Evaluation of a natural antimicrobial-based sanitizer as an alternative to chlorine for reducing foodborne pathogenic bacteria on organic produce

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I am submitting herewith a thesis written by Ellen Rebecca Simmons entitled "Evaluation of a natural antimicrobial-based sanitizer as an alternative to chlorine for reducing foodborne pathogenic bacteria on organic produce." 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.

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Evaluation of a natural antimicrobial-based sanitizer as an alternative to chlorine for reducing foodborne pathogenic bacteria on organic produce

A Thesis Presented for the
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Ellen Rebecca Simmons
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Abstract

There is a need for the development of a “natural” sanitizing agent to reduce or eliminate foodborne pathogens that meets USDA organic standards, as an alternative to chlorine. The sanitizer needs to eliminate foodborne pathogens and prevent cross contamination in rinse liquids in the presence of organic matter. In this study, the focus was to evaluate a natural antimicrobial-based sanitizing (NABS) agent in rinse liquids to determine if it was capable of eliminating foodborne pathogens on organic produce through cross-contamination studies. Five-serovar/strain cocktails of pathogenic bacteria were combined to form an inoculum cocktail, which was used to inoculate the produce. The produce was introduced into the NABS treatments (with or without organic load) or 200 ppm NaOCl and enumerated for initial reduction. To determine if cross-contamination occurred in the rinse liquids, un-inoculated produce was introduced into the shared rinse liquid container. The greatest initial reductions occurred when tomatoes inoculated with *E. coli* were introduced into the NaOCl rinse liquid (> 3.0 log CFU/g) and when the spinach samples were introduced into the 0.75% NABS (1.3 log CFU/g). Overall, cross-contamination was prevented, when compared to the water controls. Enumerating for potential survivors of pathogenic bacteria in the rinse liquids validated the prevention of cross-contamination. The addition of the organic load to the rinse liquids did not affect the efficacy of NABS, except for the case of the cantaloupes. In conclusion, NABS did not demonstrate practical initial reductions on the inoculated produce when compared to the controls, however, NABS was able to prevent cross-contamination in the rinse liquid.
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Introduction

The consumption of fresh fruits and vegetables is on the rise in the U.S. and the consumption of organic produce is growing as well. Organic produce is the top selling category in the organic industry since the organic market started retailing products almost 30 years ago (52). Also, it accounted for 43% of the U.S. organic food sales in 2012, beating out frozen foods, beverages, breads, and snack foods (15). Since, most produce does not require a kill-step for foodborne pathogens during processing and organic foods have strict United State Department of Agriculture (USDA) regulations as to what chemical sanitizers can be used for these fruits and vegetables, microbial safety can be a concern. Organic produce can become contaminated through various routes during processing including irrigation water contaminated from run-off, handlers not following Good Manufacturing Practices, soil, fertilizers, or inadequately composted manure. Also, produce that was originally free of microbial contamination can become infected in the wash tanks through cross-contamination of reused wash liquids (6, 7).

The current method for sanitizing produce is using a hypochlorite solution at concentrations up to 200 ppm (30). This method has been determined to be effective but there are major limitations to the use of chlorine. In the presence of organic matter, free chlorine in the system reacts with the organic matter and its antimicrobial activity is reduced (30). Also, at the time of discharge, after treating produce during processing, chlorine must be below 4 ppm to abide by regulations set by the Environmental Protection Agency in the Safe Drinking Water Act (16). Also, pH has a dramatic effect
A majority of organic acids are GRAS and have been shown to have high efficacy in eliminating pathogens on the surface of produce. Organic acids can be used alone or in combinations with other chemical sanitizers, other organic acids, or surfactants. In this project, a commercially available natural antimicrobial-based sanitizing agent composed of organic acids and phenolic compounds was used at various concentrations to eliminate foodborne pathogens on the surface of USDA-certified organic produce. The specific pathogens used were *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* cocktails. These cocktails were inoculated onto organic cherry tomatoes, Romaine lettuce, baby spinach, and cantaloupe samples.
Chapter I

Literature Review
Organic produce and its influence in the United States

Maintaining a diet that involves a high intake of fruits and vegetables can be extremely beneficial to human health by minimizing the risk of cardiovascular disease, hypertension, diabetes and many types of cancer (14, 35, 39). USDA and the Department of Health and Human Services, recommend that Americans increase their consumption of fruits and vegetables and decrease their consumption of sodium, calories, refined grains, solid fats and dietary cholesterol as a healthy regimen. The types of fruits and vegetables that are recommended are usually red and orange colored, dark leafy greens, and beans (49). In 2013, the World Health Organization (WHO) reported that approximately 1.7 million deaths per year are linked to low consumption of fruits and vegetables making the intake of these foods essential to a healthy diet (35, 39).

Reports note that only 6-8% of Americans actually consume their recommended daily target for fruits and vegetables and that on average they are only consuming 1.8 cups of fruits and vegetables per day (19, 39). Governmental agencies, like the USDA, are trying to encourage Americans to make more conscious decisions when sitting down to eat. Despite the low consumption rate within the last 10 years, Americans do tend to purchase fresh fruits and vegetables over canned, frozen, or dried produce and within the next 5 years fresh fruit and vegetable consumption is expected to grow by 9% (20). As the popularity of the consumption of fresh produce increases the popularity of eating organic produce also rises.
The history of organic farming goes back to the early 1900s. While directing agricultural research centers in India from 1905-1931, Sir Albert Howard from England, developed the concept of organic farming, recycling waste materials, and soil fertility. His ideas and concepts coined into the term “organic”. Passage of the Federal Organic Foods Production Act occurred in 1990 and official labeling as “USDA certified organic” started in 2002 (24). During the 1990s, organic farming became the fastest growing sector in the U.S. and European agriculture and continues to grow each year (59). Due to the attention organic farming and labeling has received over the past 20 years, consumer interest in purchasing organic produce rather than conventional produce has increased.

There are five different types of eating trends identified in America (20). For example, “short cut fuelers,” who are people that make food choices driven by convenience and “family pleasers,” who are usually women who make food choices based on children in the household. There are also “natural health embracers,” who are not driven by convenience and prefer organic foods, and natural and/or herbal remedies. The people within this trend tend to consume above average amounts of organic fruits and vegetables (20). Conventional fruits and vegetables may be genetically modified (GMOs) and are generally produced using synthetic pesticides or herbicides. This tends to give them a negative perception to some. Some consumers believe that organic produce is healthier and safer than conventional produce (33). This makes the organic produce industry more desirable to not only “natural health embracers” but to a growing population worldwide.
In a study conducted by Williams et al. (57), a majority of the 700 consumers surveyed believed that organically grown produce posed fewer risks to consumers and farmers than conventionally grown produce. Over 90% of the responses estimated lower pesticide-related mortality risks and 45% estimated lower microbial pathogen risks associated with organic produce. These are just some of the perceived risks with conventional foods that are turning consumers, not only natural health embracers, to prefer organic foods.

There are many differences between conventional and organic food production but the greatest difference is that many synthetic compounds are not allowed to come in contact with organic foods unless they are on the National List of Allowed and Prohibited Substances. U.S. regulations from the National Organic Program (NOP) require that organic foods are grown without the use of synthetic pesticides, growth hormones, antibiotics, modern genetic engineered techniques (genetically modified crops), chemical fertilizers, or sewage sludge. Instead of synthetic materials, organic farmers use animal and crop wastes, botanical or biological pest controls, and a few allowed synthetic materials that can break down quickly in the presence of oxygen and sunlight (58). These regulations can pose huge obstacles for organic farmers.

**Foodborne illness and the pathogens responsible**

In 2011, it was reported by the CDC and determined using epidemiological studies that each year an estimated 31 microbial pathogens in the U.S. caused 9.4 million episodes of foodborne illness, 55,961 hospitalizations, and 1,351 deaths (43). Of
the 9.4 million reported foodborne illnesses, 5.5 million were caused by norovirus and 1 million were caused by non-typhoidal *Salmonella enterica* spp. Shiga-toxin producing *E. coli* (STEC), while not found a leading cause of foodborne illness, does cause approximately 63,153 foodborne illnesses annually. Of the 1,351 annual deaths from foodborne outbreaks, the greatest number were attributed to *Salmonella* spp. (28%) and *Listeria monocytogenes* (19%) (43).

*Salmonella enterica* is a member of the Enterobacteriaceae family, it is a Gram-negative bacteria that is facultative anaerobic, it is a motile, and non-sporeforming rod. *Salmonella enterica* can usually be isolated from warm-blooded animals and ingesting 1,000 or more *Salmonella* bacilli results in human illness (6, 8, 42). Achlorhydric individuals, those who take antacids, children under 5 years old, 20-30 year olds, and people over 70 years old are more likely to become infected with a smaller amount of the inocua than 1,000 bacilli. Salmonellosis, caused by *Salmonella enterica* is gastroenteritis with symptoms of sudden nausea, vomiting, abdominal cramps, diarrhea, and fever. Gastroenteritis symptoms begin within 48 hours after ingestion of the bacilli and diarrhea, being the most predominant symptom, persists for up to 4 days (42). Modes of transmission of *Salmonella* include consumption of contaminated foods and water, contact with infected fecal matter, animals, or humans.

Shiga-toxin producing *E. coli* O157:H7, or STEC, is a Gram-negative, rod-shaped, facultative anaerobic bacterium, which produces Shiga toxins VT1/Stx2. The infectious dose for STEC is low with the ingestion of approximately 10 organisms can cause illness in a human (3). When a human becomes infected with STEC they may
experience abdominal cramps and pain, diarrhea, low fever, and potentially hemorrhagic colitis. Infection may lead to the sequela called hemolytic uremic syndrome (HUS), which affects the kidneys. Transmission of STEC occurs by ingesting contaminated food, which was undercooked or unpasteurized, fecal-oral transmission, person-to-person, or animal-to-person transmission (3, 6).

Listeria monocytogenes is a facultative anaerobic, Gram-positive, rod-shaped bacterium. *Listeria monocytogenes* causes the illness listeriosis in humans, which may affect the fetuses of pregnant women or persons with weakened immune systems such as those with leukemia, Hodgkin’s disease, or diabetes mellitus (29). The symptoms of listeriosis in persons with fully functioning immune systems may include fever, diarrhea, and vomiting. In pregnant women, it may cause spontaneous abortion (Listeric abortion). In the elderly and immune compromised it may cause encephalitis or TTP. *Listeria monocytogenes* has a tropism for the central nervous system, which includes the brain parenchyma causing encephalitis and the brain stem causing meningitis (41). As with the other bacteria, the modes of transmission are due to the consumption of contaminated foods, but it can also be transferred from mother to child during pregnancy, and when a human comes in contact with an infected animal (29).

**Produce involved in multistate foodborne pathogenic outbreaks**

Since fruits and vegetables are usually eaten raw and lack a kill step for foodborne pathogens during processing, contamination during growing, harvesting or handling can be of great concern as to food safety. Products like leafy greens can be
packaged as ready-to-eat (RTE), which increases the risk of a potential foodborne illness outbreak because once the produce is contaminated there are no points during processing that will abate the contamination (31). Foodborne outbreaks associated with a known fresh produce vehicle increased from less than 1.0% in the 1970s to 6% in the 1990s (44). After the 1990s, the number of produce outbreaks continued to grow up to 2015. The Centers of Disease Control and Prevention (CDC) reported that between 1998 and 2008, 46% of all foodborne illnesses were associated with fresh produce. Table 1.1 shows foodborne outbreaks associated with selected produce during the period 2004 to 2013. Within the fresh produce category, the type of produce that was associated with a large number of foodborne outbreaks was leafy vegetables, like lettuce and spinach (36). The increased number of reported outbreaks is related to several different trends, including the quality of the water that comes in contact with the produce during harvesting and the desire for fresh produce year round. In the winter, fresh produce must be transported longer distances, which can lead to contamination via multiple routes.

Since 2004, there have been three major foodborne illness outbreaks associated with tomatoes contaminated by *Salmonella enterica* serotypes. Within five years, more than 2,000 people were infected with Salmonellosis due to the consumption of contaminated tomatoes (5). An outbreak in 2008 was particularly detrimental to the tomato industry since it, and green onions, caused this widespread outbreak, which involved over 1,400 cases in 43 states.
As previously mentioned, leafy greens are of major food safety concern in the produce industry. As shown in Table 1.1, almost half of the outbreaks listed are attributed to lettuce, salad mixes, or spinach, and consumption of the latter was responsible for 3 deaths in 2006 (9, 10, 45). The majority of leafy green outbreaks are caused by *E. coli* O157:H7, however cyclosporiasis caused by the parasite *Cyclospora cayetanensis* caused one major outbreak (12). It has been determined that pathogens are hard to eliminate on the surface of leafy greens due to their complex, rough physical surface resulting in the numerous foodborne illness outbreaks reported by the CDC (13).

Cantaloupes are another produce commodity of major food safety concern in the produce industry. The largest foodborne illness outbreak associated with cantaloupe occurred as a result of handling by Jensen Farms in Colorado. The outbreak involved 147 cases of *listeriosis*, which was fatal for 33 people (11). This outbreak was the deadliest foodborne illness outbreak in the U.S. in approximately 90 years (4). Aside from this multistate outbreak, cantaloupes were also responsible for three other outbreaks associated with multiple serotypes of *Salmonella* from 2008 to 2012.

**Contamination of produce by foodborne pathogens**

During pre-harvesting procedures, pathogens may be transferred to produce by the application of inadequately composted animal manure, sewage, or soil, which was contaminated with infected fecal matter (6, 7, 54). Pathogens, like *Listeria monocytogenes*, are commonly found in the soil and in untreated sewage and contaminate fruits and vegetables when in contact. In a study by Weis and Seeliger,
they isolated 154 strains of *L. monocytogenes* from soil and plants, 16 from animal feces, 9 from wildlife feeding ground, and 8 from birds. This suggested that *L. monocytogenes* was a saprophyte, microorganisms that live on decaying organic matter, and could therefore be contracted by humans and animals (7 {Weis, 1975 #131, 56}).

Produce like tomatoes, leafy greens, and cantaloupes commonly come in contact with the soil and therefore are at a high risk for pathogenic bacteria contamination.

Produce can become contaminated with pathogens in a variety of ways. Water is a major source of microbial contamination on produce because fresh produce comes in contact with water during irrigation and post-harvest processing. Sometimes irrigation water comes from various water sources like ponds, lakes, rivers, wells, and streams that may be contaminated by run-off from nearby animal pastures. Also, the water used during post-harvest processing (rinsing, storage, cutting, etc.) can be contaminated via cross-contamination in an overhead sprinkler or dump tank. During the process of cutting, there are points of entry for microorganisms and they may become unaffected by sanitizers (47, 50). The cut surface of a leaf or a melon creates specific hiding places for pathogens like *Salmonella* or *E. coli*. Also, when cutting melons the knife may carry pathogens from the rind to the flesh where bacteria may then multiply if left unattended (6, 50, 54).

Other routes where produce may become contaminated is by ill field workers, harvesting and processing equipment, and during storage. If Good Agricultural Practices (GAPs) are used on the farm, then produce has a higher chance of becoming contaminated by pathogens. An example of a farm not keeping up with GAPs was the
cantaloupe outbreak associated with Jensen Farms in Colorado. When the Food and Drug Administration (FDA) investigated the farm, they found that the processing equipment was harboring a high population of *Listeria monocytogenes* and there was no documentation of proper cleaning of the equipment (11). If the consumption of fresh fruits and vegetables continues to increase then the awareness of the food safety for fresh produce needs to also increase to prevent outbreaks like the Jensen Farm outbreak of 2011.

**Post-harvesting handling of organic produce as a source of microbial contamination**

Organic tomatoes are either picked at the breaker stage (about ¼ of the surface at the blossom end is pink) or the vine-ripe stage (flesh is firm and almost full red in color), depending on if they are being sold directly to consumers or if they need to be transported to consumers. To remove field heat, tomatoes are cooled in a refrigerated room, a process called “room cooling,” since they are sensitive to free moisture (dew or rain). The free moisture on or around the tomatoes causes dirt and other foreign particles to adhere to the surfaces of the fruit (32). The cooling process is slow because the cold air does not circulate directly around the individual tomatoes (18, 51). After cooling, tomatoes are washed with a spray of water or by submerging in a water tank. During washing, the temperature of the liquid in the wash tank is adjusted to above the internal temperature of the tomatoes. If the water is colder than the internal temperature of the tomatoes, the fruit’s air spaces constrict creating a vacuum that draws in rinse liquids through the stem scar resulting in potential contamination by pathogens (48).
Organic tomato wash systems are often treated with an organically approved sanitizer that is listed in the USDA Approved Chemicals for Use in Organic Post Harvest Systems (58).

Organic leafy greens, like Romaine lettuce and baby spinach, are submerged in almost freezing water immediately after harvesting. This process, called hydro-cooling, is done to remove the excess field heat contained in the leaves in order to maintain product quality. If the leaves are cut for RTE mixes, they are placed in a mesh sack to keep them contained during the hydro-cooling process. Leafy greens are usually pre-washed for consumers in order to keep a high quality product once the greens are purchased. Just like the organic tomatoes, the leafy greens are either washed by a sprinkler system or placed into a wash tank that contains an organically approved sanitizer (28). Along with hydro-cooling, leafy greens can also be cooled by the vacuum cooling processes. Vacuum cooling is a process where the produce is placed in a vacuum chamber and the air is drawn out of the chamber to create a vacuum, hence, lowering the temperature of the produce. This method is very quick and can be done for large batch harvests; however, it is also one of the most expensive methods (18, 51).

For organic cantaloupe, or muskmelons, fruit is harvested when it separates from the vine and the fruit changes to a yellowish or tannish color. After harvesting, the melons are packed on the field into 40-pound cartons with 9, 12, 15, 18, or 23 melons per carton, placed on pallets, and secured with straps (46). The pallets are then transferred to a cooler where they subjected to forced-air cooling by placing the pallets around a series of fans. This cooling process reduces the temperature of the cantaloupes from
36-49°C to 25-27°C in order to maintain product quality and reduce the survival of natural background bacteria present on the cantaloupes post-harvest (approximately 1.0 log CFU/cm² reduction) (46).

The process in which produce is harvested in order to remove field heat is very important to the product quality of the organic produce industry. When the produce is washed in a wash tank, from a sprinkler system or a flume system the washing liquids are recycled to increase economical costs. This practice increases the chance of cross-contamination by foodborne pathogens and is a major safety concern.

**Sanitizers for organic produce**

The many possible routes of contamination during pre-harvesting and post-harvesting creates the need for enough sanitizer in a rinse liquids system to eliminate microbes on the surface and in the crevices of fresh produce in the case that they were contaminated during processing. Organic farmers must use uniform methods of disinfecting their produce and for handling the produce post-harvest. After produce is harvested it needs to be washed, however the only sanitizers allowed in the wash tanks, flumes, or sprinkler systems have to be approved chemicals for use in organic post harvest systems. Rinse liquids have a high probability for cross-contamination if not sanitized, so maintaining the wash liquid is imperative to the organic produce industry.

The most studied method for sanitizing organic produce is the addition of chlorine (liquid sodium hypochlorite) to a rinse liquid system. Chlorine is effective against a wide variety of microorganisms including bacteria, viruses, and fungi. The regulations set by
the NOP require that organic farmers keep the residual chlorine levels in the water at the point of discharge below 4 ppm to abide by regulations in the Safe Drinking Water Act set by the Environmental Protection Agency (EPA). However, the concentration in the rinse liquids can be up to 200 ppm at the time of produce or equipment sanitizing.

The form of chlorine that has the highest antimicrobial properties is hypochlorous acid (HOCl). When the free chlorine is in this form the free residual chlorine reacts with organic matter in the rinse liquids and depletes its efficacy as a sanitizer (16), Hua et al. (25), stated that when chlorine reacts with natural organic matter in a system, two groups of halogenated disinfection byproducts are formed, trihalomethanes (THM) and haloacetic (HAA) acids. These byproducts are potential health concerns. In Figure 1.1., Rook et al. (40) shows a resorcinol-type molecule that is oxidized by HOCl, which allows halogenation of the aromatic ring, and then a fracture of the molecule (a in Figure 1.1) forms the THM (23, 40).

HOCl is also very dependent on the pH of the wash liquid. When the pH of the wash liquid is within a range of 6.0-7.5, the majority of the chlorine is in the HOCl form. Once the pH rises above 7.5, hypochlorite ions begin to form and the antimicrobial activity in the rinse liquids decreases significantly. (40, 55) Despite these major concerns, chlorine is widely used today because it is inexpensive, effective if maintained, and easy to use on and off the produce fields.

Another approved sanitizers used in the organic produce industry is ozone. Passing oxygen across an electrical gradient generates ozone and it is then effective
against bacteria due to its oxidizing properties. Ozone creates less harmful byproducts in the rinse liquids and it has been shown to be more effective than chlorine. However, ozone has a disadvantage of being high in investment costs but low in running costs because it only requires moderate electricity to generate the ozone (38). However, ozone needs to be generated on-site and it is instable after about 20 minutes in water. Due to its instability, ozone needs to be constantly reestablished into the rinse liquids by a generator. In addition, ozone needs to be used in combination with pure water because any impurities in the water react with the ozone and consume it, also, ozone can cause corrosion of wash tanks (16, 27, 55).

Citrix, acetic, lactic, and propionic acids are generally recognized as safe (GRAS) organic acids for the use as a food ingredient and are allowed for use as sanitizers on organic fruits and (37). Organic acids are responsible for organoleptic properties of most fruits and vegetable and give them characteristics like tartness, acidity, and strong aromas (17). Organic acids exhibit antimicrobial properties but the extent of the antimicrobial activity may differ among organic acids. In a study by Akbas et al. (1), organic acids were used to eliminate E. coli O157:H7 and Listeria monocytogenes on iceberg lettuce. Other research has also been conducted using organic acids in combination with other organic acids, chlorine, or hydrogen peroxide (37, 53, 60). In a study conducted by Venkitanarayanan et al. (53), 1.5% lactic acid and 1.5% hydrogen peroxide were used to eliminate pathogenic bacteria on the surface of apples, oranges, and tomatoes. The researchers found that the organic acid in combination with the hydrogen peroxide resulted in a >5.0 log_{10} CFU per fruit when compared to a water
control. These findings suggest that in combination with other sanitizing agents organic acids have the ability to decrease the population of a foodborne pathogen significantly. The limitations with organic acids are that they are only active at low pH and generally need longer contact times in combination with higher concentrations when compared to chlorine in order to effectively reduce the microbial load on the surface of organic produce.

There are many commercially available sanitizers for use on surfaces and for produce washes (Table 1.2). Acidified sodium chlorite is commercially available (Sanova®, Ecolab) as a combination of citric acid and sodium chlorite. It is an example of using organic acids in combination with a chemical sanitizer. When the two active ingredients are combined they form active chlorine dioxide (ClO₂), which is more soluble than sodium hypochlorite (NaOCl) in water and is a better oxidizer than HOCl. Sanova is effective at 0.5-1.2 g/L and can be used on certain raw fruits and vegetables followed by a potable water rinse (26).

Peroxyacetic acid (PAA) is an organic acid-based sanitizer allowed for use in organic produce production. It is a combination of hydrogen peroxide and acetic acid and it is commercially available as Tsunami® (Ecolab) (21). It is used in wash tanks, flumes, and sprinkler systems and its optimal concentration is approximately 80 ppm. Degradation by-products of PAA acid are mainly carboxylic acids, which are not mutagenic, and are safer than those of chlorine (34). The one disadvantage of PAA is that a post-treatment wash cycle with fresh, clean water is required after use (16).
The Catallix® system (TMI Europe S.A., France), which is composed of sodium thiocyanate and hydrogen peroxide to generate hypothiocyanite (OSCN-) in the presence of the enzyme peroxidase, is another chemical sanitizer. OSCN- is the active disinfectant but it does not remain in the finished produce because it has a short half-life. Catallix is approved for use on fresh-cut produce as a processing aid and it is as effective as chlorine (2, 21). While Catallix is approved for use on organic produce, it is not often used due to its high costs.

Purac® (Purac, Bioquimica) is composed of lactic acid and is commercially available for use in wash water and produce disinfectants at 20 mL/L for 3 min. This product is reported to be just as effective as chlorine for sanitizing leafy greens (2, 21, 22), Allende et al. (2), determined that 20 mg/L of Purac and 100 mg/L chlorine reduced coliforms on fresh-cut escarole by 2.2 log CFU/g, while the other sanitizers analyzed only reduced the coliforms by approximately 1.0 log CFU/g. Purac reduced coliform counts on the surface of lettuce by 1.6 log CFU/g. It was also found that Purac was able to effectively reduce yeast and mold counts by 1.8 log CFU/g.

Citrox (Citrox Limited, Middlesbrough, UK) is a commercially available formulation containing organic acids and phenolic compounds as the active ingredients. It is recommended for use at 5.0 mL/L for 5 min to reduce bacteria on produce. This product is also reportedly a potential alternative to chlorine, especially for the case of disinfecting leafy greens (2, 21, 22). In the same study mentioned previously (2) 5.0 mg/L Citrox was able to significantly reduce the total bacteria count (P<0.001) on fresh-cut lettuce. Also, Citrox reduced the coliform counts on the lettuce by 2.3 log CFU/g.
making it more effective than chlorine. A benefit of Citrox is that it is has been certified organic in the United Kingdom so it is likely to be approved for use in the US.

Since organic produce is produced without the use of pesticides, antibiotics, genetically modified organisms, and hormones there are limited treatment options available to keep this commodity safe from pathogenic bacteria contamination. In order to find a new sanitizer for organic wash systems research has to be done to find one that works better than what is available on the markets now. Due to the public health concerns and the complex chemistry of chlorine companies have commercialized these organic acid formulations. These formulations are easy to use, relatively cheap, and show to be effective at eliminating pathogenic bacteria from organic produce.
References


47. Suslow, T. V. 2001. Water disinfection: a practical approach to calculating dose values for preharvest and postharvest applications. *Division of Agriculture and Natural Resources, University of California, Davis.*
### Appendix I

#### Tables

**Table 1.1.** Multistate foodborne outbreaks associated with selected produce from 2004 to 2013.

<table>
<thead>
<tr>
<th>Year of outbreak</th>
<th>Produce associated with the outbreak</th>
<th>Foodborne pathogen responsible</th>
<th>Cases</th>
<th>Number of US states</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004</td>
<td>Roma tomatoes</td>
<td>Multiserotype <em>Salmonella</em></td>
<td>561</td>
<td>18</td>
</tr>
<tr>
<td>2006</td>
<td>Fresh bagged spinach</td>
<td><em>E. coli</em> O157:H7</td>
<td>199 (3 deaths)</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Tomatoes</td>
<td><em>Salmonella Typhimurium</em></td>
<td>183</td>
<td>21</td>
</tr>
<tr>
<td>2008</td>
<td>Raw produce: tomatoes</td>
<td><em>Salmonella Saintpaul</em></td>
<td>1442</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Cantaloupes</td>
<td><em>Salmonella Litchfield</em></td>
<td>51</td>
<td>16</td>
</tr>
<tr>
<td>2010</td>
<td>Shredded Romaine lettuce</td>
<td><em>E. coli</em> O145</td>
<td>26</td>
<td>5</td>
</tr>
<tr>
<td>2011</td>
<td>Romaine lettuce</td>
<td><em>E. coli</em> O157:H7</td>
<td>58</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Cantaloupes</td>
<td><em>Listeria monocytogenes</em></td>
<td>147 (33 deaths)</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Cantaloupes</td>
<td><em>Salmonella Panama</em></td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>2012</td>
<td>Organic spinach and spring mix</td>
<td><em>E. coli</em> O157:H7</td>
<td>33</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Cantaloupes</td>
<td><em>Salmonella Typhimurium and Newport</em></td>
<td>261 (3 deaths)</td>
<td>24</td>
</tr>
<tr>
<td>2013</td>
<td>RTE salads</td>
<td><em>E. coli</em> O157:H7</td>
<td>33</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Fresh produce: salad mix</td>
<td><em>Cyclospora</em></td>
<td>631</td>
<td>25</td>
</tr>
</tbody>
</table>
Table 1.2. Commercially available sanitizers for use in the produce industry.

<table>
<thead>
<tr>
<th>Commercial Name &amp; Mfr.</th>
<th>Recommended Use Conc.</th>
<th>Components</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrox (Citrox Limited, Middlesbrough, UK)</td>
<td>5 mL/L for 5 min at pH 3.5</td>
<td>Organic acid formulations of Phenolic compounds</td>
<td>Organically approved in the UK, similar effectiveness to chlorine for use in wash liquids for sanitizing produce, no potable water rinse needed</td>
<td>COD* of water increases</td>
</tr>
<tr>
<td>Purac (Purac, Bioquimica)</td>
<td>20 mL/L for 3 min at pH 2.7</td>
<td>Lactic acid formulation</td>
<td>Similar effectiveness to chlorine for produce sanitation in wash liquids, no potable water rinse needed</td>
<td>COD* of water increases</td>
</tr>
<tr>
<td>Catallix (TMI Europe S.A., France)</td>
<td>40 mg/L for 5 min at pH 7.7</td>
<td>Hydrogen peroxide and thiocyanate</td>
<td>Similar effectiveness to chlorine</td>
<td>Very expensive, COD* of water increases</td>
</tr>
<tr>
<td>Sanova (Ecolab)</td>
<td>500 mg/L for 1 min at pH 2.7</td>
<td>Acidified sodium hypochlorite</td>
<td>Highly oxidative with broad-spectrum germicidal activity, more stable than NaOCl</td>
<td>Potable water rinse needed after treatment</td>
</tr>
<tr>
<td>Tsunami (Ecolab)</td>
<td>80 µL/L for 1 min at pH 5.9</td>
<td>Acetic acid, peroxyacetic acid, hydrogen peroxide</td>
<td>Active over a broad range of pH and is less sensitive to organic matter than NaOCl</td>
<td>Potable water rinse needed after treatment</td>
</tr>
</tbody>
</table>

*Chemical Oxygen Demand: total amount of all chemicals in the water that can be oxidized.
Figure 1.1. The addition of HOCl oxidized the resorcinol-type ring, resulting in halogenation of the aromatic ring and the formation of the THM (a). (1)

Chapter II

The use of a commercial naturally-occurring antimicrobial-based sanitizer to prevent cross-contamination of *Escherichia coli* O157:H7 and *Salmonella enterica* cocktails on the surface of organic cherry tomatoes
Abstract

Organic produce is a growing trend in the United States. Therefore, it is very important that the organic growers utilize practices that efficiently eliminate cross-contamination from contaminated produce to clean produce during post-harvest washing. In this study, organic cherry tomatoes were inoculated with cocktails of *Salmonella enterica* or *Escherichia coli* O157:H7 and then treated in a natural antimicrobial-based sanitizer (NABS) or 200-ppm chlorine. To determine if cross-contamination occurred in the rinse liquids un-inoculated tomatoes were placed into the shared rinse liquid treatments. The prevention of cross-contamination was verified by sampling the rinse liquids after the initial treatment of the inoculated produce. The impact of organic load (OL), time-point of inhibition, and the survival on produce during 96 h storage at ambient (21°C) conditions were additionally studied. NABS was not able to significantly reduce either of the pathogens on the surface of the tomatoes, but was able to prevent cross-contamination and was not influenced by the organic load. It was also determined that increasing the treatment time of 0.75% NABS for up to 90 min only resulted in a reduction of 1.5 log CFU/g of *E. coli* from the surface of the tomatoes, after 30 min the *E. coli* cocktail was almost fully inhibited in the 0.75% NABS rinse liquid, and storing the treated tomatoes for up to 96 h resulted in a continued effect of 0.75% NABS resulting in approximately 4.0 log CFU/g reduction. Overall, the chlorine solution had a greater initial reduction of the *E. coli* on the surface of the tomatoes than the NABS, however, NABS was able to reduce cross-contamination in the rinse liquid in the presence of the OL, which was a novel result for this experiment.
Introduction

In the United States, the consumption of fresh, organic fruits and vegetables has increased since 2002. In 2015, USDA reported that there were 19,474 USDA certified organic operations, which was a 250% increase over 2002 (33). According to USDA, organic produce is the top-selling organic category in the United States (34).

Concurrent with the increased consumption of all types of fresh produce, the Center for Disease Control has estimated there to be 48 million foodborne illness cases in the U.S. annually. 46% of these illnesses are reportedly linked to the consumption of fresh produce (9, 24, 29). During the fresh produce production chain there are many routes of contamination by foodborne pathogens. Some of the most common routes include contaminated irrigation water and post-harvest rinse liquids (11). Foodborne pathogens most often linked to foodborne illness outbreaks associated with fresh produce include Salmonella, Escherichia coli O157:H7 and norovirus (19, 30).

Tomatoes are the second most important vegetable crop with a world production of about 100 million tons (10). With so many fresh tomatoes being consumed there is also an increased threat of foodborne illness associated with that consumption. From 1990 to 2010, there were 15 multistate Salmonella outbreaks associated with tomatoes, which resulted in 1,959 illnesses, 384 hospitalizations and three deaths (7). One of the largest Salmonella outbreaks, with a confirmed link to Roma tomatoes was in 2004 and involved 429 illnesses and 129 hospitalizations (5).
Since fruits and vegetables have been implicated in outbreaks of foodborne illness, it is important to determine efficient and inexpensive methods to control the pathogens on the surface of fresh produce. One method is to use sanitizer solution to rinse produce during post-harvest handling. Solely washing or rinsing produce with tap water is not sufficiently enough to eliminate foodborne pathogens (2). In recent years, many sanitizers have been used to reduce foodborne pathogens in rinse liquids during post-harvest processing including chlorine, chlorine dioxide, ozone, chlorinated trisodium phosphate, and peroxyacetic acid (27). These sanitizers are added to ensure that the pathogenic bacterium that may be present in the rinse liquid does not spread to uninfected produce. In fresh organic produce operations, chlorine in the form of hypochlorite is used at 50-200 ppm to reduce microbial contamination (35). However, there are disadvantages to using chlorine and chlorine-based solutions for sanitization including its reduced efficacy in the presence of organic matter, difficulty monitoring free chlorine levels, sensitivity to pH, production of potentially toxic halogenated disinfection byproducts (DBPs), and USDA and EPA restrictions on concentrations used and released into the environment (1, 6, 12, 14).

While there are many sanitizers available, organic growers are limited in what they are able to use. The USDA specifies what sanitizing compounds can be used on their National List of Allowed and Prohibited Substances (26). One of the most common sanitizers used in the food industry, chlorine, is on the list but it has limits as to the concentration used and concentration allowed in effluent, the latter dictated by the Safe Drinking Water Act (32). If these regulations are not being followed, the National
Organic Program (NOP) of USDA, will investigate growers and potentially shut down an operation. Therefore it is important that the sanitizer used in the dump tanks or spray lines is both organically approved and efficient at eliminating the potential for a foodborne outbreak (20). In the organic produce industry, these issues make it highly desirable to develop a sanitizing wash system that is efficient, easy to use, and naturally derived for the farmers in order to improve safety of their products (23).

Some naturally derived essential oils and their components have been determined to have good efficacy in the reduction of foodborne pathogens on organic leafy greens (21, 22). Some of the most studied essential oils are from clove, thyme, oregano, and cinnamon. Some other naturally occurring compounds that are known to have antimicrobial properties can be derived from enzymes from animal sources e.g., lysozyme, or bacteriocins from microbial sources, e.g. nisin (17). Organic acids, such as citric, acetic, or propionic acids, are also known to exhibit antimicrobial properties (3, 25).

In the present study, a natural antimicrobial-based sanitizer (NABS) composed of organic acids and bioflavonoids derived from citrus fruits, was evaluated for its potential to eliminate foodborne pathogens on organic produce when used in a model rinse liquid system (1, 4, 13). This specific type of sanitizer was formulated to treat fruits and vegetables post-harvest or/and in a processing plant and is made from GRAS components (16). It is permitted for use in organic processing in the United Kingdom (5, 7, 10). The main objectives of the study were: (1) to evaluate the effectiveness of the commercial NABS against E. coli O157:H7 or Salmonella enterica on USDA certified organic cherry tomatoes, (2) to determine the potential for NABS to prevent cross-
contamination of foodborne pathogens in a model sanitizer wash system, (3) to
determine survival of pathogens in the contaminated model sanitizer wash system, (4)
to determine the effect of treatment time on effectiveness of the NABS, and (5) to
determine the survival of E. coli O157:H7 on the surface of the washed tomatoes during storage.

**Methods**

**Bacterial strains/serovars and inoculum preparation.** Five-strain cocktails of *Salmonella enterica* (Agona, Montevideo, Gaminara, Michigan and Saint Paul) and *E. coli* O157:H7 (932, H1730, F4546, K3995 and CDC658) were used in this study. The cultures were all obtained from frozen stocks (-18°C in 80% glycerol) from collections at the University of Tennessee in Knoxville, Tennessee. All bacteria serovars or strains were consecutively subcultured thrice in 10 mL tryptic soy broth (TSB; Bacto™ Tryptic Soy Broth; Becton, Dickinson and Company; Sparks, Maryland, USA) at 37°C for 24 h. All strains were made nalidixic acid resistant (NAR) by gradually introducing nalidixic acid (NA; Acros Organics, 99.5%, New Jersey, USA) at increasing concentrations in TSB over 24 h increments until the bacteria were resistant to 40 ppm NA. Pure cultures were isolated and new frozen stocks were prepared in 80% glycerol for the wild type (WT) and NAR strains and stored at -18°C.

Cultures were resuscitated by transferring three times in TSB with 40 ppm NA (TSBN) after which 0.3 mL of each strain was spread plated onto tryptic soy agar (TSA; Fisher Bioreagents® Granulated Agar Fisher Scientific; Fair Lawn, New Jersey, USA) with 40-ppm NA (TSAN) and incubated for 24 h at 37°C. After 24 h, each serovar or
strain was re-suspended by adding 5.0 mL of phosphate buffer solution (PBS; pH 7.2; Beckon, Dickinson and Company; Sparks, Maryland, USA) to the surface of the plates and creating a suspension. Suspensions were collected from all five serovars or strains in a sterile container to create a 25 mL cocktail with a population of approximately 9.0 log CFU/mL. The cocktails of *Salmonella* or *E. coli* were then used to inoculate tomatoes.

**Minimum inhibitory concentrations (MICs).** A commercial natural antimicrobial-based sanitizer was obtained from Phyto Innovative Products Ltd., Middlesborough, UK (Citrox 14WP ProGarda Concentrate (NABS, Batch #jj/138/e)). To determine the appropriate concentrations to use in rinse liquids, the MICs, were determined for each NAR serovar/strain of *Salmonella* or *E. coli*. The MICs were determined using sterile 96-well (250 µL maximum per well) microtiter plates. Sanitizer (NABS) solutions were made using serial twofold dilutions in TSBN. For each sample, 120 µL of NABS and 120 µL of each serovar or strain of *Salmonella* or *E. coli* (diluted to 5.0 log CFU/mL in TSBN) were used. The plates were incubated at 37ºC for 24 h. The optical density at 630 nm (OD$_{630}$) of each well was read at 0 and 24 h using a microtiter plate reader (model Synergy HT, Biotek, Winooski, VT). After 24 h the lowest concentration of sanitizer at which growth was completely inhibited, i.e., an OD$_{630}$ increase of ≤ 0.05 was defined as the MIC (31). To determine if the WT and the NAR serovars/strains had similar resistance characteristics to NABS, the MIC study was repeated with the WT strain/serovar of *Salmonella* and *E. coli*. 
**Determination of the effect of pH on the efficacy of sanitizer.** NABS samples were prepared using serial twofold dilutions to the determined MIC. They were then added to TSBN and the pH of each sample was adjusted to 3, 4, 5, 6, and 7 using 0.1 M NaOH or 0.1 M HCl using a pH meter (FisherScientific Accumet AB150, Pittsburgh, PA). Samples (120 µL) of pH adjusted NABS were added to 96-well plates and 120 µL of each strain of *E. coli* (~5.0 log CFU/mL) was added to each well. Only *E. coli* was used to determine pH effects. Positive controls consisted of 120 µL of TSBN and 120 µL of each individual strain of *E. coli* and the negative controls consisted of uninoculated TSBN and each NABS sample with the adjusted pH. The plates were incubated at 37ºC for 24 h and the absorbance was determined at 0 and 24 h. NABS samples which showed an OD ≤ 0.05 were considered negative for growth.

**Inoculation of organic cherry tomatoes.** Organic cherry tomatoes (Del Cabo Farms or Lady Moon Farms) were purchased from a local grocery store. Two cherry tomatoes constituted one “sample” (approximately 20 g samples). Each sample was spot inoculated with 200 µL of *Salmonella* or *E. coli* cocktail (10-10 µL spots or 100 µL per tomato). Once the tomato samples were aseptically inoculated with the appropriate cocktail, samples were allowed to dry on sterile surfaces in a biosafety cabinet for 2 h.

**Preparation of sanitizer rinse liquids.** In sterile beakers, 100 mL solutions of the liquid NABS was added to sterile deionized water at concentrations (v/v) of 0.0% (pH 7), 0.5% (pH 2.92), or 0.75% (pH 2.76) NABS. For the tomatoes inoculated with the *E. coli* cocktail an additional treatment of 200-ppm free residual chlorine (Clorox; pH 7.2; Oakland, CA, USA) was used as a comparison treatment. The treatment containing
chlorine was tested for free residual chlorine using a Free Chlorine & Chlorine Ultra High Range ISM (Hanna Instruments, Roonsocket, RI) immediately before use. In order to determine if there was a decrease in the sanitizer’s efficacy in the presence of organic matter, an organic load (OL) was added to the sanitizer rinse liquids that resulted in a change of pH to 4.76, 3.08, and 2.90 for 0.0, 0.5, and 0.75% NABS treatments, respectively. To prepare the OL, organic cherry tomatoes were blended with sterile deionized water using a hand-held immersion blender to produce a 20% w/w aqueous suspension. The 20% tomato suspension was added to sanitizer rinse liquids treatments at 1%. The treatment with 200-ppm free residual chlorine with OL (pH 6.4-6.8) was also tested for its free available chlorine immediately before use.

**Initial exposure of organic tomatoes to sanitizer rinse liquids.** Approximately 20-g of inoculated tomatoes were used as samples for each of the treatments. The inoculated produce samples were aseptically placed into one of the rinse liquids (0.0%, 0.5%, 0.75% NABS or 200 ppm chlorine with and without 1.0% OL) and allowed to soak for 2 min at room temperature (21ºC). Then the treated produce was removed using sterile kitchen tongs and placed into a stomacher bag. Once in the stomacher bag the produce was diluted (1:5 w/w) using phosphate buffer solution with 0.2% Tween 80 (Agros Organic; New Jersey, USA) (PBS/T80). The stomacher bag was then massaged by hand for 15 s. To enumerate for survivors, the rinsate was serially diluted in PBS, spread plated in duplicate onto TSAN, and incubated for 24 h at 37ºC. To determine the initial inoculum on the skin of the organic produce, a control sample that received no
treatment was placed into buffer, massaged, and plated for enumeration on TSAN. These experiments were repeated for a total of three times at room temperature (21°C).

**Determination of cross-contamination on organic tomatoes.** To determine if cross-contamination occurred, uninoculated produce (approximately 20-g of fresh tomatoes) was introduced into the contaminated rinse liquids from above, with and without 1.0% OL. The fresh produce commodity was allowed to soak for 2 min at room temperature and was then removed and placed into a stomacher bag. PBS/T80 was added (1:5 w/w) and then the bags were massaged by hand for 15 s. The rinsate was then serially diluted in PBS, spread plated onto TSAN in duplicate, and incubated for 24 h at 37°C. This process was repeated once more for each of the same rinse liquids using new un-inoculated produce. Both cross-contamination studies were repeated three times in total for each rinse liquid at room temperature (21°C). These two studies were referred to as Follower one (F1) and Follower two (F2).

**Determination of survivors in sanitizer rinse liquids.** To determine if there was survival of the *Salmonella* or *E. coli* cocktails in the NABS rinse liquids samples were taken from the containers after each experiment. 100 µL was removed from the containers of each treatment (with and without 1.0% OL), spread plated onto TSAN in duplicate, and incubated at 37°C for 24 h. Also, 1.0 mL was removed from the containers of each treatment and serially diluted in PBS, spread plated on TSAN in duplicate, and incubated at 37°C for 24 h. These experiments were repeated three times in total at room temperature (21°C).
Influence of treatment time on NABS inactivation of *E. coli* on organic cherry tomatoes. To determine if time was a factor in the inactivation of *E. coli* on the skin of the tomatoes, inoculated NABS-treated tomatoes were held from 2 min up to 90 min. Tomatoes were spot inoculated and allowed to dry as explained in above. Once the tomatoes were dry, one sample for each time point was added to 0.0%, 0.5% and 0.75% NABS rinse liquids. A sample was aseptically removed after 2, 4, 6, 8, 10, 30, 60, and 90 min. After each time point, the tomato sample was placed into a stomacher bag, diluted 1:5 (w/w) with PBS/T80, and stomached for 90 s at 230 rotations per min (rpm). After stomaching, the tomato mixture was diluted in PBS, spread plated onto TSAN in duplicate, and incubated for 24 h at 37ºC. This experiment was repeated thrice at room temperature (21ºC).

The effect of time on survival of *E. coli* in NABS treatment liquids. An experiment was carried out with the purpose of determining the effect of time on inactivation of *E. coli* in the NABS rinse liquids. A sample of dry, inoculated tomatoes (one sample was 2 tomatoes or approximately 20 g) was added to 0.0%, 0.5%, and 0.75% NABS rinse liquids and was allowed to soak for 2 min and then the tomatoes were aseptically removed and discarded. A 1.0 mL sample was taken after removal of the tomatoes at 0, 10, 20, and 30 min, serially diluted in PBS, spread-plated on TSAN in duplicate, and incubated for 24 h at 37ºC. *E. coli* surviving in the rinse liquids were enumerated following incubation after contact with a contaminated tomato sample. The experiment was repeated thrice at room temperature (21ºC).
The effect of tomato storage time on *E. coli* survival after NABS sanitizer treatments. The purpose of this experiment was to determine if NABS caused inactivation of *E. coli* during storage of treated tomatoes. Seven dry, inoculated samples of tomatoes (one sample was two tomatoes, approximately 20 g) were placed into 500 mL of 0.0%, 0.5%, and 0.75% NABS sanitizer solutions. After 2 min, the tomato samples were aseptically removed and placed onto sterile surfaces in a sterile container with a foil lid for storage. After 2 min (time 0), 30 min, 1, 3, 6, 24, 48, 72 and 96 h, tomato samples were placed into a stomacher bag, diluted 1:5 (w/w) with PBS/T80, and stomached for 90 s at 230 rpm. After stomaching, the diluent was serially diluted in PBS, spread plated in duplicate onto TSAN, and incubated for 24 h at 37ºC. The experiment was repeated three times in total at room temperature (21ºC).

**Experimental design and statistical analysis.** All experiments were repeated three times, in each independent replication, samples were taken in duplicate. Treatments were analyzed using the Tukey-Kramer method to determine if there were significant differences among treatments. For the cross-contamination studies, the least square means were sliced so all possible treatments could be compared within the experiments. The software used was SAS 9.3 (SAS Institute Inc., Cary, NC, USA). A level of significance of <0.05 for P values was selected to determine microbial differences.

**Results and Discussion**

**Minimum inhibitory concentrations.** The MIC study showed that NABS had inhibitory effects against the Gram-negative bacteria (Table 2.1). The MIC values for
NABS were found to be the same for all serovars of *Salmonella* and all strains of *E. coli*. Since it is ideal to use the lowest dose of an antimicrobial in a rinse liquids system the lowest possible concentration where growth was inhibited was chosen for this study, however the next highest concentration was also analyzed. The MICs were conducted for WT and NAR serovars/strains to determine if the resistance characteristics were similar. The results showed that the WT serovars/strains had the same MIC as the NAR serovars/strains.

**Effect of pH on the efficacy of the sanitizer to inhibit *E. coli***. It has been reported that organic acids present in antimicrobials work against the microorganisms due to its low pH (8, 23). To better evaluate the mode of action, the pH of NABS at 0.5% (pH 5.89) and 0.75% (pH 5.33) in TSBN was adjusted using 0.1 M NaOH and 0.1 M HCl to 3, 4, 5, 6, or 7. It was determined that for both concentrations of NABS there was still efficacy in inhibiting *E. coli* even when the pH was adjusted to 7, when compared to the controls. So at a neutral pH value the NABS components were still acting against *E. coli* (Table 2.2). Therefore, the high acidity of NABS was not the only factor playing a role in its antimicrobial activity. This characteristic of NABS makes it a novel antimicrobial for usage as an antimicrobial in a wash system for post-harvest produce. However, in the main studies for this research the aqueous rinse liquids containing NABS were not carried out using neutralized solutions (0.5% NABS\(_{aq}\) = 2.92; 0.75% NABS\(_{aq}\) = 2.76). The less additives added to the NABS was ideal in order to keep it as pure and simple as possible in accordance with regulations set by the USDA.
**Initial exposure of organic produce to rinse liquids.** Table 2.3 shows the surviving *Salmonella* on the surface of the organic cherry tomatoes after the initial treatments in 0.0%, 0.5% and 0.75% NABS (with and without OL). The initial population actually inoculated onto the surface of the untreated tomatoes (control) was determined to be 9.66±0.10 log CFU/g for the *Salmonella* cocktail. When the inoculated tomatoes were introduced into the water control (0.0% NABS) the recovery from the tomatoes was 9.33±0.03 log CFU/g, which was not significantly different from the inoculated tomatoes that did not receive treatment. The inoculated tomatoes treated in the 0.5% and 0.75% NABS treatments did not show significant differences in reduction when compared any other treatments. The influence of the 1.0% organic load on the efficacy of the washing treatments was not significant to the overall initial treatments (Table 2.3).

Table 2.4 shows the surviving *E. coli* on the surface of the organic cherry tomatoes after the initial treatments in NABS and chlorine. The initial population recovered from the untreated tomatoes was 8.78±0.43 log CFU/g. When the inoculated tomatoes were washed with water (water control with and without OL) there was not a significant log reduction when compared to the inoculated tomatoes that received no treatment (control). The inoculated tomatoes that were treated in the NABS treatments did not show significant differences in reduction when compared to the controls. In addition to the NABS treatments, a chlorinated wash system was also analyzed for comparison purposes. Initially, the chlorine system, with and without OL, had a significantly higher reduction (> 3.0 log CFU/g) when compared to the NABS treatments.
and the controls (Table 2.4). The influence of the 1.0% organic load on the efficacy of the washing treatments was not significant to the overall initial treatments.

López-Gálvez et al. (15), discovered a similar observation in a previous study concerning fresh-cut lettuce treated with NABS at 0.5%. The researchers found that NABS reduced *E. coli* on the surface of the lettuce by approximately 1.5 log CFU/g during the initial treatment under similar conditions. The reductions determined in the present study were < 1.0 log CFU/g when the tomatoes inoculated with *E. coli* were washed with 0.5% NABS with OL and 0.75% NABS without OL. In another similar study conducted by Abadias et al. (1), 0.5% NABS was used to eliminate *Salmonella* spp. and *E. coli* populations on ‘Golden Delicious’ apple plugs, it was determined that NABS caused a 1.0 log reduction when a 6.0 log CFU/mL inoculum was applied onto the apple plugs. These results are in agreement with the current study because, as mentioned previously, less than 1.0 log reduction occurred when approximately 9.0 log CFU/mL of the cocktails were utilized on the surface of the tomatoes. However, in another study where NABS was used to eliminate foodborne pathogens on escarole and fresh-cut lettuce it was found that 0.5% NABS was able to significantly eliminate the bacteria up to 2.3 log CFU/g (4). Various factors could have contributed to these variations in reducing pathogens including initially using lower population counts than 9.0 log CFU/mL, and the different physical structures escarole, lettuce and apples exhibit.

For the case of chlorine as a sanitizer, López-Gálvez et al (15), found that when they used 40-ppm chlorine they obtained an initial reduction of 2.0 log CFU/g as opposed to the 1.5 log reduction for NABS. In the present study, when the tomatoes
inoculated with *E. coli* were introduced into the 200-ppm chlorinated rinse liquid a greater initial reduction was observed when compared to the NABS. Allende et al (4), determined that 100-ppm chlorine reduced mesophilic bacteria and coliforms on fresh-cut escarole by > 1.5 log CFU/g and 2.2 log CFU/g, respectively. While 0.5% NABS was also significantly effective at reducing the microbial populations when compared to the water control. Also, they found that 100-ppm chlorine reduced mesophilic bacteria and coliforms on lettuce by approximately 1.0 log and > 1.0 log, respectively. While 0.5% NABS was the only sanitizer to significantly reduce the microbial loads on the lettuce when compared to the water control (approximately > 2.0 log CFU/g reductions). The effectiveness of NABS over chlorine could be contributed to its greater oxidizing capacities and that one of its components could be a surfactant, which would improve its delivery into the microniches of the leafy greens (28).

**Determination of cross-contamination on organic tomatoes.** To determine if cross-contamination occurred, two sample sets (F1 and F2) of un-inoculated tomatoes were introduced into the each of the systems right after the contaminated tomatoes were exposed to the rinse liquids. The population of *Salmonella* or *E. coli* transferred onto the clean surfaces of the organic cherry tomatoes is shown in Figure 2.1 and Figure 2.2, respectively. For *Salmonella*, the tomatoes introduced into the water control, with and without the OL, reveled high recoveries for F1 and F2, which indicated cross-contamination because of the absence of sanitizer. For *E. coli*, the fresh tomatoes introduced into the water control, with and without OL, also reveled high recoveries with no significant differences between F1 and F2. By determining that water alone did not
prevent cross-contamination it justified that the findings in Adams et al. (2) studies were accurate and that the need for a sanitizer in a rinse liquid modeling system is essential to the produce industry to increase food safety.

When the fresh tomatoes were introduced to the Salmonella contaminated rinse liquids with 0.5% and 0.75% NABS there were no differences between F1 and F2. Also, all of the recoveries were determined to be at or slightly above the limit of detection (2.70 log CFU/g), which indicated that cross-contamination was occurring within the NABS system. While cross-contamination was observed, all the NABS rinse liquids were found to be statistically different from the water controls demonstrating much lower rates of contamination. Overall, the 1.0% OL did not significantly effect the efficacy of the NABS at 0.5% or 0.75% for Salmonella during F1 or F2. The high inoculum level chosen for this study (9.0 log CFU/g) far exceeds what would be expected in a natural contamination event, further research should determine how the NABS system performs with lower, more realistic populations of pathogens.

For E. coli, the same prevention of cross-contamination was observed with Salmonella resulting in high recoveries in the water controls for F1 and F2, with and without OL. Also, for E. coli all of the values for 0.5% and 0.75% NABS fell at or slightly above the limit of detection (2.7 log CFU/g), once again demonstrating cross-contamination. There were no significant differences between 0.5% NABS and 0.75% NABS rinse liquids (F1 and F2), which were found to be statistically different from the water controls. Chlorine was also found to preform similar to NABS system, once again demonstrating that the project’s parameters while appropriate for monitoring inactivation,
may have been too high to draw adequate conclusions regarding the efficacy of limiting cross-contamination. The 1.0% OL did not significantly affect the efficacy of the NABS or the chlorinated system when treated against *E. coli*.

As discussed, NABS was able to prevent cross-contamination from tomatoes inoculated with a high inoculum level of foodborne pathogens to fresh, clean tomatoes when exposed to the rinse liquids with 0.5% and 0.75% NABS (with and without 1.0% OL). López-Gálvez et al. (15), determined in their cross-contamination study that approximately 1.0 log CFU/g of *E. coli* was recovered off of un-inoculated produce when introduced into contaminated rinse liquids but reported that NABS was not as effective as other sanitizers, like chlorine or Tsunami, at preventing cross-contamination. However, they did not test these other sanitizers in the presence of OL, which could have greatly affected their conclusions. Additionally, our research demonstrated that NABS preformed as well as chlorine at inhibiting *E. coli* cross-contamination. Luo et al. (18), conducted a study on determining the free chlorine concentrations needed to prevent *E. coli* cross-contamination during produce wash systems and reported that there was a change in free chlorine levels in the presence of OL in the rinse liquids. They also reported that the lower the free chlorine levels the higher the recovery of *E. coli* was observed (18). If the chlorine processing waters are not properly monitored for their free chlorine concentrations and organic load is in the rinse liquid systems this becomes a huge source of microbial contamination and leads to cross contamination and potential foodborne pathogen outbreaks. Since the OL in the NABS rinse liquids did not affect the outcome of the cross-contamination studies it can be confidently reported...
that NABS did not lose any antimicrobial activity and therefore it can be used as an effective alternative to chlorine as a prevention of microbial transfer. In this study, the OL did not affect the efficacy of the chlorinated rinse liquids and this can be explained because this experiment only represented a small-scale representation of an actual chlorinated rinse liquid system. Only two sets of fresh, un-inoculated tomatoes were tested in this experiment for a short amount of time. If the OL was increased from 1.0% to > 1.0% the antimicrobial activity of the chlorine may have been depleted due to the free chlorine reacting with the OL particles.

**Determination of survivors in NABS rinse liquids.** When the treatment liquids were examined high recoveries of *Salmonella* and *E. coli* were enumerated from the water controls with and without OL (8.63 log CFU/mL and 6.50 log CFU/mL, respectively). There was no detection of *Salmonella* in the NABS rinse liquids with and without OL (Figure 2.3). For *E. coli*, the same trend was observed, indicating that there were no survivors in the NABS rinse liquids when compared to the water controls with and without OL (Fig. 2.4). This supports NABS and chlorine as effective mitigation strategies for reducing the likelihood of cross-contamination. It was also determined that the addition of OL did not have a significant effect on the outcome of this experiment.

Abadias et al. (1) also did not recover any pathogenic bacteria populations by direct plating or enrichment in the rinse liquids after inoculated apple plugs were treated with 0.5% NABS. The researchers also concluded that NABS could prevent cross-contamination of fresh produce in the fresh-cut industry, which the present research proved it could also prevent transfer to fresh, whole tomatoes (1). Luo et al. (18) also
found that if free chlorine is used at low concentrations (1 mg/L or less) that *E. coli* was detected in the rinse liquids after contaminated produce was introduced into the system and then reported that there was cross-contamination onto fresh, clean lettuce. This data validates while chlorine may have high log reductions initially at low concentrations, it does not prevent cross-contamination in the presence of organic matter after a certain amount of time. In the current study, the chlorine solution did prevent cross-contamination of the *E. coli* onto the un-inoculated tomatoes; however, the procedure only modeled two cycles of post-harvest tomato processing. Throughout the course of a day, the chlorine’s antimicrobial properties may become depleted and cause cross-contamination as the data by Luo et al. stated. As previously mentioned, pathogenic serovars of *Salmonella* and strains of *E. coli* have been popular in foodborne outbreaks in the past so the findings in this study could greatly affect the future of produce safety. Since the pathogens were not detected in the rinse liquids after initial contamination and after the followers this results in a rinse liquid system that can be re-used for a certain amount of time to actively sanitize the organic produce post-harvest.

**Increased time of organic cherry tomatoes exposed to rinse liquids.** Only *E. coli* was chosen for the next three experiments as a model Gram-negative bacterium. It was assumed that the observations found in those experiments would have the same, or similar, outcomes for the *Salmonella* cocktail.

For the above experiments, the time the tomatoes were in contact with the rinse liquids was always held at 2 mins before the tomatoes were removed for sampling. Since, the log reductions during the initial treatments were not practical more research
was needed to investigate if increasing the treatment time improved the reductions. After holding the treatment time up to 90 mins, *E. coli* on the surface of the tomatoes was decreased by 2.14 log CFU/g in 0.0% NABS, 1.53 log CFU/g in 0.5% NABS and 2.23 log CFU/g in 0.75% NABS, as shown in Figure 2.5. This data shows that increasing the treatment time can increase the efficacy of inhibiting *E. coli* after the initial treatments of NABS by a little more than 2.0 logs CFU/mL if used at 0.75%.

**Testing of rinse liquids at various time intervals after contamination from inoculated organic cherry tomatoes.** In this study, samples of rinse liquids were tested at various time points after contaminated tomatoes were introduced into the systems after initial treatments. This was to determine the time in which the bacterium was being inhibited in the rinse liquids, since it was determined that the NABS was not having a significant effect initially, but was ultimately preventing cross-contamination. After 30 min, the *E. coli* cocktail was reduced by 0.97 logs CFU/mL in 0.0% NABS, 6.46 logs CFU/mL in 0.5% NABS and was reduced > 99.9% in 0.75% NABS (Figure 2.6). These results made it evident that within 30 min, *E. coli* would be eliminated in a contaminated rinse liquid system if NABS was used at 0.75%. During the entire rinse liquid experiments, each experiment took about 30-45 min per treatment. So, when the rinse liquids were tested for survivors and the recovery was below the limit of detection, this result was valid since it was within the time range determined in this section.

**Enumeration during storage after an initial treatment in NABS.** In addition to the above studies, a 96 h storage experiment was conducted to determine a time point in which the *E. coli* cocktail population was decreased on the surface of the tomatoes.
Since it was determined that the cocktails inoculated onto the surface of the tomatoes were not significantly inhibited, it was important to see if there was an extended effect of the NABS over time. The average population actually inoculated onto the surface of the positive controls was 7.43±0.13 log CFU/g. After 96 h, the inoculated tomatoes that were dipped in the 0.0% NABS, the water control, had a reduction of approximately 96.7% compared to the initial population. After 96 h, the tomatoes that were dipped into the 0.5% and 0.75% NABS rinse liquids had a reduction of >99.9% (Figure 2.7).

Abadias et al. (1) also conducted a storage experiment on the apple plugs to determine the effect NABS had on eliminating foodborne pathogens after 6 d. In their study, there were no significant differences among treatments after 6 d. However, they did note that NABS affected the coloring of the apples by turning them a slight brown color during storage. Whereas, another study found that NABS at 0.5% did not significantly affect the overall visual quality of lettuce and escarole after 8 d (4). In the present research, there were no significant visual changes of the tomatoes after storing for 96 h. This could have been due to the rougher skin tomatoes have as compared to leafy greens or fresh-cut apples.

**Conclusion**

In conclusion, NABS was not significantly effective at reducing the microbial load initially on the surface of the tomatoes but it was effective at reducing the amount of cross-contamination of foodborne pathogens to produce. *E. coli* inoculated tomatoes treated with a 200-ppm chlorinated system (with and without OL) achieved a >3.0 log CFU/g reduction, which was greater than the NABS system. However, with any
concentrations of chlorine in excess of 4 ppm, a potable water rinse would be required based on organic guidance, increasing the complexity and risk associated with this antimicrobial. NABS did demonstrate similar efficacy at reducing the potential for cross-contamination and was robust when evaluated with organic loading of the system.
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Appendix II

Tables

**Table 2.1.** MICs of NABS against foodborne pathogens at 37°C (1 NAR).

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella enterica</td>
<td>0.5-0.75%</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>0.5-0.75%</td>
</tr>
</tbody>
</table>
**Table 2.2.** Effect of pH on the efficacy of NABS to inhibit *E. coli* O157:H7.

<table>
<thead>
<tr>
<th>pH</th>
<th>Bacteria growth result</th>
<th>0.5% NABS</th>
<th>0.75% NABS</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>No growth</td>
<td>No growth</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>No growth</td>
<td>No growth</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Growth*</td>
<td>Growth*</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Growth*</td>
<td>Growth*</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Growth*</td>
<td>Growth*</td>
<td></td>
</tr>
</tbody>
</table>

*Less than positive control*
Table 2.3. Surviving *Salmonella enterica* on the surface of organic cherry tomatoes (log CFU/g) after treatment in 0.0%, 0.5%, or 0.75% NABS with and without organic load (OL).

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Treatment</th>
<th>Bacteria population (log CFU/g)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.0% OL</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td>None (control)</td>
<td>9.66±0.10^A</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>9.33±0.03^A</td>
</tr>
<tr>
<td></td>
<td>0.5% NABS</td>
<td>9.33±0.03^A</td>
</tr>
<tr>
<td></td>
<td>0.75% NABS</td>
<td>9.26±0.27^A</td>
</tr>
</tbody>
</table>

*Letters in same group are not statistically different.*
Table 2.4. Surviving *E. coli* on the surface of organic cherry tomatoes (log CFU/g) after treatment in 0.0%, 0.5%, or 0.75% NABS with and without organic load (OL).

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Treatment</th>
<th>Bacteria population (log CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.0% OL</td>
</tr>
<tr>
<td>None (control)</td>
<td></td>
<td>8.78±0.43&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>8.43±0.36&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>200 ppm NaOCl</td>
<td>5.06±1.17&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.5% NABS</td>
<td>8.44±0.27&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.75% NABS</td>
<td>8.17±0.51&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Letters in same group are not statistically different*
**Figure 2.1.** *Salmonella* detected on clean organic cherry tomatoes after introducing to rinse liquids with and without OL that were previously used to wash inoculated tomatoes: Follower 1 (A) and Follower 2 (B). The horizontal line represents the limit of detection equaling to 2.7 log CFU/g. Error bars represent standard deviation of three means; if no error bars are shown that means the standard deviation was equal to zero. For both plots (A and B), letters in same group are not statistically different.
Figure 2.2. *E. coli* detected on clean organic cherry tomatoes after introducing to rinse liquids with and without OL that were previously used to wash inoculated tomatoes: Follower 1 (A) and Follower 2 (B). The horizontal line represents the limit of detection equaling to 2.7 log CFU/g. Error bars represent standard deviation of three means; if no error bars are shown that means the standard deviation was equal to zero. For both plots (A and B), letters in same group are not statistically different.
Figure 2.3. Survival of *Salmonella* in the rinse liquids after the initial treatments and followers. The horizontal line represents the limit of detection equal to 0.95 log CFU/mL. Error bars represent standard deviation of three means; if no error bars are shown that means the standard deviation was equal to zero. Letters in same group are not statistically different.
Figure 2.4. Survival of *E. coli* in the rinse liquids after the initial treatments and followers. The horizontal line represents the limit of detection equal to 0.95 log CFU/mL. Error bars represent standard deviation of three means; if no error bars are shown that means the standard deviation was equal to zero. Letters in same group are not statistically different.
Figure 2.5. *E. coli* enumerated from the surface of the cherry tomatoes after soaking in 0-0.75% NABS for up to 90 min. Error bars represent the standard deviation of two means.
Figure 2.6. *E. coli* reductions in the 0-0.75% NABS after incubation at ambient temperature for up to 30 min. Error bars represent the standard deviation of three means.
Figure 2.7. Surviving *E. coli* log reductions on the surface of the organic cherry tomatoes treated with 0-0.75% NABS followed by storage for up to 96 h. Error bars represent the standard deviation of three means.
Chapter III

The use of a commercial naturally-occurring antimicrobial-based sanitizer to prevent cross-contamination of *Escherichia coli* O157:H7 cocktail on the surface of organic leafy greens
Abstract

The microbiological safety of leafy greens is a growing concern due to the fact that leafy vegetables are the leading cause of foodborne illness outbreaks with 2.2 million cases from 1998-2008. Leafy greens have complex structures and can harbor pathogens in tiny crevices and in the surfaces of fresh-cut leaves. In this study, organic Romaine lettuce and baby spinach were dip-inoculated in an *E. coli* O157:H7 inoculum bath and then placed in various concentrations of a natural antimicrobial-based sanitizer (NABS) rinse liquid with and without the presence of 1.0% organic load (OL). The efficacy of the NABS at reducing pathogenic bacteria on the leafy greens was then determined. To determine if cross contamination was occurring in the NABS rinse liquids, fresh, un-inoculated leafy greens were placed in the shared rinse liquids, samples were taken, and the microbial load from the samples was enumerated. Sampling the rinse liquids at the completion of the study was used to validate the prevention of cross-contamination. For the Romaine lettuce, the 0.75% NABS (with and without OL) was significantly effective at eliminating > 1.0 log CFU/g of the *E. coli* O157:H7. For the spinach, the 0.5% and 0.75% NABS (with and without OL) significantly eliminated ≤ 1.30 log CFU/g of the *E. coli* O157:H7. The 0.5% and 0.75% NABS (with and without OL) was also effective at eliminating the transfer of the contaminated rinse liquid to the fresh, clean lettuce and spinach leaves with > 2.0 log CFU/g reductions when compared to the water controls. This prevention of cross-contamination was validated by not detecting *E. coli* O157:H7 when sampling the rinse liquids. For both leafy greens, NABS (0.5% or 0.75%) had a significant initial reduction
of the *E. coli* O157:H7, also NABS was able to significantly reduce cross-contamination in the rinse liquids.
Introduction

It was estimated that in 2014 the organic industry would reach $35 billion in sales and would continue to grow into 2015 (20). As consumers become more aware of their health and the environment, the consumption of organic, fresh vegetables increases. Produce is known for being the top-selling category in organically grown food since 1985 and it accounted for 43% of U.S. organic sales in 2012 (6). From 1998 to 2008, it was found that out of 17 food commodities, leafy vegetables resulted in the greatest burden of foodborne illnesses with an estimated 2.2 million cases (15). In 2012, an outbreak of Shiga toxin-producing *Escherichia coli* O157:H7 (STEC) on organic spinach and spring mix blend caused 13 out of 33 people infected to be hospitalized (4, 5). From 2011 to 2013, there were two other major outbreaks concerning STEC on Romaine lettuce and Ready-to-Eat (RTE) salads (CDC 2013) (4, 17). These reported outbreaks promote this research to optimize the reduction of foodborne pathogens on raw, organic leafy greens.

Currently, organic farmers are using chlorine or other chemical disinfectants including, peroxyacetic acid or ozone, to inactivate foodborne pathogens on organic leafy greens. The chlorine levels in the organic produce rinse liquids are usually around 50-200 ppm (mg/L), in order to control microbial contaminants. The National Organic Program (NOP) has set specific regulations on keeping the residual chlorine levels in the water at the point of discharge at or below 4.0 ppm set by the Safe Drinking Water Act under the Environmental Protection Agency (EPA). Processors and farmers using chlorine as a disinfectant may not fully understand the chemistry of chlorine e.g. pH
control, free chlorine monitoring, and therefore they may not be achieving its maximum
effectiveness (7). Also, it has been found that chlorine, when used as a disinfectant,
loses its efficacy in the presence of organic matter because it reacts with the organic
materials and forms carcinogenic halogenated by-products (9, 11, 18). Once these by-
products are formed, they do not necessarily transfer onto the produce but they take
away free chlorine levels from the wash systems, therefore decreasing chlorine’s overall
sanitation effectiveness. With these disadvantages of chlorine and chlorine based
sanitizers there is a demand for a natural, stable, and safe sanitizer for the organic
produce industry. However, any sanitizer or disinfectant used on organic produce, post-
harvest, must be on the National List of Allowed and Prohibited Substances that
describes what is allowed and prohibited during the handling of organic products (21).
Moreover, other organically approved sanitizers and synthetic sanitizers have been
shown to have little effect on the reduction of pathogens. Organic acids and plant-
derived essential oils have shown to have greater effects than chlorine on the reduction
of pathogens in a wash system for produce (10, 14).

It is beneficial to have a sanitizing agent in a rinse liquid system that prevents
cross-contamination even in the presence of organic matter to avoid a potential
foodborne pathogenic outbreak. The antimicrobial components of the sanitizer used in
this study are organic acids and bioflavonoids derived from citrus fruit. The sanitizer has
been found to be effective at eliminating microorganisms and reducing microbial loads
on whole and fresh-cut produce (1, 3, 8, 12). The objective of the current study was to
used a natural antimicrobial-based sanitizer (NABS) to determine its potential to reduce
STEC on the surface of USDA-certified organic Romaine lettuce and baby spinach and to prevent cross-contamination in the rinse liquid.

Methods

Bacterial strains and inoculum preparation. Five strains of Shiga toxin-producing *E. coli* O157:H7 (932, H1730, F4546, K3995 and CDC658) were used in this study. The cultures were all obtained from frozen stocks (-18°C in 80% glycerol) from collections at the University of Tennessee in Knoxville, Tennessee. All bacteria strains were consecutively subcultured thrice in 10 mL tryptic soy broth (TSB; Beckon, Dickinson and Company; Sparks, Maryland) at 37°C for 24 h. All strains were made nalidixic acid resistant (NAR) by gradually introducing nalidixic acid (NA; Acros Organics, 99.5%, New Jersey, USA) at increasing concentrations in TSB over 24 h increments until the bacteria were resistant to 40 ppm NA. Pure cultures were isolated and new frozen stocks were prepared in 80% glycerol for the wild type (WT) and NAR strains and stored at -18°C.

Cultures were resuscitated by transferring three times in TSB with 40 ppm NA (TSBN) after which 0.3 mL of each strain was spread plated onto tryptic soy agar (TSA; Fisher Bioreagents® Granulated Agar Fisher Scientific; Fair Lawn, New Jersey, USA) with 40-ppm NA (TSAN) and incubated for 24 h at 37°C. After 24 h each strain was resuspended by adding 5.0 mL of phosphate buffer solutions (PBS; pH 7.2; Beckon, Dickinson and Company; Sparks, Maryland, USA) to the surface of the plates creating a suspension. Suspensions were collected from all 5 strains in a sterile container to
create a 25.0 mL cocktail with a population of approximately 9.0 logs CFU/mL. The cocktail composed of strains of *E. coli* was then used to inoculate the leafy greens.

**Minimum inhibitory concentrations (MICs).** A commercial natural antimicrobial-based sanitizer was obtained from Phyto Innovative Products Ltd., Middlesborough, UK (Citrox 14WP ProGarda Concentrate (NABS, Batch #jj/138/e)). To determine the appropriate concentrations to use in rinse liquids, the MICs, were determined for each NAR strain of *E. coli*. The MICs were conducted using sterile 96-well micro titer plates (250 µL maximum per well). Sanitizer solutions were made using serial twofold dilutions in TSBN. For each sample, 120 µL of the sanitizer solution and 120 µL of each strain of *E. coli* (diluted to 5.0 logs CFU/mL in TSBN) were used. The plates were incubated at 37ºC for 24 h. The optical density at 630 nm (OD₆₃₀) of each well was read at 0 and 24 h using a microtiter plate reader (model Synergy HT, Biotek, Winooski, VT). After 24 h the lowest concentration of sanitizer at which growth was completely inhibited, i.e., an OD₆₃₀ increase of ≤ 0.05 was defined as the MIC (19). To determine if the WT and the NAR strains of *E. coli* had similar resistance characteristics to the sanitizers solutions the MIC study was repeated with the WT strains.

**Inoculation of organic leafy greens.** USDA-certified organic Romaine lettuce and USDA-certified organic baby spinach were purchased from a local grocery store (The Kroger Co. Knoxville, TN). Prior to the experiments, the outer leaves and the core of the Romine lettuce were removed and the remaining leaves were cut into approximately 1.0-inch strips. The pre-washed baby spinach, as labeled, was purchased in a ready–to-eat (RTE) package. 4.0-L of 0.1% peptone water (Bacto™
Peptone; Becton, Dickinson Company; Sparks, Maryland, USA) was poured into a sterile bin and then the 25.0 mL cocktail of *E. coli* was added to create an inoculum bath for dip-inoculation. Then 250.0-g of either Romaine lettuce or baby spinach was added to the inoculum bath for 2 min. After 2 min the leaves were removed using sterilized kitchen tongs and placed onto a sterile surface to dry for 1 h. After 1 h, the leaves were placed into a salad spinner and spun for 2 cycles of 5 spins before being placed onto a clean, dry sterile surface to dry for another hour in a biosafety cabinet.

**Preparation of sanitizer rinse liquids.** In sterile beakers, 500 mL solutions of the liquid NABS was added to sterile deionized water at concentrations (v/v) of 0.0% (pH 7), 0.5% (pH 2.92), or 0.75% (pH 2.76) NABS. In order to determine if there was a decrease in the sanitizer’s efficacy in the presence of organic matter, an organic load (OL) was added to sanitizer rinse liquids. To prepare the OL, either Romaine lettuce or baby spinach was blended with sterile deionized water using a hand-held immersion blender to produce a 2% w/w aqueous solution. The 2% leady green suspension was added to the sanitizer rinse liquids at 1%.

**Initial exposure of organic leafy greens to rinse liquids.** Approximately 25.0-g of either inoculated leafy greens were used as samples for each of the treatments. The inoculated produce samples were aseptically placed into one of the NABS rinse liquids (0.0%, 0.5% and 0.75% sanitizers with and without 1.0% OL) and allowed to soak for 2 min at room temperature (21ºC). Then the treated produce was removed using sterile kitchen tongs and placed into a stomacher bag. Once in the stomacher bag the produce was diluted, 1:5 (w/w), using phosphate buffer solution with 0.2% Tween 80 (Agros
Organic; New Jersey, USA) (PBS/T80). The stomacher bag was then massaged by hand for 15 s. To enumerate for survivors the rinsate was serially diluted in PBS and spread plated in duplicate onto TSAN (for lettuce) or Cefixime Tellurite-Sorbitol MacConkey AGAR (for baby spinach) (CT-SMAC; Oxoid; Basingstoke, Hampshire, England) to grow for 24 h at 37°C. To determine the initial inoculum on the surface of the organic produce, a control sample, receiving no treatments was placed into the buffer, massaged by hand for 15 s, and was plated for enumeration. These experiments were repeated a total of three times at room temperature (21°C).

**Determination of cross-contamination on organic leafy greens.** To determine if cross-contamination was possible un-inoculated produce (approximately 25.0-g of either leafy greens) was introduced into the contaminated rinse liquids with and without 1.0% OL. The fresh produce commodity was allowed to soak for 2 min and was then removed and placed into a stomacher bag. PBS/T80 was added 1:5 (w/w) and then the bags were massaged by hand for 15 seconds. The rinsate was then serially diluted in PBS and spread plated onto TSAN or CT-SMAC in duplicate to grow for 24 h at 37°C. This process was repeated once more for each of the same rinse liquids using new un-inoculated produce. Both cross-contamination studies were repeated three times in total for each rinse liquid at room temperature. These two studies were referred to as Follower one (F1) and Follower two (F2).

**Determination of potential survivors in rinse liquids.** To determine if there was survival of the bacterial cocktails in the sanitizer rinse liquids, samples were taken after each experiment. 100 µL was removed from the containers of each treatment (with
and without 1.0% OL), spread plated onto TSAN or CT-SMAC in duplicate, and placed in the incubator at 37°C for 24 h. Also, 1.0 mL was removed from the containers of each treatment, serially diluted in PBS, spread plated onto TSAN or CT-SMAC in duplicate, and placed in the incubator at 37°C for 24 h. These experiments were repeated three times in total at room temperature (21°C).

**Statistical analysis of experimental design.** All experiments were repeated three times and in each independent repetition samples were taken in duplicate. Treatments were analyzed using the Tukey-Kramer method to determine if there were significant differences among treatments. For the cross-contamination study, the least squared means were sliced so all possible treatments could be compared within the experiments. The software used was SAS 9.3 (SAS Institute Inc., Cary, NC, USA). A level of significance of <0.05 for P values was selected to determine microbial differences.

**Results**

**Minimal inhibitory concentrations.** The MIC study showed that the sanitizer solution at 0.5% had inhibitory effects against the Gram-negative bacteria. The MIC values for the sanitizer were found to be the same for the WT and NAR strains of *E. coli*. Since it is ideal to use the lowest dose of an antimicrobial in a rinse liquid system, the 0.5% sanitizer solution was used in the wash systems, however the 0.75% sanitizer solution was also analyzed.
Initial exposure of organic leafy greens to rinse liquids. The initial population of *E. coli* from the lettuce leaves was 8.14±0.04 log CFU/g. For the inoculated Romaine lettuce introduced into the NABS treatment containers, there was no significant differences between the initial reductions of *E. coli* for the 0.5% and the 0.75% NABS resulting in initial reductions of approximately 1.0 log CFU/g. (Table 3.1). But both of the NABS treatments statistically differed from the inoculated lettuce leaves that did not receive treatment. However, the initial reduction from the 0.5% NABS did not statistically differ from the water control, while the initial reduction from the 0.75% NABS was statistically different than the water control. The 1.0% OL did not affect the antimicrobial activity of NABS for either concentration.

The initial population of *E. coli* from the organic baby spinach leaves was 7.80±0.20 log CFU/g. For the inoculated baby spinach leaves introduced into the NABS treatments, there was no significant differences between the initial reductions of *E. coli* for the 0.5% and the 0.75% NABS resulting in initial reductions of approximately 1.0 log CFU/g. But both of the NABS treatments statistically differed from the inoculated spinach leaves that received no treatment. Both of the recoveries from the spinach leaves introduced into the NABS treatments statistically differed from the water controls and the 1.0% OL did not significantly affect the overall antimicrobial activity of NABS.

Efficacy of the NABS rinse liquids to prevent cross-contamination of *E. coli* onto clean organic leafy greens. Two sets (F1 and F2) of fresh Romaine lettuce or baby spinach were added to the *E. coli* contaminated rinse liquids to determine if the NABS already present would prevent cross-contamination. When the un-inoculated,
fresh Romaine lettuce was added to the *E. coli* contaminated water control containers the recovery was approximately 5.80 log CFU/g (Figure 3.1A and 3.1B) for both F1 and F2. The addition of the 1.0% organic load did not significantly affect the recovery of the *E. coli* on the surface of the lettuce. The recovery that was determined from the lettuce after exposure to the contaminated water control showed that the absence of the NABS resulted in cross-contamination.

When the un-inoculated lettuce was added to the contaminated 0.5% and 0.75% NABS rinse liquids, for F1 and F2, the recovery values were all determined to be statistically different than populations recovered off of the leaves in the water control. All of the populations were found to be at or slightly above the limit of detection (2.70 log CFU/g), with a reduction of approximately > 99.9%, for the 0.5% and the 0.75% NABS solutions. The addition of the OL did not significantly affect the prevention of cross-contamination in the rinse liquids with sanitizer (Figure 3.1A and 3.1B).

For the baby spinach (Figure 3.2A and 3.2B), approximately 5.80 log CFU/g was recovered off of the leaves after being exposed to the contaminated system with 0.0% sanitizer for F1 and F2. The addition of the OL did not make a significant effect on the outcome of the cross-contamination detected from the leaves. When compared to the population recovered off of the spinach leaves initially, 7.26 and 7.16 log CFU/g with and without OL respectively (Table 3.1). The amount recovered from the spinach leaves after F1 and F2 verified that the absence of NABS in the rinse liquids did not prevent cross-contamination. When fresh, clean spinach was added to the contaminated 0.5% and 0.75% NABS rinse liquids, the amount of *E. coli* recovered off of the leaves was
either at or slightly above the limit of detection (2.70 log CFU/g), with all values being significantly different than the water controls (Figure 3.2A and 3.2B). This observation was true for F1 and F2 for both concentrations of the NABS. The reduction was approximately > 99.9% for both the 0.5% and the 0.75% NABS solutions. Also, the addition of the 1.0% OL did not affect the prevention of cross-contamination onto the fresh spinach leaves.

**Determination of survivors in the rinse liquids.** After the initial treatments of the inoculated leafy greens and the cross-contamination studies water samples were taken and enumerated for potential survivors. For lettuce and spinach (Figure 3.3 and 3.4), there was a population of approximately 5.30 log CFU/mL recovered from water controls with and without OL. The survival of the *E. coli* cocktail in the water controls confirmed the cross-contamination transferred onto the fresh, clean leafy greens. When samples were taken from the 0.5% and 0.75% NABS rinse liquid containers for the spinach and the lettuce the populations were below the limit of detection (0.95 log CFU/mL), supporting the efficacy of these compounds to prevent cross-contamination.

**Discussion**

The current research and in accordance with López-Gálvez et al (12), found that the initial treatment in NABS had little effect on the reduction of the microbial load on the surface of the inoculated leafy greens. *E. coli* strongly attaches to the leaf epidermis making it hard for decontamination agents to completely inactivate the pathogenic microorganisms present, but slightly reducing the overall microbial load (16). Ultimately, both of the NABS rinse liquids showed the same efficacy in the initial reduction of the *E.
coli cocktail (~8.0 log CFU/mL) on the inoculated spinach and lettuce leaves. However, Lopez-Galvez et al. (2009) found an approximate 1.50 log CGU/g reduction when fresh-cut lettuce inoculated with *E. coli* was treated 0.5% NABS. Their findings were similar to the approximately 1.30 log CFU/g reduction of the *E. coli* from the spinach leaves when washed in the 0.75% sanitizer from this study. In the study of López-Gálvez et al. (2009) inoculum level of 3.1 log CFU/g was utilized, which was less than the 8.0 log CFU/mL cocktail used in the current study. Using a higher inoculum level is beneficial because it shows that the sanitizer rinse liquid can eliminate a higher amount of microorganisms on the surface of the produce, while still producing similar reductions. Allende et al. (3) was able to achieve a greater coliform reduction, 2.3 log CFU/g, using 0.5% NABS on the surface of fresh-cut lettuce. They determined that in a rinse liquid modeling system NABS was more effective than just using water alone and in some cases it was better than other commercially available sanitizers including, Purac, Sanoxol, Sanova, Catallix, and chlorine. Also, in a study by Akbas et al. (2), they found that dipping iceberg lettuce in a solution of 0.5% citric acid or lactic acid, two widely used organic acids that could be present in NABS, caused a reduction of *E. coli* and *Listeria* that was as effective as 100-ppm free chlorine. Their study did not include the negative effects of the interaction of organic matter in the chlorine wash solution, which has been shown to dramatically impact efficacy (7, 13). Abadias et al. (1), had an objective to evaluate alternative sanitizers to chlorine in order to reduce foodborne pathogens on fresh cut apple plugs. They found that there was only a 1.0 log reduction when the apple plugs were initially treated with a NABS solution and a sodium hypochlorite (SH) solution; however, there
was prevention of cross-contamination of *E. coli*. In their study, the effect of organic matter on the efficacy of chlorine was not analyzed in order to determine if NABS was overall more effective than chlorine.

Considering, that the previous research studies conducted on NABS showed similar results as the current study, most did not test NABS in the presence of organic matter. The current study showed that the NABS was able to significantly prevent cross-contamination with and without the presence of 1.0% organic matter when compared to the water controls in both the spinach and lettuce studies. Which was verified by testing the treatment liquids after the initial treatments and after F1 and F2 (Figure 3.3A and 3.3B). This demonstrates that this new antimicrobial system inactivates foodborne pathogens as well as other commercially available systems and does not have drawbacks (e.g. organic interactions) of some widely used antimicrobials such as chlorine.

NABS has been tested as an alternative disinfectant to chlorine for the use of conventional produce but not a lot of research has been conducted for its use in the organic produce industry. It is essential to find an alternative to chlorine that meets all of the regulatory guidelines enforced by the USDA organic program. Not only does the sanitizer have to meet these guidelines but also it needs to prevent cross-contamination whether used in the presence of an organic load. NABS has proved, through this study, that it may not completely eliminate the population of *E. coli* on the surface of organic leafy greens but once fresh leafy greens are washed the NABS solution prevents cross-contamination of the highly populated inoculum in the rinse liquids.
Conclusion

Overall, the 0.5% and the 0.75% NABS did not result in practical initial reductions (log reductions > 3.0) of the *E. coli* cocktail from the leafy greens. However, for both of the leafy green experiments the NABS treatments were statistically different than the inoculated leaves that did not receive treatments. Considering the reductions were not greater than 3.0 log CFU/g, which would have been ideal, the NABS solutions did eliminate the possibility of cross-contamination in the rinse liquids. Preventing the transfer from contaminated wash liquid to fresh, un-contaminated produce makes all the difference when trying to prevent a foodborne outbreak. Also, the OL did not affect the overall antimicrobial activity of the NABS.
References


Table 3.1. Surviving bacteria on the surface of organic leafy greens (log CFU/g) after treatment in 0.0%, 0.5%, or 0.75% sanitizer treatments with and without 1.0% organic load (OL).

<table>
<thead>
<tr>
<th>Microorganism cocktail</th>
<th>Organic Produce Commodity</th>
<th>Treatments</th>
<th>Bacteria Population (log CFU/g)**</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>0.0% OL</td>
<td>1.0% OL</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Romaine Lettuce*</td>
<td>None</td>
<td>8.14±0.04^A</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>0.0% (water control)</td>
<td>7.55±0.13^B</td>
<td>7.55±0.06^B</td>
</tr>
<tr>
<td></td>
<td>0.5% NABS</td>
<td>7.15±0.22^BC</td>
<td>7.29±0.13^BC</td>
</tr>
<tr>
<td></td>
<td>0.75% NABS</td>
<td>7.05±0.09^C</td>
<td>7.10±0.21^C</td>
</tr>
<tr>
<td>Baby Spinach*</td>
<td>None</td>
<td>7.80±0.20^A</td>
<td>---</td>
</tr>
<tr>
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<td>0.0% (water control)</td>
<td>7.16±0.21^BC</td>
<td>7.26±0.28^AB</td>
</tr>
<tr>
<td></td>
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<td>6.70±0.81^D</td>
<td>6.65±0.22^CD</td>
</tr>
<tr>
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<td>0.75% NABS</td>
<td>6.52±0.13^D</td>
<td>6.53±0.28^D</td>
</tr>
</tbody>
</table>

*Romaine lettuce and Baby spinach letter groupings are not comparable to each other.

**Letters in the same group are not statistically different.
Figure 3.1. *E. coli* detected on clean organic Romaine lettuce after introducing to rinse liquids with and without OL that were previously used to wash inoculated lettuce: Follower 1 (A) and Follower 2 (B). The horizontal line represents the limit of detection equaling to 2.7 log CFU/g. Error bars represent standard deviation of three means; if no error bars are shown that means the standard deviation was equal to zero. For both plots (A and B), letters in same group are not statistically different.
Figure 3.2. *E. coli* detected on clean organic baby spinach after introducing to rinse liquids with and without OL that were previously used to wash inoculated spinach: Follower 1 (A) and Follower 2 (B). The horizontal line represents the limit of detection equaling to 2.7 log CFU/g. Error bars represent standard deviation of three means; if no error bars are shown that means the standard deviation was equal to zero. For both plots (A and B), letters in same group are not statistically different.
Figure 3.3. Survival of *E. coli* in the rinse liquids after the initial treatments and followers. The horizontal line represents the limit of detection equal to 0.95 log CFU/mL. Error bars represent standard deviation of three means; if no error bars are shown that means the standard deviation was equal to zero. Letters in same group are not statistically different.
Figure 3.4. Survival of *E. coli* in the rinse liquids after the initial treatments and followers. The horizontal line represents the limit of detection equal to 0.95 log CFU/mL. Error bars represent standard deviation of three means; if no error bars are shown that means the standard deviation was equal to zero. Letters in same group are not statistically different.
Chapter IV

The use of a commercial naturally-occurring antimicrobial-based sanitizer to prevent cross-contamination of *Listeria monocytogenes* on the surface of organic cantaloupes
Abstract

The safety of cantaloupes has been of major concern since the 2011 Jensen Farm *Listeria* outbreak. An effective sanitizer is needed to reduce the initial microbial load on the surface of cantaloupes, prevent cross-contamination, and work in the presence of organic loads. The sanitizer used in this study was a natural antimicrobial-based sanitizer, NABS, composed of organic acids and phenolic compounds. This sanitizer is approved for use on organic produce in the United Kingdom. Cantaloupe rinds were inoculated with a 5-strain cocktail of *Listeria monocytogenes* and were treated in a water control, 200-ppm chlorine, 0.5% NABS, and 0.75% NABS all with and without the presence of 2.0% organic load. To determine if cross-contamination of *Listeria* in the rinse was occurring, un-inoculated cantaloupe rinds were placed into the shared rinse liquids. The prevention of cross-contamination was verified by sampling the treatment liquids at the completion of the cross-contamination study. Initially, the 0.5% NABS and the 200-ppm chlorinated treatment were the most effective sanitizers tested because they reduced the microbial load initially by 1.0 log CFU/g. However, 0.5% NABS and 200-ppm chlorine were affected by the presence of the OL in the rinse liquids. 0.75% NABS did not have significant initial reduction when compared to the water control, however, it was less affected by the OL and prevented cross-contamination of the *Listeria* cocktail onto fresh cantaloupe rinds. This was verified by testing the treatment liquids at the end of each experiment for the potential survival of *Listeria*, indicating the prevention of cross-contamination for the 0.75% NABS rinse liquid. Further studies are needed to determine an effective method for improving the
initial reduction of the microorganism on the surface of the cantaloupe rind, which would further improve the prevention of cross-contamination in the rinse liquids.
Introduction

Cantaloupes are among some of the healthiest fruits because they are high in potassium, Vitamin A, Vitamin C, folic acid, micronutrients, and protein content (10). It has been determined that Americans consumed 8.7 pounds of cantaloupes per year due to their sweet, fresh taste and their nutritional content (5). In 2013, the U.S. value of cantaloupe production was $319 million, which results in cantaloupes being a major contributor to the produce industry. Cantaloupes are known for their complex, netted surface structure, which makes the attachment of foodborne pathogens easy and the inactivation of the pathogens difficult during post-harvest handling. This produce commodity has been known to harbor foodborne pathogens like *Listeria monocytogenes*, *E. coli* O157:H7, *Salmonella*, Norovirus, *Shigella*, and *Campylobacter*. However, most of the outbreaks associated with foodborne illnesses after consumption of cantaloupes have been associated with *Salmonella* and *Listeria*. In 2011, cantaloupes from Jensen Farms in Holly, CO caused a major outbreak of Listeriosis, resulting in the deaths of 33 people making this outbreaks the deadliest since 1924 (2). *Listeria* can be isolated from soil, sewage, and irrigation water, resulting in it being more likely to be found on the surfaces of cantaloupes since they are continuously in contact with irrigation water during pre- and post-harvesting (3). The irrigation water can come from a variety of sources including pond water, municipal water, wastewater, rivers, lakes, and wells and all of these sources have the potential to be contaminated with pathogenic bacteria. Once the cantaloupes are harvested they go through a sanitizing
step to eliminate any possible contamination that could have occurred during irrigation (11).

So far, the cantaloupe outbreaks reported were all associated with conventional cantaloupes but a major concern develops when microbiological safety is evaluated for organic cantaloupes. The United States Department of Agriculture (USDA) has set regulations regarding what can be used as a sanitizer on organic produce in order for it to remain organic. These regulations put organic produce at a higher risk of pathogenic contamination than conventional produce. The most common type of sanitizer allowed to be used on organic produce is chlorine at concentrations ranging from 100-200 ppm, however, there are other sanitizers used including ozone, peracetic acid, and chlorine dioxide. Even though chlorine is widely used in the organic industry, free residual chlorine available in the sanitizing solution reacts with organic matter also present and produces carcinogenic organochlorine compounds (7). Essential oils and organic acids have been found to exhibit natural antimicrobial properties and are generally-recognized-as-safe (GRAS) making it easy for them to be approved as sanitizers for organic produce (8, 15). These essential oils and/or organic acids have low water solubility and usually need to be used in combination with surfactants to create stable emulsions. These surfactants also work in combination to help deliver the antimicrobial to the crevices and ridged surfaces of organic produce like cantaloupes.

The first objective of this research was to use a commercial sanitizer composed of citrus fruit derived organic acids and a surfactant in the presence and absence of an organic load to eliminate a cocktail of *L. monocytogenes* strains on the surface of
organoc cantaloupe rinds. The second objective was to determine if the commercial sanitizer was more effective than 200-ppm chlorine in the presence and absence of an organic load at eliminating the *Listeria* cocktail. Also, it was determined if the commercial sanitizer was effective at preventing cross-contamination in the rinse liquids after the contaminated cantaloupes were introduced into the system.

**Methods**

**Bacterial strains and inoculum preparation.** A five-strain cocktail of *L. monocytogenes* (LM1, 310, Scott A, V7, and LM2) was used in this study. The cultures were all obtained from frozen stocks (-18°C in 80% glycerol) from collections at the University of Tennessee in Knoxville, Tennessee. All bacteria strains or serovars were consecutively subcultured thrice in 10 mL tryptic soy broth with 0.6% yeast extract (Bacto™ Tryptic Soy Broth and Bacto™ Yeast Extract; Beckon, Dickinson and Company; Sparks, Maryland, USA) (TSBYE) at 32°C for 24 h. All strains were made nalidixic acid resistant (NAR) by gradually introducing nalidixic acid (NA; Acros Organics, 99.5%, New Jersey, USA) at increasing concentrations in TSBYE over 24 h increments until the bacteria were resistant to 40 ppm NA. Pure cultures were isolated and new frozen stocks were prepared in 80% glycerol for the wild type (WT) and NAR strains and stored at -18°C.

Cultures were resuscitated by transferring three times in TSBYE with 40 ppm NA (TSBNYE) after which 0.3 ml of each strain was spread plated onto tryptic soy agar with 0.6% yeast extract and 40-ppm NA (TSA; Fisher Bioreagents® Granulated Agar Fisher Scientific; Fair Lawn, New Jersey, USA) (TSANYE) and incubated for 24 h at 32°C.
After 24 h each strain was re-suspended by adding 5.0 mL of phosphate buffer solution (PBS; pH 7.2; Beckon, Dickinson and Company; Sparks, Maryland, USA) to the surface of the plates and creating a suspension. Suspensions were collected from all five strains in a sterile container to create a 25.0 mL cocktail with a population of approximately 9.0 log CFU/mL. The cocktail of *Listeria* was then used to spot inoculate the cantaloupe rind squares.

**Minimum inhibitory concentrations (MICs).** A commercial natural antimicrobial-based sanitizer was obtained from Phyto Innovative Products Ltd., Middlesborough, UK (Citrox 14WP ProGarda Concentrate (NABS, Batch #jj/138/e)). To determine the appropriate concentrations to use in rinse liquids, the MICs, were determined for each NAR strain of *Listeria*. The MICs were conducted using sterile 96-well (250 µL maximum per well) microtiter plates. Sanitizer solutions were made using serial twofold dilutions in TSBNYE. For each sample, 120 µL of the NABS solution and 120 µL of each serovar of *Listeria* (diluted to 5.0 log CFU/mL in TSBNYE) were used. The plates were incubated at 37°C for 24 h. The optical density at 630 nm (OD\textsubscript{630}) of each well was read at 0 and 24 h using a microtiter plate reader (model Synergy HT, Biotek, Winooski, VT). After 24 h the lowest concentration of sanitizer at which growth was completely inhibited, i.e., an OD\textsubscript{630} increase of ≤ 0.05 was defined as the MIC (12).

To determine if the WT and the NAR serovars had similar resistance characteristics to the sanitizer the MIC study was repeated with the WT strain of *Listeria*.

**Inoculation of organic cantaloupes.** Organic cantaloupes (imported from Mexico) were purchased from a local grocery store (Whole Foods, Knoxville,
Tennessee). The surfaces of several cantaloupes were marked with 2.5 x 2.5 cm squares and then the squares were aseptically cut out. The attached cantaloupe pulp was trimmed away leaving a thin square of the rind. Then the squares were placed in a water bath at 71°C for 5 min to inactivate any pre-existing background micro flora. Subsequently, the squares were removed and allowed to dry in a biosafety cabinet for 1 h. After this hour the squares were spot inoculated with 200 µL of the Listeria cocktail (10-10 µL spots or 100 µL per square). Once the cantaloupe rind samples were aseptically inoculated with the appropriate cocktail, samples were allowed to dry on sterile surfaces in a biosafety cabinet for 1 h.

**Preparation of sanitizer rinse liquids.** All sanitizer rinse liquids were prepared as 100 mL solutions (v/v%, prepared with sterile de-ionized water) in sterile beakers. Eight different rinse liquids were prepared: 200 ppm free residual chlorine (Clorox; Oakland, CA, USA), 200 ppm free residual chlorine with 2.0% organic load (OL), 0.0% NABS (pH 7), 0.0% NABS with 2.0% OL, 0.5% NABS (pH 2.92), 0.5% NABS with 2.0% OL, 0.75% NABS (pH 2.76), and 0.75% NABS with 2.0% OL. In order to determine if there was a decrease in the sanitizer’s efficacy in the presence of organic matter, an OL was added to the sanitizer rinse liquids. To prepare the OL, organic cantaloupe pulp and rind were blended using a hand-held immersion blender with sterile deionized water to produce a 20.0% w/w aqueous suspension. The 20.0% (w/w) cantaloupe suspension was added to sanitizer rinse liquids treatments at 2.0%. The two treatments containing chlorine were tested for free residual chlorine using a Free Chlorine & Chlorine Ultra High Range ISM (Hanna Instruments, Roonsocket, RI) immediately before use.
Initial exposure of organic cantaloupes to sanitizer rinse liquids. Two of the inoculated cantaloupe squares were used for each treatment. The inoculated produce samples were aseptically placed into one of the rinse liquids (200 ppm free residual chlorine, 0.0%, 0.5% and 0.75% NABS with and without 2.0% OL) and allowed to soak for two min. Then the treated produce was removed using sterile kitchen tongs and placed into a stomacher bag. Once in the stomacher bag the produce was diluted, 1: 5 (w/w), using phosphate buffer solution with 0.2% Tween 80 (Agros Organics; New Jersey, USA) (PBS/T80). The stomacher bag was then rubbed by hand for 15 s. To enumerate for survivors the rinsate was serially diluted in PBS and spread plated in duplicate onto TSANYE to grow for 24-48 h at 32ºC. To determine the initial inoculum on the skin of the organic produce a control sample, that received no treatments, was placed into the buffer, massaged by hand for 15 s, and was plated for enumeration. These experiments were repeated a total of three times at room temperature (21ºC).

Determination of cross-contamination on organic cantaloupes. To determine if cross-contamination was possible un-inoculated produce (two fresh, un-inoculated cantaloupe squares) was introduced into the contaminated rinse liquids with and without 2.0% OL. The fresh produce commodity was allowed to soak for 2 min and was then removed and placed into a stomacher bag. PBS/T80 was added 1:5 (w/w) and then the bags were rubbed by hand for 15 s. The rinsate was then serially diluted in PBS and spread plated onto TSANYE in duplicate to grow for 24 h at 32ºC. This process was repeated once more for each of the same rinse liquids using new un-inoculated produce. These two studies were referred to as Follower one (F1) and
Follower two (F2) because they followed behind the initial treatment. Both cross-contamination studies were repeated three times in total for each rinse liquid at room temperature.

**Determination of survivors in sanitizer rinse liquids.** To determine if there was survival of the *Listeria* cocktail in the NABS or chlorine rinse liquids, samples were taken from the rinse liquids containers after each experiment. 100 µL was removed from the containers of each treatment (with and without 2.0% OL), spread plated onto TSANYE in duplicate, and placed in the incubator at 32ºC for 24 h. Also, 1.0 mL was removed from the containers of each treatment, serially diluted in PBS, spread plated on TSANYE in duplicate, and placed in the incubator at 32ºC for 24 h. These experiments were repeated three times in total at room temperature (21ºC).

**Statistical analysis of experimental design.** All experiments were repeated three times and in each independent repetition samples were taken in duplicate. Treatments were analyzed using the Tukey-Kramer method to determine if there were significant differences among treatments. For the cross-contamination study, the least squared means were sliced so all possible treatments could be compared within the experiments. The software used was SAS 9.3 (SAS Institute Inc., Cary, NC, USA). A level of significance of <0.05 for P values was selected to determine microbial differences.
Results

**Minimum inhibitory concentrations.** The MIC study showed that NABS had inhibitory effects against the Gram-positive bacteria. The MIC values, 0.5-0.75% NABS, were found to be the same for all WT and NAR serovars of *L. monocytogenes*. Since it is ideal to use the lowest dose of an antimicrobial in a rinse liquids system, the lowest possible concentration where growth was inhibited, 0.5% NABS, was chosen for this study, however the next highest concentration (0.75% NABS) was also analyzed.

**Initial exposure of organic cantaloupes to sanitizer rinse liquids.** The inoculated squares that received no treatment exhibited the initial population of the *Listeria* cocktail on the surface of the organic cantaloupe squares, which was 9.35±0.16 log CFU/g. When the inoculated cantaloupe squares were introduced into the rinse liquids containing the water control, with or without the organic load, the recovery from the squares was not significantly different than the samples that did not receive treatment. Compared to the water control, the most significant recovery occurred when the samples were treated with 0.5% NABS, showing approximately 1.0 log CFU/g reduction of the *Listeria*. 0.75% NABS was not as effective as 0.5% NABS and 200 ppm chlorine as it was not statistically different than the samples that did not receive treatment or the water control. The NABS manufacturer’s recommended dose was reported to be 0.5% NABS and this result proves that it is more effective at this concentration than at higher concentrations (1). The chlorine rinse liquid was able to inhibit < 1.0 log CFU/g on the inoculated samples introduced into the system when compared to the water control. The chlorine treatment was not statistically different than
the 0.5% NABS treatment. Overall, the addition of the organic load to the rinse liquids did statistically affect the reduction of the *Listeria* for the 0.5% NABS and this recovery was not statistically different than the controls. Since the 0.5% NABS was affected by the OL, the 0.75% NABS and the 200-ppm chlorine treatments were more effective at initially reducing the *Listeria* from the cantaloupe rinds.

**Determination of cross-contamination on organic cantaloupes.** To determine if cross-contamination occurred fresh, un-inoculated cantaloupe rind squares were added to the same rinse liquids after the initial treatments. Two sets of fresh produce were added, which were referred to as Follower one (F1) and follower two (F2). For the water control samples, the population of the *Listeria* cocktail transferred from the contaminated rinse liquids to the fresh produce was 6.80±0.32 log CFU/g for F1 and 6.50±30 log CFU/g for F2. The addition of the 2.0% OL did not affect this recovery for the water control. The high recoveries from the water controls indicated that there was transfer from the contaminated rinse liquids to the fresh cantaloupe squares. This confirmed that water alone in a rinse liquids system in not efficient enough to eliminate the transfer of foodborne pathogens.

When the fresh cantaloupe samples were added to the contaminated 0.5% NABS, 0.75% NABS, and the chlorine treatments, the log reductions were greater than what was determined for the water control treatments. All of the treatments were statistically different than the water controls. However, for F1, the 0.5% NABS and the chlorine treatment were affected by the OL and the 0.75% NABS was not affected by the OL. For F2, only the chlorinated treatment was affected by the OL. For the cross-
contamination studies without the OL the chlorine was able to prevent the transfer of the *Listeria* onto the clean cantaloupe rinds. Nonetheless, 0.75% NABS was less affected by the OL when compared to the other treatments, however, it was not significantly different than the 0.5% NABS.

**Determination of survivors in rinse liquids.** After the initial treatments and the cross-contamination study, samples of the rinse liquids were taken in order to determine if the *Listeria* cocktail was still active, hence, causing cross-contamination. In the water control samples almost full recovery of the *Listeria* cocktail was enumerated from the treatment containers. In the 0.5% and the 0.75% NABS rinse liquid containers, with and without the organic load, a lower population of *Listeria* was recovered from the solutions when compared to the water controls, which is the reason there was some recovery during F1 and F2. For the chlorine treatment solutions, there was zero recovery of the *Listeria* cocktail in the container without the organic load and 5.36 log CFU/mL was recovered from the container with the organic load. Hence, verifying the cross-contamination of the cocktail to the squares in the chlorine treatment with organic load.

**Discussion**

The 0.5% NABS treatment resulted in a 1.0 log CFU/g initial reduction of the *Listeria* cocktail and the 200-ppm chlorine solution also resulted in an initial reduction of approximately 1.0 log CFU/g. NABS at 0.5% was the only case where the addition of the organic load affected the recovery after the initial treatment. The minimal log reductions can be attributed to the attachment of the *Listeria* to the rind of the cantaloupe. Once the cocktail was inoculated onto the rind and allowed to dry, the cells
dried into the crevices of the netted cantaloupe surface and escaped the contact of the disinfectant. The addition of the organic load did not improve the contact of the sanitizer to the surface of the melon. In a study by the California Cantaloupe Advisory Board, they determined that scrubbing the cantaloupes with a wash of 200-ppm hypochlorite, or an equally effective sanitizer, to get the maximum reduction of potential surface contamination (11). The scrubbing would help to loosen the *Listeria* that was attached to the rind, while the sanitizer would eliminate the viability of the pathogen. However, this method would be time consuming because a handler would have to scrub each cantaloupe during processing. In a study by Ukuku et al. (14), different methods for applying sanitizers to whole-cantaloupes were analyzed. It was determined that when 200-ppm chlorine was added to a rinse liquid with agitation or rubbing, the *Salmonella* cocktail was significantly reduced when compared to dipping or dipping with rotation methods. This result is consistent with the findings of Sapers et al. (9) who determined that washing alone cannot achieve a level of decontamination exceeding 99.9% (3 log) population reduction of pathogens on apples and cantaloupe rinds.

Overall, the NABS and the chlorine solutions without the organic load were able to reduce the transfer of the cocktail to the fresh, un-inoculated cantaloupe samples. In decreasing order are how effective the treatments were in preventing cross-contamination: 200 ppm Cl > 0.5% NABS > 0.75% NABS > 0.75% NABS OL > 0.5% NABS OL > 200 ppm Cl OL > 0.0% NABS OL > 0.0% NABS. The 200-ppm Cl solution was ultimately the best sanitizer to eliminate cross-contamination, however, when the organic load was added the antimicrobial properties of the solution were depleted and
cross-contamination occurred. Luo et al. (7), proved that when organic matter is introduced into a chlorinated system the free chlorine concentrations change immediately and cross-contamination will occur. The measured free chlorine is defined as the residual chlorine available after satisfying the chlorine demand of organic materials. The chlorine present in the system was consumed in the reaction with the organic load, leaving minimal residual free chlorine in the rinse liquids and resulting in cross-contamination of the *Listeria* cocktail as seen in Figure 4.1 A and 4.1B. In Figure 4.2, the recovery of the cocktail in the chlorinated treatment with the organic load verified that the free chlorine was tied up in the reaction with the organic mater and the same was not true for the chlorinated system without the organic load.

In a similar study, under identical conditions with a variance in produce type, it was determined that chlorine was affected by the organic matter in the rinse liquids when eliminating *Salmonella* from the surface of organic cherry tomatoes (4). In another study by Ukuku et al. (13), the researchers investigated the inactivation of attached *Listeria monocytogenes* from cantaloupe surfaces using 1,000-ppm chlorine and 5% hydrogen peroxide. The authors also studied the efficacy of the sanitizers to prevent transfer of the pathogen to fresh cantaloupe rinds. It was determined that 1,000-ppm chlorine and 5% hydrogen peroxide caused significant reductions of the pathogenic bacteria and for their cross-contamination study, they found that the cantaloupe plugs that contained 3.26 log CFU/cm² of the pathogen was transferred to the fresh cantaloupe rinds. These observations are consistent with the current research because
cross-contamination was observed when 200-ppm chlorine was used as a sanitizer rinse liquid.

It was proven that the recommended dosage for NABS, 0.5%, was the most effective dose for the elimination of the cocktail on the surface of the cantaloupe samples. As shown in Figure 4.1, the 0.5% NABS solution was not significantly different than the chlorine treatment meaning it was just as effective at preventing cross-contamination, except for the case of 0.5% NABS with OL in Figure 4.1B. For most cases during the cross-contamination study the 0.75% NABS solution was significantly different than the chlorine solution. In either case, it was not as effective as the chlorine; however, it was not statistically different than the 0.5% NABS. It was determined, for a similar formulation of NABS (BC30 versus 14WP), that it was not effective at inhibiting oral microorganisms when tested at concentrations higher than 1.0% (6). When the organic load was added to the treatments, there was cross-contamination recovered from all treatments but the chlorine and the water controls recovered the highest amounts of bacteria, making NABS, at 0.75%, the most effective sanitizer for the elimination of *Listeria* on the surface of the cantaloupes. Further research would need to be conducted to determine if scrubbing would improve the overall effect of the 0.75% NABS for the safety of organic cantaloupes.
Conclusion

Washing cantaloupes with water alone is not sufficient to maintain the safety of this problematic produce commodity. The gold standard of produce disinfecting, 200-ppm chlorine, was also proved to not be sufficient enough to be used as a sanitizer when an organic load is present in the rinse liquids system. 0.75% NABS was determined to be the most effective sanitizer even in the presence of the organic load. More research needs to be conducted in order to determine a better method to inhibit the microbial load from the surface of the cantaloupes. If the initial microbial load is reduced then the potential for cross-contamination in the treatment liquids will also be reduced. However, an effective sanitizing agent will still be recommended in rinse liquids to prevent any foodborne pathogen related outbreaks.
References

Appendix IV

Tables

Table 4.1. Surviving Listeria cocktail on the surface of organic cantaloupes (log CFU/g) after treatment in 0.0%, 0.5%, 0.75%, or 200 ppm NaOCl sanitizer treatments with and without 1.0% organic load (OL).

<table>
<thead>
<tr>
<th>Microorganism cocktail</th>
<th>Organic Produce Commodity</th>
<th>Treatments</th>
<th>Bacteria Populations (log CFU/g)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.0% OL</td>
<td>1.0% OL</td>
</tr>
<tr>
<td><strong>Listeria</strong></td>
<td>Cantaloupes</td>
<td>None</td>
<td>9.35±0.16^A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200 ppm NaOCl</td>
<td>8.77±0.20^BC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0% (water control)</td>
<td>9.42±0.19^A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5% NABS</td>
<td>8.49±0.31^C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.75% NABS</td>
<td>9.15±0.23^AB</td>
</tr>
</tbody>
</table>

* Letters in the same group are not statistically different.
Figure 4.1. *Listeria* detected on clean organic cantaloupes after introducing to rinse liquids with and without OL that were previously used to wash inoculated cantaloupe rinds: Follower 1 (A) and Follower 2 (B). The horizontal line represents the limit of detection equaling to 2.7 log CFU/g. Error bars represent standard deviation of three means; if no error bars are shown that means the standard deviation was equal to zero. For both plots (A and B), letters in same group are not statistically different.
Figure 4.2. Survival of *Listeria* in the rinse liquids after the initial treatments and followers. The horizontal line represents the limit of detection equal to 0.95 log CFU/mL. Error bars represent standard deviation of three means; if no error bars are shown that means the standard deviation was equal to zero. Letters in same group are not statistically different.
Conclusion

The collective objective of this study was to analyze a natural antimicrobial-based sanitizer, NABS, for its abilities to reduce the population of pathogenic bacteria attached to the outer surface of organic produce. In addition to NABS’s initial abilities to reduce pathogenic bacteria, it was also analyzed for its potential to reduce or prevent cross-contamination in a model rinse liquid, with the addition of organic matter, onto fresh organic produce. The prevention of cross-contamination was validated by enumerating potential survivors in the rinse liquid at the completion of the study. It was determined that NABS did not have a practical initial reduction, > 3.0 log reduction, of the attached pathogenic bacteria from the surface of the produce. However, it was determined that when 200-ppm chlorine was used to eliminate E. coli from the surface of organic cherry tomatoes that there was a practical reduction compared to NABS and the water controls.

Nonetheless, in the rinse liquid, NABS was able to prevent cross-contamination onto fresh produce once the pathogenic bacteria was introduced into the system. Also, NABS was only affected by organic matter in the rinse liquids for the case of the Listeria inoculated cantaloupes, where chlorine was also affected by the organic matter interacting with hypochlorous acid. Since the organic matter did not affect the prevention of cross-contamination for the case of the tomatoes and the leafy greens, this makes NABS a potential alternative to chlorine as a sanitizer for organic produce. The recommendation is further strengthened by the advantages of NABS because it is easy to use, its pH does not need to be maintained, and it has low long-term costs when compared to chlorine.
Vita

Ellen Rebecca Simmons was born in Fredericksburg, VA on February 7, 1991 to parents Barbara and Michael Simmons. Ellen grew up in Fredericksburg and graduated in 2009 from Massaponax High School. In 2009, Ellen moved to Newport News, VA to attend Christopher Newport University, where she received her Bachelor of Science degree in Chemistry with a minor in Organismal Biology. During her undergraduate degree, Ellen worked part-time as a pharmacy technician at CVS/Pharmacy, while simultaneously conducting independent research in an Organic Chemistry laboratory. In this laboratory, Ellen got her first experience with Food Science. In 2013, Ellen moved to Knoxville, Tennessee to attend the University of Tennessee at Knoxville. She moved to Knoxville to obtain her Master of Science degree in Food Science and Technology with a focus on Food Microbiology, which she obtained in December 2015.