



5-2015

Sanitization Effectiveness of Alkaline-Dissolved Essential Oils as Organic Produce Washing Solutions

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I am submitting herewith a thesis written by Marion Lewis Harness III entitled "Sanitization Effectiveness of Alkaline-Dissolved Essential Oils as Organic Produce Washing Solutions." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Faith Critzer, Major Professor

We have read this thesis and recommend its acceptance:

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Sanitization Effectiveness of Alkaline-Dissolved Essential Oils as Organic
Produce Washing Solutions

A Thesis Presented for the
Master of Science
Degree

The University of Tennessee, Knoxville

Marion Lewis Harness III

May 2015

Acknowledgements

There are many people I would like to thank for their support, which has allowed me to get to this point. First and foremost, I want to thank Dr. Critzer, my advisor and mentor. The opportunity she gave me was more than I could have hoped for. With her instruction and encouragement throughout my graduate school experience, I always knew my direction. These last three years would have been much more difficult, I am sure, with anyone else at the helm.

Next, I need to thank the professors at the University of Tennessee. From the time I was first introduced to food science as an undergraduate, teachers here have both helped me discover my love for food science and continue my understanding. I am convinced I am in the right field, and it is thanks to them.

I would also like to extend my thanks to the rest of the Food Science Department, especially the students and my lab-mates. They made our University a welcome and fun place to work and were willing to help me at any time. The friendships and relationships I have made at UT are very important to me, and I hope that they will continue through the next stages of my life.

Finally, I want to extend my heartfelt gratitude to my family and friends. My parents' support means a lot to me, and my graduation will be a day for all of us to celebrate, because they endured this with me. My amazing friends, both from Memphis and Knoxville, were the reason I got through each week lightheartedly. I have them to thank for who I am today.

Abstract

Produce is often rinsed immediately post-harvest to remove dirt and debris. Rinse water can be a point of cross-contamination if no antimicrobials are present. While plant essential oils (EOs) are recognized as antimicrobials, their hydrophobicity makes them difficult to implement in rinsing solutions. In this study, the efficacy of emulsified EOs were examined against *Salmonella* on the surface of cherry tomatoes and *Escherichia coli* O157:H7 on the surface of baby spinach. Contaminated produce samples were rinsed in an emulsions of clove bud oil or thyme oil at 0.2 and 0.5% (v/v), as well as free chlorine at 200 ppm and sterile de-ionized water as controls. These treatments were also tested for their vulnerability to organic loading in the system, by adding 1% (w/v) organic load (OL) in the form of blended produce (spinach or tomato). Wash solutions were also tested for their ability to inhibit pathogen transfer onto uninoculated produce samples. To accomplish this, clean produce was immersed in rinse water immediately following contaminated samples. Finally, the wash solutions were enumerated for any viable pathogens.

Emulsified clove bud oil with whey protein at 0.5% was the most effective at reducing levels of *Salmonella* from tomato surfaces, while 0.5% thyme oil with gum arabic, next most effective, proved more resistant to the influence of 1% organic matter. Chlorine, commonly used as an antimicrobial in the produce industry, lost all measureable effectiveness in an organically loaded system. However, against *E. coli* O157:H7 on spinach surfaces, 0.5% thyme oil emulsion was the best EO treatment. Although chlorine was more effective in a clean system, 0.5% emulsified thyme oil was the next most effective against *E. coli* and was not vulnerable to 1% OL, unlike chlorine.

Overall, when testing organically loaded systems that simulate realistic conditions in dump tanks, emulsified EO systems were more effective at reducing pathogen levels and were better at inhibiting pathogen transfer and survival. These data establish potential for these emulsions to be employed as alternative antimicrobials for produce sanitizing systems.

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Introduction

Consumption of fresh produce has increased over the years in the United States (16, 18, 68). Because there is usually no heat treatment or kill-step before consuming most produce, any pathogenic microorganism introduced in the field or during post-harvest handling is likely to be present when eaten. Pathogens attributed to outbreaks are often recovered from a variety of different reservoirs, from soil and irrigation water to processing equipment and processing water (83). The many potential sources of contamination and the absence of a kill step are some of the main reasons that fresh and minimally processed produce are common agents of human pathogen transmission (10, 76).

Post-harvest washes are common in the produce industry, primarily to clean off soil residues, but best practices dictate the use of disinfectants to prevent cross-contamination during this processing step. While any effective sanitizer will inhibit the cross-contamination of clean produce that shares the same wash, the surface of plant materials often protects pathogenic microbes from aqueous solutions, due to small crevices and often hydrophobic micro-niches (11, 23, 39, 81). Even when incorporated during washing, many sanitizers are vulnerable to environmental influences, such as exposure to light, air and organic matter, making them less effective at inactivating target pathogens.

Plant-based essential oils (EOs) have garnered greater attention lately as antimicrobial agents, since many researchers have demonstrated activity in vitro against target pathogens and there is a growing market demand for clean-label antimicrobials (23). This research has shown that several EOs retain their antimicrobial activity at low

concentrations and are relatively stable and resistant to outside influence. EOs have been tested for efficacy as produce rinses in the past, but because of their hydrophobic nature, they are difficult to implement into aqueous systems. Therefore, it is hypothesized that emulsifying these antimicrobial compounds could significantly increase the effectiveness of such a sanitizer (28, 98, 100).

The objective of this study was to examine the efficacy and resilience of emulsified EOs to inactivate *Salmonella* on the surfaces of cherry tomatoes and *E. coli* O157:H7 on the surface of baby spinach and limit cross-contamination of uninoculated produce, as compared to chlorinated water.

Chapter I

Literature Review

Organic produce in the United States. Fruits and vegetables are well recognized as essential for any diet designed for good health. The US Department of Agriculture, along with Health and Human Services, now suggests that consumers fill half their plates exclusively with fruits and vegetables (88). Research has indicated that produce consumption helps in preventing heart disease, strokes, certain forms of cancer, hypertension, diabetes, obesity, birth defects, cataracts, and has even been shown to improve lung capability in asthma patients (26). Because of these health benefits, organizations like the USDA and the World Health Organization constantly push to increase the global consumption of fruits and vegetables (88, 91, 96).

Over the last few decades, consumption of fruits and vegetables has increased, especially in their fresh form (68). This increase in demand can be attributed, at least partially, to the influence of the aforementioned organizations. Using data collected from the USDA, Figure 1.1 shows the usages of fresh fruits and vegetables in the United States. This data indicates that the combined consumption of fresh fruits and vegetables in the US increased from 176 pounds per capita in 1976 to 254 pounds per capita as of 2012, a nearly 70% increase (16, 18). As the demand for fruits and vegetables increases, suppliers are expected to deliver a wider variety of produce farther distances, regardless of the time of year.

The introduction of “organic” agriculture dates back to the 1940’s and can be credited to Jerome Rodale and Lord Northbourne (66, 72). It was not until the last three decades, however, that it became a household term and such items became available at conventional retail stores instead of solely health food stores. As consumers tend to see synthetic compounds and genetically engineered foods with more of a negative perception, the organic foods market is expected to continue its expansion.

Organic foods are grown, harvested, and processed differently than conventional foods. “Organic” is a labeling term meaning that an agricultural product was certified by the USDA National Organic Program to have been produced using approved methods. Among other restrictions, the use of synthetic fertilizers, ionizing radiation, sewage sludge, and genetic engineering are prohibited for a product to be classified as “organic”. The purpose of these organic practices is ultimately to preserve biodiversity, nurture ecological balance and also to maintain a healthy succession of resources (87). These restrictions were first regulated in 2002, known as the National Standards on Organic Agricultural Production and Handling, or the Organic Rule, and were set in place by the National Organic Standard Board. The Organic Rule also made it clear that these regulations were not meant to ensure the safety of these products (32). Sales of organic food increased from \$12 billion in 2004 to \$24 billion by 2012 and show every sign of continuing to grow in the foreseeable future (42). Since organic commodities are becoming a larger player in the fresh and minimally processed produce segment, it is crucial that their safety is thoroughly assessed.

Multi-state foodborne outbreaks associated with selected produce.

Food safety is vital to any branch of the industry, but it is especially problematic when it comes to foods that are minimally processed or eaten raw. Of the outbreaks reported with a known food medium in the US, outbreaks involving fresh produce increased from 0.7% in the 1970’s to 6% in the 1990’s (76). The CDC reported that during the years 1998-2008, leafy vegetables accounted for 13% of the outbreaks associated to specific commodities, while fruits and nuts accounted for 11%, indicating a continued upward trend. It is likely that increasing consumption rates, as indicated in Figure 1.1,

compounded with improved outbreak detection have played a large role in the frequency of produce-related outbreaks over the past two decades (16, 18).

As shown in Table 1.1, there have been multiple multi-state foodborne outbreaks associated with tomatoes, leafy greens and cantaloupes over the last decade. Since 2005, three multistate outbreaks associated with tomatoes stand out, all involving *Salmonella*. Two separate outbreaks in 2006 caused a combined 300 people to become ill, each outbreak spreading over 19 states (89). An outbreak of *Salmonella* Saintpaul in 2008 significantly affected the tomato industry, since tomatoes were the first commodity suspected of contamination, which eventually sickened over 1400 people across 43 states (6). Leafy greens have been involved in even more multistate outbreaks since 2005, usually involving *E. coli* O157:H7. The only outbreak of *Cyclospora*, however, resulted in over 600 illnesses (17, 21). Leafy greens are a difficult commodity to control, since they are often packaged with a variety of vegetables, like salad mixes, and are commonly served as a garnish. Their prevalence in foodborne outbreaks, as shown in Table 1.1, attests to this. Four multistate outbreaks involving cantaloupe in the last decade, listed in Table 1.1, have caused more consideration to be paid to that segment of the food industry. The deadliest foodborne outbreak in the US in almost 90 years was because of cantaloupes contaminated with *Listeria monocytogenes* from Colorado in 2011, causing 147 illnesses and 33 deaths (14, 82). These multistate outbreaks have put increasing pressure on the produce industry to improve their food safety practices in the field and post-harvest. Science-based research has focused on mechanisms of contamination and strategies to inactivate foodborne pathogens.

Foodborne contamination of fresh and minimally-processed produce.

Historically, two of the most important pathogens involved with fresh produce contamination have been *Salmonella enterica* and *Escherichia coli* O157:H7 (8, 67). Scallan et al. (73) used data collected throughout 2000-2008 to estimate foodborne pathogen burden. Out of the 55,961 hospitalizations and 1,351 deaths annually caused by contaminated food in the US, non-typhoidal *Salmonella* was the number one cause of both, responsible for 35% of the hospitalizations and 28% of the deaths, on average. *Salmonella enterica* is a Gram-negative, enteric bacteria and is considered one of the most deadly bacterial foodborne pathogens. *Escherichia coli* is a species of bacteria that is normally harmless, but depending on serotype, can cause human illness. These pathogenic serotypes are classified into pathotypes, with the most infamous being Shiga toxin-producing *E. coli* (STEC), which includes the most well-known serogroup, *E. coli* O157:H7. STEC or enterohemorrhagic *E. coli* (EHEC) consist of the strains of *E. coli* that, by producing Shiga toxin, can cause hemolytic uremic syndrome (HUS), a type of renal failure that can be lethal, especially in children (55). By Scallan et al.'s estimations, foodborne O157 STEC illnesses caused 2,138 hospitalizations (3rd among bacteria) and 20 deaths annually in the US from 2000-2008 (73). Outbreak data from 1998 to 2008, collected and analyzed by Batz et al. (8), showed that 18.1% of the 258 *E. coli* O157:H7 associated illnesses and 16.6% of the 1288 non-typhoidal *Salmonella* associated illnesses were linked to produce. Produce was ranked 4th highest in public health impact among food categories, according to the study (8).

Produce can become contaminated through multiple ways before being harvested. Some of the most recognized sources of contamination include wildlife, nearby livestock, irrigation water, manure and compost. Contamination of crops can

happen directly, through wildlife or cattle feces, or else indirectly through contaminated fertilizers, compost or irrigation water. While fencing can deter most livestock and wildlife from accessing crops, something as small as a fruit fly can spread foodborne pathogens to produce surfaces (52).

Irrigation water is one of the most notorious vectors of pathogenic transfer onto produce. While testing irrigation water quality is considered key to preventing the use of contaminated water, most of these tests examine fecal indicator microorganisms, commonly generic *E. coli*, which has been the case since the early 1900's (30). More recently, since generic *E. coli* has now been found to occur naturally in environments other than the intestinal tracts of animals, using them as fecal indicators has been called into question (49). While there are no other bacteria that might easily or feasibly replace *E. coli* as a reliable fecal indicator, it is important to note that simply testing water quality, while recommended, does not entirely solve all potential safety problems.

During the harvest and processing of produce, entirely new vectors of contamination are introduced, including the field workers themselves, harvesting equipment, rinse water, processing equipment and even storage and packaging equipment. If proper hygiene of workers is not maintained, it is easy for a pathogen like *Salmonella* to spread to produce via the fecal-oral route, particularly when workers are ill. Additionally, improper cleaning and sanitation in the packinghouse have been linked to likely causes of foodborne outbreaks since pathogens can transfer quickly from the equipment surfaces to produce surfaces, especially when biofilms form. The FDA found deficiencies in the Jensen Farm cantaloupe packinghouse with several food contact surfaces heavily burdened with the outbreak strains of *L. monocytogenes* and a lack of sufficient documentation for cleaning and sanitizing of these surfaces (35). This

outbreak is a recent example of likely biofilm establishment, which have been demonstrated to be almost impossible to remove by routine cleaning and sanitizing activities.

As consumption increases, the chance of contamination of fresh produce does likewise, due to increased exposure, transportation times, extended storage and encroaching wildlife onto more crowded farmland. While contamination of individual fruits and vegetables is ultimately inevitable over time, cross-contamination of other raw produce can turn these events into large-scale outbreaks and is avoidable in many cases.

Most fresh produce does not undergo a thermal treatment or kill-step prior to being eaten. Any mishandling throughout the life of the plant can be potentially life threatening to consumers. Even in its whole form, produce can harbor pathogenic bacteria in wounds or tiny crevices present in many fruits and vegetables (95). Studies have demonstrated that pathogenic bacteria that come into contact with produce in the field can survive for months. Islam et al. (50, 51) established that avirulent mutagens of *E. coli* O157:H7 and *Salmonella* Typhi were able to survive in the phyllosphere of lettuce and parsley and the fields they were grown for three and six months, respectively. When damaged through disease, or during cutting and slicing, there is an even greater concern for microbial growth, since the newly exposed surface can be a source of nutrients for microorganisms (32). Due to increased survival at low populations in the field, there are numerous recognized sources of contamination that have briefly been discussed here. However, it is important to keep in mind that one contaminated piece of produce does not cause a multistate outbreak. One prevalent point of cross-contamination that can impact a lot of produce is through the use of a post-harvest rinse, particularly if there

are not adequate disinfectants present. If pathogens are able to survive within this rinse water, all incoming produce are likely to become contaminated as well, possibly leading to a foodborne outbreak.

Post-harvest washing as a source of produce contamination. There are multiple methods used to remove “field heat” and clean produce post-harvest (19, 1). If the produce commodity is sensitive to moisture, the simplest pre-cooling method is room cooling, where produce is simply left in a refrigerated room for a given amount of time. This is fairly inefficient and is typically only used for very small amounts of produce. Forced-air cooling is another method used on produce sensitive to moisture changes, but it is significantly faster due to fans that force the cold air to come into contact with as much produce as possible. Finally, one of the most complex and expensive dry systems used to pre-cool produce is vacuum cooling, where a vacuum draws out the air from the chamber containing the produce. Under a vacuum, the atmospheric pressure is lowered and water evaporates faster, causing the temperature to drop as well. This is most common with leafy vegetables (19, 1).

If the produce is not likely to be damaged by moisture changes, post-harvest hydro-cooling is the simplest method. In hydro-cooling, produce comes into contact with cold water, which lowers the temperature rapidly and cleans off some of the dirt and debris from the field. With dump tank systems, produce is simply deposited into a large container of shared water and removed, usually via a conveyer system. Water flumes are much the same, ordinarily involving agitation to make the process more effective. While these two techniques are efficient at cooling and cleaning debris from produce, the shared water can act as the perfect vector for cross-contamination if there are not sufficient antimicrobials present. Sprays bars, while still hydro-cooling produce,

do not share water throughout the batch. While antimicrobials are still strongly recommended for spray hydro-cooling methods, one contaminated piece of produce will not necessarily contaminate the entire harvest unless the water is recirculated (19,1).

One concern that is often emphasized within the realm of produce safety is the ability of bacteria to internalize into produce. For example, tomato specific guidelines set forth by the FDA forbid tomatoes from being submerged in wash water that is not at least 10° F higher than the pulp temperature of the tomatoes (34). This is due to the fact that when tomatoes are submerged into water that is colder than their internal temperature, they can draw water in, allowing pathogenic microorganisms to harbor inside (7). This is exceptionally dangerous, since studies have proven that human pathogens like *Salmonella* (common in tomato outbreaks, see Table 1.1) can not only survive, but grow inside tomatoes at 68° F, well within the normal storage temperature range of tomatoes (101). *Salmonella* has also been shown to internalize into cantaloupe via damage or stem scar openings (70). Studies have also examined *E coli* O157:H7 internalizing through the surface of iceberg lettuce deep enough to be protected from subsequent chlorine treatments up to 20-200 ppm for five min (74, 80).

Because of wash water's potential for cross-contamination, the presence of antimicrobials is extremely important. The most commonly used and studied antimicrobial agent for these rinses is chlorine (64). Chlorine is effective against all known forms of microorganisms, including bacteria, fungi, viruses, protozoa and even bacterial spores (85). "Active" chlorine is measured in free residual chlorine or free available chlorine, which is the concentration of hypochlorous acid (HClO).

Hypochlorous acid is the form of chlorine that has the most antimicrobial activity and is responsible for disinfection when chlorine is utilized. These compounds can be quickly

consumed throughout their exposure to organic matter, oxygen and sunlight. The efficacy of chlorine is also heavily dependent on pH. The recommended pH range for washing fresh produce is 6.0-7.5, since this is the range at which most chlorine should be in the form of HClO, the most activated form of chlorinated solutions (94). As the pH rises, the formation of hypochlorite ions are preferred, which have little to no antimicrobial activity compared to hypochlorous acid. Chlorine is the most common antimicrobial used in hydro-cooling because it is widely available, relatively cheap and very easy to use, although it is often criticized for its susceptibility to organic matter and its off-gassing of dangerous by-products, such as chloroform and bromodichloromethane, which are classified as possible human carcinogens by the International Agency for Research on Cancer (15).

Other useful antimicrobials utilized and studied in produce washing include chlorine dioxide, peroxyacetic acid (PAA), ozone, hydrogen peroxide and organic acids. Chlorine dioxide stays dissolved in solution without hydrolyzing and is less influenced by pH than chlorine compounds, but it must be generated on-site, as it cannot be shipped because of its explosive potential under pressure (2, 31). Peroxyacetic acid is allowed as a fresh or fresh cut produce water additive, and is praised for its relative tolerance to organic matter and pH changes (94). The main disadvantage of PAA in comparison to other sanitizers is its substantial cost. Ozone has reportedly been used since 1893, but is typically only used in fresh-cut operations (69). While not as strongly dependent on the solution's pH, ozone is remarkably sensitive to organic matter and might, like chlorine, potentially form unwanted by-products (41). For example, bromate formation is one concern, because of its proven carcinogenicity in some animals (71). It is also more expensive than conventional chlorine methods. Hydrogen peroxide, while

showing promise in studies testing its antimicrobial abilities with fresh and minimally processed produce, is actually not approved by the FDA in these systems, unless it is in the form of PAA. Organic acids, which are approved for use in a wide array of applications, are expensive to use, mainly because of their strict pH requirements.

Challenges with organic produce systems. Because the most important difference between organic produce and conventional is the lack of synthetic ingredients, most analysis comparing the two types actually analyzes chemical differences, such as pesticide residue or micronutrient content (4, 5, 22). While this is essential to the target audience of organic foods, who prioritize these specific benefits, some have claimed that the use of manure and the absence of fertilizers, pesticides or preservatives simply increase the risks of foodborne illness (78). The major concern for the biosafety of organic produce is the use of manure, in which pathogens like *E. coli* O157:H7 and *Salmonella* have been reported to survive from 70 to 260 days (37, 46, 47, 65, 93). The Organic Rule allows raw manure to be used if it is applied at least 90-120 days before harvest, depending on if the edible portion of the produce comes into contact with the soil (3). This specific waiting time seems to be chosen arbitrarily, as literature indicates that pathogens can survive in manure much longer (37, 46, 47, 65, 93). For example, Forshell et al. (37) found that *Salmonella* was able to survive in “cold” (not composted) cattle manure for as long as 204 days. Himathongkham et al. (46) found that *Salmonella* could survive more than 3 months in poultry manure, depending on not just temperature, but also water activity. Wang et al. (93) found that *E. coli* O157:H7 was able to survive in bovine feces for as many as 70 days at 5 °C, 56 days at 22 °C and 49 days at 37 °C. Nicholson (65) only noted that while *Salmonella*, *E. coli* O157:H7, *Listeria monocytogenes* and *Campylobacter* could all survive more than a

month in livestock manure after it was spread on land, none were detectable after 9 months.

The most common treatment of animal waste in organic farming is “composting,” which is essentially allowing microorganisms to break down the materials in the manure into forms that are more bioavailable for the plants. This is done by heating the manure for a given amount of time (commonly three days at 131-170 °F) (32). Windrow composting is commonly done with larger quantities of manure. In this system, the pile is turned at least five times after it has reached internal temperatures of 131-170 °F for three days, with a cumulative composting time of 15 days, by NOP standards (3). This method should inactivate all pathogens, but turning must be done in such a way as to fully incorporate the outer layer to the inner core since pathogens on the outer surface will not be inactivated (57).

In regards to organic post-harvest wash systems, there are also strict limitations as to what can be used, according to the NOP. Chlorine, the gold standard of wash water antimicrobials, is allowed, but must be at levels below 4 ppm (mg/l) at the point of discharge. This does not necessarily limit how much is used throughout the process, as long as most of the chlorine is used up by the time the wash water is discarded (79). While ozone and peroxyacetic acid are also permitted for use as produce surface disinfectants, they have their own restrictions and can be expensive for small scale produce production. These compounds, either because of their expensive costs, strict limitations or reputations for off-gassing, are not always used by farmers. Some producers choose to only use water to wash their produce post-harvest instead, saving time and money but drastically increasing food safety risks.

Overview of essential oils. Essential oils (also called volatile or ethereal oils) are aromatic and oily liquids obtained by the extraction typically by steam distillation of plant materials (90). There are over 300 essential oils (EOs) used commercially today, mostly in the pharmaceutical, cosmetic and food industries, and there are around 3,000 known (12). In relation to food safety, some of the most important chemicals in essential oils are secondary metabolites. They are “secondary” because they are not necessary for plant life, but they are important. These compounds usually play a role in plant-pathogen defense, which might explain why they display antimicrobial activity.

Some of the most pertinent EO derivatives to food safety are eugenol (from clove oil) and thymol (from thyme or oregano oil). While EOs can have a complex make-up of as many as 45 individual constituents, the most active compounds are usually the phenylpropenes, terpenes, terpenoids, and “other” secondary metabolites (48). Clove bud oil is made of 75-95% eugenol, while thyme oil contains anywhere from 10-64% thymol (9, 56, 59). Essential oils are well recognized to be effective at low concentrations against a broad spectrum of microbes (12).

Antimicrobial activity of essential oils. While the need for a universal way to test and compare the efficacy of essential oils has been noted, there is still none recognized (24). Thyme oil is sometimes considered more bactericidal than clove oil, but both are considered to be two of the most effective essential oils against bacteria (12, 29, 38, 54). Other notable candidates include cinnamon bark oil (which contains cinnamaldehyde) and oregano oil (which contains carvacrol and some thymol). In a review by Sara Burt, MIC's (minimum inhibitory concentrations) of these essential oils were compiled from multiple studies for comparison (12). Against *E. coli*, clove oil and thyme oil had similar MIC's, with ranges of 0.4-2.5 µl/ml for clove and 0.45-1.25 µl/ml

for thyme (13, 20, 33, 43, 77). Against *Salmonella*, however, thyme oil seemed the favored antimicrobial agent, with MIC's reported as low as 0.45 µl/ml, but sometimes as high as clove oil, >20 µl/ml (20, 43). This could be due to the changing components of EOs, depending on factors like harvesting seasons and geographical locations (12).

Studies of the mechanisms of EO antimicrobial action usually focus on the effects of the target microorganism's cytoplasmic membrane. Thymol and eugenol have been studied extensively to uncover their specific modes of action against bacteria. Their antibacterial activity is certainly linked to their ability to interact with membrane proteins. Mis-folding and even disintegration of the lipopolysaccharide layer leads to an increased permeability, as evidenced by potassium and ATP leakages (27, 44, 45, 48, 53, 92).

Research by Moore-Neibel et al. (61) found that lemongrass oil was able to reduce populations of *Salmonella enterica* from organic leafy greens immediately after rinsing by up to ~2 log CFU/g from organic iceberg lettuce and organic baby spinach with two min dip treatments at 0.5%. Continued exposure from residual lemongrass oil during storage lowered *Salmonella* levels over the three day sampling period (60). In a separate study, Moore et al. (62) tested olive extract (up to 5%), hibiscus concentrate (up to 30%), apple extract (up to 5%) and hydrogen peroxide (at 3%) against *Salmonella enterica* and like-wise found them time and concentration dependent. The most effective at day 0 was olive extract, which was able to reduce the population by >2.5 log CFU/g from iceberg lettuce after a two min dip treatment at only 3% (62). A study by Todd et al. (84) found cinnamon leaf oil similarly effective, with 0.5% cinnamon oil reducing *Salmonella* Newport by up to 2 log CFU/g on day 0 after a two min rinse from romaine lettuce surfaces. Again, romaine lettuce was the easiest leafy green to disinfect

by these essential oil solutions, and residual effects of the antimicrobial was able to lower the levels of the bacteria throughout the three sampling days after the treatment (84). Another study by Moore-Neibel (60) discovered that oregano oil was the most effective yet, with >4 log CFU/g reductions of *Salmonella enterica* from all four organic leafy greens tested after only one min exposure at 0.5% oregano oil. Oregano oil is similar in make-up to thyme oil (both containing thymol and carvacrol), and both are hailed as two of the more antimicrobial essential oils. Yossa et al. (99) also tested the efficacy of essential oil solutions on leafy green surfaces, evaluating them against *E. coli* O157:H7 as well as *Salmonella enterica*, and continuing to sample up to 14 days after treatment. They also used an emulsifier (Tween 20) to potentially improve the disinfectant abilities of the solutions and compared these treatments to chlorine at 5 ppm, finding that the antibacterial effects of the essential oil solutions (cinnamaldehyde and a proprietary mix of clove, rosemary and thyme oil) were comparable to that of chlorine on lettuce surfaces (99).

Surfactants and emulsifiers in sanitizers. The most vital component of any post-harvest produce sanitizer is the antimicrobial agent, as it should serve as a preventative measure to cross-contamination, even if it is not effective enough to completely eliminate the pathogen at the point of contamination. However, when measuring the effectiveness of a sanitizer by its lethality on the surface of a particular piece of produce, it is important to note that the surface of the produce itself can serve as a protective barrier for the pathogen (81). Cuts, crevices, stem scars and the overall roughness or texture of the plant surface can all make a big difference to the accessibility of micro-niches by sanitizers (39). The hydrophobicity of certain areas of the plant surface alone can deter aqueous sanitizers from being effective (11). While EOs are

hydrophobic by nature, they are likely to avoid this complication, but they still need to be applicable in an aqueous solution. Since emulsions are often used to stabilize mixtures of hydrophobic and hydrophilic compounds, there is interest in finding emulsifiers or emulsifying processes that might allow essential oils to be effective as post-harvest produce rinsing agents. Studies have been done in the past, utilizing emulsions to enhance the antimicrobial capabilities of essential oils (28, 58, 98, 100). Research done by Zhang et al. established that EO emulsions had enhanced wetting abilities, crucial for surface disinfectants (100).

Since the present study was to propose an EO based post-harvest wash for organic produce, an approved emulsifying agent was essential. The problem with most common, synthetic emulsifiers is that the National Organic Standards Board (NOSB) prohibits all synthetic substances from being used in organic crop production, unless specifically allowed. Natural alternatives are therefore necessary. Whey protein is a group of the naturally occurring proteins in milk. While whey protein is better known for its nutritional content, and is often marketed as a dietary supplement, its functional properties, including foam-stabilizing, fat-binding and emulsifying abilities, are well-documented (25, 36, 75). Its emulsifying capabilities have been studied as early as 1973 (63). Gum arabic, or acacia gum, is another naturally occurring substance. It is formed from the hardening of the sap from acacia trees, commonly found in the Sudan. It is a food stabilizer, thickening agent and emulsifier. It is mostly used in either confectionary products to prevent sugar crystallization and control texture or else in beverages as an emulsifier or for flavor encapsulation (97). These two natural emulsifiers were chosen based off of previous work conducted by Luo et al., which studied different substances' abilities to self-emulsify alkaline-dissolved essential oils (58).

With fresh, minimally processed and organic produce, there is much work to be done with regard to microbiological safety. It is important to maintain proper hygiene and sanitation on the farm and in the packinghouse to inhibit the chances of pathogen contamination. Post-harvest rinses are already common, used to clean and cool produce quickly before it is processed and packaged. The presence of a sanitizer is recommended to prevent cross contamination, but many small scale and organic farms find most sanitizers difficult to implement. If alternative disinfectants are to be utilized, they should be naturally-derived compounds that can be easily dispersed into an aqueous system in order to achieve organic approval and retain activity against target pathogens. In this study, plant-based essential oils were emulsified with natural compounds using low-cost emulsification technology and the resulting solutions were examined for their produce surface disinfection abilities versus associated pathogens.

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Appendix I

Figures

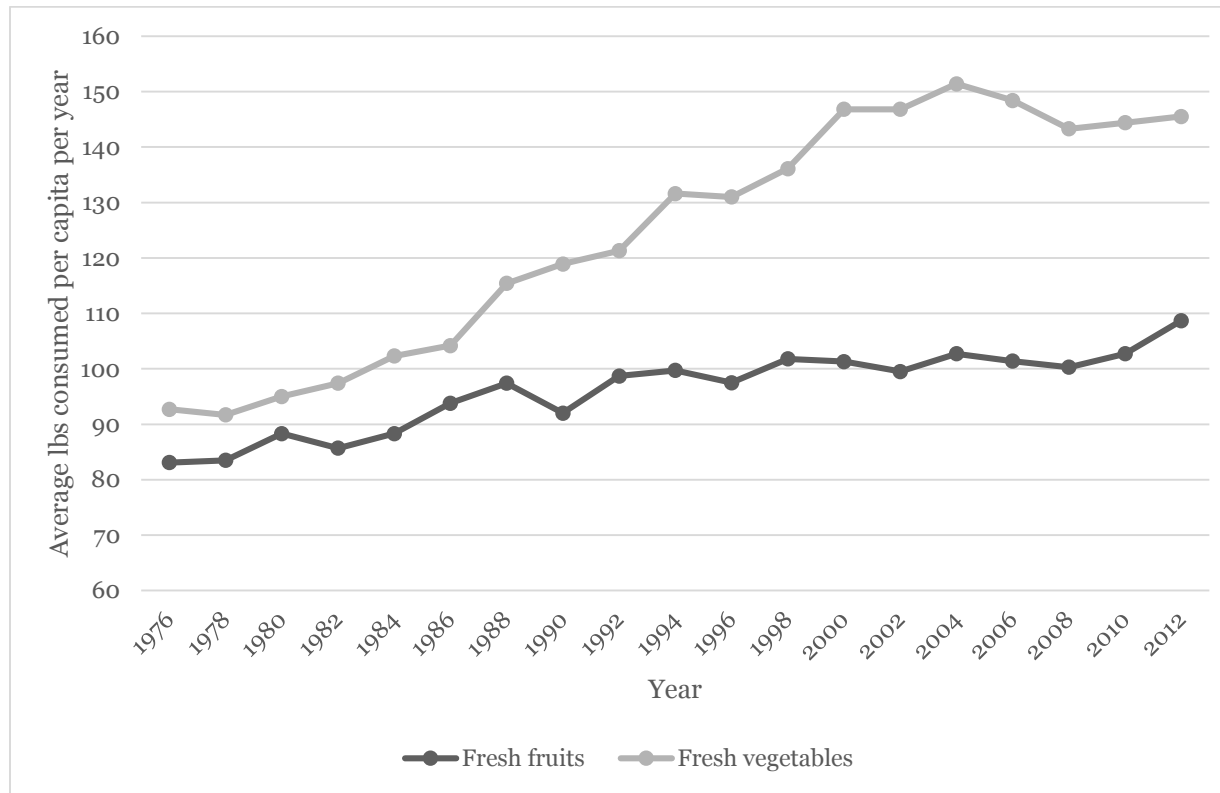


FIGURE 1.1. *Changes in fresh produce consumption in the U.S. since 1976. Data was collected from the 2014 Fruit and Tree Nuts Yearbook and the 2014 Vegetables and Pulses Yearbook, made public by the CDC.*

Tables

TABLE 1.1. *Multi-state foodborne pathogen outbreaks associated with select produce commodities*

Year	Commodity	States affected	Number ill	Pathogen
2006	Tomatoes	21	183	<i>Salmonella</i> Typhimurium
2006	Tomatoes	19	115	<i>Salmonella</i> Newport
2006	Leafy greens	26	183	<i>E. coli</i> O157:H7
2008	Tomatoes	43	1442	<i>Salmonella</i> Saintpaul
2008	Cantaloupes	16	51	<i>Salmonella</i> Litchfield
2010	Leafy greens	2	9	<i>Listeria monocytogenes</i>
2010	Leafy greens	5	26	<i>E. coli</i>
2011	Leafy greens	4	7	<i>Listeria monocytogenes</i>
2011	Leafy greens	10	60	<i>E. coli</i> O157:H7
2011	Cantaloupes	28	147	<i>Listeria monocytogenes</i>
2011	Cantaloupes	10	20	<i>Salmonella</i>
2012	Leafy greens	5	28	<i>E. coli</i> O157:H7
2012	Cantaloupes	24	261	<i>Salmonella</i>
2013	Leafy greens	4	33	<i>E. coli</i> O157:H7
2013	Leafy greens	25	631	<i>Cyclospora</i>

Chapter II

Utilization of Emulsified Clove Bud Oil and Thyme Oil to

Inactivate *Salmonella* on Cherry Tomatoes

Abstract

Emulsions of thyme oil with gum arabic and clove bud oil (CBO) with whey protein were tested for their bactericidal activity against *Salmonella* on the surface of cherry tomatoes. These solutions were compared to water and chlorine at 200 ppm free residual chlorine as controls. All these solutions were also exposed to 1% (w/v) organic loading (OL), in the form of blended cherry tomatoes to determine their vulnerability to an organically loaded system. Additionally, uninoculated tomatoes were passed through the treatment solutions after inoculated produce to determine the likelihood of *Salmonella* cross-contamination. 0.5% CBO emulsion (v/v) was the most effective compound, while 0.5% (v/v) thyme oil emulsion showed the most resilience to organic loading. Chlorine was just as effective as 0.5% thyme oil emulsion \pm 1% OL and 0.5% CBO emulsion with 1% OL, but was completely ineffective in the presence of 1% organic matter. All treatments, other than the water controls, showed less than 1.65 log CFU/g (the highest detection limit) *Salmonella* transfer onto the clean tomatoes and had less than -0.8 log CFU/ml in the treatment liquids, showing no meaningful differences between them. These data indicate that emulsified essential oils show promise as post-harvest rinses for produce.

Introduction

With outbreaks associated with fresh produce increasing in recent years, improved food safety practices in post-harvest handling are becoming more important to the produce industry (26). Plant surfaces can harbor pathogenic bacteria introduced from the environment, including irrigation water, wildlife, and bioaerosols, for months (2). These plant surfaces' topography, including damaged areas, crevices and

hydrophobicity can protect the bacteria from removal with water (10, 30). Post-harvest washes of produce are often utilized to remove field heat and debris, but should contain some antimicrobial agents if they are to protect the produce from pathogen cross-contamination when contaminated produce enters the washing system.

There are many antimicrobials already in use as post-harvest wash water sanitizers, including chlorine, peroxyacetic acid (PAA) and ozone. Chlorine is the most widely used because of its broad-spectrum activity, low costs and simplicity of implementation, as well as its availability. PAA and ozone are good alternatives to chlorine in many ways, but are too expensive for many producers to consider, especially when producing small yields. Chlorine is allowed in organic produce rinses, but there are restrictions on its uses. Alternative post-harvest washing solutions would benefit the organic produce industry, since their choices are so severely limited to restrictions by the NOP, as well as cost restraints when dealing with small scale operations.

Plant-derived essential oils have become the subject of increased research as antimicrobials in recent years instead of just flavor additives, as consumers push for more natural ingredients and become more apprehensive toward preservatives (5). They are remarkable for their effectiveness at low levels and their stability (4, 12, 28). Thyme and clove oils are considered two of the most effective essential oils against bacteria (3, 7, 9, 16). They have been reported to increase membrane permeability, reduce membrane potential and deplete intracellular ATP when examined against bacteria (6, 12-15, 29).

The objective of the current study was to evaluate emulsified thyme and clove bud oil as a post-harvest antimicrobial for laboratory simulated water immersion washing, as compared to chlorine and no antimicrobial controls. These systems were

tested with cherry tomatoes that were inoculated with *Salmonella enterica* and analyzed for their ability to lower the populations of *Salmonella*, inhibit cross-contamination onto uninoculated cherry tomatoes and determine their susceptibility to organic matter.

Materials and Methods

Bacterial cultures and maintenance. A five-strain *Salmonella* cocktail was used, containing the following serovars: Agona (alfalfa sprout associated outbreak), Montevideo (tomato associated outbreak), Gaminara (orange juice associated outbreak), Michigan (cantaloupe associated outbreak) and Saintpaul (pepper associated outbreak). All strains were made resistant to 40 ppm nalidixic acid (NA; Acros Organics, Geel, Belgium) so they could be distinguished from the background microflora of tomatoes. All NA resistant strains were evaluated for susceptibility to essential oils and chlorine as compared to the wild type to assure no differences in susceptibility existed. All cultures were kept in 15% glycerol stocks at -80 °C for long-term storage.

Media preparation. Tryptic soy agar (Becton, Dickinson and Company, Sparks, MD) with added 6.8 g/l sodium thiosulfate, 0.8 g/l ammonium ferric citrate (Thermo Fisher Scientific, Waltham, MA) was mixed, sterilized (121 °C for 15 min) and cooled to ~55 °C before sterile NA (Acros Organics, Geel, Belgium) from a stock solution was added to get a final concentration of 40 ppm NA, and the molten agar was poured into Petri dishes. The resulting agar, TSANSA, was used to enumerate *Salmonella*.

Inoculum preparation. Cultures were individually revived by three consecutive 24 h transfers into tryptic soy broth with nalidixic acid (TSBN; Becton, Dickinson and Company, Sparks, MD) and incubated at 37 °C. A 300 µl aliquot of each of these cultures was individually spread onto tryptic soy agar plates with 40 ppm nalidixic acid (TSAN) and incubated at 37 °C for 24 h to form a lawn. Each plate was

then flooded with 5 ml phosphate buffer pH 7.2 (Becton, Dickinson and Company, Sparks, MD) with 0.2% Tween 80 (Thermo Fisher Scientific, Waltham, MA) to collect cells. Equal volumes of each culture were combined for the inoculum.

Sample inoculation and preparation. Cherry tomatoes (Naturesweet Tomatoes, San Antonio, TX) purchased from local retail outlet, were spot inoculated with 10 μ l of the *Salmonella* inoculum. Tomatoes were dried inside a biosafety cabinet for 2 – 3 h.

Essential oil emulsion preparation. To prepare a stock solution of alkaline-dissolved thyme oil and gum arabic, first a glycerol bath was heated to $\sim 125^{\circ}\text{C}$. A solution that was 10% (v/v) thyme oil (Sigma-Aldrich, St. Louis, MO) and 90% 3M NaOH (Thermo Fisher Scientific, Waltham, MA) was heated to boiling $\sim 115^{\circ}\text{C}$ and left to boil for 10 min. Separately, a 10% (w/v) gum arabic (GA) (Acros Organics, Geel, Belgium) solution was purified by centrifugation at $4,500 \times g$ for 10 min before the supernatant was collected. Once these two solutions were ready, a mixture was made that was 0.5% (v/v) alkaline-dissolved thyme oil and 0.5% (v/v) purified gum arabic. The pH of this 1% thyme oil emulsion stock solution was then lowered to 7.0 (± 0.1) using 3M, 1M and 0.1M citric acid (Sigma-Aldrich, St. Louis, MO). Throughout the experiment, pH was measured using an accumet XL 15 pH/mV/Temperature Meter (Thermo Fisher Scientific, Waltham, MA).

Similarly, for a stock solution of alkaline-dissolved clove bud oil and whey protein, a glycerol bath was heated to $\sim 120^{\circ}\text{C}$. A 10% (v/v) CBO (Sigma-Aldrich, St. Louis, MO) in 3M NaOH solution was heated to boiling $\sim 110^{\circ}\text{C}$ and allowed to boil for 10 min. Separately a 2% (w/v) whey protein concentrate solution brought to a pH of 4.0 (± 0.1) using 3M, 1M, 0.1M citric acid was centrifuged at $4,500 \times g$ for 10 min and the

supernatant was collected before it was brought back to a neutral pH of 7.0 (± 0.1) using 3M NaOH. These two solutions were mixed to create a final stock solution of 0.5% (v/v) alkaline-dissolved CBO and 0.5% (v/v) purified whey protein before the pH was brought down to 7.0 (± 0.1), again using 3M, 1M and 0.1M citric acid. This is termed 1% CBO emulsion for the remainder of the experiment.

Organic loading of wash systems. Cherry tomatoes were blended and added to sterile de-ionized water until the mixture was 20% (w/w) blended cherry tomato. This was termed Organic Load (OL) and was added to different solutions to get a final concentration of 1% OL to check a solution's susceptibility to the presence of organic compounds.

Preparation of wash systems. Wash liquids containing one of 12 treatments were prepared: sterile deionized water, DI water with 1% OL, 200 ppm free residual chlorine, a chlorinated solution that was 200 ppm free residual chlorine until 1% OL was added, 0.2% thyme oil emulsion, 0.2% thyme oil emulsion with 1% OL, 0.5% thyme oil emulsion, 0.5% thyme oil emulsion with 1% OL, 0.2% CBO emulsion, 0.2% CBO emulsion with 1% OL, 0.5% CBO emulsion and 0.5% CBO emulsion with 1% OL. All wash liquids containing chlorine were tested for free residual chlorine using a Free Chlorine & Chlorine Ultra High Range ISM (Hanna Instruments, Roonsocket, RI) immediately after being mixed and before being used. All 12 treatments were individually dispersed in 100 ml volumes in sterile glass beakers.

Simulated post-harvest washing of tomatoes and recovery of *Salmonella*. Inoculated tomato samples (two tomatoes each, ~20 g) were dipped into the prepared wash liquids and left for 2 min. Samples of inoculated tomatoes were not dipped in any wash liquid prior to recovery to determine the initial counts present on

the produce. After treatment, tomatoes were placed into stomacher bags and diluted 1:5 (w/w) with phosphate buffer plus 0.2% Tween 80 using a Baby Gravimat gravimetric diluter (Microbiology International, Frederick, MD). Stomacher bags were shaken by hand for 15-20 s. The rinsate was diluted in buffered peptone water (BPW; Becton, Dickinson and Company, Frederick, MD) and spiral plated (WASP 2 Spiral Plater, Microbiology International, Frederick, MD) in duplicate onto TSANSA to enumerate *Salmonella*.

Subsequently, four consecutive uninoculated tomato samples were washed with each of the same wash liquids to test for *Salmonella* transfer. These samples were diluted in PBS with Tween 80 and rinsed as well, but for each of these sets of followers, the same rinsate was reused so that the final rinsate represented all of the potential transfer from one inoculated sample. This rinsate was then diluted, and plated as described above. Wash liquids were also enumerated for *Salmonella* after all tomato samples were rinsed by filtering 10 ml of the rinsate through a 0.45 µm membrane filter using a Millipore filter system (EMD Millipore Corporation, Billerica, MA).

Data Analysis. Each treatment was replicated four times with two samples each and duplicate subsampling (n=16). Statistical analyses were conducted using the generalized linear mixed model procedure (Proc GLIMMIX) of SAS 9.4 (SAS Institute Inc., Cary, NC) with significance levels set at $P < 0.05$. Analysis of variance was run to test for differences of populations of *Salmonella* between treatments. Analyses was done separately on inoculated samples, uninoculated following samples and liquid wash samples.

Results

Efficacy of emulsified EOs on inoculated tomatoes. The effect of treatment wash solutions was found to significantly influence the subsequent *Salmonella* populations rinsed from the inoculated samples (Figure 2.1; $P < 0.01$). *Salmonella* was recovered from inoculated tomato samples at levels ranging from 5.31 to 7.41 log CFU/g. Initial populations of *Salmonella* on tomatoes were 7.41 log CFU/g (Figure 2.1), which was not statistically different from tomatoes rinsed in DI water (7.12 log CFU/g), chlorine with 1% OL (at 7.23 log CFU/g) as well as 0.2% CBO emulsion with 1% OL (7.06 log CFU/g; $P > 0.05$). The 1% OL in the chlorinated washes lowered the free residual chlorine from 200 ppm to an average of 136 ppm.

Without 1% OL, hypochlorous acid was able to lower the *Salmonella* levels to 6.32 log CFU/g (1 log reduction; Figure 2.1). This reduction was similar to 0.5% thyme oil emulsion with 1% OL (6.62 log CFU/g), 0.2% CBO emulsion (6.50 log CFU/g), 0.5% thyme oil emulsion (6.49 log CFU/g) and 0.5% CBO emulsion with 1% OL (6.12 log CFU/g). The 0.5% CBO emulsion was statistically the most effective treatment at lowering *Salmonella* levels, resulting in a 2 log reduction (5.31 log CFU/g). The efficacy of these treatments to reduce *Salmonella* populations on inoculated cherry tomatoes were as follows: 0.5% CBO emulsion > 200ppm chlorine = 0.5% thyme oil emulsion with and without 1% OL = 0.2% CBO emulsion = 0.5% CBO emulsion with 1% OL > No treatment control = DI water = 200ppm chlorine with 1% OL = 0.2% CBO emulsion with 1% OL.

Prevention of *Salmonella* cross-contamination. Wash treatments also had a significant effect on the cross-contamination of *Salmonella* to uninoculated cherry tomatoes that followed ($P < 0.01$; Figure 2.2). Uninoculated samples rinsed in DI water

resulted in an average *Salmonella* population of 5.37 log CFU/g, which was significantly different than all other wash treatments, with the exception of water with 1% organic load, which had no significant impact on the bacterial recovery ($P>0.05$; data not shown). All other treatments were found to have populations below the limit of detection 1.65 log CFU/g.

Finally, the wash liquids were also enumerated for *Salmonella* and the treatments were shown to significantly affect the results ($P<0.01$). *Salmonella* was recovered from water at an average of 6.64 log CFU/ml, which again was significantly higher than all other treatments except for water containing 1% OL (Figure 2.3). Chlorine with OL had one sample that was positive for *Salmonella* from the six samples that were analyzed, resulting in an average recovery of -0.84 log CFU/ml. In all other treatments, *Salmonella* was not recovered and were thus below the limit of detection (-1.05 log CFU/ml). All treatments that contained an antimicrobial treatment were found to be similar for *Salmonella* populations ($P>0.05$).

Discussion

Chlorine at 200 ppm was just as effective as the highest level of thyme oil emulsion tested (0.5%), but the presence of 1% blended tomatoes revealed a vulnerability to organic loading that was not present (or at least as significant) in the emulsified essential oil (EO) treatments. 0.5% thyme oil emulsion showed no significant decrease when in the presence of 1% OL, whereas chlorine with 1% OL was no better than the control, a water rinse. While chlorine with 1% OL was measured to still have 136 ppm free residual chlorine on average, the introduction of tomato samples (more organic matter) seems to have reduced the chlorine's bactericidal effects. The

0.5% CBO emulsion sample was susceptible to the presence of organic matter, significantly decreasing in efficacy, however the 0.5% CBO emulsion with 1% OL was still just as effective as chlorine at 200 ppm. So not only was 0.5% CBO emulsion the most effective against the *Salmonella* present on the tomatoes, but it was also fairly resistant to organic loading, although it could be said that the 0.5% thyme oil emulsion treatment was even more impervious to organic matter, as it did not significantly change when introduced to organic loading.

Even though few studies have been done with self-emulsified, alkaline-dissolved essential oils, many have been done testing the basic bactericidal effects of EOs independently. For example, a study done by Friedman et al. in 2002 (9) indicated that even though clove bud oil and thyme oil were two of the more effective EOs against *Salmonella enterica* RM1309, thyme oil showed the lower BA50 value (the concentration at which there is a 50% decrease in bacterial recovery) of 0.045%. Similarly, Olasupo et al. (24) found that the MIC (minimum inhibitory concentration) of thymol, the main antimicrobial component in thyme oil, was lower than that of eugenol, the main antimicrobial component in clove oil, against *Salmonella* Typhimurium (1.0 mmol⁻¹ versus 3.0 mmol⁻¹, respectively). A study performed by Oussalah et al. in 2007 (25) found the MIC's of thyme oil and clove bud oil to be the same against *Salmonella* Typhimurium, at 0.1% (v/v). The lethal effects of thymol and eugenol against *Salmonella* were compared as long ago as 1987, by Karapinar et al., who observed that at 50 µg/ml eugenol, the growth of 10⁷ *Salmonella* Typhimurium was inhibited after 24 h, where it took 500 µg/ml thymol to achieve the same results (16). The greater bactericidal activity of eugenol contrasts the other studies.

While these studies differ, thyme oil is usually observed to be just as lethal, if not more-so, against *Salmonella*. The current study's results indicate that the emulsified version of clove bud oil can be significantly more effective than the emulsified version of thyme oil. Whether this is because of the emulsifying capabilities of whey protein versus gum arabic on the tomato surface is unclear. Both treatments (thyme oil and CBO emulsions) did not have any recovered *Salmonella* on uninoculated tomatoes. This similarity in performance was also evident when enumerating the wash liquids. All were shown to be equally bactericidal against *Salmonella* with >6 log CFU/ml difference between these treatments and the water rinse survival.

Chlorine's efficacy in reducing levels of *Salmonella* from tomato surfaces (a 1.09 log CFU/g reduction after two min in 200 ppm chlorine) in the current study corresponds with research done by Zhuang et al. (31), which recorded a reduction of *Salmonella* Montevideo of 1.23 log CFU/cm² of the tomato surface after a two min dip in 210 ppm free chlorine. Beuchat et al. (1) found that chlorine at 200 ppm was able to reduce levels of *Salmonella* by 2.04 log CFU/cm² of lettuce leaf surface after only 60 s exposure, indicating that chlorine may be more effective at reducing bacteria from leafy greens, but was still within 1 log of the current study's results. Beuchat et al. worked with tomato surfaces as well, but the reduction of *Salmonella* was undetermined (1). Results from the present study differ, however, from those of Felkey et al. (8), which indicated that chlorine at 150 ppm was able to reduce levels of *Salmonella* by 6.36 log CFU/ml of the rinsate from tomato surfaces after 120 s of contact time at 25 °C.

Research by Das et al. (4), on the effects of ozone treatment against *Salmonella* on the surface of tomatoes found that a five min treatment of 20 ppm ozone was able to reduce levels of *Salmonella* Enteritidis by ~4 log CFU/tomato. A study comparing

commercial alkaline cleaners and acidified chlorinated cleaners by Kenney et al. (17) found that the best reduction of *Salmonella muenchen* possible was 3.11 log CFU/apple after a 60 s rinse. In regards to cross-contamination, research from Nou et al. (23) found that only 20 ppm chlorine was necessary to inhibit any detectable *Salmonella* survival within the wash water, after a 30 s rinse of contaminated lettuce samples, until levels of organic loading (shredded lettuce) reached 1% (v/v).

Moore-Neibel et al. (19-21), through multiple studies, did research on the efficacy of lemongrass oil, hibiscus concentrate, olive extract, apple extract, hydrogen peroxide, and oregano oil against *Salmonella* on the surface of different organic leafy greens. They found oregano oil most effective of these, reducing populations of *Salmonella enterica* by >4 log CFU/g after a one min rinse of 0.5% oregano oil. They found all essential oil solutions significantly effective at reducing *Salmonella* populations, and usually dependent on concentration and exposure duration (19-21). A similar study, by Todd et al. (27), tested the effects of cinnamon leaf oil and found it able to reduce levels of *Salmonella* Newport by as much as 2 log CFU/g from romaine lettuce surfaces.

When compared to the efficacy of other post-harvest washes and other essential oil research, the results of the present study indicate that emulsified essential oils show promise as future alternative organic produce wash solutions to inhibit cross-contamination and reduce the likelihood of pathogen survival on produce surfaces.

Stability is always a concern when evaluating any compound. Thyme and clove oils are known to be relatively stable throughout storage (11, 28). Thyme oil specifically has been shown to be remarkably resistant to the effects of light and temperature during long term storage, as denoted by Turek et al. in 2012 (28). Therefore it is not altogether unexpected to find that these compounds were more resilient than chlorine when it

comes to the influence of organic matter, one of chlorine's biggest weaknesses.

Additionally, there have been studies indicating that when oxidized, phenolic essential oil compounds may increase in antimicrobial activity (22).

Research by Luo et al. (18) compared the efficacy and stability of alkaline-dissolved clove bud oil self-emulsified with whey protein, gum arabic, soy lecithin and their combinations. The results from this study indicated that the solutions containing WPC, as the only emulsifying agent or in combination with others, were statistically unchanged in hydrodynamic diameter and polydispersability index throughout seven days of storage, proving their exceptional stability. Gum arabic, reportedly because of its low surface hydrophobicity and high molecular weight in comparison to protein emulsifiers, proved to be less efficient in EO entrapment, and less stable across the seven day storage period. Nevertheless, gum arabic did better than its alternative, lecithin, which caused aggregation when used as the sole emulsifier following neutralization (18). These data demonstrate that the EO emulsions have the potential for extended storage after production due to their stability and limited inactivation when used in wash systems, but further studies should be conducted to investigate these aspects.

Ultimately, the emulsified essential oil solutions proved to be as bactericidal against *Salmonella* on tomato surfaces as chlorine, if not more-so, and much more resistant to organic loading. These findings suggest that more studies should be done on these solutions, examining their physiological effects on produce, any organoleptic changes to the product and economic analysis.

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Appendix II

Figures

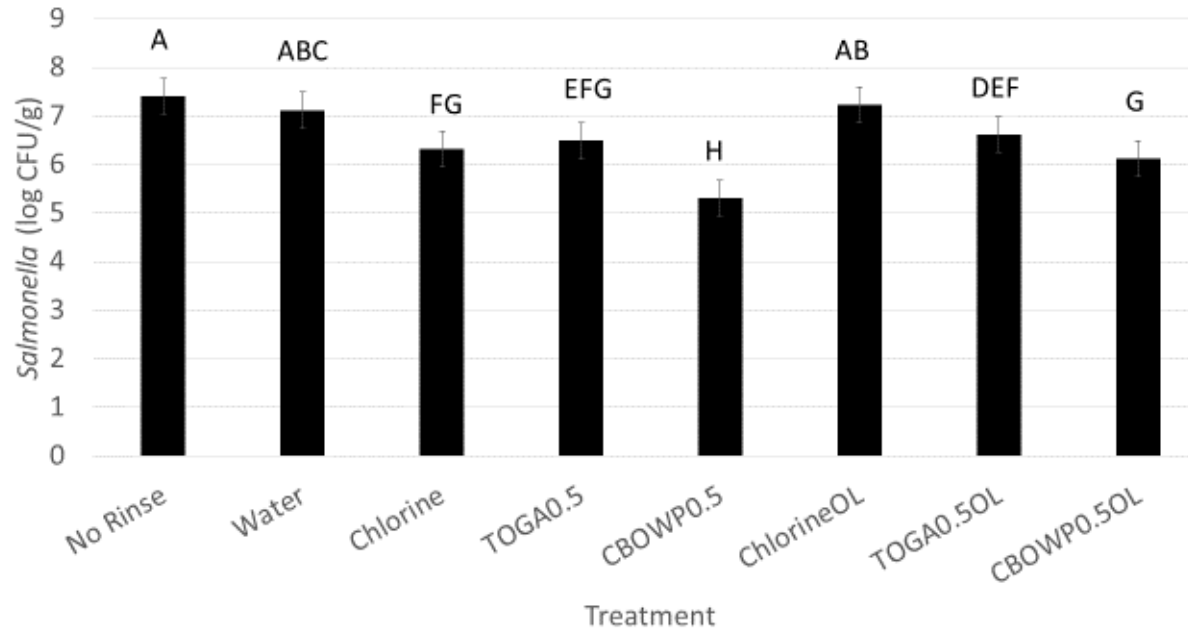


FIGURE 2.1. *Salmonella* recovered from inoculated cherry tomatoes after treatment rinse (n=16). Treatments with different letters signify significant differences ($P \leq 0.05$).

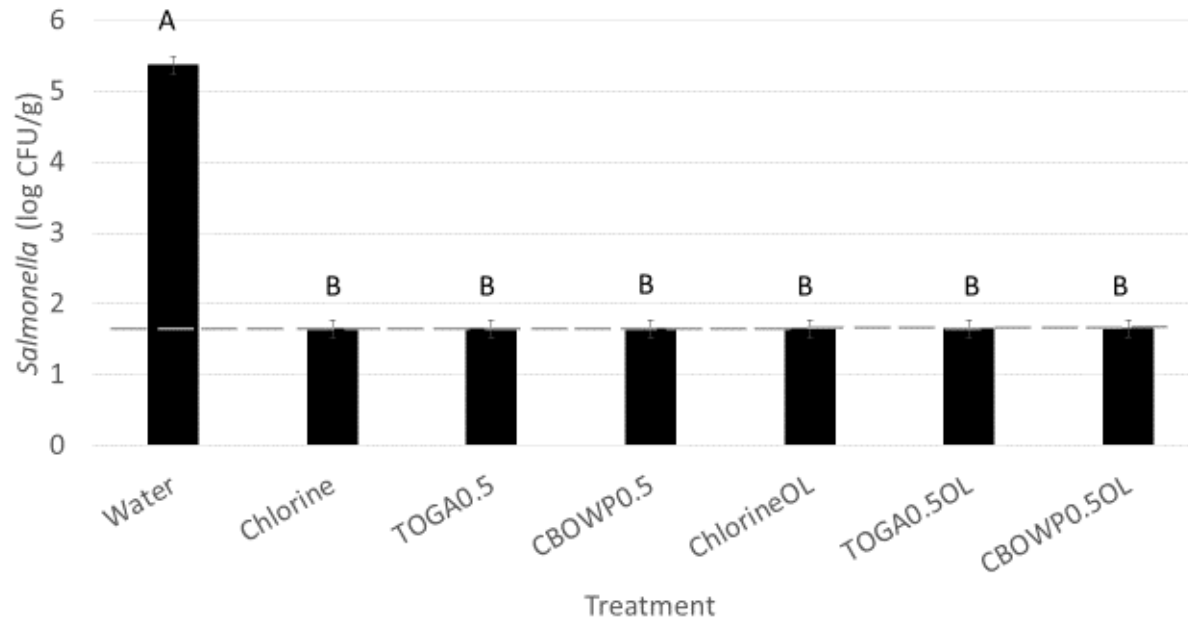


FIGURE 2.2. *Salmonella* recovered from uninoculated cherry tomatoes after shared rinse with contaminated produce (n=16). Treatments with different letters signify significant differences ($P \leq 0.05$). Dotted line represents limit of detection.

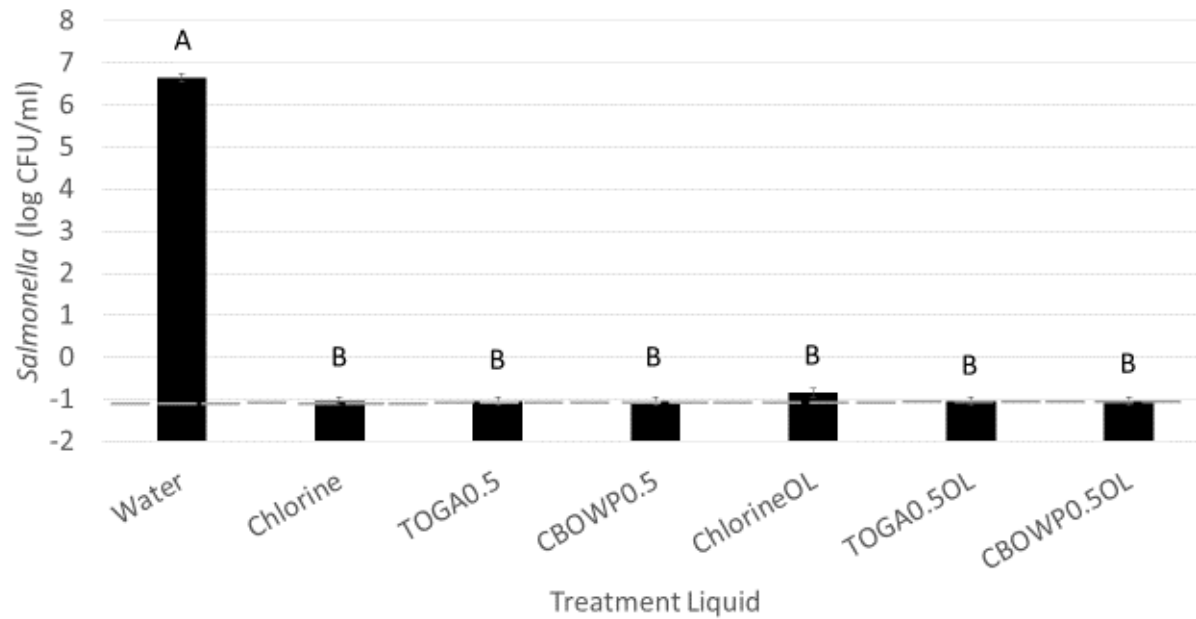


FIGURE 2.3. *Salmonella* recovered from rinse liquid after washing inoculated and uninoculated cherry tomatoes (n=16). Treatments with different letters signify significant differences ($P \leq 0.05$). Dotted line represents limit of detection.

Chapter III

Utilization of Emulsified Clove Bud Oil and Thyme Oil to

Inactivate *Escherichia coli* O157:H7 on Baby Spinach

Abstract

Emulsified solutions of clove bud oil (CBO) with whey protein and thyme oil with gum arabic were analyzed for their antibacterial properties against *Escherichia coli* O157:H7 on the surface of baby spinach. Contaminated spinach samples were rinsed in these solutions as well as solutions of chlorine at 200 ppm and water as controls. These treatment rinses were also investigated for their susceptibility to organic matter, by adding 1% (w/v) organic load (OL) in the form of blended spinach. All treatment liquids were also tested for their ability to transfer *Escherichia coli* O157:H7 from contaminated samples to uncontaminated spinach. The treatment wash liquids were also tested for *E. coli* O157:H7 survival after all spinach samples had been rinsed. In a system without organic loading, chlorine at 200 ppm was the most effective with over a 4 log reduction. However, 0.5% (v/v) thyme oil emulsion was the second most effective, with a 3 log reduction, and seemed completely invulnerable to organic loading while chlorine with 1% OL was significantly less effective than all thyme oil emulsions. A 0.5% (v/v) CBO emulsion with and without 1% OL was just as effective as chlorine with 1% OL, with >1 log reduction. Results from testing for *E. coli* O157:H7 transfer were much the same, with chlorine being the most effective but significantly hindered by the presence of organic matter. 0.5% thyme oil emulsion with 1% OL was the next most effective, even more effective than the 0.5% thyme oil emulsion in a clean system, which was observed to have the third best antimicrobial activity. Chlorine with 1% OL and CBO emulsion with and without 1% OL all had similar activity, with over 6 log CFU/g recovered from the clean spinach samples. When evaluating the recovery of *E. coli* O157:H7 from the treatment liquids, only chlorine with 1% OL had counts significantly above the detection limit, with the exception of the controls. These results indicate potential for the use of

essential oil emulsions as post-harvest rinses for leafy greens, as they consistently showed more resilience against organic loading than chlorine.

Introduction

The need for adequate produce safety practices is now more imperative than ever, with increased consumption and a rise in produce related outbreaks in recent years (21, 25). Disease causing pathogens can take advantage of the rough and sometimes hydrophobic surfaces of produce, like the creases and indentions in the phyllosphere of leafy greens, to protect them from harm (1, 3, 26). While chlorine is a common disinfectant agent, it is much less effective in systems with organic matter that readily builds up during production from plant material and soil. Those using chlorine must therefore continually test chlorine levels to achieve desired ranges of free chlorine and in many cases simultaneously acidify water to assure formation of hypochlorous acid. Even with adequate amounts of chlorine, the sanitizer may still have trouble coming into contact with the hydrophobic outer membrane of bacterial cells. Alternatives to chlorine that are commonly studied and used throughout the produce industry include peroxyacetic acid (PAA) and ozone. While these compounds are comparable to chlorine in their broad-spectrum antimicrobial activity, they are significantly more expensive to implement, which can be a deciding factor to small and organic farmers.

Because of the well-documented antimicrobial activity and stability of essential oils, they have garnered considerable attention as alternative rinsing agents for produce (4, 6, 9, 28). Thyme and clove oils are lethal against most pathogenic bacteria, including pathogenic *E. coli*, even at low concentrations (4, 7, 8, 10). The main drawback is that

such oily compounds are difficult to implement into aqueous systems, particularly without some emulsifying agent dispersing them evenly throughout the system.

Therefore the purpose of this study was to examine emulsified thyme and clove bud oil solutions as rinsing agents for baby spinach contaminated with *E. coli* O157:H7. These EO emulsions, as well as chlorine and water controls, were tested for their ability to lower *E. coli* O157:H7 populations, inhibit their transfer onto uninoculated samples, and determine their overall susceptibility to the presence of organic matter.

Materials and Methods

Bacterial cultures and maintenance. A five-strain *E. coli* O157:H7 cocktail was used, containing the following strains: H1730 (lettuce associated outbreak), F4546 (alfalfa sprout associated outbreak), K3995 (spinach associated outbreak), 932 (human feces) and CDC 658 (cantaloupe associated outbreak). All strains were first made resistant to 40 ppm nalidixic acid (NA; Acros Organics, Geel, Belgium) so as to differentiate them from the background microflora of spinach. All NA resistant strains were screened for their susceptibility to chlorine and essential oils in comparison to their wild type to assure there were no differences.

Media preparation. Sorbitol MacConkey Agar (Oxoid, Basingstoke, UK) was sterilized (121 °C for 15 min) before being cooled to 65 °C and cefixime (Sigma-Aldrich, St. Louis, MO) and potassium tellurite (Chem-Impex International, Wood Dale, IL) were added to concentrations of 0.05 mg/l and 2.5 mg/l, respectively. The final agar (CT-SMAC) was then poured into Petri dishes and used for the enumeration of *E. coli* O157:H7.

Inoculum preparation. Cultures were individually recovered by three consecutive 24 h transfers into tryptic soy broth (Becton, Dickinson and Company, Sparks, MD) with nalidixic acid (TSBN) incubated at 37° C before a 300 µl portion of each strain was individually spread plated onto tryptic soy agar with 40 ppm nalidixic acid (TSAN). These plates were incubated for 24 h at 37° C so that a lawn of bacteria could form on each surface. These lawns were then individually flooded with 5 ml of phosphate buffer pH 7.2 (PBS; Becton, Dickinson and Company, Sparks, MD) with 0.2% Tween 80 (Thermo Fisher Scientific, Fair Lawn, NJ) and mixed in equal portions to make the cocktail. The cocktail was then poured into a sterile high-density polyethylene pan (Thermo Fisher Scientific, Waltham, MA) and mixed with 4 liters of 0.1% peptone (Becton, Dickinson and Company, Sparks, MD).

Sample Inoculation and Preparation. Organic baby spinach was purchased from a local retail outlet. The spinach was then submerged in the inoculum using sterile tongs and slotted spoons and left there for 2 min before being removed and laid out on a sterile tray to dry, inside a biosafety cabinet. The spinach was allowed to dry for 1 h on the tray before being carefully removed and spin-dried in clean plastic salad spinners (Progressive International, Kent, WA) to remove excess liquid. Each load of spinach was spun with ten drawstring pulls to promote uniformity. The spinach was then transferred onto a new sterile tray and allowed to dry 1 h more before being used.

Essential oil emulsion preparation. Stock solutions were made of the two emulsified essential oil washes within 48 h of use. For the first solution, a glycerol bath was heated to ~125° C in a glass beaker. A 10% (v/v) thyme oil (Sigma-Aldrich, St. Louis, MO) in 3M NaOH (Thermo Fisher Scientific, Waltham, MA) was mixed and heated until boiling ~115° C for 10 min. Concurrently a solution of 10% (w/v) gum

arabic (Acros Organics, Geel, Belgium) was centrifuged for 10 min at 4,500 x *g*, and the supernatant was collected for purification. The resulting liquids were combined and sterile de-ionized (DI) water was added so that the final solution was 0.5% (v/v) purified gum arabic and 0.5% (v/v) alkaline-dissolved thyme oil. 3M, 1M and 0.1M citric acid (Sigma-Aldrich, St. Louis, MO) was added to this 1% thyme oil emulsion solution until the pH reached 7.0 (± 0.1). During the course of the experiment, an accumet XL 15 pH/mV/Temperature Meter (Thermo Fisher Scientific, Waltham, MA) was used to measure pH.

For the other solution, the glycerol bath was heated to only $\sim 120^\circ\text{C}$. A solution of 10% (v/v) clove bud oil (CBO; Sigma-Aldrich, St. Louis, MO) in 3M NaOH was heated in the glycerol until it boiled ($\sim 110^\circ\text{C}$) for 10 min. A 2% (w/v) whey protein concentrate solution (w/v) was mixed before being acidified to pH 4.0 (± 0.1). The acidified whey protein solution was then centrifuged for 10 min at 4,500 x *g* and the supernatant was collected. 3M NaOH was then added to the collected supernatant until it reached a pH of 7.0 (± 0.1) which yielded a 2% purified whey protein solution. The two liquids were then mixed with sterile DI water to produce a stock solution that was 0.5% (v/v) purified whey protein and 0.5% (v/v) alkaline-dissolved CBO. This mixture, termed 1% CBO emulsion, was then acidified with 3M, 1M and 0.1M citric acid until the pH was 7.0 (± 0.1).

Organic loading of wash systems. Blended spinach leaves were diluted 1:5 (w/w) in sterile DI water and the subsequent liquid was used, as a 20% stock solution of Organic Load (OL), to test the treatment solutions' susceptibility to the influence of organic compounds.

Preparation of wash systems. Washing solutions of 12 different treatments were prepared: sterile DI water, sterile DI water with 1% OL, 200 ppm chlorine, chlorine originally at 200 ppm with 1% OL, 0.2% CBO emulsion, 0.2% CBO emulsion with 1% OL, 0.5% CBO emulsion, 0.5% CBO emulsion with 1% OL, 0.2% thyme oil emulsion, 0.2% thyme oil emulsion with 1% OL, 0.5% thyme oil emulsion, and 0.5% thyme oil emulsion with 1% OL. Free residual chlorine was measured for the chlorinated solutions with a Free Chlorine & Chlorine Ultra High Range ISM (Hanna Instruments, Roonsocket, RI) before being used. The chlorinated liquids with organic load were checked before and immediately after the organic loading to see the quantifiable effect on free chlorine. Two samples of 500 ml of each of the 12 wash treatment liquids were poured into sterile glass beakers before each experimental replication.

Simulated post-harvest washing of spinach and recovery of *E. coli* O157:H7. Inoculated spinach was weighed out to 25 g (± 1 g) before being submerged in the treatment wash liquids, or else rinsed immediately in the case of control samples. After 2 min in the wash treatments, samples were transferred to a stomacher bag and diluted 1:5 (w/w) in phosphate buffer solution with 0.2% Tween 80 via a Baby Gravimat gravimetric diluter (Microbiology International, Frederick, MD). These bags were then shaken for 15-20 s by hand. The rinsate was then enumerated for *E. coli* O157:H7 on CT-SMAC by first diluting the samples in buffered peptone water (BPW; Becton, Dickinson and Company, Sparks, MD) before spiral plating (WASP 2 Spiral Plater, Microbiology International, Frederick, MD) the samples in duplicate.

Three following consecutive uninoculated 25 g spinach samples were then washed in each treatment liquid after the inoculated samples to examine *E. coli* O157:H7 transfer. The rinsate from each following uninoculated sample was also diluted in BPW

and enumerated using the WASP spiral plater. 10 ml were filtered through a 0.45 µm membrane filter with a Millipore filtering system (EMD Millipore Corporation, Billerica, MA). Similarly, the treatment solutions were enumerated for *E. coli* O157:H7 and filtered as described above after being used.

Data analysis. Each treatment was replicated three times each with two samples and duplicate subsampling (n=12). Statistical analyses were conducted with a significance level set to $P \leq 0.05$ using SAS 9.4's (SAS Institute Inc., Cary, NC) generalized linear mixed model procedure (Proc GLIMMIX). Analysis of variance was also run to determine differences in survival of *E. coli* O157:H7 between treatments on inoculated produce, uninoculated produce and rinse systems.

Results

Efficacy of emulsified EOs on inoculated spinach samples. Wash treatment type was found to significantly affect the recovery of *E. coli* O157:H7 from inoculated spinach samples (Figure 3.1; $P < 0.01$). Initial populations of *E. coli* averaged 7.44 log CFU/g, which was not significantly different from samples rinsed in water with and without 1% OL (with averages of 6.90 log CFU/g and 6.96 log CFU/g, respectively; Figure 3.1). Chlorine at 200 ppm was the most effective at reducing *E. coli* O157:H7, resulting in an average of 2.34 log CFU/g. Chlorine in the presence of 1% OL (which lowered the free chlorine in the system from 200 ppm to an average of 179 ppm) yielded 6.06 log CFU/g of *E. coli*, which was not significantly different from treatment washes of 0.2% thyme oil emulsion (6.46 log CFU/g), 0.5% CBO emulsion with 1% OL (6.22 log CFU/g), or 0.5% CBO emulsion (5.96 log CFU/g). The 0.5% thyme oil emulsion treatment lowered *E. coli* O157:H7 levels to an average of 3.96 log CFU/g, which was

significantly different from all other treatments, except for 0.5% thyme oil emulsion with 1% OL (at 4.44 log CFU/g). As shown in Figure 3.1, the efficacy of these treatments to reduce *E. coli* O157:H7 populations from inoculated baby spinach samples were as follows: 200 ppm chlorine > 0.5% thyme oil emulsion with and without 1% OL > 0.5% CBO emulsion with and without 1% OL = 200 ppm chlorine with 1% OL > DI water = No treatment control.

Prevention of *E. coli* O157:H7 cross-contamination. In regards to bacterial transfer, wash treatment significantly affected the recovery of *E. coli* onto uninoculated spinach samples (Figure 3.2; $P < 0.01$). Levels of *E. coli* were recovered from spinach samples rinsed in contaminated DI water at an average of 7.19 log CFU/g, which was not significantly different from samples rinsed in cross-contaminated water containing 1% OL (at 7.10 log CFU/g), 0.2% CBO emulsion (at 7.06 log CFU/g) or 0.2% CBO emulsion with 1% OL (at 7.04 log CFU/g). Uninoculated spinach samples exposed to contaminated chlorine at 200 ppm yielded an average of 1.79 log CFU/g, which was the least amount of cross-contamination observed ($P < 0.05$). Chlorinated water with 1% OL, however, showed that an average of 6.70 log CFU/g *E. coli* was transferred onto the clean samples, which was statistically the same as 0.2% CBO emulsion with 1% OL (at 7.04 log CFU/g), 0.2% thyme oil emulsion (at 6.83 log CFU/g), 0.5% CBO emulsion with 1% OL (at 6.64 log CFU/g), 0.5% CBO emulsion (at 6.59 log CFU/g) and 0.2% thyme oil emulsion with 1% OL (at 6.52 log CFU/g). 0.5% thyme oil emulsion transferred an average of 5.96 log CFU/g onto uninoculated baby spinach, which was statistically not as effective as its organically loaded counterpart (0.5% thyme oil emulsion with 1% OL) which reduced the levels of *E. coli* to 5.44 log CFU/g, although in reality these values may not dramatically impact food safety risk.

Finally, when evaluating the recovery of *E. coli* in the wash liquids, the effect of treatment was found to significantly influence the outcome (Figure 3.3; $P < 0.01$). *E. coli* O157:H7 was recovered from the de-ionized water at an average of 6.33 log CFU/ml, which was the same as the liquid samples containing only DI water with 1% OL (at 6.42 log CFU/ml). Recovery of *E. coli* from the chlorinated wash with 1% OL resulted in an average of 0.19 log CFU/ml, which was significantly different from every other treatment at all levels (Figure 3.3). The rest of the treatments were all statistically the same, with the 200 ppm chlorine treatment averaging -0.50 log CFU/ml, 0.5% CBO emulsion at -1.05 log CFU/ml, 0.5% CBO emulsion with 1% OL at -1.05 log CFU/ml, 0.5% thyme oil emulsion at -0.65 log CFU/ml, and 0.5% thyme oil emulsion with 1% OL at -1.05 log CFU/ml, all of which were very near or at the detection limit of -1.05 log CFU/ml.

Discussion

Studies indicate that in regards to the inhibition and antibacterial activity on *E. coli*, thyme oil, or its primary component thymol, is more effective than clove bud oil, or its primary component eugenol, whether in terms of MIC (minimum inhibitory concentration), MBC (minimum bactericidal concentration), MTC (maximum tolerated concentrations) or BA50 (concentration resulting in 50% decrease in population) (5, 8, 18-20, 24). However, there are a few contradictory studies that show lower MIC's for CBO than for thyme oil against *E. coli* O157:H7 (10, 27). Results from the current study indicated that in the form of microemulsions and on the surface of spinach leaves, thyme oil is more effective than clove bud oil, agreeing with the vast majority of in vivo studies.

Comparable to research by Rodgers et al. (22) which indicated that chlorine at 200 ppm was able to reduce levels of *E. coli* O157:H7 by >5.0 log CFU/g from whole apple surfaces and 4.6 log CFU/g shredded lettuce after a five min rinse, the current study resulted in a 5.1 log CFU/g *E. coli* O157:H7 reduction after a two min rinse. Other studies have found chlorine less effective against *E. coli* O157:H7 in comparison to the results of the current study. Velazquez et al. (29), for example, recorded that a one min rinse of 200 ppm chlorine resulted in only a 2.65 log CFU/tomato decrease on tomato samples and 1.4 log CFU/leaf reduction on lettuce surfaces. Beuchat et al. (2) reported a 2.63 log CFU/cm² reduction of *E. coli* O157:H7 from lettuce leaves, after a one min rinse of 200 ppm chlorine. These differences in efficacy when compared to results from the current study can be attributed, at least partially, to the lower contact time of the chlorinated wash and possibly differences in susceptibility of strains used.

Research done on alternative disinfectants denoted similar effectiveness. Kenney et al. (11) compared five different commercial alkaline, acidic, and chlorinated apple cleaners, the most effective reducing populations of *E. coli* O157:H7 by 2.27 log CFU/apple. Rodgers et al. (22) recorded that ozone, at 3 ppm, was able to decrease *E. coli* O157:H7 by >5 log CFU/g from both apples and shredded lettuce after a five min wash. Similar to the present study, Nou et al. (17) indicated that chlorine, even at 20 ppm for 30 s contact time, was enough to inhibit cross-contamination of *E. coli* O157:H7 through the wash water, at least until 0.5% (v/v) organic loading was added.

The resilience of the essential oil solutions to organic matter cannot be attributed to their emulsified forms, as both thyme oil and clove bud oil have been shown to be relatively stable compounds and virtually unaffected by environmental factors, such as light, temperature, and time, tested during storage (9, 28). Plant-derived oils have a

general reputation for being reasonably stable, likely due to their radical-scavenging and overall antioxidant capabilities (16, 23). Results from a previous study by Zhang et al. (30) indicated that emulsified EOs showed enhanced wetting capabilities. These emulsified EO compounds were chosen based off of previous work by Luo et al. (12), which tested and compared the stability and efficacy of different combinations of emulsifying agents including gum arabic and whey protein with clove bud oil.

Three different studies by Moore-Neibel et al. (13-15) examined the efficacy of lemongrass oil, olive extract, apple extract, hibiscus concentrate, hydrogen peroxide and oregano oil against *Salmonella* on the surfaces of organic romaine and iceberg lettuce and organic baby and mature spinach. These results showed that these essential oil solutions were significantly effective, while both concentration and duration dependent. Far and away, the most effective was oregano oil, which reduced *Salmonella* populations by 4 log CFU/g after a one min rinse in 0.5% oregano oil (13-15).

While the chlorine and the essential oil emulsion treatments of the present study significantly lowered populations of *E. coli* O157:H7 from baby spinach, it should be noted that significantly more of the target pathogen was able to survive and transfer onto uninoculated samples, when compared to *Salmonella* on tomato surfaces from Chapter 2, as evidenced by Figure 3.2 and Figure 2.2. The large difference in surface area of a 25 g sample of baby spinach versus two cherry tomatoes is most likely solely responsible for this difference. The results from the inoculated samples, as shown in Figures 2.1 and 3.1 show that the difference is not because of a resistance to the treatments by *E. coli* O157:H7. Solutions that were able to reduce populations by up to 3 log CFU/g *E. coli* O157:H7 from spinach only lowered *Salmonella* from tomatoes by ~1.5 log CFU/g, at most. These results indicate that even in the presence of the best

disinfectants, a high enough load of pathogenic bacteria can cross-contaminate leafy green surfaces in a post-harvest wash, reinforcing the importance of good agricultural practices pre-harvest.

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Appendix III

Figures

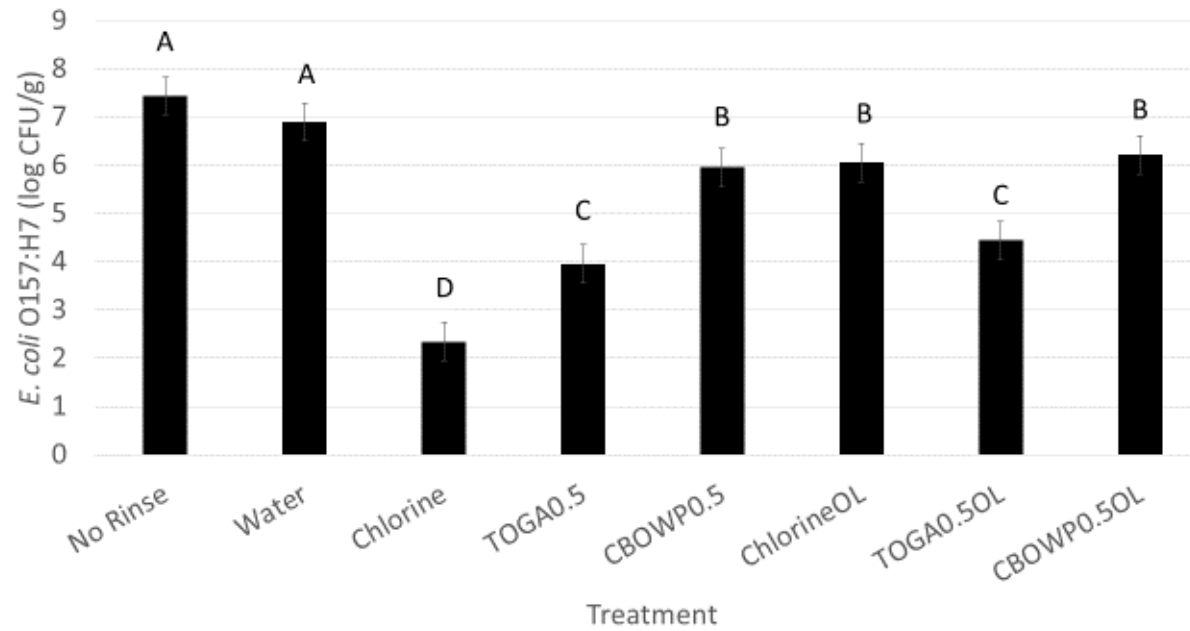


FIGURE 3.1. *E. coli* O157:H7 recovered from inoculated baby spinach samples after treatment rinse (n=12).

Treatments with different letters signify significant differences ($P \leq 0.05$).

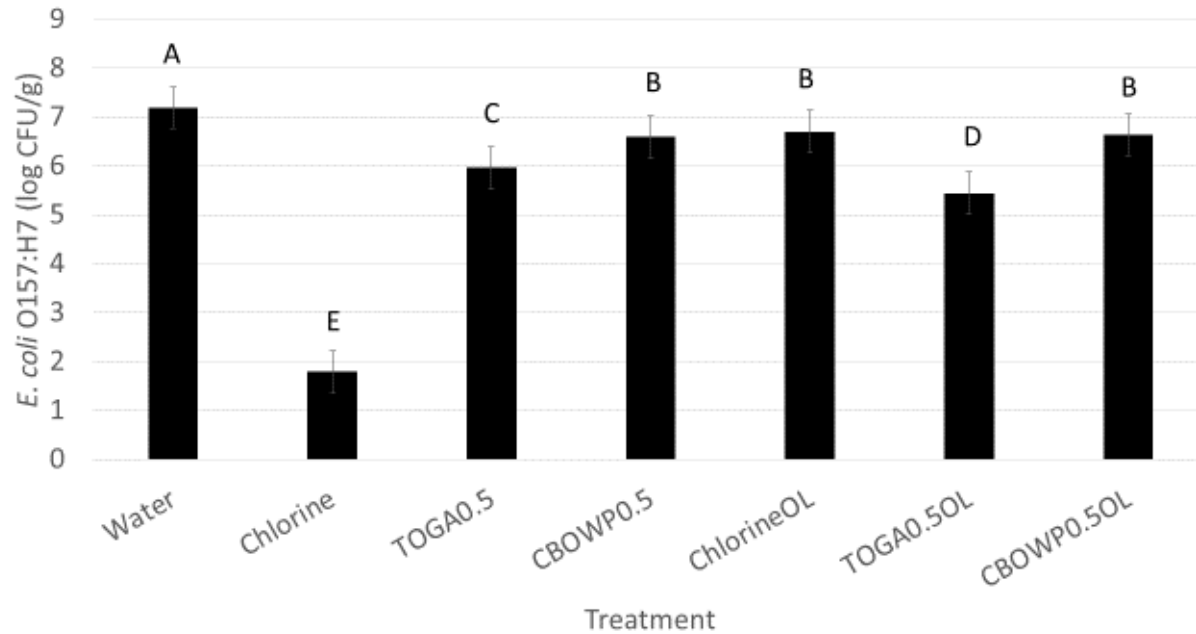


FIGURE 3.2. *E. coli* O157:H7 recovered from uninoculated baby spinach samples after shared rinse with contaminated produce (n=12). Treatments with different letters signify significant differences ($P \leq 0.05$).

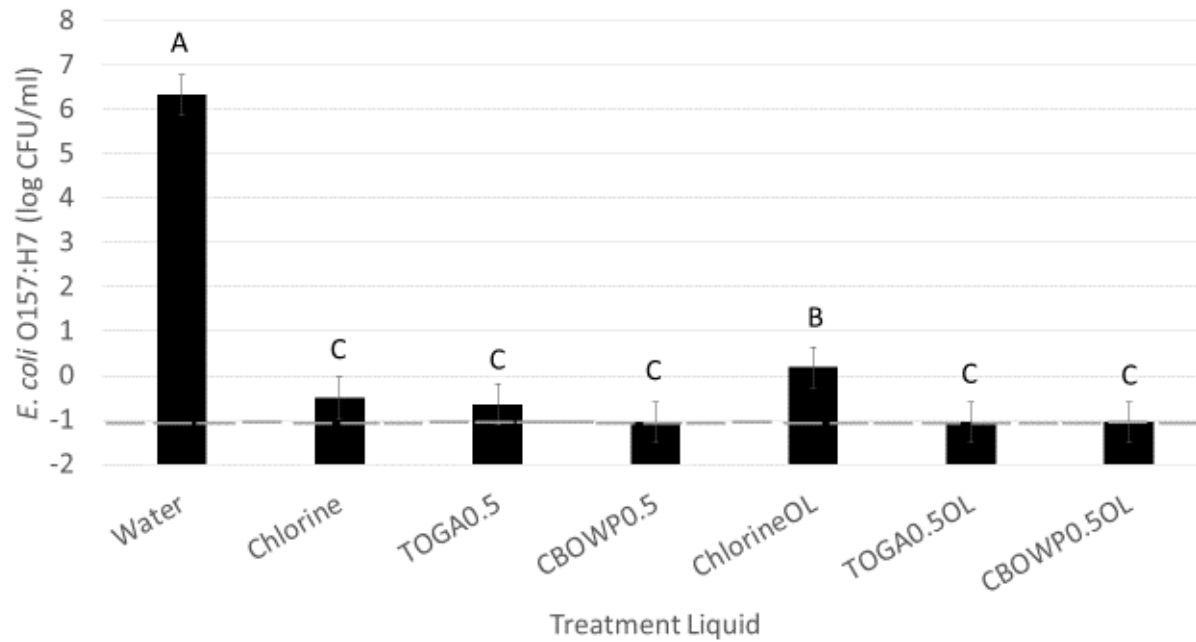


FIGURE 3.3. *E. coli* O157:H7 recovered from rinse liquid after washing inoculated and uninoculated baby spinach (n=12). Treatments with different letters signify significant differences ($P \leq 0.05$). Dotted line represents limit of detection.

Conclusions

While chlorine was very effective in a clean system, the emulsified thyme oil solution proved to be the most reliable throughout the study. Emulsified EOs proved relatively resistant to organic loading and significantly effective at reducing levels of *E. coli* O157:H7 from baby spinach surfaces, but chlorine in a clean system was the most effective. This study provides evidence for continued research on emulsified essential oils as produce sanitizers, including sensory evaluations, physiological concerns to the produce and overall cost.

Appendix IV

Gum arabic's effectiveness in enhancing the activity of chlorine against *Salmonella* on organic cherry tomatoes

Materials and Methods

Bacterial cultures and maintenance. A *Salmonella* cocktail of five strains was used, containing the following serovars: Agona (alfalfa sprout associated outbreak), Montevideo (tomato associated outbreak), Gaminara (orange juice associated outbreak), Michigan (cantaloupe associated outbreak) and Saintpaul (pepper associated outbreak). Serovars were first made resistant to 40 ppm nalidixic acid (Acros Organics, Geel, Belgium) so that they could be identified among the background microbes on tomatoes. Nalidixic acid resistant serovars were tested for their susceptibility to chlorine to ensure nalidixic acid would not weaken the strains in comparison to wild type serovars. The cultures were kept in 15% glycerol stocks at -80 °C for long term storage.

Media preparation. Tryptic soy agar (tryptic soy broth and granulated agar; Becton, Dickinson and Company, Sparks, MD) with added sodium thiosulfate, ammonium ferric citrate (Fisher Scientific, Fair Lawn, NJ) was mixed and sterilized (121 °C for 15 min) before sterile nalidixic acid (Acros Organics, Geel, Belgium) was added, and the molten agar was poured into Petri dishes. The resulting agar, TSANSA, was used to enumerate *Salmonella*.

Inoculum preparation. After three consecutive 24 h transfers into tryptic soy broth with nalidixic acid at 37 °C, a 300 µl aliquot of each of the *Salmonella* cultures was spread onto tryptic soy agar plates with nalidixic acid and incubated at 37 °C for another 24 h to form a lawn of bacteria. Plates were then flooded with 5 ml phosphate buffer pH 7.2 (Becton, Dickinson and Company, Sparks, MD) with 0.2% Tween 80 (Fisher Scientific, Fair Lawn, NJ) which resulted in a liquid cocktail culture of the five *Salmonella* serovars.

Sample inoculation and preparation. Certified Organic cherry tomatoes (del Cabo, Vernon, California, USA) imported from Baja California, Mexico, were purchased from a local grocery store, and were spot inoculated with 100 µl of the *Salmonella* inoculum. Tomatoes were dried inside a biosafety cabinet for 2 – 3 h.

Preparation of wash systems. Gum arabic (Acros Organics, Geel, Belgium) at 10% was purified via centrifugation at 4,500 x g for 10 min and the supernatant collected. Wash liquids containing one of four treatments were prepared: sterile deionized water, 200 ppm chlorine, 0.1% purified gum arabic with 200 ppm chlorine, or 1% purified gum arabic with 200 ppm chlorine. All wash waters were tested for free residual chlorine using a Free Chlorine & Chlorine Ultra High Range ISM (Hanna instruments, Roonsocket, RI) after being mixed with the gum and before being used. 100 ml of each of these wash liquids was dispensed into multiple sterile glass beakers.

Simulated post-harvest washing of tomatoes and recovery of *Salmonella*. Inoculated tomato samples (two tomatoes each, usually ~20 g) were dipped into the prepared wash liquids and left for 2 min. Inoculated controls were not dipped in any wash liquid prior to recovery. After treatment, tomatoes were placed into stomacher bags and diluted 1:5 (w/w) with phosphate buffer plus Tween 80 using a Baby Gravimat gravimetric diluter (Microbiology International, Frederick, MD). Stomacher bags were oscillated at 3000 rpm in a Pulsifier (Microbiology International, Frederick, MD) for 15 s. The rinsate was diluted and spiral plated (WASP 2 Spiral Plater, Microbiology International, Frederick, MD) in duplicate onto TSANSA to enumerate *Salmonella*.

Four consecutive uninoculated tomato samples were washed with each of the same wash liquids to test for *Salmonella* transfer. These samples were rinsed, diluted,

and plated like the inoculated samples. Wash liquids were also enumerated for *Salmonella* after all tomato samples were rinsed. Free residual chlorine was then measured with the chlorine meter in those liquid washes that initially contained chlorine to find the amount that was used.

Data analysis. Each treatment was replicated four times with two samples each and duplicate subsampling (n=16). Statistical analyses was conducted on the inoculated samples using the generalized linear mixed model procedure (Proc GLIMMIX) of SAS 9.3 (SAS Institute Inc.; Cary, NC) with significance levels set at $P < 0.05$. Analysis of variance was run to test for differences of populations of *Salmonella* between treatments.

Results

Efficacy of chlorinated solutions on inoculated tomatoes. The wash liquid treatments applied to the inoculated tomatoes were found to be statistically significant ($P < 0.05$). The treatment means are illustrated in Figure 4.1. *Salmonella* was recovered from inoculated tomatoes that were not dipped into any wash liquid at 7.19 log CFU/g of tomato sample. Those that were dipped into a wash liquid containing only deionized water showed *Salmonella* levels of 6.77 log CFU/g. Inoculated tomatoes that were dipped into wash liquid that contained 200 ppm chlorine and 1% gum arabic showed *Salmonella* levels of 6.90 log CFU/g. The means of these treatments were found not to be significantly different from each other using Fisher's LSD ($P > 0.05$). Inoculated tomatoes that were treated with 200 ppm chlorine and 0.1% gum arabic showed levels of *Salmonella* of 5.66 log CFU/g, which was significantly less than the

previous treatment means ($P < 0.05$). However, the wash liquid containing only 200 ppm chlorine proved to be the most effective of all ($P < 0.05$), as the inoculated tomatoes that were rinsed with it showed *Salmonella* levels of 4.96 log CFU/g. The efficacy of these treatments to reduce *Salmonella* populations from inoculated organic cherry tomatoes were as follows: 200 ppm chlorine > Chlorine with 0.1% gum arabic > Chlorine with 1% gum arabic = Water control = No treatment control.

Prevention of *Salmonella* cross-contamination. Samples that followed the washing treatments of contaminated tomatoes were also analyzed to test for the amount of *Salmonella* transfer onto clean tomatoes. Their means are illustrated in Figure 4.2. Water was statistically the least effective of these according to Fisher's LSD with an average of 4.57 log CFU/g *Salmonella* transferred. Chlorine with 1% gum arabic was statistically the next least effective, transferring *Salmonella* at an average of 1.53 log CFU/g. The other two treatment washes were not significantly different in effectiveness at preventing the transfer of *Salmonella*, with numbers from 1.08 log CFU/g (chlorine + 0.1% gum arabic) to 1.24 log CFU/g (chlorine) ($P > 0.05$).

In regards to surviving *Salmonella* in the rinse liquid after rinsing tomato samples, only the water control treatment allowed survival significantly above the detection limit, with an average of 6.90 log CFU/ml *Salmonella* recovered from the water. As shown in Figure 4.3, all treatments containing chlorine were able to inhibit *Salmonella* survival at least as high as the detection limit of 0.95 log CFU/ml. Levels of free residual chlorine, measured after all tomato samples had gone through the system, were as follows: 200 ppm free residual chlorine in the chlorine treatment, 111 ppm in the chlorine treatment with 0.1% gum arabic and 59 ppm in the chlorine treatment with 1% gum arabic.

The results from this study indicate that gum arabic, while known to have some surfactant abilities, does not enhance the antimicrobial activity of chlorine. This investigation shows that its presence lowers levels of free residual chlorine and hinders chlorine's ability to eliminate bacteria from the surface of produce. More research is needed to test other surfactants' abilities to improve the efficacy of chlorine, but gum arabic is not a promising candidate based off of this data.

Figures

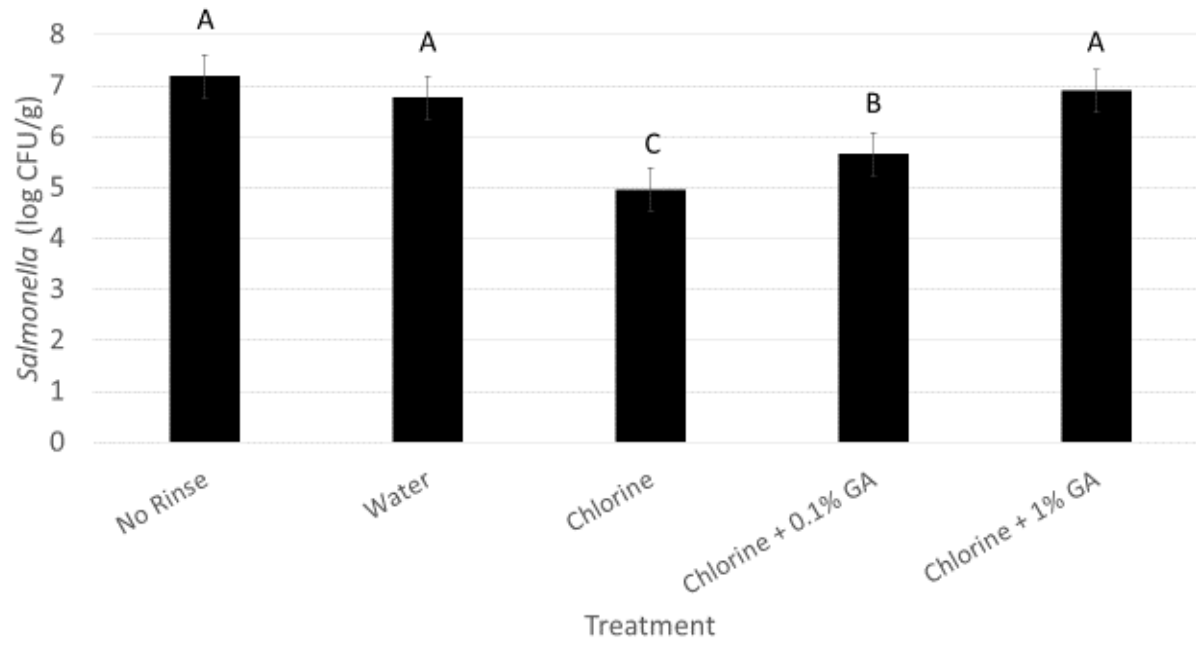


Figure 4.1. *Salmonella* recovered from inoculated cherry tomatoes after treatment rinse (n=16). Treatments with different letters signify significant differences ($P \leq 0.05$).

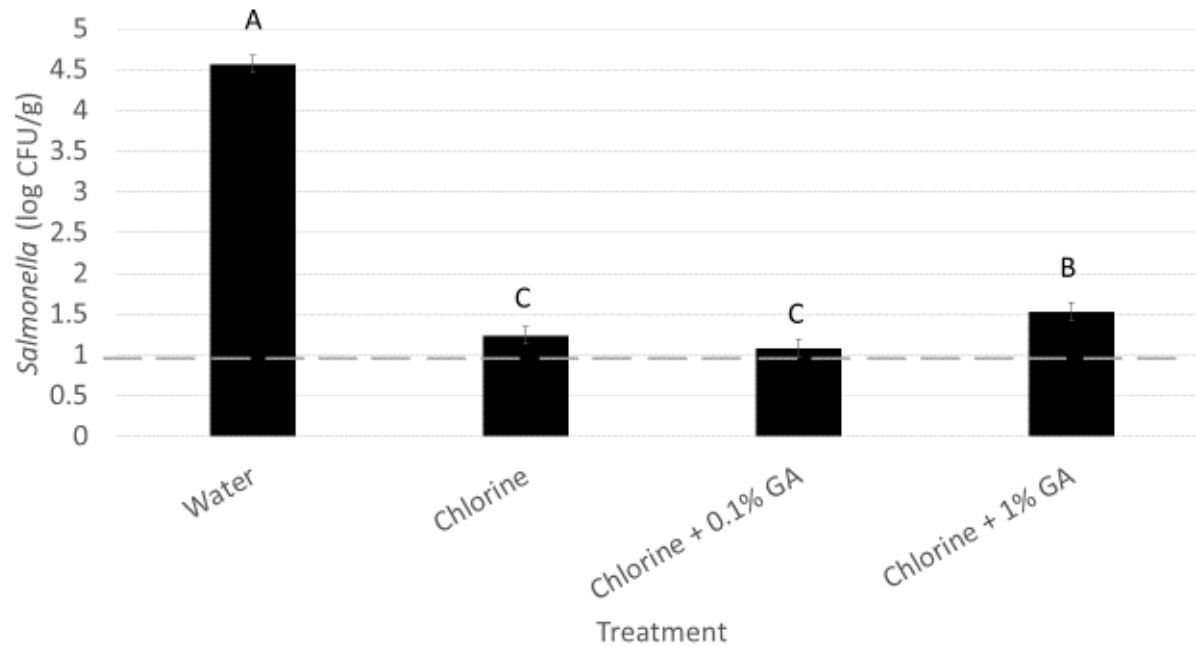


Figure 4.2. *Salmonella* recovered from uninoculated cherry tomatoes after shared rinse with contaminated produce (n=16). Treatments with different letters signify significant differences ($P \leq 0.05$). Dotted line represents limit of detection.

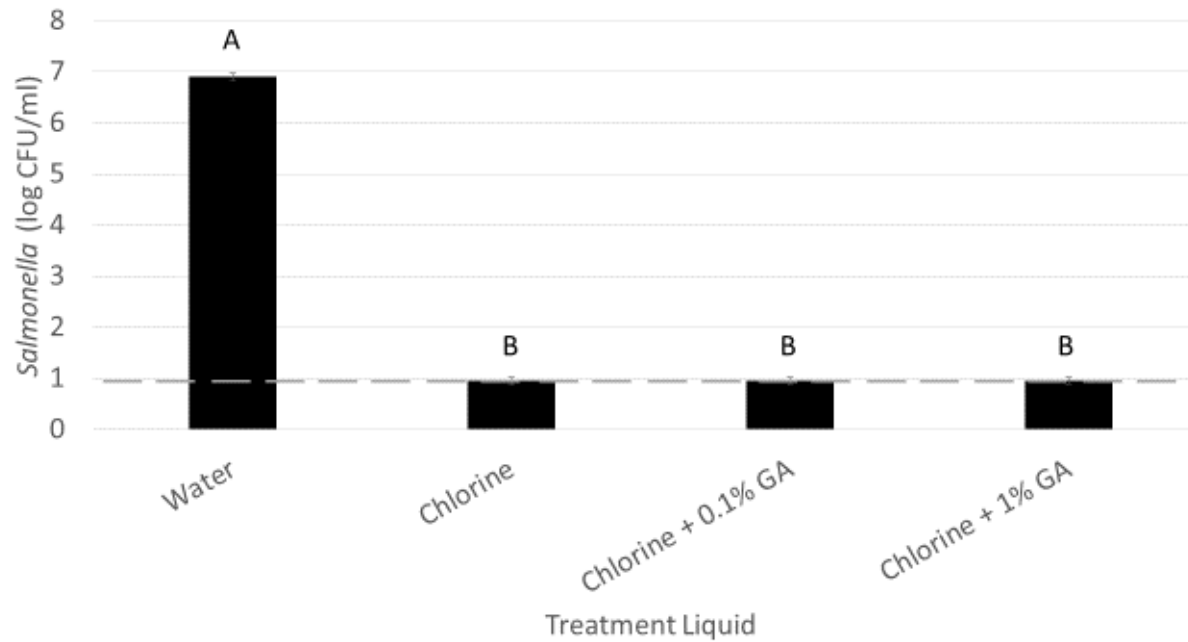


Figure 4.3. *Salmonella* recovered from rinse liquid after washing inoculated and uninoculated cherry tomatoes (n=16). Treatments with different letters signify significant differences ($P \leq 0.05$). Dotted line represents limit of detection.

Vita

Marion Lewis Harness III (Trey) was born in Memphis, Tennessee on October 22, 1989 to parents Marion and Cathy Harness. Trey grew up in Memphis and graduated from White Station High School in 2008. He then moved to Knoxville, Tennessee to attend the University of Tennessee, where he received his Bachelor of Science degree in Food Science and Technology, with a concentration in Pre-Pharmacy, in 2012. Trey also worked part-time for the last year of his undergraduate degree as a Pharmacy Technician at Walgreens in Seymour, Tennessee, while simultaneously volunteering in a Food Chemistry laboratory as well as a Food Microbiology laboratory. Trey entered the University of Tennessee Food Science and Technology Graduate School to study Food Microbiology in 2012 to earn his Master of Science degree.