min. The cocktail of five *Salmonella* serovars were pooled from 18-24 h cultures in 2 ml volumes, diluted with TSB, and added to test solutions for a final concentration of 5 $\log_{10}$ CFU/ml. Solutions were incubated in screw cap glass tubes with minimal head space at 35ºC for 20-24 h. Turbidity was evaluated and samples were plated onto TSA and incubated for 20-24 h at 35ºC prior to enumeration of colonies. The minimum inhibitory concentration (MIC) was defined as the lowest concentration resulting in no visible growth in broth but viable when plated onto TSA. The minimum lethal concentration (MLC) was defined as the lowest concentration resulting in no visible growth in broth or agar plates.

Oregano Chemical Analysis. Gas chromatography-mass spectrophotometry analysis was conducted following a previously described method *(24)*. The oregano EO was analyzed using an Agilent GC (model 6890) and MS (model 5973) with an DB-5 capillary column (30 m length x 0.32 mm i.d. x 0.25 μm d_f). Thirty microliters of oil were diluted into 1.5 ml hexane (HPLC grade; Fisher Scientific) and 1 μl was injected into the GC-MS sampling port in the split mode (ratio 15/1). The temperature program was set at 60°C initially for 1 min and then increasing the temperature to 90°C at 2°C/min, and finally an increase at 210°C at 5°C/min. Conditions were set as follows: capillary direct interface temperature, 280; ionization energy 70 eV; mass range, 33-330 amu; EM voltage (Atune+200); scan rate, 5 scan/s. Helium was the carrier gas, at a flow rate of 1.5 ml/min. Total analysis time was 41 min. Components were identified by comparing mass spectra with those in the NIST library (2002 version).

Whole Lettuce Study

Antimicrobial Wash Solution Preparation. Treatments of 1% acetic acid (glacial, J.T. Baker, Pleasant Prairie, WI), 0.02% oregano essential oil, 1% acetic acid + 0.02% oregano essential oil, 50 ppm free chlorine from household bleach (Inter-American Products, Cinncinnati, OH) or deionized (DI) water were prepared. Unacidifed chlorine was used in throughout this study to mimic conditions used by a small grower or consumer. Individual antimicrobials were added to 500 ml DI water, shaken vigorously for 1 min, and then transferred to a large wash bin (18 L capacity) for a final volume of 6 L. Solutions were stirred for 1 min. All treatments were conducted at 22±1ºC and initial and post treatment pH (Accumet AB15 pH meter, Fisher Scientific) were measured. Oxidation-reduction potential (ORP; Orion QuikChek meter, Fisher and Hanna Instruments combo pH/ORP/temperature tester, Woonsocket, RI) was measured for oregano EO and chlorine treatments. Parts per million (ppm) of free chlorine levels were

verified by QuanTab chloride test strip (Hach Co., Loveland, CO). Untreated inoculated and uninoculated lettuce samples were used as positive and negative controls.

Leaf Preparation and Inoculation. To evaluate the transfer of *Salmonella* during washing, whole lettuce leaves were used. Romaine lettuce (*Lactuca sativa* var. longfolia) was purchased locally at retail (Kroger® Fresh Selections). Two or three layers of outer leaves were removed from each head and inner leaves were rinsed in DI water and stored at 4ºC up to 2 h prior to experimentation. Individual leaves were inspected for consistency in approximate size and weight. Browned or torn leaves were excluded.

Lettuce leaves to be inoculated were spun dry in salad spinner (Progressive International® Corp, Kent, WA) and placed into a sterile metal pan inside a laminar flow biosafety cabinet (Nuaire Class II). Approximately 30 cm^2 on the abaxial surface was inoculated with 100 µl in 20-30 spots with the five-strain culture suspension to achieve an initial concentration of 7 $\log_{10}$ cfu/leaf. Leaves were held in the biosafety cabinet for 1-2 h prior to washing to allow for bacterial attachment. Stems of the inoculated leaves were marked with black permanent marker to be distinguished from uninoculated leaves during washing.

Cross-Contamination Evaluation. Eighteen uninoculated and 3 inoculated lettuce leaves were added to the wash bin and stirred for one min. After contact times of 2, 5, and 10 min, 6 uninoculated and 1 inoculated leaf were removed aseptically and separately placed into sterile sampling bags (VWR, Radnor, PA) with 60 ml of Dey-Engley broth (DE; Becton Dickinson). Lettuce was then pummeled in a stomacher (400C circulator system, Seward, West Sussex, UK) at 230 rpm for 1 min. After the same contact intervals, 1 ml of wash water solution was pipetted into 9 ml DE broth to measure residual *Salmonella* levels.

Detection of Microorganisms. Serial dilutions from lettuce bags and wash water tubes were plated on TSA+Rif (100 μg/ml) with an automated plater (Autoplate 4000; Spiral Biotech Inc., Norwood, MA). Plates were incubated at 35ºC for 24 h. Colonies were enumerated using an automatic counter (Q-count®; Advanced Instruments Inc., Norwood, MA).

To determine the presence of *Salmonella* below the detection limit, bagged lettuce samples were enriched with 60 ml double strength TSB and incubated overnight at 35ºC for 24 h. Wash water samples were enriched by transferring 1 ml from DE broth into 9 ml TSB with the same incubation conditions. Subsequently 1 ml was transferred from enrichment solutions to 9 ml Rappaport-Vassiliadis *Salmonella* enrichment broth (RV; Becton Dickinson) and incubated at 42ºC for 24 h. After, a loopful (approx. 3 μl) was streaked onto XLD agar and incubated at 35ºC for 24 h. Three colonies of presumptive *Salmonella* strains were randomly selected from each treatment for confirmation by triple sugar iron (TSI; Becton Dickinson) and lysine iron agar (LIA; Becton Dickinson) slants incubated at 35ºC for 24 h.

Fresh Cut Lettuce Study

To compare the novel oregano treatment with other standard produce washes, the reduction of *Salmonella* levels was evaluated using fresh cut lettuce. Romaine lettuce was obtained from Zaxby's research and development facility and the outer 2 to 3 leaves were removed, inner leaves were rinsed under running DI water, and aseptically chopped into pieces (ca. 5 by 5 cm). Two pieces for each treatment were spun dry and placed in sterile empty Petri dishes in a biosafety cabinet. The remaining lettuce was stored at 4ºC for up to 2 h prior to the experiment.

Similarly to the previous inoculation method, 100 μl per treatment of *Salmonella*+Rif cocktail suspension were spotted onto the abaxial side of the lettuce leaves and then held in the

biosafety cabinet to allow bacterial attachment. After 1-2 h the inoculated leaf pieces were mixed in sterile stomacher bags with uninoculated pieces for 50 g total weight.

Acetic acid + oregano essential oil (10 min, based on previous experimental results), chlorine (1 min + 1 min water rinse), and DI water (1 min) treatments were prepared as described previously. Additionally Chemstar Liquid Fruit and Vegetable Wash (Chemstar Corp., Lithia Springs, Ga; 90 s) and Fit Antibacterial Fruit and Vegetable Wash (Health Pro Brands Inc., Cincinatti, OH; 30 s plus water rinse) were prepared based on manufacturers instructions. Treatment solutions in 400 ml volumes were added to bags and they were gently agitated for 1 min (except 30s for Fit treatment). After contact time, 1 ml of wash water was added to 9 ml DE broth, spiral plated on TSA+Rif which were incubated at 35ºC for 24 h prior to enumeration. The remaining residual wash water was drained from the bag. Two hundred ml of DE neutralizing broth was added to the lettuce bags and they were stomached at 230 rpm for 1 min. Serial dilutions from lettuce bags were plated on TSA+Rif, incubated, and enumerated as described above. *Salmonella* confirmation and wash water enrichment followed the same procedures described in the whole leaf study.

Wash Solution Neutralization Test

All treatments were tested for DE broth's neutralization capacity following a modified method previously described by Zhang et al *(146).* Briefly, in the whole leaf study, uninoculated lettuce leaves were separately washed in treatment solutions for 5 min, removed and added to a sterile stomacher bag with 60 ml DE neutralizing broth. A 0.1 ml volume of *Salmonella* test suspension was added and bags were pummeled in stomacher at 230 rpm for 1 min. A control bag containing 60 ml DE , 0.1 ml *Salmonella*, and 1 unwashed uninoculated lettuce leaf was also pummeled. Samples were spiral plated on TSA+Rif, incubated at 35ºC for 24 h, and enumerated

using an automatic counter. The DE broth was determined to effectively neutralize the solution when differences in *Salmonella* levels between the treatments and the control were within ± 15%. Similarly, to test the neutralization of the water samples in the whole leaf study and the treatments used in the fresh cut study, 1 ml of treatment solution was added to 9 ml DE broth with 0.1 ml of *Salmonella* suspension. The surviving *Salmonella* levels (as determined above) were compared to the control containing 10 ml DE and 0.1 ml of *Salmonella* suspension.

Statistical Analysis

Bacterial reduction and cross-contamination transfer data were analyzed after $\log_{10}$ transformation. Three replicates (unless noted otherwise) of each treatment were pooled before data analysis. In the whole leaf study, a two-way ANOVA was run using SAS Software Release 9.13 (SAS Institute Inc., Cary, NC) to compare the mean reduction (Tukey-Kramer method) on inoculated leaves and transfer to uninoculated leaves and residual water across the various treatments and time points. When values from cross-contamination studies were below the enumeration limit, median values were compared using the Kruskal-Wallis test. In the fresh cut lettuce study, a one-way ANOVA was run using Minitab (version 16.1, State College, PA) to compare the reductions (Tukey-Kramer method) across the various treatments. The value of significance was reported with the level $\alpha = 0.05$.

CHAPTER 4

RESULTS

Antimicrobial and Chemical Oregano Essential Oil Characterization

The antibacterial activity of the oregano essential oil was determined by the broth dilution assay. The minimum inhibitory concentration, defined as the lowest concentration resulting in no visible growth in broth but viable on agar plates, was 430-859 mg/L. The minimum lethal concentration, when the bacterial growth was completely inhibited in broth and on agar, was 602-860 mg/L. The major components of the oregano essential oil obtained by GC-MS were carvacrol (61.05%), p-cymene (14.35%), and γ-terpinene (7.36%) (Table 2).

Treatment Optimization

Different concentrations of oregano essential oil, acetic acid, and combinations were tested to determine the most effective treatment for further analysis (Table 3). Oregano essential oil was more effective at reducing the levels of *Salmonella* on inoculated leaves and preventing cross-contamination to uninoculated leaves as concentrations increased. The highest *Salmonella* reduction (4.91 $\log_{10}$ cfu/leaf) was shown after 2 min of exposure to 0.05% oregano EO and the *Salmonella* transfer to uninoculated leaves was below the detection limit. However, visible leaf browning occurred at concentrations above 0.02% (Fig. 1).

Increasing the acetic acid concentration from 0.5% to 1% caused negligible differences in the average transfer of *Salmonella* to the uninoculated leaves (5.27 and 5.18 $\log_{10}$ cfu/leaf, respectively). However, combining acetic acid with oregano EO decreased the transfer. On

average, the combination treatment of 0.01% oregano EO + 1% acetic acid was more effective than the 0.03% oregano EO treatment at reducing the *Salmonella* transfer to uninoculated leaves without browning the lettuce leaves.

The 0.02% oregano essential oil + 1% acetic acid, reduced *Salmonella* levels up to 4.23 $\log_{10}$ cfu/leaf and was the most effective treatment that did not cause negative browning effects. Transfer to all 18 uninoculated leaves was below the detection limit (3.08 $\log_{10}$ cfu/leaf); however, all tested positive for *Salmonella* after enrichment. These results suggest this combination has potential to prevent *Salmonella* cross-contamination in wash water and reduce surface contamination. This treatment was used in further investigations.

Whole Lettuce Study

Generally the combination treatment of 1% acetic acid + 0.02% oregano essential oil was more effective than its singular components to reduce *Salmlonella* levels on lettuce leaves and transfer to water and leaves (Tables 4, 5, and 6). After 2 min, the combination treatment showed a significantly higher reduction in *Salmonella* transfer to the wash water ($p < 0.05$) than the oregano EO treatment and water control (Table 6). With 5 min or more of exposure time, the combination treatment was outperforming the acetic acid treatment. Acetic acid and oregano treatments did not differ compared the control in the amount of *Salmonella* transferred to the wash water, with the exception of the 10 min oregano treatment.

Similarly the combination treatment significantly reduced *Salmonella* levels ($p < 0.05$) more on the inoculated leaves after 5 min of exposure (3.35 $\log_{10}$ cfu/leaf) than the acetic acid treatment (1.32 $\log_{10}$ cfu/leaf) and water control (1.53 $\log_{10}$ cfu/leaf; Table 4). Extending the time from 2 to 10 min further reduced the *Salmonella* levels recovered from the inoculated

leaves with only the 10 min exposure time (4.04 $\log_{10}$ cfu/leaf) showing significant differences ($p < 0.05$) from the oregano treatment alone (2.58 $\log_{10}$ cfu/leaf).

After 2 min of exposure, the combination treatment significantly reduced the amount of *Salmonella* transferred ($p < 0.05$) to uninoculated leaves as compared to its singular component treatments and water (Table 5). Increasing the exposure time from 2 to 5 min significantly reduced the transfer amount ($p < 0.05$) but increasing from 5 to 10 min showed no further reductions. In many of the combination treatments, the amount of *Salmonella* transferred to uninoculated leaves was below the enumeration limit but detected after enrichment.

Alone, the acetic acid treatment performed similarly to the water control and did not significantly reduce the *Salmonella* population recovered from inoculated leaves nor the transfer to uninoculated leaves or water ($p > 0.05$) compared to the control. Extending the exposure time from 2 to 10 min did not affect the *Salmonella* levels recovered.

The oregano treatment showed marginal differences from the water control. The average *Salmonella* transfer to uninoculated leaves (5.11 $\log_{10}$ cfu/leaf) was significantly ($p = 0.0499$) decreased in comparison to water (5.27 $\log_{10}$ cfu/leaf), but the estimated *Salmonella* reduction on inoculated leaves was not significantly different ($p > 0.05$) compared to the control (Tables 4 and 5). Furthermore, neither the amount of *Salmonella* transferred to wash water, uninoculated leaves, nor the estimated *Salmonella* reduction on inoculated leaves was significantly different from the acetic acid treatment ($p > 0.05$).

In comparing median values, the reduction in *Salmonella* transfer to uninoculated leaves caused by the chlorine treatment was not significantly different ($p > 0.05$) than that caused by the combination treatment after 5 and 10 min; however, more *Salmonella* was detected from the combination treatments after enrichment (Table 5). At all exposure times, the chlorine treatment

reduced *Salmonella* populations in wash water and on uninoculated leaves so that they were never present in numbers above the enumeration limit and rarely present after enrichment (Tables 5 and 6). The estimated log reduction of *Salmonella* populations caused by chlorine was not significantly different ($p > 0.05$) from the combination treatment after 5 min but was lower than the acid, oregano, and water control treatments at time 2 and 5 (Table 4). However, the increased recovery shown after 10 min of the chlorine treatment, was found not to be statistically different ($p > 0.05$) from all other treatments with the same exposure time. Additionally, the physiochemical properties of the wash water treatment solutions were determined pre- and post-treatment (Table 7).

Fresh Cut Lettuce Study

The combination oregano and acetic acid treatment showed a significant reduction in viable *Salmonella* recovered from fresh-cut lettuce leaves compared to the Chemstar solution and water control (Table 8). The combination treatment resulted in reductions that were not significantly different from the chlorine and Fit washes. No significant reductions were caused by Chemstar, Fit, and chlorine treatments, and the *Salmonella* population reduction caused by Chemstar washes was not significantly different from water.

The transfer of *Salmonella* to residual wash water was not detectable by enumeration after any treatment except the control (5.59 $\log_{10}$ cfu/ml). Enrichment of samples below the enumeration limit found 1 chemstar sample out of the 6 tested to be positive and 1 combination treatment out of 6 to have viable *Salmonella* remaining in the residual wash water.

Table 2. Oregano essential oil constituents determined by GC-MS analyses

Peak	Constituent	%	RT (min)
1	α-thujene	0.41	3.66
2	β-pinene	0.42	4.80
3	myrcene	1.09	5.22
4	terpeinolene	0.82	5.97
5	p-cymene	14.35	6.32
6	eucalyptol	0.16	6.45
7	γ-terpinene	7.36	7.52
8	2-carene	0.14	8.60
9	linalool	2.74	9.26
10	borneol	0.15	12.12
11	thymol methyl ether	0.21	16.44
13	thymol	5.19	19.01
14	carvacrol	61.05	19.67
17	β-caryophyllene	0.97	23.06
18	β-bisabolene	0.14	25.77
19	caryophyllene oxide	0.93	27.57

Table 3. *Salmonella* recovered from inoculated and uninoculated whole romaine lettuce leaves after various treatments to optimize combination treatment conditions[x]

Treatment	Concentration	Inoculated leaves (log cfu/leaf) Exposure time (min)			Uninoculated leaves (log cfu/leaf) Exposure time (min)		
		2	5	10	2	5	10
Oregano Essential Oil	0.01%	6.68	6.67	6.55	5.63	5.67	5.59
	0.02%	6.15	5.84	5.30	5.21	5.18	5.10
	0.03%	5.32	5.77	5.51	4.73	4.42	3.58
	0.05%	3.26	4.29	3.69	< 2.78[y]	< 2.78[z]	< 2.78[y]
Acetic Acid	0.50%	6.31	6.35	6.40	5.24	5.28	5.28
	1.00%	6.72	6.41	6.56	5.07	5.17	5.28
Oregano Essential Oil + Acetic Acid	0.01% + 0.50%	6.60	5.69	6.13	4.60	4.35	4.36
	0.01% + 1.00%	6.55	5.90	6.26	4.21	4.18	4.18
	0.02% + 1.00%	5.96	4.70	4.09	< 3.08[y]	< 3.08[y]	< 3.08[y]

[x]Initial inoculum (n = 3) ca. 8 $\log_{10}$ cfu/leaf; inoculated leaf values (n = 1); uninoculated leaf mean values (n = 6)
[y]Estimated value with some below enumeration limit (1cfu x dilution factor x ml DE diluent)

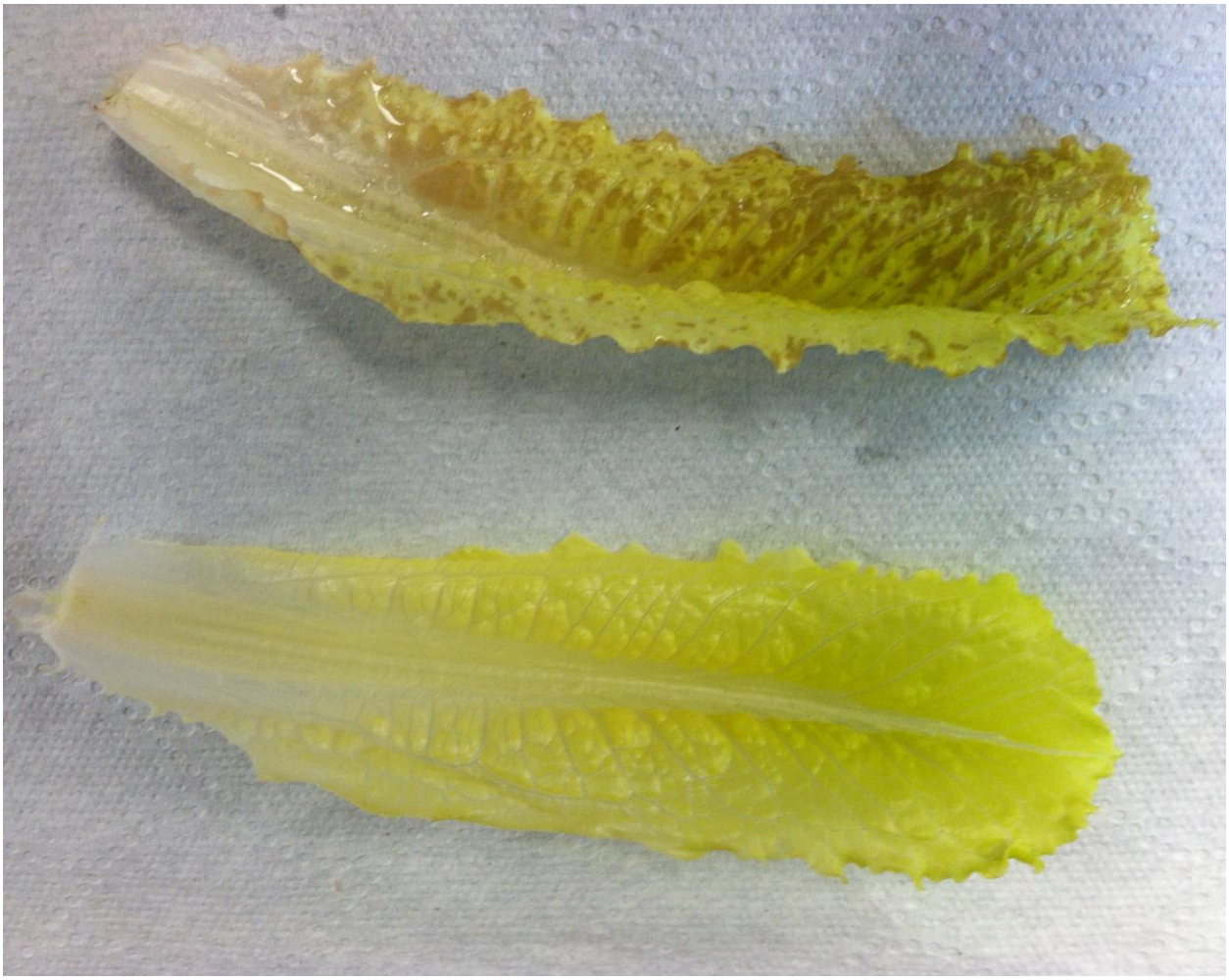


Figure 1. Quality comparison after 1 min exposure to 0.05% oregano essential oil (top leaf) and water control (bottom leaf)

Table 4. *Salmonella* recovered from inoculated whole romaine lettuce leaves before and after washing with various treatments

	Inoculated leaves (log cfu/leaf) Exposure time (min)			
Treatment	0	2	5	10
1% Acetic Acid	8.09	6.80A	6.76A	6.49A
0.02% Oregano Essential Oil	8.20	6.16A	5.85A	5.62A
1% Acetic Acid + 0.02% Oregano Essential Oil	8.22	5.67A	4.88B	4.18BC
50 ppm Free Chlorine	8.10	3.18B	3.80C	5.24AC
DI Water	8.19	6.46A	6.66A	6.27A

ABC Mean values (n = 3) within columns followed by different letter are significantly different ($p < 0.05$); Mean values (n = 9) of initial inoculum, time = 0 min

Table 5. *Salmonella* transfer to uninoculated whole romaine lettuce leaves and detection below enumeration limit after various wash treatments

Treatment[b]	Uninoculated leaves[a] (log cfu/leaf) Exposure time (min) 2	5	10	# Uninoculated Leaves with Viable *Salmonella* / # Leaves Tested Exposure time (min) 2	5	10
1% Acetic Acid	5.26	5.14	5.15	18/18	18/18	18/18
0.02% Oregano Essential Oil	5.23	5.09	5.01	18/18	18/18	18/18
1% Acetic Acid + 0.02% Oregano Essential Oil[x]	< 3.34[y]	< 3.11[y]	< 3.08[y]	18/24	18/24	18/24
50 ppm Free Chlorine	< 3.08[z]	< 3.08[z]	< 3.08[z]	0/18	1/18	1/18
DI Water	5.23	5.25	5.33	18/18	18/18	18/18

[x]Mean values (n = 18) except combination treatment (n = 24)
[y]Estimated value with some below enumeration limit (1 cfu x dilution factor x 60 ml DE diluent)
[z]Value below enumeration limit

Table 6. *Salmonella* transfer to residual wash water and detection below enumeration limit after various wash treatments

	Residual Wash Water (log cfu/ml)			# Water Samples with Viable *Salmonella* / # Water Samples Tested		
	Exposure time (min)			Exposure time (min)		
Treatment	2	5	10	2	5	10
1% Acetic Acid	4.66AB	4.01AB	4.22AB	3/3	3/3	3/3
0.02% Oregano Essential Oil	4.9A	4.78A	4.64A	3/3	3/3	3/3
1% Acetic Acid + 0.02% Oregano Essential Oil	4.28B	3.33B	< 2.30[x]C	4/4	4/4	4/4
50 ppm Free Chlorine	< 2.30[x]	< 2.30[x]	< 2.30[x]	0/3	0/3	0/3
DI Water	4.89A	4.90A	4.88B	3/3	3/3	3/3

ABC Mean values (n = 3) within column followed by different letter are significantly different ($p < 0.05$)

[x]Value below enumeration limit (1 cfu x dilution factor)

Table 7. Physiochemical properties of various treatment solutions used to wash whole romaine lettuce leaves

Treatment	ORP[x]		pH range	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
1% Acetic Acid	ND[z]	ND[z]	2.65-2.71	2.62-2.69
0.02% Oregano Essential Oil	322.5 ± 3.5	310 ± 34.3	6.24-6.59	5.86-6.09
1% Acetic Acid + 0.02% Oregano Essential Oil	453.6 ± 9.7	471.6 ± 20.0	2.71-2.89	2.67-2.78
50 ppm Free Chlorine	577.3 ± 20.0	627 ± 36.1	8.35-8.70	8.60-8.96
DI Water	ND[z]	ND[z]	6.55-6.60	5.90-6.61

[x]Mean values (n = 6) ± SD
[y]Not Determined

Table 8. *Salmonella* log reduction from inoculated fresh cut romaine lettuce leaves, wash solution applications, and properties after washing with selected treatments

Treatment	Exposure time	pH range[x]		Log Reduction (cfu/g)
		Pre-treatment	Post-treatment	
0.02% Oregano Essential Oil + 1% Acetic Acid	10 min	2.67-2.75	2.71-2.78	2.41 A
Chemstar[y]	90 s	2.56-2.74	2.57-2.63	1.64 BC
Fit[y]	30 s[z]	3.05-3.10	3.05-3.12	1.87 AB
50 ppm Free Chlorine	1 min[z]	8.52-9.25	8.58-8.95	1.83 AB
Water	1 min	6.23-7.89	5.51-6.32	1.00 C

ABC Mean values (n = 6) within columns followed by different letter are significantly different ($p < 0.05$); initial inoculum levels ca. 6 $\log_{10}$ cfu/g

[x]Mean values (n = 6) ± SD

[y]Solutions made according to manufacturer's instructions

[z]Treatments followed by 1 min water rinse

CHAPTER 5

DISCUSSION

In this study, we investigated a novel antimicrobial wash, a combination of oregano essential oil and acetic acid, and its ability to prevent cross-contamination and remove pathogens during the washing of romaine lettuce compared to its singular components and other sanitizers. Our results show that the combination treatment was able to reduce *Salmonella* on whole lettuce leaves up 4-log cfu/leaf and prevent cross-contamination to other leaves and the wash water. Compared to its singular components, the combination treatment significantly reduced the cross-contamination to uninoculated leaves after 2 min, the residual amount of *Salmonella* in the wash water after 5 min and in 10 min, showed a greater log reduction on inoculated leaves.

Maintaining the bacteriological quality of wash water and thus preventing cross-contamination is the primary goal of wash water sanitizers *(9)*. Both the combination treatment and chlorine effectively prevented cross-contamination and when compared, did not transfer a significantly different amount of *Salmonella* to uninoculated lettuce. However, we can conclude chlorine is likely more effective at reducing cross-contamination on whole leaves because the *Salmonella* bacterial populations in wash water and on uninoculated leaves were never detected in numbers above the enumeration limit (Tables 5 and 6).

After 5 min there were no significant differences in the reduction of *Salmonella* levels from the combination treatment compared to chlorine, and after 10 min the combination treatment significantly reduced the levels. The reduction in *Salmonella* levels on lettuce shown

by the chlorine (up to 4.9 log cfu/leaf) and the combination treatment (up to 4.0 log cfu/leaf) suggest both treatments are acceptable intervention strategies to inactivate pathogens on produce. Doyle and Erickson suggest that any treatment with at least a 3 log reduction of enteric pathogens meets the general standard *(29)*.

The 1% acetic acid treatment did not lead to a significant reduction in *Salmonella* from inoculated leaves or reduce the cross-contamination to uninoculated leaves and wash water as compared to the water control. This agrees with Huang and Chen who reported that 1% acetic acid did not significantly reduce *E. coli* levels on inoculated spinach leaves when washed at room temperature *(57)*. While higher concentrations of acetic acid can be more effective at reducing the presence of pathogens *(62, 140)*, dipping lettuce in concentrations above 1% may cause organoleptic quality losses *(2, 134)*.

Previous works have demonstrated the potential to remove pathogens when washing produce with essential oils *(47, 71, 102, 111)*. Investigations on the inhibitory effects of oregano EO and its constituents against a range of organisms, indicate that antimicrobial activity occurs as an additive effect of its major components *(17, 66)*. Carvacrol (61%), p-cymene (14%), and γ-terpinene (7%) were found as the major components of the oregano essential oil used throughout this study (Table 2), which coincides with previous studies that consistently found carvacrol as a major component of *Origanum vulgare* subsp. *hirtum (3, 19, 135)*. The MIC and MLC values of oil against *Salmonella* were 430-859 and 602-860 mg/L falling within the upper range of inhibition, 100-800 mg/L, shown by previous broth dilution studies *(17, 33, 66, 91, 97)*. While there are several published methods used to measure antibacterial activity, disk diffusion, agar wells, agar dilution and broth dilution, the latter was chosen because it is most sensitive *(13, 116)*.

Most hypotheses suggest essential oils inhibit bacteria by cellular membrane disruptions. Because the hydrophobicity of the solute determines their permeability through the cellular membrane, essential oils, lipophilic compounds, can integrate into the membrane with relative ease *(66).* Confocal scanning electron microscopy *(66)* and liposomal membrane models *(129)* have shown that carvacrol physically distorts the cellular membrane allowing for cellular leakage. Ultimately these changes in the membrane fluidity will dissipate the pH gradient (proton motive force) and electrical potential, impairing cellular processes and possibly causing death *(66, 130).*

When optimizing our treatment, oregano essential oil concentrations above 0.03% (276 ppm) were found to cause visible lettuce browning (Fig. 1). However, other studies found washing lettuce with a higher concentration, 300 ppm, had no adverse sensory affects *(50).* This contradiction suggests the oil composition contributes to its browning capability. In attempts to lower the concentration of essential oil but maintain the antimicrobial activity, essential oils have been assessed for synergistic activity with other hurdles. Oregano essential oils or components in combination with heat, high pressure, and other antimicrobials have proven successful *(57, 63, 70, 141).* It is also known that interactions between organic acids and polyphenolics enhance their inhibitory effects *in vitro*. Dimitrijević et al *(27)* showed that sublethal combinations of lactic acid and oregano oil increased the inhibition of *L. monocytogenes*. Oliveira et al *(25)* found a synergistic inhibition of *Staphylococcus aureus* between lactic acid and thymol or carvacrol. Zhou et al *(145)* showed that acetic acid and thymol or carvacrol had a synergistic effect against *Salmonella*. They found that ¼ the carvacrol or thymol in combination with acetic acid was needed to give the same effect as the carvacrol or thymol alone.

While the mechanism of combined action remains unclear, both organic acid and

polyphenolic inhibition are thought to similarly disrupt the cellular membrane. Some have suggested the addition of organic acids decreases the essential oil's hydrophobicity making insertion into the membrane easier *(61).* However, further studies are needed to fully understand this mechanism.

In our fresh cut lettuce study, we compared the novel combination wash treatment (0.02% oregano essential oil + 1% acetic acid for 10 min) to other available sanitizers. All treatments except the Chemstar significantly reduced the *Salmonella* levels when compared to the water control. Statistical comparisons showed the combination treatment was as effective as the chlorine treatment. While there were differences in treatment application and inoculum levels, the reduction shown on whole lettuce leaves was much higher than the reduction shown in the fresh cut study. This is likely explained because bacteria tend to accumulate at cut edges and their internalization into the plant tissues protects them from wash treatments *(120).*

The results from the study show that the combination treatment of acetic acid and oregano essential oil is generally a more powerful wash additive than its singular components. In addition, this novel wash treatment composed of natural ingredients may serve as a potential alternative to chlorine or other surfactant based sanitizers.

CHAPTER 6

CONCLUSIONS

In general, small farmers need more training on wash water sanitation and cross-contamination risks. Chlorine is the most commonly used sanitizer to control wash water quality; however, the popularity of "natural" products, especially among those participating in the "local food movement" addresses a need for natural alternative sanitizers. The results of the present study show that a combination treatment of plant derived oregano essential oil and GRAS certified acetic acid are capable of significantly reducing *Salmonella* surface contamination on whole lettuce leaves up to 4 logs and preventing a significant reduction in cross-contamination to other lettuce leaves and residual wash water. The combination treatment was generally more effective than its singular components and close in efficacy to chlorine. In fresh cut leaves the reduction of *Salmonella* shown after washing with chlorine was no different than that of the natural combination sanitizer. Together these results suggest this novel wash treatment may serve as a potential natural alternative to chlorine. However, the effect of the natural sanitizer on sensory properties has not been determined nor has its practical application been assessed such as its stability when in contact with high organic loads or extended time periods.

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