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Improving Microbial Safety of Locally Grown Produce in West Virginia Area

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Improving Microbial Safety of Locally Grown Produce in West Virginia Area

Ka Wang Li

Dissertation submitted to the
Davis College of Agriculture, Natural Resources, and Design
at West Virginia University

Doctor of Philosophy in
Animal and Food Sciences

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2021

Keywords: Foodborne pathogen, Surrogate, Farmers' market, Produce, Sanitation, West Virginia
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ABSTRACT

Improving Microbial Safety of Locally Produced Food in West Virginia Area

Ka Wang Li

There is a growing concern regarding the microbial safety of farmers' markets as fresh produce consumption in the United States is gaining popularity. The CDC reported 46% of foodborne illnesses are related to fresh produce. Consuming fresh produce raw removes a processing step, increasing the risk of cross-contamination, leading to illness. However, this risk can be mitigated by washing produce with antimicrobial solutions during post-harvest processes. To reduce the microbial load on fresh produce, the triple-wash process, where the produce is rinsed with water twice and lastly an antimicrobial solution (WWA). WWA is recommended by the WVU Extension Service Small Farm Center, but its efficacy has yet to be evaluated.

The objectives of this project were: 1.) to evaluate the two triple-wash procedures (WAW/WWA) with commercial antimicrobials to inactivate foodborne pathogens and surrogate bacteria on cucumbers, tomatoes, and spinaches; 2.) to evaluate the two triple-wash procedures with SaniDate-5.0 on microbial quality of butternut squashes; 3.) to determine the economic feasibility of the triple-wash application in a processing plant, and 4.) to assess how produce growers handle containers and evaluate the survival of *Salmonella* and *Listeria monocytogenes* on various commonly used produce container surfaces under refrigerated and room conditions.

Results showed Triple-wash by WWA with antimicrobials achieved additional reductions ($P < 0.05$) of pathogens by 0.38-0.56 log₁₀ CFU/cucumber, 0.71 -1.48 log₁₀ CFU/tomato, 0.35-1.07 log₁₀ CFU/g spinach, and 0.7-2.0 log₁₀ CFU/squash than the WAW procedure. The estimated annual operating cost of the triple-wash process with SaniDate-5.0 ranges from \$487.05 to \$1,977.33 for growers producing 1,000-5,000 squashes. Pathogens decreased slower ($P < 0.05$) at 3.2°C on pressed-card and wood surfaces than at 22.5°C on a plastic surface. At 22.5°C, *Salmonella* and *L. monocytogenes* reduced ($P < 0.05$) to <1.30 log (detection limit) after 3 days, and 12 days on plastic, respectively.

Collectively, results indicate that WWA can achieve additional pathogen reduction, SaniDate-5.0 could be used as an alternative antimicrobial agent in the triple-wash process for small local produce growers. It showed the WWA method was an economically feasible approach for produce growers to improve microbial safety during postharvest processing of squash.

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CHAPTER 1

Introduction

Fresh produce including fruits and vegetables is an important component of a healthy and balanced diet. The demand for locally produced food has created growth opportunities for farmers' markets in the USA, its national count increased from 1,755 in 1994 to 8,771 in 2019 (USDA AMS, 2020). With 93 farmers' markets operating at over 112 locations throughout West Virginia, full-time produce growers generate an average annual revenue of approximately \$40,000, and over half a million customers are served annually through West Virginia's Farmers' markets (Li et al., 2018). However, fruits and vegetables that are consumed raw, are increasingly being recognized as important vehicles for the transmission of foodborne pathogens. The Centers for Disease Control and Prevention (CDC) concluded nearly half of foodborne illnesses are caused by pathogens on fresh produce in the United States (CDC, 2019).

Ensuring fresh produce safety at farmers' markets is crucial for long-term growth in the local foods sector in WV. The West Virginia Small Farm Center suggested produce growers use a three-step wash procedure to eliminate pathogens from produce surfaces if their produce is consumed raw or grown close to the ground since 2017. The triple-washing process (two water rinses, followed by an antimicrobials dip) has been recognized as an effective approach for inactivating pathogens from food surfaces and improving food safety in farm production environments (Strohbehn et al., 2013). Recent studies demonstrated that the mixture of peroxyacetic acid and hydrogen peroxide (SaniDate-5.0) effectively reduced *Salmonella* and *Listeria monocytogenes* on cucumbers, tomatoes, and butternut squash (Leifert et al., 2008; Li et al., 2020), and local produce growers have shown interest in incorporating SaniDate-5.0 into

their post-harvest processing to reduce microbial load, extend product shelf-life, as well as the cost of applying this sanitation approach in, small-scale, local produce grower settings.

Fresh produce needs to be handled properly to reduce the risk of foodborne pathogen cross-contamination from sources such as humans, animals, and the environment through growing, harvesting, processing, shipping, and handling. During post-harvest storage, produce that is stored in unsensitized containers could serve as a potential pathogens reservoir and thereby harbor and transfer foodborne pathogens such as *Salmonella* spp. and *L. monocytogenes* (Cossentine et al., 2004; Higbee et al., 2001; Killinger & Adhikari, 2014; Randall et al., 2011). Material used in the storage containers, i.e., wood, pressed-card, or plastic, are subject to weathering or has rough surfaces that can provide a niche for pathogen survival. The role of storage container material as a potential source of pathogen contamination at local farmers' markets is not well-understood, especially for pathogen survival rate on various material surfaces, therefore: the objectives of this research project were:

- 1.) To evaluate the two triple-wash procedures with commercial antimicrobials including an H₂O₂-peroxyacetic-acid blend, a lactic/citric acid blend, and chlorine water to inactivate foodborne pathogens and surrogate bacteria on cucumbers, tomatoes, and spinaches.
- 2.) To evaluate the two triple-wash procedures with an H₂O₂-peroxyacetic-acid mixture (SaniDate-5.0) to improve microbial safety and quality of butternut squashes
- 3.) To determine the feasibility of the triple-wash application in a processing plant.
- 4.) To assess how small produce growers handle storage containers and evaluate survival of *Salmonella* and *Listeria monocytogenes* on various produce container surfaces commonly used at farmers' markets, under storage conditions at refrigerated and room temperature.

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CHAPTER 2

Literature Review

2.1. Foodborne illness and outbreaks

According to a survey from FAO and WHO, annual global produce production increased from 500 million to nearly 3 billion tons from 1980 to 2004 (FAO & WHO, 2008). From 1970 to 2017, supply of fresh produce in the U.S. increased dramatically from 154.4 to 202.6 pounds per capita availability (USDA-ERS, 2020). Fresh produce is vulnerable to foodborne pathogen contamination since it is often consumed raw without a “killing” step, even with interventions to reduce the microbial contamination from farm to the table. Foodborne illness remains a challenging public health issue, causing approximately 9.4 million cases in the United States annually (Scallan et al., 2011). The Centers for Disease Control and Prevention (CDC) defines a foodborne outbreak as two or more people contracting the same disease by consuming the same product (CDC, 2018a). From 1996 to 2010, the U.S. Food and Drug Administration (FDA) reported 131 outbreaks associated with over 20 different fresh produce commodities in the U.S. resulting in 14,350 illnesses, 1,382 hospitalizations, and 34 deaths (U.S. FDA, 2016a). In 2014, the CDC’s FoodNet Surveillance Program identified 19,542 cases of Foodborne infection, 4,445 hospitalizations, and 71 deaths (CDC, 2015a). A USDA ERS report determined the cost of 15 major foodborne illnesses in the U.S. increased from \$15.5 billion (2013) to \$17.6 billion (2018). *Salmonella*, *Toxoplasma*, *Listeria*, Norovirus, and *Campylobacter* accounted for \$15.7 billion in 2018, about 90% of the total costs (USDA ERS, 2021).

2.2. Foodborne pathogen and recent outbreaks

The Interagency Food Safety Analytics Collaboration (IFSAC;2020), consists of CDC, FDA, and USDA, identified 3,981 outbreaks related to *Salmonella*, *Escherichia coli* O157, *Listeria*, or *Campylobacter* through 1998 to 2018. Of which 1,459 outbreaks could be assigned

to a single food category: 905 was confirmed or suspected to be caused by *Salmonella*, 255 by *E. coli* O157, 44 by *Listeria*, and 255 by *Campylobacter*, respectively. *Salmonella* illnesses were the most evenly distributed among various food commodities across the four pathogens, while *E. coli* O157, *Listeria*, and *Campylobacter* were mostly attributed to few food categories (IFSAC, 2020).

2.2.1. *Escherichia coli*

Escherichia coli is a Gram-negative, rod-shaped bacterium., *E. coli* is a facultative anaerobic, non-spore-forming, mesophilic bacterium belonging to the family *Enterobacteriaceae*. *E. coli* replicates rapidly (~20 mins) under optimal conditions (37°C) and can be found in the lower intestines of warm-blooded animals including human and ruminants and environment samples (Bertoldi et al., 2015; CDC, 2016a; Jay et al., 2005).

There are well-recorded pathogenic *E. coli* strains, including Shiga toxin-producing *E. coli* (STEC), Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC) and Diffusely Adherent *E. coli* (DAEC). All pathotypes represent collections of strains that have similar virulence factors and cause similar diseases (Jang et al., 2017; Robins-Browne et al., 2016). Gerba (2009) had captured the relationship between intestinal pathogenic *E. coli* in Figure 2.1. de Oliveira Elias et al. (2019) reported that the most severe diseases recorded during produce-associated outbreaks are caused by EHEC, which includes the Shiga-toxin-producing *E. coli* (STEC) or verocytotoxin-producing *E. coli* (VTEC) serotypes. Their related diseases include bloody diarrhea, thrombotic thrombocytopenic purpura, hemorrhagic colitis, and hemolytic-uremic syndrome (de Oliveira Elias et al., 2019).

E. coli O157:H7, a STEC, as well as six other serogroups: O26, O45, O103, O111, O121, and O145, are responsible for more than 160,000 cases of foodborne illnesses annually in the United States (Bertoldi et al., 2015; CDC, 2016a). These pathogens can contaminate fresh produce during production where water is contaminated with infected animal feces and cross-contaminations from equipment, surfaces, and/or handlers (Yeni et al., 2016). The infectious dose was as low as 2 cells as reported by Hara-Kudo (2010) or less than 700 cells (Hara-Kudo & Takatori, 2011; Tuttle et al., 1999).

In recent years, over 75% of *E. coli* O157:H7 illnesses were related to vegetable crops, such as leafy greens, and red meat products especially beef (IFSAC, 2020). Romaine lettuce contaminated with *E. coli* O157:H7 appears to be a recurring item in multistate outbreaks, resulting in 167 cases in 27 states with 85 hospitalizations in early 2020 (CDC, 2020a). Previously, romaine lettuce contaminated with *E. coli* O157:H7 caused 16 hospitalizations in 2019, and 96 hospitalizations with five deaths in 2018 (CDC, 2019b, 2019a). In 2015, an *E. coli* O157:H7 outbreak linked to a food truck serving several farmers' markets in Seattle, WA, resulted in 6 illnesses and 3 hospitalizations (Food Safety News, 2015). In 2011, strawberries contaminated with *Escherichia coli* O157:H7 sold at a farmers' market in Oregon sickened 16 people and caused one death (Food Safety News, 2011).

2.2.1.1. Shiga toxin

Several species of *Shigella* including *Shigella flexneri*, *boydii*, *sonnei*, and *dysenteriae* are gram-negative bacilli that cause shigellosis, with symptoms including fever, stomach pain, hemorrhagic colitis, and hemolytic-uremic syndrome (CDC, 2020f; Melton-Celsa, 2014). Disease transmission by fewer than 100 CFUs, Shigellosis mainly affects children in developing countries through fecal to oral transmission or human to human transmission from contaminated

water, food, or feces (Bennish et al., 2020; CDC, 2020d; Khan et al., 2013). Out of *S. flexneri*, *boydii*, *sonnei*, and *dysenteriae*, *S. dysenteriae* type 1 is the only species reported to generate Shiga toxin, an exotoxin that leads to the most severe shigellosis, and has the highest mortality rate out of the four species (Bennish et al, 2020; Khan et al., 2013).

Shiga toxin-producing *Escherichia coli* (STEC) is a subset of serotypes inside of Enterohaemorrhagic *Escherichia coli* (EHEC), a pathotype of *E. coli* that produces Shiga-like toxin I and II (Stx 1 and Stx 2) that molecularly relate to that from *Shigella dysenteriae* type 1 (Schüller, 2011). Scheutz et al. subtyped three Stx1 (Stx1a, Stx1c, and Stx1d) and seven Stx2 (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g) by PCR sequencing (Scheutz et al., 2012). The Stx(s) enter the intestine, bind with the glycosphingolipid Gb3, halt protein synthesis within the cell and induce apoptosis; they can alter gene or protein expression in epithelial, endothelial, mesangial cells, and monocytes (Melton-Celsa, 2014). Stx(s) damage the microcirculation, causing infarction of the mucosa, that leads to bleeding into the bowel and bloody diarrhea (Johannes et al., 2010).

2.2.2. *Salmonella*

The CDC estimated *Salmonella* spp. was responsible for a million foodborne illnesses annually in the United States (CDC, 2012a). It was the leading cause of death by foodborne illness in the United States (Scallan et al., 2011). Previous outbreaks in the United States, with produce as delivery vehicle, included tomatoes, sprouts, cantaloupes, etc. (James M. et al., 2006a).

First isolated in 1885 from swine cholera (FDA-NSTA, 2009), *Salmonella* has more than 2500 serotypes identified since 2015 (CDC, 2015c); these serotypes are divided into six subspecies (Su & Chiu, 2007), and most are identified as *S. enterica* which is commonly

recognized as a foodborne pathogen (Le Minor & Popoff, 1987). *Salmonella* is a Gram-negative, rod-shaped, motile, facultative anaerobe, with diameters around 0.7 to 1.5 μm and length 2 to 5 μm (Doyle & Buchanan, 2013; U.S. FDA, 2014a). The most common reservoirs for non-typhoidal salmonellae are cattle poultry, mainly chickens and turkeys, pigs, and wild animals. For typhoid and enteric fevers-inducing strains like *Salmonella enterica* serovar Typhimurium, there is no significant animal reservoir since their mode of spreading mainly involves fecal-oral transmission typically with fecal contaminated water (Chaudhuri et al., 2018; Giannella, 1996).

The infectious dose of *Salmonella* is dependent on the status of the immune system, serotype, and the composition of food as the delivery vehicle, but records show that salmonellosis can be caused by less than 10 vegetative cells (D'aoust et al., 1985). Hara-Kudo (2010) analyzed 11 *Salmonella* outbreaks and concluded the infectious dose could be as low as 363 MPN (Most Probable Number) (Hara-Kudo & Takatori, 2011).

Salmonella infections can be acquired through mainly the consumption of contaminated food and water (Morris & Potter, 2013). In healthy individuals, defensive mechanisms against *Salmonella* includes gastric acid, short-chain fatty acids produced by normal intestinal microflora (Giannella, 1996). Salmonellosis occurs when *Salmonella* cells invade the digestive tract spreading from the intestines to other body parts. *Salmonella* colonizes the ileum and colon after ingestion, and then they invade the epithelium cells in the small intestine and multiply intracellularly. They spread through lymph nodes via circulation and cause an inflammatory response that could lead to ulcers responsible for damaging the intestine, causing gastroenteritis, including diarrhea, fever, and abdominal cramps with a short onset period of 12 to 72 hours after infection which could also last as long as 4 to 7 days (Giannella, 1996; James M. et al., 2006a). Giannella (1996) summarizes the invasion process as shown in Figure 2.2. Severe cases of the

infection usually occur in infants, elderly, and immune-compromised patients, which leads to detrimental diarrhea that requires the patient to be hospitalized even after antibiotic treatment. Patients with less severe infections can recover in less than a week when rehydrated (Coburn et al., 2007).

Although the majority of *Salmonella* infections are from poultry products, a CDC study reported that fresh produce has been associated with approximately 50% of illnesses caused by contaminated produce. Over 75% of illnesses were attributed to food groups including chicken, seeded vegetables such as tomatoes, pork, eggs, and turkey (IFSAC, 2015, 2020; Painter et al., 2013). A study in 2014 reported that one *Salmonella* spp. cells were isolated on fresh herbs from 13 farmers' markets located on the west coast of the U.S. (Levy et al., 2015). Since 2013 the CDC has recorded multiple outbreaks related to *Salmonella* present on pre-cut fruits and melons, papayas, coconuts, spouts, and cucumbers; these foods caused more than 1700 reported cases, more than 400 hospitalizations, and 8 deaths (CDC, 2020b). In 2020, *Salmonella* Enteritidis in peaches caused a multistate outbreak, infecting 101 people from 17 states, leading to 28 hospitalizations (CDC, 2020g). In the same year, *Salmonella* Javiana in cut fruit lead to an outbreak of 165 cases in 14 states, with 73 hospitalizations (CDC, 2020c). *Salmonella* Newport in red, white, and yellow onions caused 1,127 cases and 167 hospitalizations from 48 states in 2020 (CDC, 2020e). *Salmonella* Carrau in pre-cut melons caused 137 cases and 38 hospitalizations from 10 states in 2019 (CDC, 2019d).

2.2.3. *Listeria*

Each year *L. monocytogenes* is estimated to cause 1400 hospitalizations and 250 deaths in the United States and was reported to be one of the leading causes of death from foodborne illness in the U.S.(19%) followed by *Salmonella* spp. (28%) (Scallan et al., 2011). *Listeria* spp.

contains six species with 17 serotypes; 13 serotypes represent *L. monocytogenes*, 2 serotypes represents *L. innocua*. *L. innocua* is considered to be a non-pathogenic variant of *L. monocytogenes* (James M. et al., 2006b). Non-pathogenic species of *Listeria* can be indicative of poor hygienic practices, or as an indicator organism for *L. monocytogenes* (Gurtler et al., 2017). *L. monocytogenes* is a Gram-positive, non-spore-forming, psychrotrophic, facultative anaerobic motile short rod (Farber & Peterkin, 1991). They are 0.4 to 0.5 μm in diameter and 0.5 to 2 μm in length. (Schuchat et al., 1991). *L. monocytogenes* can cause listeriosis, an invasive illness in humans. Once the bacterium enters the host's monocytes, macrophages, or polymorphonuclear leukocytes, it reproduces and spreads directly from cell to cell in the host, without needing to spread interstitially to reach other cells. Protein groups on the *L. monocytogenes* cell surface enable its survival in phagocytic cells and enhance its spread from cell to cell (FDA, 2012). *L. monocytogenes* was first demonstrated to transmit via contaminated food in 1983 and can be found in the environment, as well as domestic livestock, ruminants, wild animals, and birds (FAO & WHO, 2008; Schlech et al., 1983).

The psychrotrophic property of *L. monocytogenes* allows it to survive during storage; grow at a refrigerated temperature as low as -0.4°C ; and reach a typical infective dose of around $3 \log_{10}$ CFU/g for listeriosis or 5.28 to $9 \log_{10}$ CFU/g to result in gastroenteritis (Chan & Wiedmann, 2009; Gurtler et al., 2017). *L. monocytogenes* respond to cold stress by decreasing metabolic rates, increasing unsaturated fatty acids in membrane lipids, increasing cold shock protein expression, and import of cryoprotective compounds (Bucur et al., 2018). It is known that temporary temperature abuse from improper handling can increase the growth rate of *L. monocytogenes* (Bibek Ray & Arun Bhunia, 2013).

L. monocytogenes can be isolated from many environmental samples including water, soil, decaying vegetation, and animal feces (Farber & Peterkin, 1991). Food-borne outbreaks of listeriosis have been related to contaminated unpasteurized milk and their products in 2016 and 2017, causing 10 hospitalizations and 3 deaths (CDC, 2018c, 2018b); ready-to-eat deli meats caused 12 cases with hospitalizations and one death over four states, in 2020, and 10 cases with hospitalizations and one death over five states in 2019 (CDC, 2019f, 2021a); packaged salads produced at Ohio caused 19 cases over nine states, 19 hospitalizations, and one death (CDC, 2016b); cantaloupes from Colorado caused 147 cases in 28 states, with 143 hospitalizations and 33 deaths (CDC, 2012b). Other food vehicles include caramel apples, bean sprouts, Enoki mushrooms, and more (CDC, 2020d; Garner & Kathariou, 2016; USFDA, 2016; Zhu et al., 2017).

The U.S.-FDA indicated that 5% of vegetables and salads, and 5.7% of RTE meats contain *L. monocytogenes* (Hitchins, 1996). Due to several outbreaks in the 1980s, the U.S. FDA and the USDA FSIS regulated that RTE foods are classified as adulterated if *L. monocytogenes* is detected at > 1 cell/25g food samples (U.S. FDA, 2004). The IFSAC reported over 75% of *L. monocytogenes* associated illnesses were attributed to dairy and fruits and 90% of illnesses were attributed to non-meat food categories (IFSAC, 2020).

Listeriosis symptoms for healthy adults are mainly diarrhea and fever, which would cause abortion and stillbirth on pregnant patients and meningitis, pneumonia for newborns (Gaschignard et al., n.d.; James M. et al., 2006b; Okike et al., 2013). Pregnant women are about 13 times more likely to contract listeriosis than the general population (Silk et al., 2012) symptoms include meningitis, septicemia, abortion, and stillbirths (CDC, 2017). The most effective way to prevent the disease is to avoid high-risk foods during pregnancy. These foods

include 1.) unpasteurized milk and its products such as soft cheese; 2.) raw or slightly cooked spouts; 3.) Cut melons that are left at room temperature for more than 4 hours; 4.) ready-to-eat foods including deli meats, cold cuts, lunch meats, hot dogs, or fermented sausages and; 5.) Fish products including cold-smoked fish (CDC, 2019e).

2.2.4. *Campylobacter*

Campylobacter is a microaerophilic curved Gram-negative rod that has corkscrew motility and is carried in the intestine of many warm-blood animals, especially poultry (Laughlin et al., 2019; Oyarzabal et al., 2017). It is a commensal organism colonized in the intestinal tract (cecum) of poultry. Cecum and colon are the main reservoirs of *Campylobacter* spp. which can leak or rupture during processing and cause contamination to the carcass (Berrang et al., 2001). Chantarapanont et al, (2003) showed that the chicken skin provides a favorable environment for survival of *Campylobacter* spp., even under frozen conditions, and can form viable but non-culturable cells under poor growth conditions (Chantarapanont et al., 2003). *Campylobacter* is commonly thermophilic, they can grow between 37 and 42°C with an optimum temperature of 41.5°C. Its microaerophilic nature limits growth to low oxygen concentrations (between 3% to 15%). In laboratory settings *Campylobacter* spp. is isolated with 5% oxygen, 10% carbon dioxide, and 85% nitrogen (Oyarzabal et al., 2017). *Campylobacter* is sensitive to dry conditions; optimal water activity (a_w) of *Campylobacter* growth is 0.997, growth is inhibited when a_w is lower than 0.987 and concentrations of sodium chloride (NaCl) are above 2% w/v or pH is below 4.9 (Silva et al., 2011).

Campylobacter spp. have been known to cause diseases in animals since the early 20th century, but they were not recognized as a foodborne pathogen until the 1980s (Silva et al., 2011). The CDC reported 1.5 million cases of *Campylobacter* infections annually in the U.S, and

cases are more common in the summer (CDC, 2019h). Over half of non-dairy *Campylobacter* illnesses were attributed to Chicken (58.3%) and up to 70% of human campylobacteriosis can be attributed to the consumption of poultry products or unpasteurized milk and unchlorinated waters (Acheson & Allos, 2001; CDC, 2019g; IFSAC, 2020). According to the CDC, the infectious dose of *Campylobacter* is as low as a few cells. Campylobacteriosis causes diarrhea, bloody diarrhea, fever, stomach cramps, nausea, vomiting, and complications including irritable bowel syndrome, arthritis, and Guillain-Barré syndrome. It has an incubation period of 2-5 days and possibly extends to one week (CDC, 2019h). Campylobacteriosis is generally self-limited and lasts a week or less; antimicrobial treatments, including fluoroquinolone and azithromycin, can be used to decrease the duration of symptoms and bacterial shedding (Laughlin et al., 2019).

The CDC revealed that *Campylobacter* outbreaks are not commonly reported due to the high frequency of this infection with an average of 30 outbreaks annually in recent years. In 2008, *Campylobacter* contamination in bagged raw peas caused 18 people to become sick at five south-central Alaska farmers' markets (Gardner et al., 2011). Recent *Campylobacter* foodborne outbreaks include instances related to consumption of unpasteurized milk in Utah caused 99 cases with 10 hospitalizations, and one death were reported (Davis, 2016); undercooked chicken livers served in Ohio and Oregon caused two presumptive cases and three confirmed cases of campylobacteriosis (CDC, 2015a); and contaminated tap water near the border of Arizona and Sonora, Mexico caused 26 cases of Guillain-Barré syndrome (Jackson et al., 2014).

2.2.5. Enterococcus

Enterococcus is a genus for a group of lactic acid bacteria (LAB) that contains both commensal and pathogenic microorganisms. *Enterococci* are Gram-positive, non-spore-forming, facultative anaerobic cocci that appear in a single formation, pairs, or chains. They are

ubiquitous in the environment and can be found in the guts of animals (Bennett et al., 2015). They grow optimally at a temperature of 35 °C, although most species can grow at temperatures of 10 to 45 °C (Franz et al., 1999).

Enterococcus spp. can be adapted to the fermentation activity of cheese and dry sausages due to their tolerance for a high concentration of salts (up to 40% bile salts) and a wide pH range of 4.6 to 9.9 (Foulquié Moreno et al., 2006). When meat is improperly processed or stored, *Enterococci*, including *E. faecalis* and *E. faecium*, cause spoilage in processed meats (Franz et al., 1999). *Enterococcus* infects the human body and causes urinary tract infections, sepsis, endocarditis, and wound infection (Oprea & Zervos, 2007; Poh et al., 2006). *Enterococci* lack some virulence factors and are not as intrinsically virulent as other foodborne pathogens. Resistance to a variety of antibiotics raises concern. Vancomycin-resistant enterococci are one of the leading causes of nosocomial infections (Caballero et al., 2017; A. Khan et al., 2019). From 2009-2015, the CDC reported one *Enterococcus faecalis* outbreak, causing 13 cases of illness (Dewey-Mattia et al., 2018).

Recent data reported by the IFSAC revealed that outbreaks and sporadic infections caused by the four priority pathogens were generally demographically similar, attributing illnesses to each of 17 food categories. They emphasized that interventions to reduce illnesses from these pathogens need to target a variety of food categories, including *Salmonella* in multiple food categories, *E. coli* O157 in vegetables and beef, *Listeria* in dairy and fruits, and *Campylobacter* in multiple food categories, especially in chicken (IFSAC, 2020).

2.3. Surrogate Roles in Food safety studies

Foodborne pathogens are generally handled in biosafety level 2 (BSL-2) facilities by trained personnel. For organizations without access to BSL-2 laboratories or who wish to study a

pathogen in food processing environments, surrogate microorganisms are an alternative to the respective pathogens for intervention treatments studies, researchers can determine inactivation kinetics, while preventing introduction of pathogen contamination in food processing facilities. Based on this constraint, a proper surrogate should be non-pathogenic, behaves similarly during inactivation and susceptibility to injury, possess comparable kinetics to target pathogen, is simple to prepare and genetically stable (FDA, 2018).

When selecting an appropriate surrogate strain for fresh produce studies, Busta *et al.* (2013) recommended an ideal strain should have stable and consistent growth patterns, is easy to cultivate to high populations with stability until usage, is inexpensive to enumerate, can be differentiated from background microflora, does not induce spoilage, has similar attachment characteristics, and similar susceptibility to injury to that of the target pathogen.

Regression analysis can be used to validate a surrogate bacteria in a tested temperature range. Ceylan and Bautista evaluated *Pediococcus acidilactici* ATCC 8042 and *Enterococcus faecium* NRRL B-2354 as thermal surrogate microorganisms for *Salmonella* in low-moisture pet food products. Inoculated samples were treated at 76.7, 82.2, and 87.88°C. After enumeration, log-transformed plate counts were plotted against time for each temperature. D-values indicated *P. acidilactici* 8042 was more heat resistant than the *Salmonella* control but less heat resistant than *E. faecium* B-2354, validating the use of *P. acidilactici* 8042 and *E. faecium* B-2354 as surrogates for *Salmonella* in dry pet food products that are thermally processed at 76.7 to 87.88°C (Ceylan & Bautista, 2015).

2.3.1. *Enterococcus faecium* as a Pathogen Surrogate

Enterococcus faecium NRRL B-2354 is commonly used and recommended as a surrogate bacterium for *Salmonella* Enteritidis PT 30 in validation of almond thermal processing in (Jeong

et al., 2011). Previously known as *Pediococcus* NRRL B-2354 and *Micrococcus freudenreichii*, *E. faecium* NRRL B-2354 (ATCC 8549) was reclassified with the NRRL and ATCC designations (Ma et al., 2011)

Kopit et al. evaluated the safety of *E. faecium* NRRL B-2354 based on its genomic and functional characteristics, including detection of virulence factors; biofilm formation and adherence, antibiotic susceptibility; survival at low pH; high temperature, and in the presence of ethanol. Researchers reported that strain- and application-specific evaluations of *E. faecium* NRRL B-2354 as a conservative surrogate was needed. (Kopit et al., 2014)

Bianchini et al. (2014) validated *E. faecium* NRRL B-2354 as a surrogate in the extrusion of carbohydrate-protein meals in place of *Salmonella* spp. by processing contaminated meal mixtures containing chicken meal, rice flour, potassium chloride, and potassium sorbate at 73.78°C resulted in a 5-log reduction of the surrogate, 80.38°C resulted in brought the *E. faecium* counts to below detection limits (<10 CFU/g). For comparison, a cocktail included *Salmonella enterica* Branderup NVSL 96-12528, *Salmonella enterica* Oranienburg NVSL 96-12608, *Salmonella enterica* Typhimurium ATCC 14028, *Salmonella enterica* Enteritidis IV/NVSL 94-13062, and *Salmonella enterica* Heidelberg/ Sheldon 3347-1 was treated with the same extrusion procedure. The control treatment showed a 5-log reduction was achieved at 60.6°C, and below detection limits at 68.8°C, showing the surrogate was more heat resistant than *Salmonella* spp., which suits as a safer, conservative alternative for these validation studies.

Ceylan and Bautista validated *E. faecium* NRRL B-2354 against a seven-strain cocktail of *Salmonella* Anatum, Montevideo, Senftenberg 775w, Tennessee, Schwarzengrund, Infantis, and Mbandaka during thermal processing of dry pet food with moisture levels of 9.1, 17.9, and 27.0%, heated at 76.7 and 87.88°C. At 9.1% moisture, D-values for the *Salmonella* spp. and *E.*

faecium NRRL B-2354 were 6.54 and 11.66 min at 76.7°C, 2.66 and 4.08 min at 82.2°C, and 1.07 and 1.69 min at 87.8°C respectively. Findings suggested the thermal inactivation characteristics of *E. faecium* NRRL B-2354 were suitable to use as a conservative surrogate for *Salmonella* spp. in dry pet food. (Ceylan & Bautista, 2015)

Enache et al. compared the heat resistance and survival of *Salmonella* Tennessee with *E. faecium* NRRL B-2354 by dry inoculation using talc to remove moisture after growth in growth media (on a plate or in broth), before being introduced into a model peanut butter matrix. The matrix was then heated to 85°C to determine thermal death time. Researchers found no significant difference in thermal resistance when using plate-cultured or broth-cultured *Salmonella*, but found *E. faecium* had greater heat resistance when cultured in the broth compared to cells grown on agar. Regardless of what cell type was used for dry inoculum preparation, *E. faecium* had significantly ($P < 0.05$) greater heat resistance than *Salmonella* Tennessee, as the researchers concluded that *E. faecium* is an appropriate conservative surrogate for *Salmonella* under the tested conditions (Enache et al., 2015).

Jeong et al. (2011) evaluated *E. faecium* NRRL B-2354 as a surrogate for the thermal inactivation of *Salmonella* Enteritidis PT30 with moist air, convection heating for almonds. The results showed at various time, temperature, and humidity levels, thermal inactivation on *E. faecium* was reduced by 0.6 log and 1.4 log, lower than the 3 log and 5 log reduction on *Salmonella* Enteritidis, showing *E. faecium* could function as a conservative moist-air heating surrogate for *Salmonella* Enteritidis PT 30 on almonds.

To be applied in food safety studies, surrogates need to be validated by obtaining growth and resistance data for a microorganism and evaluating the efficacy of an intervention or inactivation process before experimental studies. Ideal surrogates behave similarly to the targeted

pathogen in their inactivation kinetics, growth parameters, and survivability under given conditions as determined with appropriate statistical analyses, and are nonpathogenic and genetically stable.

2.4. Microbial Safety in Farmers' Markets Produce

The natural microflora on fresh produce is usually nonpathogenic, but produce can be contaminated with bacteria, yeasts, and molds during growth, harvest, post-harvest processing and handling, and transportation from human, animal, or environmental sources (Ahvenainen, 1996; Sánchez et al., 2012). During processing, produce is prone to mechanical damage, which may increase tissue senescence, reducing their resistance to microbial spoilage and product shelf-life (Trias et al., 2008). The microbiological quality of produce varies widely based on the types of commodity. Methods used in microbiological quality studies may not be directly comparable, but they show types of commodities and tests conducted. The common evaluation methods use different microbiological measurements as an indicator of microbial quality and cleanliness of the sample, including aerobic plate count, total/fecal coliforms, generic *E. coli*, and common foodborne pathogens.

There are few publicly available studies contrasting the microbial quality of produce sourced from grocery stores and farmers' markets. Roth et al. (2018) compared the microbial quality of tomatoes, leafy greens, berries, and spinach from 9 farmers' markets (301 samples) and 12 supermarkets (100 samples) in Florida between 2016 to 2017. Farmers' market produce showed a higher total coliform prevalence at 50.8% (153 of 301) and 34% (34 of 100) of supermarket produce. Farmers' market leafy greens ($2.3 \log_{10}$ CFU/g) and spinach ($2.4 \log_{10}$ CFU/g) also had higher total coliform counts than their supermarket counterparts, at $1.1 \log_{10}$ CFU/g and $0.70 \log_{10}$ CFU/g, respectively. Generic *E. coli* was detected in 2.3% (7 of 301) of

farmers' market produce, and none in supermarket samples. Generic *E. coli* was detected in 5.8% of spinach, 2.6% of leafy greens, and 2.2% of tomatoes samples from farmers' markets, averaging at $<1 \log_{10}$ CFU/g. *L. monocytogenes* was found in spinach (3.9%, 2 of 52) and leafy greens (2.6%, 2 of 77) from farmers' market. *Salmonella* was detected in one farmers' market tomato and one supermarket berry sample. No *E. coli* O157:H7 was detected.

Scheinberg et al. (2017) surveyed leafy green produce from Pennsylvania farmers' markets in 2017. *E. coli* was detected in 28% of kale (15 of 54), 29% of lettuce (15 of 52), and 17% of spinach samples (8 of 46). *Listeria* spp. were found in 2% of kale (1 of 54), 4% of lettuce (2 of 52) samples, and 7% of spinach samples (3 of 46). Authors concluded that food safety training and public health oversight would be beneficial for farmers' market vendors.

Levy et al. (2015) surveyed 13 farmers' markets in Los Angeles and Seattle; 133 fresh herbs (basil, cilantro, and parsley) were sampled for microbial quality. They reported that 112 of 133 fresh herbs samples were coliform positive. Thirty-two samples were generic *E. coli* positive, with up to 3.15 and 4.15 $\log_{10}$ CFU/g for coliform and *E. coli*, respectively. Based on guidelines for microbial quality of RTE foods established by the Public Health Laboratory System of the United Kingdom (PHLS), 16 samples out of 133 contained more than 2 $\log_{10}$ CFU/g of generic *E. coli*, which was unsatisfactory (Gilbert et al., 2000).

Wood et al. (2015) collected 68 Romaine lettuce from five farmers' markets in Vancouver, Canada for the level of aerobic bacteria, total coliforms, and *E. coli* in 2015. The mean aerobic plate count (APCs) of lettuce samples was 6.3 $\log_{10}$ CFU/g and ranged from 4.8 to 7.8 $\log_{10}$ CFU/g. While 72% (49) of samples contained coliforms at a mean of 1.9 $\log_{10}$ CFU/g, 13% (9) of the lettuce contained approximately 0.7 $\log_{10}$ CFU/g *E. coli*, highlighting the potential concerns for microbial safety in farmers' market produce.

Pires et al. (2020) evaluated food safety risks associated with consuming fresh produce; authors purchased produce from 44 farmers' markets in Northern California to assess the prevalence of *Salmonella* and *E. coli*. Researchers did not find *Salmonella* on 128 produce samples while *E. coli* was found in 40 (31.3%) out of 128 fresh produce samples, with the contamination ranging from 0 to 2.96 log₁₀/mL, with an overall average of 0.13 log₁₀/mL.

Iturriaga, et al. (2007) studied the impact of relative humidity and storage temperature on *Salmonella* growth in tomatoes. Tomatoes were inoculated with 3.8 log₁₀ CFU/tomato *Salmonella* Montevideo. Inoculated tomato samples were stored at 22 or 30°C for 10 days, at various levels of relative humidity (60, 75, 85, or 97%). Results showed high relative humidity (97%) promoted the growth of *Salmonella* and the biofilm formation. *Salmonella* could survive for 10 days with around 2 log₁₀ CFU increased population on the surface of tomatoes. Even when relative humidity decreased to 60%, *Salmonella* maintained between 3.5 to 4.5 log₁₀ CFU/tomato throughout the experimental period.

Colás-Medà et al., (2017) studied *L. monocytogenes* and *Salmonella* on fresh-cut pears for their survival against refrigerated conditions (consistent 4°C) and temperature abuse (4°C for the first 3 days, 8°C for the rest of the study) for 8 days. Storage conditions had no effect ($P>0.05$) on each pathogens, *L. monocytogenes* showed a 1.5-2.0 log₁₀ CFU/g increase in population, and *Salmonella* showed a slight decline of 0.5 log₁₀ CFU/g. This experiment showed the psychrophilic properties of *L. monocytogenes* and the bacteria preserving nature in refrigerated environments. Controlling storage temperature was shown to be ineffective in reducing microbial load on fresh produce.

Application of antimicrobials is not the only efficient way to decontaminate fresh produce by inactivating the pathogen cells. An alternative method includes detaching pathogen

cells from fresh produce's surface with the use of ultrasound. Jose et al. (2014) tested the adherence and inactivation of *Salmonella* Enteritidis and *E.coli* on green peppers and melons with organic acid and ultrasound. By using the combination of 40 kHz Ultrasound, 1% lactic acid, and 1% acetic acid at a 2-minute duration, ultrasound caused a similar reduction of both pathogens on produce ($1.8 \log_{10}$ CFU/cm³ reduction). But combining ultrasound with organic acid showed a synergistic effect ($2.1\text{-}3.0 \log_{10}$ CFU/cm³).

Although most outbreaks reported are focused on the food items, sanitizing contaminated food contact surfaces also needs to be considered, since cross-contamination could occur when clean produce comes in contact with a contaminated surface (Yi et al., 2020).

2.4.1. Farmers' practices

Fresh produce in outdoor markets is more difficult to control when compared to grocery stores. Multiple variables contribute to this difficulty, including sanitation, temperature control, and worker hygiene. Limited access to power, toilets, handwashing stations, and non-uniform cleaning and sanitation procedures create hygiene issues (Worsfold et al., 2004). Khouryieh et al. investigated consumers' awareness of safety in farmers' markets fresh produce in Kentucky; out of 239 responses, more than half (65%) of the respondents expressed concerns about the safety of perishable farmers' market purchases, and 43% of respondents perceived farmers to be the most responsible to their products' safety (Khouryieh et al., 2019).

A detailed survey was conducted to study food safety practices of farmers and farmers' market managers from Georgia, Virginia, and South Carolina. Among 55 surveyed markets, 22 (49%) markets consisted of more than 15 vendors at their peak season, while 7 markets had 5 or fewer vendors. More than half (128/226) of the surveyed small-to-medium-sized farmers used manure as fertilizers. Thirty-one percent (69/226) of the farmers used untested irrigation sources

such as streams, ponds, untested wells, and rainwater. Only one-third (73/226) of the farmers always cleaned the produce transport containers. This lack of hygiene raises concerns of cross-contamination when reusing those containers during transport. Additionally, 35% (79/226) of farmers did not control for temperature (refrigeration) during transportation of the goods, albeit placing produce in a portable refrigerator or storing in a large cooler was the most popular way to cool produce during transport to farmers' markets (125/226) (Harrison et al., 2013).

Harrison et al. (2013) found 19 out of 45 (42%) of the surveyed farmers' market managers did not establish food safety standards for the market. Less than a quarter of the markets offered sanitation training to workers and vendors. Only 3 out of 45 (6%) market managers asked if there were any sanitation training courses available to the vendors; this lack of interest by market managers regarding vendors' food safety practices coupled with the lack of food safety standards suggest a limited understanding of potential risks. Half of the markets surveyed revealed vulnerability to potential foodborne outbreaks and inability to address these risks (Harrison et al., 2013).

Norwood et al. (2019) surveyed 300 market managers and vendors in Houston, Texas to identify food safety practices and educational needs. A total of 66% of the 59 survey participants believed that food safety training was beneficial, and 48% did not have any previous experience food safety training or education. Fifty-four percent of participants reported that management required them to follow food safety practices but only 36% of the vendors received food safety training. Positive practices such as designated eating areas and booths equipped with tent covering were noted; however, researchers also observed high-risk behaviors including pets defecating near produce booths, produce box container reuse, and the presence of live farm animals (goats and chicken).

Refrigeration at or below 41°F (5 °C) during post-harvest procedures, transportation, and display are required for Temperature Control Safety (TCS foods), as listed in the FDA food code, to maintain microbial quality in produce including “raw seed sprouts, cut melons, cut leafy greens and cut tomatoes or mixtures of tomatoes that are not modified in any way so that they are unable to support pathogenic microorganism grow or toxin formation” (FDA, 2013).

2.4.2. The Food Safety Modernization Act

The Guide to Minimize Microbial Food Safety Hazards of Fresh-cut Fruits and Vegetables, published by the FDA, recommended implementing Good Agricultural Practices (GAPs) to reduce the risk of foodborne pathogen contamination of fresh produce (FDA, 2018).

The FSMA produce safety rule is part of the FDA Food Safety Modernization Act (FSMA) that was published in 2015; outlined in Section 105 of FSMA, this legislation establishes minimum standards for safe production and harvesting of fresh fruits and vegetables. These standards are based on a foundation of Good Agricultural Practices (GAPs). Standards include 1.) worker health, hygiene, and training; 2.) agricultural water used for production and post-harvest; 3.) biological soil amendments like compost and manure; 4.) animals, domesticated and wild; 5.) Equipment, tools, buildings, and sanitation; and 6.) Production of sprouts (FDA, 2020; Produce Safety Alliance, 2021a).

The FSMA produce safety rules went into effect 60 days after publication (November 27, 2015) in the Federal Register. Depending on size of businesses and farms, will be applied at different times: 1.) Large businesses, with a three year average annual gross produce sales greater than \$500,000, have 2 years from the effective date of the rule to comply; 2.) small businesses, with a three year average annual gross produce sales between \$250,000 and \$500,000, have 3 years from the effective date of the rule to comply; 3.) very small businesses,

with a three year average annual gross produce sales between \$25,000 and \$250,000, have 4 years from the effective date of the rule to comply. Producers with a three-year average annual gross produce sales of less than \$25,000 are exempt from this rule (FDA, 2017; Produce Safety Alliance, 2021b).

Good Agricultural Practices (GAP) is used by multiple national and international organizations. The Food and Agriculture Organization of the United Nations (FAO) defined GAP as “a collection of principles to apply for on-farm production and postproduction processes, resulting in safe and healthy food and non-food agricultural products, while taking into account economic, social and environmental sustainability” (FAO, 2016). In the United States, Good Agricultural Practices is a voluntary audit that verifies that fruits and vegetables are produced, packed, handled, and stored to minimize risks of microbial food safety hazards, by adhering to the recommendations made in the U.S. Food and Drug Administration’s (FDA) Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables (FDA, 2020; Gravani, 2009).

Hultberg et al. (2012) assessed adherence to Good Agricultural Practices (GAPs) in vegetable growers from Minnesota in 2012 via survey by mail. Out of the 32% response rate (274/855), over 65% of respondents reported compliance to proper worker hygiene practices, including 1.) harvest container and tool sanitization and cleaning, 2.) water treatment to reduce the potential for microbial contamination, and 3.) protection of growing and storing vegetables from contamination. The researchers concluded that Minnesota farmers believe they adhere to many recommended food safety best practices but are lagging in important areas such as 1.) treating wash and processing water, 2.) taking measures to keep animals out of production fields, and 3.) cleaning and disinfecting harvesting tools and containers on a scheduled basis.

Inconsistency in adherence to GAPs was also observed from a recent survey of 160 Kentucky farmers. While 90% of respondents claimed to be familiar with GAPs, only 47% practiced water quality GAPs, and 55% adhered to soil amendment GAPs. Farmers also lacked understanding of potential microbiological contamination sources, less than half (41%) identified soil as a source of pathogenic contamination, and 51% recognized irrigation water as a potential source. Only 28% of participants recognized refrigeration and cooling to be a source of contamination (Sinkel et al., 2018).

2.4.3. Pathogen survival on food contact surfaces

Cross contamination has been identified as a major factor contributing to foodborne illness (Bloomfield & Scott, 1997; Ross & Guzewich, 1999). Kusumaningrum et al., (2003) tested the survival of *Salmonella* Enteritidis, *Staphylococcus aureus*, and *Campylobacter jejuni* at different inoculation levels (50, 12, 119 CFU/cm² respectively) on stainless steel, and the transfer efficiency from stainless steel to cucumber and chicken fillets. Results showed that *S. Enteritidis* and *S. aureus* were able to survive for at least 2 days at a contamination level of 3 log₁₀ CFU/cm². *C. jejuni* was reduced to below detectable limits (0.62 CFU/100 cm² log₁₀ unit) within one to two hours, supporting its need for an microaerophilic environment to survive. Pathogens had transfer rates of 20-100% from stainless steel to cucumber and chicken fillets.

Pathogens adhere to different surfaces in different manners. Silva, et al. (2008) evaluated adherence of *L. monocytogenes* to eight materials found in kitchen environments, in addition to the viability of adhered cells. Stainless steel 304, marble, granite, glass, polypropylene from two sources, and two types of silestone were inoculated with 10 strains of *L. monocytogenes* after 48 hours of growth (8 log₁₀ cells/mL). Adhesion assays were performed after 2 hours at 37°C. Scanning electron microscopy revealed that *L. monocytogenes* strains adhere to every tested

surface material; it adhered tightly to granite and marble and loosely on polypropylene. Although *L. monocytogenes* attached to polypropylene loosely, nearly 100% of its samples were viable compared to the silestone with the lowest recovery rate of 18.5%.

Choi et al. (2012) studied survival of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* on stainless steel and concluded that tested pathogens could survive for seven days at conditions of 25 °C and approximately 70% RH. *E. coli* O157:H7 reduction was between 2 to 5 log₁₀ CFU/mL during storage compared to 2 to 3 log₁₀ CFU/mL reduction for *S. Typhimurium* and 2 to 4 log₁₀ CFU/mL for *L. monocytogenes*.

Since pathogens can survive on numerous surfaces for a prolonged periods, effective sanitation methods that reduce cross-contamination between fresh produce and food contact surfaces are important to improve microbial safety in fresh produce.

2.4.4. Sanitation on contact surfaces

Potential presence of pathogens in farmers' markets should not be overlooked. Characterizing foodborne pathogens and selecting an effective sanitizing method for food contact surfaces to keep fresh produce from foodborne pathogens contamination is a priority. Pathogen control processes for post-harvest produce are important for small growers to reduce pathogens on produce surfaces.

Cleaning and sanitizing are essential to reducing risk of cross-contamination during post-harvest processing and display of produce in farmers' markets (Alzamora et al., 2000; FDA, 2013). Cleaning removes germs, soil, and impurities from a surface, using detergent and water. Sanitizing reduces microorganisms on a surface to a safe level; cleaning and sanitizing the surface improves microbial quality and reduces the risk of infection (CDC, 2021b). Barker et al. (2003) demonstrated that using only detergent was not sufficient to restore food contact surfaces

(workspace, chopping board, cloth) to a hygienic state by comparing the reduction of *S. Enteritidis* when treated with detergent and different concentration of chlorine solutions on 8 different surfaces. These surfaces included fingers, chopping boards, cloths, knife-handles, door handles and more. Detergent-based cleaning could not consistently restore the tested surfaces to a hygienic state, and 500 ppm of hypochlorite was not satisfactory for food hygiene purposes. But a high concentration (5000ppm) of hypochlorite was effective.

In a study of farmers and farmers' market practices, Harrison et al. (2013) found few (88/226) farmers sanitized food contact surfaces at their farms. The majority of survey participants who claimed they sanitized surfaces could not identify the difference between a sanitizer and a detergent. Vinegar, detergent, soap, diluted bleach solutions, sulfur solutions, citric acid solutions, and ammonia-based solutions were chosen to sanitize food contact surfaces (Harrison et al., 2013). Detergents soften the food soils and promote their dispersion and removal, while sanitizers reduce the population of spoilage and pathogenic microorganisms without sterilizing the produce contact surfaces (Marriott & Gravani, 2006).

Establishing proper sanitation processes is important for reducing risk of cross-contamination. Surfaces that include buckets and bins for harvesting and transport; utensils and benchtops for post-harvest production, tabletops and containers for display in the market have direct contact with fresh produce and should be made of surfaces that can be easily cleaned like non-porous plastic or metal. While stainless steel is commonly used in food processing, its usage is not commonly observed in the farmers' market environment. Wood and cardboard are commonly used in farmers' markets but these materials cannot be cleaned as easily due to the crevices on the surfaces that as hold soil and moisture.

Biofilms have significant implications for microbial food contamination and transmission of foodborne diseases because pathogens can colonize surfaces, forming multicellular microbial communities that protect those pathogens from adverse conditions, therefore increasing their resilience (González-Rivas et al., 2018). Biofilm is a major problem in sanitation, it is more resistant to sanitation than single cells and requires physical removal; otherwise it could cause cross-contamination (Gupta et al., 2016). Bae, et al. (2012) evaluated survival of foodborne pathogens on stainless steel surfaces in various attached forms (adhered cells, biofilm produced in TSB, biofilm produced at RH 100%) stored at a different relative humidity (RH 23%, 43%, 68%, 85%, and 100%). Efficacy of chemical sanitizers on inhibition of different foodborne pathogen biofilms on stainless steel surfaces. A mixture of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Staphylococcus aureus*, and *Cronobacter sakazakii* was used to form different biofilms at different relative humidity levels on stainless steel coupons. Coupons were then treated with water, chlorine-based (Clean Shot, Chemical Leader Co., Seoul, Korea), and alcohol-based sanitizers (Alpet-F, Ildong Pharmaceutical Co., Seoul, Korea) for 1 or 5 minutes. The alcohol-based sanitizer was effective on *E. coli* in the biofilm, with 1 minute of treatment, it reduced the *E. coli* population from around 6 log₁₀ CFU/coupon to undetectable (~5 log₁₀ reduction). Alcohol treatment was less effective against *S. aureus* because it only produced an ~4 log₁₀ reduction when treated for 1 minute; albeit, this treatment reduced *S. aureus* to undetectable (~5 log₁₀ reduction) after 5 minutes of treatment. The chlorine sanitizer performed relatively poorly, reducing on *E. coli* and ~1 to 2 log₁₀ reductions on *S. aureus* by ~4 and ~1 to 2 log₁₀, respectively. In general, cells attached via adhesion were more susceptible to chemical sanitizers compared to biofilm counterparts.

Presterl et al. (2007) enhanced the effects of alcohol-based, hydrogen peroxide-based, and povidone-iodine-based sanitizers on *Staphylococcus epidermidis* biofilm using the static microtitre plate model. They concluded that hydrogen peroxide (at 3% and 5%), and alcohols rapidly eradicate *S. epidermidis* biofilms achieving a >5 log reduction after 5 minutes; whereas, a 3 log survival was observed in the 30 minutes povidone-iodine treatment.

While individual sanitizer treatments have some effect on biofilms, it is possible to combine a chemical treatment with a physical treatment like steam to improve biofilm inactivation. Ban and Kang (2016) studied inactivation of biofilms formed by *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* on stainless steel using sodium hypochlorite, hydrogen peroxide, iodophor, and benzalkonium chloride with steam. The use of steam resulted in an additional reduction of up to 2.78 log₁₀ units.

As part of the Food Safety Modernization Act, the Produce Safety Rule provided nationwide standards during growth, harvest, and handling of fresh produce as a measure to reduce associated foodborne outbreaks. However, growers with a three-year average annual gross produce sales of less than \$25,000 are exempt from this rule. Regardless of exemption, vendors providing fresh produce options should follow good agricultural practices to minimize microbial hazards.

2.5. Sanitizers

After effective cleaning with detergents, soil is removed, but this process does not ensure removal of pathogens. Sanitation is used to disinfect surfaces and reduce microbial load. Physical sanitizers disinfect by exposing surfaces to heat, including hot water, pressurized steam, hot air, or radiation. Chemical sanitizers are more commonly used than physical sanitizers; they differ in their general attributes, as well as their antimicrobial properties. Sanitizers are selected

based on like stability, ease of use, non-toxicity, non-corrosiveness, impact on product quality, and cost (Lentsch et al., 1979).

2.5.1 Chlorine

Sodium hypochlorite is the active compound found in household bleach. It is generally recognized as safe (GARS), fast-acting, low cost, and easy to use. However, chlorine-based sanitizers are unstable at higher temperatures, corrosive to metals, can oxidize food affecting its color and lipid, are less effective in hard water, and the reaction between free chlorine and organic matter promotes the formation of a small number of harmful by-products, including possible carcinogens like haloacetic acids (HAA) and trihalomethanes (THMs) (Alzamora et al., 2000; Shen et al., 2013).

Hypochlorous acid (HOCl) is the most effective form of chlorine for sanitation, this form is present predominantly in a pH range of 4.0 to 6.0; pH is commonly adjusted by addition of citric acid. (USDA-AMS, 2015). Hypochlorous acid is effective against vegetative cells of bacteria, yeasts and molds, spores, and viruses. It hydrolyzes cell membranes, reacts with amino acids, and interferes with metabolism and enzyme functions in cells (Estrela et al., 2002).

The FDA approved the use of chlorine in washing fresh produce, and this regulatory body recommended applying a 40-200mg/L solution for 1-2 minutes as sanitizing treatments for produce and equipment (U.S. FDA, 2014b, 2016b). It is typically used at levels of 200 ppm for food contact surfaces, and 400 ppm for non-food contact surfaces such as floors and walls (Alzamora et al., 2000).

Efficiency of chlorine-based sanitizers has been studied with different application methods and food product types. Chaidez et al. (2007) observed a 3 log₁₀ CFU/unit reduction in *S. Typhimurium* inoculated on fresh tomatoes by spraying chlorine solution (200mg/L) holding

for 30 seconds; a 3.61 log₁₀ CFU/unit reduction was observed by immersing the tomatoes in the same chlorine solution. Another study on the dynamic effects of free-chlorine concentration, reported a 4 to 5 log₁₀ CFU/mL reduction for a mixture of *Salmonella* and *E. coli* strains at 30 seconds contact time in the presence of fresh produce extract (Shen et al., 2013).

Beuchat et al. (1998) tested efficiency of spraying as a chlorine application on apples, tomatoes, and lettuce compared to traditional dipping. *E. coli* O157:H7, *L.monocytogenes*, and *Salmonella* were inoculated onto produce samples. Sodium hypochlorite at 200 and 2000 ppm, room condition were applied at different times (0, 1, 3, 5, or 10 min) before analysis. A greater concentration of (2000ppm) chlorine yielded greater reduction, and inactivation by chlorine reached 0.35 – 2.30 log₁₀ CFU/cm²) after one minute of application. Results also suggested that spraying could be an alternative to immersion application because it generated similar reductions (Beuchat, et al., 1998).

2.5.2 Peroxyacetic acid

Peroxyacetic acid (PAA), or peracetic acid, is a colorless liquid, has a pungent odor and low pH similar to vinegar, is an oxidizer, and is generally recognized as safe (GRAS). It is synthesized from the reaction between acetic acid and hydrogen peroxide and is considered to be a stronger biocide than hydrogen peroxide (McDonnell & Russell, 1999; USDA-AMS, 2016).

PAA is widely used in food and beverage and health care industries as an antimicrobial agent (USDA- FSIS, 2021). It can also be used for sanitizing produce, leafy greens, poultry, and meats. (FAO & Ma. Patricia V. Azanza, 2004) PAA is an effective sanitizer against both gram-positive and gram-negative bacteria, as well as yeasts and molds; it causes cell membrane disruption, and it oxidizes sulfhydryl and sulfur bonds in proteins, enzymes, and other metabolites (CDC, 2019c).

PAA is stable in the 100-200 ppm range. It has a low tolerance to soil and is less compatible with hard water. It has higher organic material tolerance than chlorine-based sanitizers (Marriott & Gravani, 2006; Omar A. Oyarzabal & Steffen Backert, 2011). The FDA limits PAA use in wash water to no more than 80ppm when assisting the peeling of fruits and vegetables, followed by rinsing with potable water (U.S. FDA, 2016b). Concentrations of 100-200ppm are permitted for sanitizing food-processing equipment or food contact articles (U.S. FDA, 2011).

The efficacy of PAA has been studied for different food products. Applying 500ppm PAA to almonds without agitation caused up to a 1.93 log₁₀ CFU/g reduction on *Salmonella enterica* (Pao et al., 2006). While combining 80ppm PAA with the rolling process for 60 seconds, *Salmonella* was reduced by up to 5.5 log₁₀ CFU/mL (Chang & Schneider, 2012). Singh et al. (2018) reported that between 5-minute treatments of PAA, chlorine-based sanitizer, and lactic acid, PAA achieved the highest reduction on *E. coli* O157:H7 (lettuce, lemon, tomato, and blueberry at 2.2, 5.7, 5.5, and 6.7 log₁₀ CFU/g, respectively), *S. Typhimurium* (lemon, tomato, cantaloupe, blueberry at 5.4, 6.8, 4.5, and 5.9 log CFU/g, respectively), and *L. monocytogenes* (lettuce and cantaloupe at 2.4 and 4.4 log₁₀ CFU/g, respectively).

2.5.3 Lactic acid

Organic acids are naturally found in fruit and food products as by-products of fermentation, examples are malic acid in apples, lactic acid from dairy products, tartaric acid from grapes, citric acid from lemon, and acetic acid from vinegar (Theron & Lues, 2011). These acids are generally recognized as safe (GRAS) by the FDA, and they are used as preservatives, antioxidants, and flavorings. They can be found in soft drinks, desserts, salad dressings, and acidulants in low acid foods (Mircea Enachescu Dauthy, 1995; Theron & Lues, 2011).

Organic acids are effective sanitizers against gram-positive and negative bacteria, as well as yeasts and molds depending on concentration, pH, and method of application (Theron & Lues, 2011). They can cross the cell membrane in their undissociated form, dissociate into ions inside the cell, and disrupt the electron transport chain (ETC) and DNA synthesis (Doyle & Buchanan, 2013; Gómez-García et al., 2019; Lambert & Stratford, 1999). These effects have been shown to damaged the membrane of *Escherichia coli*, lengthened the lag phase, and decreased the growth rate of cells (Axe & Bailey, 1995; Theron & Lues, 2011).

Lactic acid is naturally produced by lactic acid bacteria through fermentation, it is primarily used to control pH and as flavoring to food products (Doyle & Buchanan, 2013). Effectiveness of lactic acid was studied on multiple agricultural products with different foodborne pathogens. A 5-minute wash with 1% lactic acid reduced *E. coli* by 2.7 log₁₀ CFU/g on baby spinach (Huang & Chen, 2011). A 10-minute treatment of 2% lactic acid reduced a cocktail of *E. coli*, *S. Typhimurium*, and *L. monocytogenes* by up to 3.42 log₁₀ unit on apples, and up to 2.54 log₁₀ CFU/units on lettuce (Park et al., 2011).

While acid-based sanitizers are generally effective on bacteria, strains like *Salmonella Typhimurium* and *S. Enteritidis* can adapt to acid stress and improve survival in lactic acid fermented foods and in the presence of common food preservatives like EDTA (Ethylenediaminetetraacetic acid), at low dosage (Leyer & Johnson, 1992, 1997).

Lactic acid was shown to inhibit spore former including *Clostridium butyricum* (BRACKETT, 1987; Hirsch et al., 1952; Minor & Marth, 1970). In a comparison of *Bacillus coagulans* inhibition in tomato juice, lactic acid was most effective compared to acetic, malic, and citric acids (Rice & Pederson, 1954). Another study showed lactic, malic, and citric acids

were more effective than acetic and propionic acids for inhibition of a mixture of *E. coli*, *S. Typhimurium*, and *L. monocytogenes* (Park et al., 2011).

Conclusion

Foodborne disease outbreaks that originate from farmers' markets will negatively impact the perception of locally grown fresh produce and in turn reduce economic viability, whether produce is grown conventionally or organically. Food safety risks could be mitigated by incorporating proper safety and sanitation practices during production and in farmers' market operations. Effectiveness of different sanitation methods depends on microbial sensitivity, sanitizer used, and methods of application, resulting in variable reported outcomes. Further investigation on pathogen-produce combinations, effectiveness of sanitizing agent applications, and operating with verified sanitizing agents are needed.

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Figure 2.1: Relationship between different pathotypes of *Escherichia coli*. (Gerba, 2009, p. 22)

Figure 2.2: *Salmonella* invading intestinal mucosa (Giannella, 1996).

CHAPTER 3

Compare the Efficacy of Two Triple-Wash Procedures with Sodium Hypochlorite, Lactic-Citric Acid Blend, and Mixer of Peroxyacetic Acid and Hydrogen Peroxide to Inactivate *Salmonella*, *Listeria monocytogenes*, and surrogate *Enterococcus faecium* on Cucumbers and Tomatoes

This study aimed to evaluate the two triple-wash procedures with commercial antimicrobials to inactivate foodborne pathogens and surrogate bacteria on cucumbers and tomatoes. Fresh West Virginia locally grown cucumbers and tomatoes were dip-inoculated with *Salmonella* Typhimurium and Tennessee, *Listeria monocytogenes* (3-strain), and *Enterococcus faecium*. Produce were washed through two triple-wash steps (10 s each) including water dip, antimicrobial dip, and water dip (WAW), or water dip, water dip, and antimicrobial dip (WWA), followed by draining (2 min) on aluminum foil paper. The WWW process was also included as water-only control. Tested treatments were 1) water, 2) sodium hypochlorite (SH; 100 ppm, pH-8.2 or ASH pH-6.8 adjusted by citric acid); 3) lactic and citric acid blend (LCA; 2.5%); and 4) an H₂O₂-peroxyacetic-acid mixer (SaniDate-5.0, SD-0.0064, 0.25 and 0.50%). Surviving bacteria were recovered on XLT-4 (*Salmonella*), MOX (*L. monocytogenes*), and bile esculin agar (*E. faecium*). Data (2 replicates/4 samples/replicate) were analyzed using the Mixed Model Procedure of SAS ($P=0.05$). Counts of *Salmonella*, *L. monocytogenes*, and *E. faecium* on unwashed cucumbers and tomatoes were 5.42 to 6.23, 6.31 to 6.92, and 6.05 log₁₀CFU/produce, respectively. Triple-wash with water only reduced all three tested bacteria by 0.45 to 1.36 log₁₀CFU/fruit. Triple-wash by WWA with antimicrobials achieved additional reductions (LsMeans) of 0.38 log₁₀CFU/cucumber (*Salmonella*), 0.56 log₁₀CFU/cucumber (*E. faecium*), 1.48 log₁₀CFU/tomato (*Salmonella*), 1.09 log₁₀CFU/tomato (*L. monocytogenes*), and 0.71

$\log_{10}$ CFU/tomato than the WAW procedure. Applying SD-0.25% and SD-0.50% solutions in triple-washing cucumbers and tomatoes indicates similar ($P > 0.05$) reductions to ASH and greater ($P < 0.05$) reductions than SH and LCA. *E. faecium* behaved less susceptible ($P < 0.05$) or no difference ($P > 0.05$) to *Salmonella* in most cases except for tomatoes in the WWA process. Results of this study indicate that SD could be used as an alternative antimicrobial agent for chlorine water in triple-wash processing at locally small produce plants. Future pilot plant validation studies and cost-effectiveness analysis are needed for applying SD solutions in triple-wash by WV locally small produce growers.

Keywords: Postharvest wash, Triple-wash, Antimicrobials, Pathogens, Surrogate, Cucumbers, Tomatoes

3.1. Introduction

Among common foodborne pathogens, *Salmonella* is the second common cause of foodborne illnesses (Dewey-Mattia et al., 2018). A multi-state outbreak of *Salmonella* Poona on sliced cucumber from 2015 to 2016 caused more than 900 infected cases, six deaths recorded according to the Center for Disease Control and Prevention (CDC) (CDC, 2019). In 2006, an outbreak across 21 states in the United States caused 183 people ill, of which 22 patients were hospitalized (CDC, 2006). In 2017, Denmark, Finland, Germany, Ireland, and the United Kingdom were affected by the outbreak of *Salmonella* on cucumbers (EFSA and ECDC, 2018). Another popular fresh produce commodity, tomatoes, has been associated with *Salmonella* outbreaks as well. Tomatoes were also linked to a recent *Salmonella* outbreak in Sweden, with 71 identified infections/illnesses (Colombe et al., 2019). In 2015, tomatoes served at Chipotle restaurants in Minnesota were reported to be contaminated with *Salmonella* (Minnesota Department of Health, 2015).

Listeria monocytogenes is another pathogen of concern reported by the United Fresh Produce Association (UFPA) due to the higher fatality rate of listeriosis (UFPA, 2018). In Iran, the prevalence of *Listeria monocytogenes* was reported to be 18% of sampled cucumbers (Hosseini et al., 2013). Depending on the area, the prevalence of *L. monocytogenes* was ranged from 3.78% (Li et al., 2017b) to 17.5% (Strawn et al., 2013) on cucumber sampled from the Farmers' market. Although outbreaks caused by *L. monocytogenes* on tomatoes are uncommon, tomatoes were recognized as a common food crop that susceptible to food pathogens (Honjoh et al., 2016).

The consumption of fresh produce (fresh vegetables and fruits) has increased to 345 pounds (per capita availability) in 2017 (USDA ERS - Food Availability (Per Capita) Data

System, 2019) In the United States; however, there has been increasing concern regarding the microbial safety of farmers' market sold produce (Scheinberg et al., 2017). Fresh produce was accounted for 46% of foodborne illnesses according to a comprehensive analysis by CDC (Painter et al., 2013). A recent study in West Virginia and Kentucky farmers' markets showed that 18.6% of spinach, 10.9% of tomatoes, 18.5% of peppers, and 56.3% of cantaloupes were tested positive for *Salmonella*, and 3.78% of the produce samples were positive for *Listeria* spp. (Li et al., 2017b). Fresh produce may be eaten without further processing, therefore, cross-contamination during transportation or handling becomes a serious issue to ensure produce safety. As the demand for locally produced foods has increased nationwide, estimating to achieve the \$20 billion target by 2019 (USDA, 2015), produce safety becomes a recurring problem especially in regions where raw consumption of fresh produce is common practice.

In 1998, the United States Food and Drug Administration (FDA) published the "Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables" as the main guidelines for Good Agricultural Practices (GAP), which identified that antimicrobial chemicals in processing water may reduce microbial load on the surface of produce (U.S.-FDA, 1998). The West Virginia University (WVU) Extension Service Small Farm Center encourages small produce growers to apply a triple-wash process during their post-harvest processing if their produce is eaten raw or grown on the ground (Strohbehn et al., 2013). Although more evidence suggests washing is more a cross-contamination resource than a pathogen reduction step, the triple-wash process (water rinse, water rinse, and final antimicrobials dip) is still recommended for removing pathogens from food surfaces and improving on-farm food safety with the assumption of clean water being used in each wash step (Strohbehn et al., 2013). The effectiveness of the triple-wash depends critically on the antimicrobial solutions used. Sodium

hypochlorite (referred to as chlorine water) has been well recognized and used extensively as an effective and low-cost sanitizer during the post-harvest washing process (Shen et al., 2013). The antimicrobial effect of chlorinated water against *Salmonella* was observed when spraying 200 ppm chlorinated water onto tomato surfaces (Bari et al., 2003). However, chlorinated water has obvious disadvantages including easily being degraded by organic matter and generating chlorine by-products (Shen et al., 2016). Therefore, local produce growers are interested in learning the efficacy of new antimicrobial solutions. For example, Preston County Workshop Inc., a local small produce processor, is currently using SaniDate-5.0 (a mixer of peroxyacetic acid and H₂O₂) in their triple-wash tanks to control foodborne pathogens of their produce. According to our recent internal survey from locally small produce growers at 2018, West Virginia, Small Farm Conference produce safety training workshop, approximately half of the participants (9/20) currently choose water dip-antimicrobial dip-water dip (WAW) and the other half (10/20) pick water dip-water dip-antimicrobial dip (WWA) procedure. Recently, there is growing recognition that post-harvest washing reduces cross-contamination with no expectation of achieving log₁₀ count reductions of pathogens on produce (Gombas et al, 2017). Therefore, the concentration level to be used and the antimicrobial efficacy of the triple-wash procedures is needed to be investigated.

The efficacy of antimicrobial solutions during triple-wash should be tested in real local small produce commercial settings because the dynamics of processing conditions applied by local produce growers could be less controlled than laboratory conditions. Almost no local small produce plants want to use a real pathogen in their processing lines, instead, they usually apply alternative methods such as ensuring a sufficient active sanitizer within the wash tank. The use of a pathogen surrogate is a possible valid approach and the target surrogate needs to be validated in

the laboratory conditions first (Hu and Gurtler, 2017). *Enterococcus faecium*, a Gram-positive chain shape coccus, has been studied in our WVU poultry farm as a safer alternative for *Salmonella* during steam conditioning, antimicrobial inclusion, standard/thermal aggressive pelleting during broiler feeds manufacture (Boney et al., 2018; Boltz et al., 2019). *E. faecium* has also been validated as a surrogate for *Salmonella* in almond pasteurization (Jeong et al., 2011). However, this *Salmonella* surrogate has not been validated on fresh produce and no publications identified the ideal surrogate for *Salmonella* during the post-harvest produce washing process. As the outbreaks associated with *Salmonella* and *L. monocytogenes* are of concern among fresh produce, a more comprehensive evaluation of the washing procedure should be carried to fulfill the requirement of produce microbial safety. Therefore, the objectives of this study were to evaluate two triple-wash procedures with three commercial antimicrobials to inactivate *Salmonella*, *L. monocytogenes*, and the surrogate *E. faecium* on WV locally grown cucumbers and tomatoes.

3.2. Materials and Methods

3.2.1. Fresh produce sample preparation and background microflora elimination

Fresh cucumbers and tomatoes were purchased from WV Morgantown Farmers' market and then overnight stored in the refrigerated cooler. Before the experiment, the population of natural microflora on produce surfaces were determined by adding one cucumber or tomato into 200 ml buffered peptone water (BPW) with shaking 30 s following by spread-plating onto tryptic soy agar after 10-fold serial dilution and incubated at 35°C for 48 h. Results indicated that there were approximately 5-6 log₁₀ CFU/produce of background microflora on cucumbers and tomatoes surfaces. It was noticed that the microflora on cucumbers interfered with the results of the *E. faecium* experiment as the selective media used for the *E. faecium* experiment was not

selective enough. Therefore, cucumber samples were decontaminated before subjected to *E. faecium* inoculation. To reduce microflora on cucumber in *E. faecium* experiment, a pre-wash procedure was conducted. Fresh cucumbers were first rinsed with tap water and then submerged in chlorinated solution (40 mL bleach in 2 L water) for a minute, followed by submerged in boiling water for 5 seconds. This surface disinfection method was verified by showing no colonies on bile esculin agar after shaking cucumbers with 200 ml BPW following by spread-plating. The decontaminated cucumber samples were air-dried in a biosafety cabinet before inoculation.

3.2.2. Preparation of inoculum

Salmonella Typhimurium ATCC 14028, *Salmonella* Tennessee ATCC 10722, *L. monocytogenes* strains L2624 and L2625 (cantaloupe outbreak serotype 1/2b, donated by Dr. Joshua Gurtler, USDA-ARS, Wyndmoor, PA), and surrogate *E. faecium* ATCC 8459 were used in this study. Both *Salmonella* and *L. monocytogenes* strains were used in the previous related farmers' market produce safety projects (Li et al., 2017b, 2018), and the strain of *E. faecium* has been studied on poultry meat projects (Lemonakis et al., 2017; Boltz et al., 2019). *Salmonella*, *L. monocytogenes*, and *E. faecium* retrieved from frozen stock cultures were streak-plated onto Xylose Lysine Tergitol-4 (XLT-4), Modified Oxford (MOX), and bile esculin (BEA) agar, respectively, and then incubated at 35°C for 48 h to generate single colonies. Before the experiment, a single colony picked from XLT-4 (*Salmonella*), MOX (*L. monocytogenes*), and BEA (*E. faecium*) agar of each strain were enriched in 10 mL tryptic soy broth (TSB; Alpha Biosciences, Baltimore, MD, USA) at 35°C for 24 h. Then, the individual cultured strain was centrifuged ($5,000 \times g$) for 15 min (VWR Symphony 4417, VWR International, Radnor, PA). Each centrifuged strain was then washed triplicate in 0.1% BPW followed by re-suspending in

10 ml of 0.1% BPW. Inoculum of *Salmonella* and *L. monocytogenes* were made from the combination of the four-strain cocktail, where two *Salmonella* strains and two *L. monocytogenes* strains were combined (Li et al., 2017b; 2018). After a four-strain cocktail was made, the inoculum with approximately $6 \log_{10}$ CFU/mL was made by adding the mixed 40 ml of the cocktail into 3 L 0.1% BPW solution for the dip inoculation process. The inoculum level of *E. faecium* was $6.5 \log_{10}$ CFU/mL by adding 40 ml of triplicate washed strains into 3 L 0.1% BPW solutions.

3.2.3. Inoculation of fresh produce samples

Tomatoes and cucumbers were inoculated by placing the product in a metal bowl containing 3 L of *Salmonella* or *Listeria* inoculum with gentle stirring for 5 min, followed by placement in a biosafety cabinet for 15 mins to allow for the pathogen attachment. According to our preliminary studies, *E. faecium* was inoculated onto tomato and cucumber samples by pipetting 1 mL of inoculum onto the samples and fully covering the surface using food-grade plastic wrap for 30 seconds, then air-dried in a biosafety cabinet for 15 mins.

3.2.4. Triple-wash produce with antimicrobials

Before the triple-washing process, the Inoculated produce (cucumbers and tomatoes) were tested the surface temperatures using a scan thermometer, and the surface temperatures of both products were $46.76 \pm 0.6^{\circ}\text{F}$ (8.2°C). Then, inoculated samples were left unwashed (control) or triple washed in three metal containers with 3L of solution each. Each treatment contained 6 samples randomly and evenly split into 2 groups, each sample contains either one tomato or one cucumber. Two triple-wash procedures were applied to the samples, including WAW or WWA. Each step in the triple wash procedure was completed by dipping the samples into the solutions with manual rotation for 10 s with agitation at ~ 200 rpm (Li et al., 2017). Treatments tested

include: i) tap water only (pH=6.9, 15.4°C); ii) sodium hypochlorite (free available chlorine 100 ± 0.6 ppm, pH=8.2-SH, or pH=6.8-adjusted by 10% citric acid-ASH, 14.4°C; Birko, Henderson, CO, USA, iii) a lactic/citric acid blend (LCA, Veggiexide®, 2.5%, pH=5.1, 15.4°C, Birko), and iv) a H₂O₂-peroxyacetic-acid mixer (SaniDate-5.0, SD, 15.2°C; Arbico Organics, Tucson, AZ, USA) with the concentrations of 0.0064 (pH 6.25), 0.25 (pH 5.52) and 0.50% (pH 3.75). Free chlorine concentration was measured using the N, N diethyl-1,4 phenylenediamine sulfate method with a chlorine photometer (CP-15, HF Scientific, Inc., Ft. Myers, FL). Temperatures of all wash solutions were ranged from 57.92 to 59.72°F (14.4 to 15.4°C), which meets the U.S. FDA advisory that wash water needs to be 10°F higher than the produces (46.76°F) being washed (U.S.-FDA, 2018). After triple-wash procedures, samples were drained and dried on aluminum foil paper for 2 min.

3.2.5. Microbiological analysis

Each unwashed and washed produce sample was placed into a sterile sample bag (Nasco, Fort Atkinson, WI, USA) and rinsed in 200 ml of BPW, followed by vigorously shaking for 60 s to detach microorganisms from the surface. Sample rinse solutions were then 10-fold or 100-fold serially diluted in 0.1% BPW and then spread-plated (adding 0.1 ml onto 1 plate or 1.0 ml equally split onto 3 plates) on XLT-4, MOX, and BEA agar to enumerate *Salmonella*, *L. monocytogenes*, and *E. faecium* cells, respectively. All agar plates were then incubated at 35°C for 24 h (XLT-4) or 48 h (MOX and BEA) and were manually counted for colony-forming-unites (CFU) after the incubation period. Three different dilutions were used for the spread-plating of each sample including “0” dilution by adding 1 ml of 200 ml BPW diluent equally split onto 3 agar plates (0.33 ml each). Colonies from each of the three plates were added together after incubation, therefore the detection limit is 2.3 log₁₀ CFU/fruit (=log₁₀ 200

CFU/fruit). The presumptive positive colonies were confirmed by using an Oxoid latex agglutination test kit (Oxoid Ltd, Basingstoke, Hampshire, UK).

3.2.6. Data analysis

The triple-wash studies were duplicated with 4 cucumbers and 4 tomatoes per treatment per repeat with a total of 8 samples per treatment for each bacterium. The experimental design is a randomized 2×6 factorial design with 2 triple-wash procedures (WAW or WWA) and 6 antimicrobial treatments. The survival and reduction of *Salmonella*, *L. monocytogenes*, and *E. faecium* was analyzed using the mixed model procedure of SAS (version 9.2, SAS Institute, Cary, NC), including individual factors of triple-wash procedures, antimicrobial treatments, and their interactions. The comparison of reductions between *Salmonella* and the surrogate *E. faecium* was also analyzed using the mixed model procedure. The reduction data were determined by a reduction ratio of $\log_{10} (N_0/N)$, which includes N_0 , the average control plate counts, and N , the plate count of each antimicrobial treated sample (Adler et al., 2016). The means were determined by Tukey HSD at an $\alpha = 0.05$ significance level.

3.3. Results and Discussion

3.3.1. Comparison of WAW and WWA procedures

In general, the least-squares mean (LsMean) values across the 6 tested antimicrobial treatments (water only wash were not included) indicate that triple-wash with WWA procedure is more effective ($P < 0.05$) than WAW in reducing *Salmonella* (2.39 vs 2.01 $\log_{10}$ CFU/cucumber) and *E. faecium* (2.16 vs 1.60 $\log_{10}$ CFU/cucumber) on cucumbers, and reducing *Salmonella* (2.82 vs 1.34 $\log_{10}$ CFU/tomato), *L. monocytogenes* (2.35 vs 1.26 $\log_{10}$ CFU/tomato), and *E. faecium* (2.81 vs 2.10 $\log_{10}$ CFU/tomato) on tomatoes. Although there is a statistical difference between WWA and WAW process, the $\log_{10}$ reductions of the difference are ranged

from 0.38 to 1.44 log₁₀ CFU/fruit, which is still relatively low. Applying WWA or WAW procedures on cucumbers showed no difference in reductions (1.39 vs 1.35 log₁₀ CFU/cucumber) for *L. monocytogenes*.

3.3.2. Efficacy of triple-wash with antimicrobials against *Salmonella*

Survivals and reductions of *Salmonella* on cucumbers are shown in Table 3.1. All 6 antimicrobial treatments were more effective ($P < 0.05$) in reducing *Salmonella* than the water-only wash (1.26 to 1.36 log₁₀ CFU/cucumber, Table 3.1). Compared to SH, ASH increased reductions of the pathogen by 0.41 ($P < 0.05$, WAW) and 0.32 log₁₀ CFU/cucumber ($P = 0.06$, WWA). As expected, antimicrobials applied in the WAW procedure for cucumbers significantly ($P < 0.05$) reduced the *Salmonella* population (survivals of 3.36 to 4.17 log₁₀CFU/cucumber) than the untreated control (5.80 log₁₀CFU/cucumber), with the reductions ranging from 1.63 (SD-0.0064%) to 2.44 (SD-0.50%) log₁₀ CFU/cucumber (Table 3.1). Compared to the WAW, SH, SD-0.0064%, and SD-0.25% applied in the WWA process achieved an additional ($P < 0.05$) reduction of *Salmonella* by 0.4 to 0.5 log₁₀ CFU/cucumber (Table 3.1). However, no significant ($P > 0.05$) difference of reductions between WAW and WWA in ASH, LCA, and SD-0.50% washed samples (Table 3.1). Compared to SH and LCA, adding SD (0.0064, 0.25, 0.50%) into triple-wash process showed similar or greater ($P < 0.05$) reductions (1.63-2.44 vs 1.82-2.00 log₁₀ CFU/cucumber for WAW; 2.09-2.66 vs 2.14-2.43 log₁₀ CFU/cucumber for WWA, Table 3.1). Compared to ASH, SD-0.25% and SD-0.50% showed similar ($P > 0.05$) reductions ranging from 2.18 to 2.44 log₁₀ CFU/cucumber (WAW) and 2.43 to 2.75 log₁₀ CFU/cucumber (WWA).

As shown in Table 3.2, triple-washing tomatoes in antimicrobials by WAW process significantly ($P < 0.05$) reduced *Salmonella* with the survival populations ranging from 3.72 to 4.80 log₁₀ CFU/tomato compared to 5.70 log₁₀ CFU/tomato (unwashed control). All 6 tested

antimicrobials were more effective ($P < 0.05$) for reducing the pathogen than the water only wash, **except for LCA**. Again, ASH achieved additional ($P < 0.05$) reduction of 0.66 log₁₀ CFU/tomato than SH washed samples. SD-0.25% and -0.50% treated samples showed similar ($P > 0.05$) reductions (1.63 to 1.98 log₁₀ CFU/tomato) compared to ASH (1.98 log₁₀ CFU/tomato), and greater ($P < 0.05$) reductions than SH (1.32 log₁₀ CFU/tomato) and LCA (0.90 log₁₀ CFU/tomato). The reduction of SD-0.0064% is similar ($P > 0.05$) to SH and LCA, but less ($P < 0.05$) than ASH. Compared to the WAW, applying WWA process increased ($P < 0.05$) the reduction levels from 1.32 to 3.14, 1.98 to 3.30, 0.90 to 3.28, 1.13 to 1.72, 1.98 to 3.35, and 1.63 to 3.26 log₁₀ CFU/tomato for SH, ASH, LCA, SD-0.0064%, SD-0.25%, and SD-0.50%, respectively (Table 3.2).

3.3.3. Efficacy of triple-wash with antimicrobials against *L. monocytogenes*

Survivals and reductions of *L. monocytogenes* on cucumbers and tomatoes are shown in Tables 3 and 4, respectively. As shown in Table 3.3, triple-washing cucumbers in 6 antimicrobials by WAW process significantly ($P < 0.05$) reduced *L. monocytogenes* with the survival populations ranging from 4.44 to 5.55 log₁₀ CFU/cucumber compared to the 6.31 log₁₀ CFU/cucumber of the control (Table 3.3). For the WAW process, reductions of SH, ASH, LCA, SD-0.25%, and SD-50% are greater than the water control (0.59 log₁₀ CFU/cucumber) except for SD-0.0064% showing a similar reduction (0.76 log₁₀ CFU/cucumber). Among the tested antimicrobial treatments, no difference ($P > 0.05$) was found between the reductions (1.56 to 1.87 log₁₀ CFU/cucumber) caused by SH, ASH, SD-0.25%, and SD-0.50%, which are greater ($P < 0.05$) than LCA (1.23 log₁₀ CFU/cucumber) and SD-0.0064% (0.76 log₁₀ CFU/cucumber) treatments. Applying WWA process increased ($P < 0.05$) reductions from 1.64 to 2.25, 1.87 to 2.41, and 0.76 to 1.30 log₁₀ CFU/cucumber for SH, ASH, and SD-0.0064% washed samples,

respectively (Table 3.3). However, the WWA process did not increase reductions of the pathogen on cucumbers washed in LCA, SD-0.25% and SD-0.50% as compared to the WAW process (Table 3.3).

The efficacy of antimicrobials to inactivate *L. monocytogenes* on tomatoes has not been widely studied. As shown in Table 3.4, significantly lower survivals ($4.42\text{--}5.46 \log_{10}$ CFU/tomato) were observed on tomatoes washed in 6 antimicrobials using the WAW process than the untreated control ($6.39 \log_{10}$ CFU/g) and water wash control ($5.96 \log_{10}$ CFU/g). SD-0.25% and SD-0.50% reduced the pathogen counts by 1.51 to $1.72 \log_{10}$ CFU/tomato, which were slightly ($P > 0.05$) lower than ASH ($1.97 \log_{10}$ CFU/tomato) and greater ($P < 0.05$) than the reductions of SH ($0.93 \log_{10}$ CFU/tomato), LCA ($1.18 \log_{10}$ CFU/tomato), and SD-0.0064% ($0.94 \log_{10}$ CFU/tomato, Table 3.4). Compared to the WAW process, applying WWA procedures ($P < 0.05$) increased reductions of all 6 tested antimicrobial treatments by 0.49 to $1.40 \log_{10}$ CFU/tomato (Table 3.4).

3.3.4. Comparison of *Salmonella* verse Surrogate *E. faecium*

The response of surrogate *E. faecium* to antimicrobials on cucumbers and tomatoes is shown in Tables 3.5 and 3.6, respectively. Triple-wash with only water reduced ($P < 0.05$) the surrogate by $0.56 \log_{10}$ CFU/cucumber and $0.45 \log_{10}$ CFU/tomato compared to the unwashed control. ASH showed greater ($P < 0.05$) and similar ($P \geq 0.05$) reductions than SH for cucumbers and tomatoes, respectively. For the WAW process, reductions obtained after washing in SH, ASH, LCA, SD-0.0064%, SD-0.25%, and SD-0.50% ranged from 0.80 to $2.27 \log_{10}$ CFU/cucumber (Table 3.5) and from 1.34 to $2.90 \log_{10}$ CFU/tomatoes (Table 3.6). Like *Salmonella*, the application of the WWA process with 6 tested antimicrobials resulted in additional ($P < 0.05$) reductions of 0.25 to $0.83 \log_{10}$ CFU/cucumber and 0.49 to $1.22 \log_{10}$

CFU/tomato, respectively, compared to the WAW process. For cucumbers, the application of SD-0.0064%, -0.25% and -0.50% solutions showed similar ($P > 0.05$) reductions (1.82-2.27 $\log_{10}$ CFU/cucumber for WAW, 2.48-2.96 $\log_{10}$ CFU/cucumber for WWA) compared to ASH (2.08 $\log_{10}$ CFU/cucumber for WAW, 2.39 $\log_{10}$ CFU/cucumber for WWA), but better ($P < 0.05$) reductions than SH and LCA treatments in both WAW (0.80-0.92 $\log_{10}$ CFU/cucumber) and WWA (1.05-1.63 $\log_{10}$ CFU/cucumber) process (Table 3.5). For tomatoes, SD-0.25% and 0.50% indicated greater ($P < 0.05$) reductions (2.48-2.90 $\log_{10}$ CFU/tomato) than SH (1.88 $\log_{10}$ CFU/tomato), ASH (1.37 $\log_{10}$ CFU/tomato), and LCA (1.34 $\log_{10}$ CFU/tomato) treated samples using WAW process. Applying the WWA process, SD-0.50% was the most effective ($P < 0.05$) in reducing the pathogen surrogate from tomatoes (3.53 $\log_{10}$ CFU/tomato, Table 3.6) followed by SD-0.25%, SH, LCA, and ASH, which showed similar reductions of 2.50 to 2.97 $\log_{10}$ CFU/tomato.

In this study, the mixed model procedure was applied to compare the reductions of *Salmonella* verse the surrogate *E. faecium* as shown in Tables 7 (cucumbers) and 8 (tomatoes). For cucumbers, the reductions of *E. faecium* after the application of WAW or WWA process with the 6 tested antimicrobial treatments were less (LsMeans 1.60 vs 2.01 $\log_{10}$ CFU/cucumber for WAW, $P < 0.05$) or not different (LsMeans 2.16 vs 2.39 $\log_{10}$ CFU/cucumber for WWA, $P > 0.05$) than *Salmonella*. Specifically, application of SH, ASH, and LCA indicated lower ($P < 0.05$) reductions, which ranged from 0.80 to 2.08 $\log_{10}$ CFU/cucumber (WAW) and 1.05 to 2.39 $\log_{10}$ CFU/cucumber (WWA) for *E. faecium* compared to *Salmonella* reduced by WAW (1.82 to 2.41 $\log_{10}$ CFU/cucumber) and WWA (2.14 to 2.75 $\log_{10}$ CFU/cucumber) process (Table 3.7). SD-0.25% and SD-50% reduced *E. faecium* by 1.82 to 2.27 $\log_{10}$ CFU/cucumber (WAW) and

2.65 to 2.96 log₁₀ CFU/cucumber (WWA), which were similar ($P > 0.05$) to the reductions of *Salmonella* (Table 3.7).

For tomatoes, the reductions for *E. faecium* from WAW with antimicrobials were greater ($P < 0.05$) than the *Salmonella* results (LsMeans 2.10 vs 1.39 log₁₀ CFU/tomato), but they were the same ($P > 0.05$) to the reductions of *Salmonella* in WWA processed samples (LsMeans 2.80 vs 2.81 log₁₀ CFU/tomato). Specifically, *E. faecium* on tomatoes washed through WWA with SD-0.0064%, 0.25%, and 0.50% behaved no significant difference ($P > 0.05$) to *Salmonella* with reductions of 2.19 to 3.53 log₁₀ CFU/tomato (*E. faecium*) compared to the reductions of 1.72 to 3.35 log₁₀ CFU/tomato (*Salmonella*) (Table 3.8).

3.3.5. Discussion

Results from this study suggest that apply WWA produce during triple-wash is a better approach than WAW in reducing foodborne pathogens. This conclusion could be possibly explained by the that the residual sanitizers after the WWA process further inactivate pathogens on the product samples since the neutralization step was absent from this study. benefits the final antimicrobial wash. The WWW control showed reductions of 0.5 to 1.2 log₁₀ CFU/fruit across all tested pathogens on cucumbers and tomatoes in this study, which are similar to Wang and Ryser (2014) reporting that 1.0 log₁₀ CFU/g reduction of *Salmonella* yielded by water wash for 15 s in a pilot-scale processing line.

As the physiochemical properties of cucumbers and tomatoes' surface are greatly different, it is plausible that the same sanitizer may not result in the same level of antimicrobial effect. Our results suggest it is critical to consider the types of fresh produce when choosing the sanitizer to reduce the foodborne pathogens, as the antimicrobial effect of one sanitizer may

vary. Plus, the amount of organic matter such as dust, soil, and debris of produce surfaces could impact the washing process.

Chlorine is a common sanitizer used in fresh produce due to the economic feasibility and strong antimicrobial ability (Shen et al., 2013). When chlorinated water is used as a sanitizer in fresh produce, the maximum concentration regulated by U.S.-FDA is 200 ppm. However, even 100 ppm chlorinated water demonstrates strong antimicrobial activity. An earlier study reported that tomatoes dipped in 100 ppm chlorinated water for 30 s showed significant reduction against *Salmonella*, and the level of reduction was not different between 30 s, 1 min, or 2 min (Wei et al., 1995). A recent study by Sreedharan et al. (2017) showed that 100 ppm of free chlorine reduced *Salmonella* by $> 4.5 \log_{10}$ CFU/tomato in a model flume water for 30 s. It suggested that a longer treatment time of chlorinated water was not necessary on fresh produce against *Salmonella*, which is beneficial in a real production pipeline. When immersing tomatoes into 200 ppm chlorinated water, its activity was significantly higher than 1 or 2 ppm ozonated water on *Salmonella* under different levels of turbidity of the water (Chaidez et al., 2007). Currently, there are no small produce growers (contacted with 8 local growers in WV) that acidified pH of chlorine water before triple-washing their produce in WV (personal communication with Dr. Tom McConnell, Program Leader of the WV Small Farm Center). However, during the large industry scale produce washing process, adjusting the pH of chlorine solutions to 6.8 with citric acid is recognized to ensure the protonated form of hypochlorite predominates in the wash solution (Luo et al., 2012). Therefore, chlorine solution with near-neutral pH at 6.8 adjusted by citric acid was included as a test treatment in this study. Results of this study suggest that the antimicrobial efficacy of chlorinated water in the concentration of 100 ppm was significant against *Salmonella*, and the reductions were improved when the pH of SH was adjusted to 6.8 in

most cases in this study except for *E. faecium* on tomatoes. A previous study by Wang and Ryser (2014) also reported that chlorine plus citric acid (pH 6.0) yielding a greater reduction ($3.1 \log_{10}$ CFU/g) for *Salmonella* on tomatoes than chlorine at alkaline status ($2.1 \log_{10}$ CFU/g). This is because hypochlorous acid, the most effective antimicrobial component, predominates in chlorine water at near-neutral pH, whereas the hypochlorite would be in the ionic form rather than the antimicrobial protonated state at alkali pH solution (White, 2010). The antimicrobial effect of SH- or ASH-100 ppm can also be maximized when the WWA triple wash procedure was used on cucumbers and tomatoes, as the reduction of *Salmonella* was significantly higher in WWA than WAW process.

The antimicrobial activity of chlorinated water is not limited to *Salmonella. L. monocytogenes.*, another common food pathogen, was also sensitive to chlorinated water treatment on the surface of fresh produce. On cucumbers, 1 min and 2 min of 200 ppm chlorinated water bath demonstrated the same level of *Salmonella* reduction (Yuk et al., 2006). Spraying 200 ppm chlorinated water on tomatoes significantly reduces *L. monocytogenes* on tomatoes (Beuchat et al., 1998; Bari et al., 2003). Surprisingly, although *L. monocytogenes* outbreak was reported on cucumber before (Meldrum et al., 2009; Ponniah et al., 2012; Hossein et al., 2013), the antimicrobial effect of chlorine water against *L. monocytogenes* is relatively lacking in the current literature. Considering the easy accessibility of chlorinated water, chlorinated water without or with neutralizing pH was included in this study to better contribute to the current database of antimicrobial effect against *L. monocytogenes* on cucumbers (Table 3.3). Our results suggest that 100 ppm chlorinated water (SH) is an effective antimicrobial against *L. monocytogenes* on cucumbers, and neutralizing pH to 6.8 by citric acid and the WWA procedure maximizes the antimicrobial effect of chlorinated water as the significant higher

reduction was observed. On tomatoes, although SH-100 ppm is still an effective sanitizer (Table 3.4), the antimicrobial efficacy of SH against *L. monocytogenes* was significantly increased when the pH was adjusted to 6.8 (ASH), which is similar to SD-0.25 and SD-0.50% and greater than SD-0.0064% and LCA.

Recently, there is a growing interest for produce processors to apply other antimicrobial chemicals for replacing chlorinated water since chlorine water easily reacts with water constituents and generates chlorine byproducts after repeated replenish with new chlorine solutions (López-Gálvez et al., 2012; Shen et al., 2016). Local small produce growers in WV are also losing interest in chlorine use due to the concern of natural and organic processes of fresh produce (personal communication with Dr. Tom McConnell, Program Leader of the WV Small Farm Center). LCA, a buffered mixture of the lactic and citric acid solution, was introduced by the food chemical industry about a decade ago and reported to effectively reduce *Salmonella* on poultry carcasses and avoid discoloration of chicken meat caused by lactic acid solution (Laury et al., 2009). The only study of LCA on produce demonstrated that spraying 2.5% LCA onto Jalapeno peppers through commercial cabinet reduced the natural flora, *Salmonella*, and the surrogate generic *Escherichia coli* by 1.3, 1.1, and 0.8 log₁₀ CFU/g, respectively (Adler et al., 2016). The mechanism of LCA to inhibit bacteria survival is the combined effect of lactic and citric acid. Lactic acid decreases the ionic concentration within the bacterial cell membrane of its exterior cell wall and citric acid can diffuse through the cell membrane and penetrate the weak non-dissociated acid. The combination of both acids leads to an accumulation of the acid within the cell cytoplasm, acidification of the cytoplasm, disruption of the proton motive force, and inhibition of substrate transport (Vasseur et al., 1999). Results showed that similar reductions (<0.5 log₁₀ CFU/g) were achieved by LCA compared to the SH for against *Salmonella*, *L.*

monocytogenes, and the surrogate *E. faecium* for most tests in this study. However, LCA was less effective than ASH for inactivating *Salmonella* on tomatoes and *E. faecium* on cucumbers.

SaniDate-5.0 is a mixed antimicrobial solution composed of 23% H₂O₂, 5.3% peroxyacetic acid (PAA), and 70% unknown ingredients (likely water), which has been recommended by the WV Small Farm Center to wash fresh produce processed from local small farms (Li et al., 2017b) since the major wholesale buyer in WV Appalachian Harvest require the use of SaniDate-5.0 as part of the post-harvest protocol for growers selling to their business especially for an organic farming process (personal communication with Dr. Tom McConnell, Program Leader of the WV Small Farm Center). Like other oxidizing chemicals, SaniDate-5.0 oxidizes bacterial cells, denatures protein, and further disrupts cell wall structure to kill or inhibit bacteria (Block, 2011). A previous study by Brinez et al. (2006) reported that the mixer of H₂O₂ and PAA reduced non-pathogenic strains of *Staphylococcus*, *Listeria* spp. and *E. coli* by more than 5 log₁₀ CFU/ml after 10-min contact even with the organic matter present in solutions (Bríñez et al., 2006). Results of this study suggested similar ($P > 0.05$) reductions of *Salmonella*, *L. monocytogenes*, and *E. faecium* on cucumbers and tomatoes achieved by SD-0.25% and SD-50% as compared to ASH, which was greater ($P < 0.05$) than the reductions of SH and LCA solutions. The market price of a 5-gallon pallet is \$330 for SaniDate-5.0 compared to \$12 for SH and \$108.5 for LCA, therefore agricultural economic cost-effectiveness analysis is needed to verify that SaniDate-5.0 is economically feasible for locally small produce growers as an alternative antimicrobial solution to chlorine water.

E. faecium has been previously studied and validated as a potential *Salmonella* surrogate in almonds (Jeong et al., 2011), a balanced carbohydrate protein meal (Bianchini et al., 2014), and pet foods (Ceylan and Bautista, 2015) during the thermal activation process. Our recent

study also confirmed that *E. faecium* could be a nonpathogenic surrogate of *Salmonella* for in-plant antimicrobial validation studies on broiler carcasses (Lemonakis et al., 2017). To evaluate the suitability of choosing surrogate microorganisms for foodborne pathogens when exposed to antimicrobials, the surrogate needs to be behaved equally well or better (resistant to interventions) than the target pathogen in challenge studies (Adler et al., 2016). Therefore, side-by-side comparisons of reduced levels of *Salmonella* and *E. faecium* after a triple wash through WAW or WWA with antimicrobials on cucumbers and tomatoes were presented in Tables 7 and 8. Results indicate that the *E. faecium* strain used in this study could potentially be a surrogate of *Salmonella* for validating triple-wash with commercial antimicrobials on cucumbers in locally small produce processing settings, however, more studies are still needed to confirm its use on tomatoes as a *Salmonella* surrogate since opposite results generated from WAW verse WWA process. Other nonpathogenic bacteria such as generic *Escherichia coli* (ATCC BAA-1427, ATCC BAA-1428, ATCC BAA-1429, ATCC BAA-1430, and ATCC BAA-1431) could be surrogates for *Salmonella* on different produce commodities including tomatoes (Adler et al., 2016). Because our previous pilot plant trial study showed that spraying 50 ppm SH or 1.0% LCA reduced the generic *E. coli* on jalapeno peppers by 0.8 to 1.0 log₁₀ CFU/g and were not different from the reductions (0.5 to 1.1 log₁₀ CFU/g) of *Salmonella* (Adler et al., 2016).

3.4. Conclusions

Under the condition of this study, the triple-wash WWA procedure is better than the WAW to inactivate *Salmonella*, *L. monocytogenes*, and *E. faecium* on cucumbers and tomatoes. SaniDate-5.0 at the concentration of 0.25% and 0.50% indicates similar or better antimicrobial efficacy than chlorine water without or with pH adjustment. *E. faecium* could be a potential *Salmonella* surrogate used for validation studies of antimicrobial treatments during the post-

harvest washing process on produce. Results from this study provide important information for local small produce growers who are interested to adopt the triple-wash during postharvest processing. Future studies are needed to validate the same procedure in commercial pilot plant settings and cost-effectiveness is necessary to evaluate whether SaniDate-5.0 is economically feasible for locally small produce processors.

Limitations of this study

The authors are recognizing the following limitations of this study. First, this extension validation study is valuable for locally very small produce growers in WV, which is not representing/stimulating large industry commercial-scale produce processing. Second, the extent of cross-contamination in the different wash regimes of the triple-wash process was not reported in this study. It is well established that preventing cross-contamination is more critical than the reduction of pathogens during the produce washing process (Gombas et al, 2017). The cross-contamination study of triple-wash in three washing tanks with or without antimicrobials should be included in our future related studies. Third, the cucumbers tested for *E. faecium* were pre-treated to remove background microorganisms, which may not be well representing the cucumbers' surface characteristics. An antibiotic marker should be introduced into *E. faecium* to solve this issue in our future related studies. Fourth, the absence of a neutralization step from the WWA process may further reduce the pathogens by the residual sanitizer on produce samples. We realize that D/E neutralizing broth with a more general neutralizing effect should be included in our studies.

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Ch.3 Tables

Table 3.1. Survival and reduction of *Salmonella* Typhimurium and Tennessee on cucumbers ($\log_{10}$ CFU/cucumber) by triple wash procedure water dip-antimicrobial dip-water dip (WAW) or water dip-water dip-antimicrobial dip (WWA) in water, sodium hypochlorite (SH, 100 ppm, pH 8.2), ASH (SH, 100 ppm, pH 6.8 adjusted by citric acid), lactic and citric acid blend (LCA, Veggiexide®, 2.5%), a PAA and hydrogen peroxide mixer (SD, SaniDate-5.0, 0.0064, 0.25 and 0.50%).

Treatment	Survival		Reduction	
	WAW	WWA	WAW	WWA
Control	$5.80 \pm 0.28a$	$5.42 \pm 0.33a$	—*	—*
Water	$4.54 \pm 0.05b$	$4.06 \pm 0.05b$	$1.26 \pm 0.05cA$	$1.36 \pm 0.05cA$
SH-100 ppm	$3.80 \pm 0.44cd$	$3.00 \pm 0.23cd$	$2.00 \pm 0.44bA$	$2.43 \pm 0.23abB$
ASH-100 ppm	$3.39 \pm 0.38e$	$2.67 \pm 0.42d$	$2.41 \pm 0.38aA$	$2.75 \pm 0.42aA$
LCA-2.5%	$3.98 \pm 0.54cd$	$3.28 \pm 0.26c$	$1.82 \pm 0.54bA$	$2.14 \pm 0.26bA$
SD-0.0064%	$4.17 \pm 0.55bc$	$3.33 \pm 0.43c$	$1.63 \pm 0.55bcA$	$2.09 \pm 0.43bB$
SD-0.25%	$3.62 \pm 0.73de$	$2.76 \pm 0.12d$	$2.18 \pm 0.73abA$	$2.66 \pm 0.20aB$
SD-0.50%	$3.36 \pm 0.59e$	$2.79 \pm 0.19d$	$2.44 \pm 0.59aA$	$2.63 \pm 0.19aA$

—* indicates reduction data are not available

Mean values with different letters within a column are significantly different ($P < 0.05$)

Mean values with different capital letters within a row are significantly different ($P < 0.05$)

Table 3.2. Survival and reduction of *Salmonella* Typhimurium and Tennessee on tomatoes ($\log_{10}$ CFU/tomato) by triple wash procedure water dip-antimicrobial dip-water dip (WAW) or water dip-water dip-antimicrobial dip (WWA) in water, sodium hypochlorite (SH, 100 ppm, pH 8.2), ASH (SH, 100 ppm, pH 6.8 adjusted by citric acid), lactic and citric acid blend (LCA, Veggiexide®, 2.5%), a PAA and hydrogen peroxide mixer (SD, SaniDate-5.0, 0.0064, 0.25 and 0.50%).

Treatment	Survival		Reduction	
	WAW	WWA	WAW	WWA
Control	5.70 ± 0.19a	6.23 ± 0.42a	—*	—*
Water	4.96 ± 0.08b	5.45 ± 0.05b	0.74 ± 0.08eA	0.78 ± 0.05cA
SH-100 ppm	4.38 ± 0.31cd	3.09 ± 0.50d	1.32 ± 0.31bcA	3.14 ± 0.50aB
ASH-100 ppm	3.72 ± 0.44d	2.93 ± 0.73d	1.98 ± 0.44aA	3.30 ± 0.73aB
LCA-2.5%	4.80 ± 0.15b	2.95 ± 0.12d	0.90 ± 0.15deA	3.28 ± 0.12aB
SD-0.0064%	4.57 ± 0.25bc	4.51 ± 0.20c	1.13 ± 0.25cdA	1.72 ± 0.20bB
SD-0.25%	3.72 ± 0.39d	2.88 ± 0.28d	1.98 ± 0.39aA	3.35 ± 0.28aB
SD-0.50%	4.07 ± 0.1d	2.97 ± 0.32d	1.63 ± 0.13abA	3.26 ± 0.32aB

—* indicates reduction data are not available

Mean values with different letters within a column are significantly different ($P < 0.05$)

Mean values with different capital letters within a row are significantly different ($P < 0.05$)

Table 3.3. Survival and reduction of *Listeria monocytogenes* on cucumbers ($\log_{10}$ CFU/cucumber) by triple wash procedure water dip-antimicrobial dip-water dip (WAW) or water dip-water dip-antimicrobial dip (WWA) in water, sodium hypochlorite (SH, 100 ppm, pH 8.2), ASH (SH, 100 ppm, pH 6.8 adjusted by citric acid), lactic and citric acid blend (LCA, Veggiexide®, 2.5%), a PAA and hydrogen peroxide mixer (SD, SaniDate-5.0, 0.0064, 0.25 and 0.50%).

Treatment	Survival		Reduction	
	WAW	WWA	WAW	WWA
Control	6.31 $\pm$ 0.28a	6.92 $\pm$ 0.44a	—*	—*
Water	5.72 $\pm$ 0.37b	6.20 $\pm$ 0.13b	0.59 $\pm$ 0.37cA	0.72 $\pm$ 0.13cA
SH-100 ppm	4.67 $\pm$ 0.46c	5.87 $\pm$ 0.49c	1.64 $\pm$ 0.46abA	1.05 $\pm$ 0.46bcB
ASH-100 ppm	4.44 $\pm$ 0.72c	4.52 $\pm$ 0.43d	1.87 $\pm$ 0.72aA	2.41 $\pm$ 0.43aB
LCA-2.5%	5.08 $\pm$ 0.40c	6.17 $\pm$ 0.43c	1.23 $\pm$ 0.40bA	0.75 $\pm$ 0.43cB
SD-0.0064%	5.55 $\pm$ 0.46b	5.62 $\pm$ 0.39c	0.76 $\pm$ 0.46A	1.30 $\pm$ 0.39bB
SD-0.25%	4.56 $\pm$ 0.49c	5.64 $\pm$ 0.35c	1.75 $\pm$ 0.49aA	1.28 $\pm$ 0.35bA
SD-0.50%	4.75 $\pm$ 0.49c	5.76 $\pm$ 0.40c	1.56 $\pm$ 0.49abA	1.16 $\pm$ 0.40bA

—* indicates reduction data are not available

Mean values with different letters within a column are significantly different ($P < 0.05$)

Mean values with different capital letters within a row are significantly different ($P < 0.05$)

Table 3.4. Survival and reduction of *Listeria monocytogenes* on tomatoes ($\log_{10}$ CFU/tomato) by triple wash procedure water dip-antimicrobial dip-water dip (WAW) or water dip-water dip-antimicrobial dip (WWA) in water, sodium hypochlorite (SH, 100 ppm, pH 8.2), ASH (SH, 100 ppm, pH 6.8 adjusted by citric acid), lactic and citric acid blend (LCA, Veggiexide®, 2.5%), a PAA and hydrogen peroxide mixer (SD, SaniDate-5.0, 0.0064, 0.25 and 0.50%).

Treatment	Survival		Reduction	
	WAW	WWA	WAW	WWA
Control	6.39 $\pm$ 0.16a	6.41 $\pm$ 0.16a	—*	—*
Water	5.96 $\pm$ 0.03b	5.99 $\pm$ 0.03b	0.43 $\pm$ 0.03dA	0.42 $\pm$ 0.03cA
SH-100 ppm	5.21 $\pm$ 0.30c	3.74 $\pm$ 0.39d	1.18 $\pm$ 0.30bcA	2.67 $\pm$ 0.39aB
ASH-100 ppm	4.42 $\pm$ 0.14d	4.03 $\pm$ 0.54d	1.97 $\pm$ 0.14aA	2.38 $\pm$ 0.54aA
LCA-2.5%	5.46 $\pm$ 0.15c	4.08 $\pm$ 0.20d	0.93 $\pm$ 0.15Ac	2.33 $\pm$ 0.20aB
SD-0.0064%	5.45 $\pm$ 0.97c	4.79 $\pm$ 0.16c	0.94 $\pm$ 0.97Abc	1.62 $\pm$ 0.16bB
SD-0.25%	4.88 $\pm$ 0.19d	3.76 $\pm$ 0.40d	1.51 $\pm$ 0.19abA	2.65 $\pm$ 0.40aB
SD-0.50%	4.67 $\pm$ 0.06d	3.93 $\pm$ 0.41d	1.72 $\pm$ 0.06aA	2.48 $\pm$ 0.41aB

—* indicates reduction data are not available

Mean values with different letters within a column are significantly different ($P < 0.05$)

Mean values with different capital letters within a row are significantly different ($P < 0.05$)

Table 3.5. Survival and reduction of *Enterococcus faecium* on cucumbers ($\log_{10}$ CFU/cucumber) by triple wash procedure water dip-antimicrobial dip-water dip (WAW) or water dip-water dip-antimicrobial dip (WWA) in water, sodium hypochlorite (SH, 100 ppm, pH 8.2), ASH (SH, 100 ppm, pH 6.8 adjusted by citric acid), lactic and citric acid blend (LCA, Veggiexide®, 2.5%), a PAA and hydrogen peroxide mixer (SD, SaniDate-5.0, 0.0064, 0.25 and 0.50%).

Treatment	Survival		Reduction	
	WAW	WWA	WAW	WWA
Control	6.05 ± 0.59a	6.05 ± 0.71a	—*	—*
Water	5.49 ± 0.27b	5.49 ± 0.27b	0.56 ± 0.27cA	0.56 ± 0.27eA
SH-100 ppm	5.25 ± 0.39b	5.00 ± 0.26c	0.80 ± 0.39bcA	1.05 ± 0.26dA
ASH-100 ppm	3.97 ± 0.49d	3.66 ± 0.41e	2.08 ± 0.49aA	2.39 ± 0.41bA
LCA-2.5%	5.13 ± 0.19b	4.42 ± 0.41d	0.92 ± 0.19bA	1.63 ± 0.41cB
SD-0.0064%	3.86 ± 0.52d	3.57 ± 0.60e	2.19 ± 0.52aA	2.48 ± 0.60bA
SD-0.25%	4.23 ± 0.30c	3.40 ± 0.57ef	1.82 ± 0.30aA	2.65 ± 0.57abB
SD-0.50%	3.78 ± 0.51d	3.09 ± 0.44f	2.27 ± 0.51aA	2.96 ± 0.44aB

—* indicates reduction data are not available

Mean values with different letters within a column are significantly different ($P < 0.05$)

Mean values with different capital letters within a row are significantly different ($P < 0.05$)

Table 3.6. Survival and reduction of *Enterococcus faecium* on tomatoes ($\log_{10}$ CFU/tomato) by triple wash procedure water dip-antimicrobial dip-water dip (WAW) or water dip-water dip-antimicrobial dip (WWA) in water, sodium hypochlorite (SH, 100 ppm, pH 8.2), ASH (SH, 100 ppm, pH 6.8 adjusted by citric acid), lactic and citric acid blend (LCA, Veggiexide®, 2.5%), a PAA and hydrogen peroxide mixer (SD, SaniDate-5.0, 0.0064, 0.25 and 0.50%).

Treatment	Survival		Reduction	
	WAW	WWA	WAW	WWA
Control	6.05 $\pm$ 0.36a	6.05 $\pm$ 0.51a	—*	—*
Water	5.60 $\pm$ 0.05b	5.60 $\pm$ 0.05b	0.45 $\pm$ 0.05e	0.45 $\pm$ 0.05e
SH-100 ppm	4.17 $\pm$ 0.75d	3.27 $\pm$ 0.46de	1.88 $\pm$ 0.75cA	2.78 $\pm$ 0.46cA
ASH-100 ppm	4.68 $\pm$ 0.23c	3.55 $\pm$ 0.12cd	1.37 $\pm$ 0.23dA	2.50 $\pm$ 0.12cdB
LCA-2.5%	4.71 $\pm$ 0.82c	3.49 $\pm$ 0.39cd	1.34 $\pm$ 0.82dA	2.56 $\pm$ 0.39cdB
SD-0.0064%	4.12 $\pm$ 0.47d	3.86 $\pm$ 0.62c	1.93 $\pm$ 0.47cA	2.19 $\pm$ 0.62dA
SD-0.25%	3.57 $\pm$ 0.50e	3.08 $\pm$ 0.41e	2.48 $\pm$ 0.50bA	2.97 $\pm$ 0.41bB
SD-0.50%	3.15 $\pm$ 0.67f	2.52 $\pm$ 0.44f	2.90 $\pm$ 0.67aA	3.53 $\pm$ 0.20aB

—* indicates reduction data are not available

Mean values with different letters within a column are significantly different ($P < 0.05$)

Mean values with different capital letters within a row are significantly different ($P < 0.05$)

Table 3.7. A comparison of the reduction of *Salmonella* and surrogate *Enterococcus faecium* on cucumbers ($\log_{10}$ CFU/cucumber) by triple wash procedure water dip-antimicrobial dip-water dip (WAW) or water dip-water dip-antimicrobial dip (WWA) in water, sodium hypochlorite (SH, 100 ppm, pH 8.2), ASH (SH, 100 ppm, pH 6.8 adjusted by citric acid), lactic and citric acid blend (LCA, Veggiexide®, 2.5%), a PAA and hydrogen peroxide mixer (SD, SaniDate-5.0, 0.0064, 0.25 and 0.50%).

Treatment	WAW		WWA	
	<i>Salmonella</i>	<i>E. faecium</i>	<i>Salmonella</i>	<i>E. faecium</i>
Water	1.26 $\pm$ 0.05A	0.56 $\pm$ 0.27B	1.36 $\pm$ 0.05A	0.56 $\pm$ 0.27B
SH-100 ppm	2.00 $\pm$ 0.44A	0.80 $\pm$ 0.39B	2.43 $\pm$ 0.23A	1.05 $\pm$ 0.26B
ASH-100 ppm	2.41 $\pm$ 0.38A	2.08 $\pm$ 0.49B	2.75 $\pm$ 0.42A	2.39 $\pm$ 0.41A
LCA-2.5%	1.82 $\pm$ 0.54A	0.92 $\pm$ 0.19B	2.14 $\pm$ 0.26A	1.63 $\pm$ 0.41B
SD-0.0064%	1.63 $\pm$ 0.55A	2.19 $\pm$ 0.52B	2.09 $\pm$ 0.43A	2.48 $\pm$ 0.60A
SD-0.25%	2.18 $\pm$ 0.73A	1.82 $\pm$ 0.30A	2.66 $\pm$ 0.20A	2.65 $\pm$ 0.57A
SD-0.50%	2.44 $\pm$ 0.59A	2.27 $\pm$ 0.51A	2.63 $\pm$ 0.19A	2.96 $\pm$ 0.44A

Mean values with different capital letters within a row under WAW or WWA column are significantly different ($P < 0.05$)

Table 3.8. A comparison of the reduction of *Salmonella* and surrogate *Enterococcus faecium* on tomatoes ($\log_{10}$ CFU/tomato) by triple wash procedure water dip-antimicrobial dip-water dip (WAW) or water dip-water dip-antimicrobial dip (WWA) in water, sodium hypochlorite (SH, 100 ppm, pH 8.2), ASH (SH, 100 ppm, pH 6.8 adjusted by citric acid), lactic and citric acid blend (LCA, Veggiexide®, 2.5%), a PAA and hydrogen peroxide mixer (SD, SaniDate-5.0, 0.0064, 0.25 and 0.50%).

Treatment	WAW		WWA	
	<i>Salmonella</i>	<i>E. faecium</i>	<i>Salmonella</i>	<i>E. faecium</i>
Water	0.74 ± 0.08A	0.45 ± 0.05B	0.78 ± 0.05A	0.45 ± 0.05B
SH-100 ppm	1.32 ± 0.31A	1.88 ± 0.75B	3.14 ± 0.50A	2.78 ± 0.46B
ASH-100 ppm	1.98 ± 0.44A	1.37 ± 0.23B	3.30 ± 0.73A	2.50 ± 0.12B
LCA-2.5%	0.90 ± 0.15A	1.34 ± 0.82A	3.28 ± 0.12A	2.56 ± 0.39B
SD-0.0064%	1.13 ± 0.25A	1.93 ± 0.47B	1.72 ± 0.20A	2.19 ± 0.62A
SD-0.25%	1.98 ± 0.39A	2.48 ± 0.50A	3.35 ± 0.28A	2.97 ± 0.41A
SD-0.50%	1.63 ± 0.13A	2.90 ± 0.67B	3.26 ± 0.32A	3.53 ± 0.20A

Mean values with different capital letters within a row under WAW or WWA column are significantly different ($P < 0.05$)

CHAPTER 4

Inactivation of Foodborne Pathogens (*Salmonella* and *Listeria monocytogenes*) on Locally Processed Spinaches by Three-Step Wash with Antimicrobials

A three-step wash with commercial antimicrobials including an H₂O₂-peroxyacetic-acid mix, chlorine water, and a lactic/citric acid blend was conducted to inactivate foodborne pathogens on spinaches. Fresh spinaches from West Virginia small growers were artificially contaminated with a 4-strain mixture of *Salmonella* and *Listeria monocytogenes*. Inoculated spinaches were three-step washed in water, antimicrobial, and water (WAW) or water, water, and antimicrobial (WWA) with 10 s of each step. Antimicrobial treatments are sodium hypochlorite (SH; 100 ppm, pH-6.8), lactic/citric acid blend (LCA; 2.5%), and a H₂O₂-peroxyacetic-acid mixer (SaniDate-5.0, 0.0064, 0.25 and 0.50%). Microbial populations were analyzed on XLT-4 (*Salmonella*) and MOX agars (*L. monocytogenes*) with a total of 9 samples (3 replicates) followed by analysis using SAS (Mixed Model Procedure, $P=0.05$). Unwashed spinaches recovered 4.57-5.10 and 6.68-6.73 log₁₀CFU/g of *Salmonella* and *L. monocytogenes*, respectively. WWA procedure obtained additional reductions of the pathogens by 0.35-1.07 log₁₀CFU/g (LsMeans) than the WAW procedure. Three-step wash in 0.25% and 0.50% of SaniDate-5.0 solution showed at least similar or even greater ($P < 0.05$) reductions of the pathogens on spinaches than those of SH and LCA treated samples. Results suggested that SaniDate-5.0 is a promising antimicrobial agent that could be suggested to WV small spinach growers during the post-harvest three-step wash process.

Keywords: Three-step wash, Antimicrobials, *Salmonella*, *Listeria monocytogenes*, Spinach

4.1. Introduction

Locally grown/processed food products are growing dramatically in the recent 20 years with the number of farmers' markets nationwide reaching 8,268 in 2014 which increased by almost 5 folds than the amount in 1994 (1,755) (USDA-ERS, 2020). In the state of West Virginia (WV), 93 farmers' markets are over 112 locations statewide creating annual sales valued at ~\$9.1 million (Li et al., 2018) and providing the average annual income for full-time and part-time produce farmers ranged from \$20,000 to \$41,200 (Scheinberg, 2012). The increasing requirement for locally produced foods is partially due to the fresh produce from the farmers' market is believed to be more "organic" and safer than conventional wholesale stores (Scheinberg, 2012; Scheinberg et al., 2017). This is typically for WV, a state with a high obesity rate, the push to increase fresh produce consumption for healthier lifestyles triggers the importance of safe processing of locally grown produce.

Although purchasing fresh produce from local farmers' markets is very popular among residents in WV, food safety of produce from farmers' market is a major concern since a recent study of 5 locally grown produce commodities selling at WV and KY farmers' markets showed 10.9-56.3% of tested produce samples were positive for *Salmonella* and 3.8% of tested samples are positive for *Listeria* (Li et al., 2017b). The population of aerobic plate counts and total coliforms on spinach is more than $5 \log_{10}$ CFU/g and greater than those on tomatoes, peppers, cucumbers, cantaloupes. Plus, 22.6% of spinach samples obtained from WV Morgantown farmers' market were tested for the presence of *Salmonella enterica* spp. (Li et al., 2017b). The production of produce, including leafy greens, typically requires some form of irrigation water during the growing process. For the locally small-scale growers, irrigation water sometimes comes from rainwater, groundwater, and well water, which provides a good opportunity for

foodborne pathogens such as *Salmonella* and *Listeria* from soil to be contaminated onto produce surfaces (Leifert et al., 2008). To maintain the confidence of local consumers regarding the microbial safety of produce purchased from farmers' markets, a three-step wash process is highly recommended for WV locally small produce growers to implement during post-harvest processing if their produce is grown from the soil or uncooked (Li et al., 2018; 2020a; 2020b). The types of antimicrobial agents play an important role in the effectiveness of the three-step wash. Large industry scale post-harvest processing usually adds chlorine water in the washing tanks to inactivate foodborne pathogens on the surfaces of produce since chlorine water is easy to be prepared (dissolving Clorox or calcium-hypochlorite into waters) and low cost with only \$12/per 5-gallon pallet (Shen et al., 2013). However, locally small produce growers are preferring to use other antimicrobial chemical agents due to the concerns of chlorine by-products (Shen et al., 2016) and may not be approving for the organic process.

Small produce growers in WV are very interested to apply a mixture of peroxyacetic acid and H₂O₂ (referred to as SaniDate-5.0) during their post-harvest processing. Our recent studies demonstrated that SaniDate-5.0 effectively reduce *Salmonella* and *Listeria monocytogenes* counts on cucumbers, tomatoes (Li et al., 2020a), and butternut squashes (Leifert et al., 2008) from WV Morgantown Farmers' market and increase the shelf life period of butternut squash during a pilot plant trial study (Li et al., 2020b). In our three-step wash training workshops for local small produce growers at the 2019 and 2020 WV Small Farm Conference, five participants are interested to know the antimicrobial efficacy of SaniDate-5.0 on leafy greens such as spinach, since there is a growing recognition among produce growers that washing process should be effective to decreased rates of cross-contamination but with no expectation to dramatically reduce foodborne microbial pathogens on surfaces of leafy greens (Gombas et al.,

2017). Therefore, this study was designed to evaluate the efficacy of two three-step wash procedures (water + water + antimicrobial agents vs water + antimicrobial agents + water) to decrease microbial populations of *Salmonella* and *L. monocytogenes* on WV locally grown spinach.

4.2. Materials and Methods

4.2.1. Preparation of inoculum

Bacterial strains used in this study are the same ones in our previous triple-wash validation studies (Li et al., 2020a; 2020b) including two *Salmonella* strains which are *Salmonella* Typhimurium ATCC 14028 and Tennessee ATCC 10722 and two *L. monocytogenes* strains L2624 and L2625. The two *L. monocytogenes* are both serotype 1/2b isolated from cantaloupe outbreaks and donated from Dr. Joshua Gurtler, Senior Microbiologist from United States Department of Agriculture-Agricultural Research and Services at Wyndmoor, Pennsylvania. All 4 bacterial strains were maintained as frozen stock cultures and activated by streak-planting onto the selective medium Xylose-Lysine-Tergitol-4 (XLT-4, Hardy Diagnostics, MD, U.S.A) and Modified Oxford (MOX, Hardy Diagnostics) agar to generate single colonies (pure cultures) of *Salmonella* and *L. monocytogenes*, respectively, after incubating at 35°C for 24-48 hours. On the day of the experiment, two single colonies of each strain were picked from XLT-4 and MOX agars followed by cultivating in 10 mL pre-manufactured sterile tryptic soy broth (Hardy Diagnostics) at 35°C for 24 h. The individual 24 h fresh cultured strain was then duplicate-washed in diluted 0.1% buffered peptone water (BPW, Hardy Diagnostics) by centrifuging with the moderate speed of $5,000 \times g$ for 15 min (VWR Symphony 4417, VWR International, Radnor, PA) and re-suspending by vortexing in a 15 ml tube containing 10 ml of new sterile 0.1% BPW solutions. After that, all four 10 ml of re-suspended *Salmonella* and *L.*

monocytogenes solutions were then combined to make a total of 40 ml of a four-strain cocktail inoculum (Li et al., 2017b; 2018; 2020a; 2020b). The final inoculum solution used for spinach was prepared by adding the 40 ml prepared mixture into a fresh sterile 3 L of 0.1% BPW solution in a 5 L autoclaved metal bowl. The microbial population of *Salmonella* and *L. monocytogenes* in the inoculum was approximately 5-6 log₁₀ CFU/ml based on the counts from XLT-4 and MOX agars, respectively.

4.2.2. Inoculation of spinach samples

Fresh spinach samples were obtained from Morgantown Farmers' market every Saturday at noon and stored at a walk-in refrigerator (5°C) for 18-24 h. Before inoculation, spinach leaves were first weighted to create 20 batches with 300 grams of each batch. Inoculation was conducted by placing each batch of spinach (300 gram) into a pre-autoclaved 5 L volume metal bowl with 3 L of the aforementioned inoculum of *Salmonella* and *Listeria* with gentle stirring with a magnetic stir (~100 rpm) for 5 min. After that, the 300-gram inoculated spinach samples were allowed to air-dry for 1 h in a biosafety cabinet to allow for the attachment of the pathogens before the three-step washing experiment. The 3 L inoculum solution was re-prepared after the triplicate was used.

4.2.3. Triple-wash produce with antimicrobials

After inoculation, each 300 gram of inoculated spinach leaves was randomly picked and washing by three steps in another three fresh pre-autoclaved metal containers. Each container was filled with 3 L of the solutions (tap water or antimicrobial agents) to create a wash ratio of 10:1 for the wash solution verse spinach leaves. Two different three-step wash procedures were conducted in this study. They are first water dip, second antimicrobial agent dip, and final water dip referred to as WAW; or first water dip, second water dip, and final antimicrobial dip referred

to as WWA. Every step was conducted by completely immersing inoculated spinaches into the solutions with manual rotation by hands with gloves for 10 s with agitation speed at approximately 200 rpm (Li et al., 2020a; 2020b) and continued for the three bowls referred to as three-step wash. Details of the wash solutions without or with commercial antimicrobials including tap water (WVU campus municipal), sodium hypochlorite (SH, Birko, Henderson, CO, USA), a lactic/citric acid blend (LCA, Veggiexide®, Birko), and SaniDate-5.0 (Arbico Organics, Tucson, AZ, USA) solutions are described in Table 4.1. Temperatures of all wash solutions were 10°F higher than the spinach sample (46.76°F) which followed the recommendation from the U.S. Food and Drug Administration (FDA) advisory board (Center for Food Safety and Applied Nutrition, 2020). After a three-step wash, spinach leaves were placed on aluminum foil papers in a biohazard for 5 min to drain out the extra water.

4.2.4. Microbiological analysis

Three 25-g of unwashed or washed spinach samples were randomly picked and weighted from the whole 300-g of each batch. Every 25-g sample was aseptically transferred into a food sample bag (Nasco, Fort Atkinson, WI, USA) which was pre-added with 200 ml of BPW. The sample bag was then homogenizing for 1 min in a Masticator (Microbiology International, Frederick, MD, U.S.A). The homogenizer was then appropriately diluted in several 9.0 (10-fold) or 9.9 ml (100-fold) of 0.1% BPW solutions followed by plating onto XLT-4 and MOX agar for analyzing cell counts of *Salmonella* and *L. monocytogenes*, respectively. XLT-4 agar plates were incubated at 35°C for 24 h and MOX agar plates were incubated at 35°C for 48 h. Agar plates for *Salmonella* and *L. monocytogenes* in the range of 20-200 colony-forming-unites (CFU) were picked and used the Oxoid latex agglutination test kit (Oxoid Ltd, Basingstoke, Hampshire, UK) to test and confirm 2 presumptive positive *Salmonella* and *L. monocytogenes* colonies from

every plate. Then all plates were manually counting for CFU with the detection limit is $0.9 \log_{10}\text{CFU/g}$.

4.2.5. Data analysis

This three-step study was conducted triplicate with the sample unit of 25 g of spinach $\times$ 3 in each treatment of each repeat with a total of 3 repeats. Therefore, there were 9 samples in each treatment for *Salmonella* or *L. monocytogenes*. The design of the experiment in this study is a randomized 2×6 factorial design with 2 types of three-step wash procedures (WAW or WWA) and 6 treatments of water or antimicrobial agents. The mixed model procedure of SAS (version 9.2, SAS Institute, Cary, NC) was used to analyze the difference among the survival and reduction of the pathogens, including individual factors of three-step wash procedures, antimicrobial treatments, and their interactions. Data of pathogen reductions of each treatment were calculated in the Excel spread sheet by a reduction ratio of $\log_{10} (N_0/N)$, which includes N_0 (the average CFU counts of the untreated control samples) and N (the CFU counts of each sample treated by different antimicrobials) based on our previous study (Adler et al., 2016). Comparison of means of various treatments was determined by Tukey HSD with significance level set at $\alpha = 0.05$.

4.3. Results and Discussion

4.3.1. Three-step wash by WAW verse WWA procedures

Analysis from the mixed model procedure showed that the antimicrobial efficacy of triple-wash against *Salmonella* and *L. monocytogenes* was determined by the three-step wash procedures (WAW or WWA, $P < 0.05$) and types of antimicrobial agents ($P < 0.05$). The interaction between wash procedures and antimicrobials was also significant ($P < 0.05$). In this study, the least-squares mean (LsMean) values of the 5 tested antimicrobial treatments (not

including the water-only treatment) were calculated to compare the overall antimicrobial efficacy between WAW and WWA procedures. Results indicate that applying the WWA procedure in a three-step wash is better ($P < 0.05$) than WAW in decreasing the cell counts of *Salmonella* and *L. monocytogenes* on spinaches with LsMeans of 1.86 vs 0.79 log₁₀CFU/g and 1.37 vs 1.02 log₁₀CFU/g, respectively.

4.3.2. Three-step wash with antimicrobials against *Salmonella*

Data in Figure 4.1 shows the survivals and reductions of *Salmonella* on spinaches without or after triple-wash treatments. Triple-wash with tap water without antimicrobials only slightly ($P > 0.05$) reduces *Salmonella* of spinach samples by 0.15 to 0.28 log₁₀CFU/g compared to the control (unwashed samples). Three-step washing spinach samples in 5 different antimicrobial treatments showed greater ($P < 0.05$) reductions of the pathogen than the WAW treatment regardless of WAW or WWA procedures except for SaniDate-5.0-0.0064% in WWA showing similar reductions (0.44 vs 0.28 log₁₀ CFU/g) than the only water washed samples. As expected, three-step wash with antimicrobials in the WAW procedure significantly ($P < 0.05$) decreased the cell counts of *Salmonella* (survivals of 3.15 to 4.24 log₁₀CFU/g) than the control (4.57 log₁₀CFU/g), and the reductions were ranged from 0.33 (SaniDate-5.0-0.25%) to 1.42 (LCA-2.5%) log₁₀CFU/g (Figure 4.1). Compared to the WAW procedure, SH-100 ppm, SaniDate-5.0-0.25% and SaniDate-5.0-0.50% in WWA procedure achieved an additional ($P < 0.05$) reduction of 0.25, 1.48, and 1.28 log₁₀CFU/g, respectively (Figure 4.1). However, LCA-2.5% and SaniDate 5.0-0.0064% did not show a difference ($P > 0.05$) of the pathogen reductions when the wash procedure was switched from WAW to WWA (Figure 4.1). For WAW procedure, adding SaniDate-5.0-0.0064, -0.25, or -0.50% into triple-wash solutions showed less ($P < 0.05$) reductions than those of SH-100 and LCA-2.5% (0.33-0.52 vs 1.19-1.42 log₁₀CFU/g, Figure

4.1). For WWA procedure, samples washed in SaniDate-5.0-0.25% and -0.50% solution showed similar ($P > 0.05$) reductions of the pathogen than LCA-2.5% (1.76-1.81 vs 1.86 log₁₀CFU/g) and greater ($P < 0.05$) reductions than those from SH-100 ppm (1.76-1.81 vs 1.44 log₁₀CFU/g).

4.3.3. Three-step wash with antimicrobials against *L. monocytogenes*

Figure 4.2 shows the reductions of *L. monocytogenes* on spinaches after a three-step wash. After inoculation, the population of *L. monocytogenes* on unwashed spinach was 6.68 to 6.73 log₁₀CFU/g. Three-step wash without antimicrobials (only tap water) decreased ($P < 0.05$) the pathogen populations by 0.42 to 0.47 log₁₀CFU/g than the untreated control (Figure 4.2). Applying triple-wash for spinach with WAW procedure with 5 antimicrobials dramatically ($P < 0.05$) decreased the counts of *L. monocytogenes* than the control (survivals of 5.56 to 5.75 vs 6.68 log₁₀CFU/g, Figure 4.2). Applying the WAW process with all 5 tested antimicrobial treatments reduced *L. monocytogenes* by 0.93 to 1.12 log₁₀CFU/g, which is greater ($P < 0.05$) than the water-only treatment (0.47 log₁₀ CFU/g) but similar ($P > 0.05$) after comparison with each other (Figure 4.2). Compared to the WAW process, the WWA procedure enhanced ($P < 0.05$) reductions of the pathogen in the treatments of LCA-2.5%, SaniDate-5.0-0.25%, and -0.50% by 0.36, 0.65, and 0.74 log₁₀CFU/g, respectively (Figure 4.2). However, applying the WWA process with SH-100 ppm and SaniDate 5.0-0.0064% did not increase ($P > 0.05$) reductions of the pathogen on spinaches after switching to the WAW procedure (Figure 4.2). Among the tested concentrations of SaniDate-5.0, reductions of SaniDate-5.0-0.25% (1.67 log₁₀CFU/g) and -0.50% (1.65 log₁₀CFU/g) were greater ($P < 0.05$) than those of SH-100 ppm (1.17 log₁₀CFU/g) and LCA-2.5% (1.35 log₁₀CFU/g); the reduction of SaniDate-5.0-0.0064% is close ($P > 0.05$) to those of SH-100 ppm but less ($P < 0.05$) than the LCA-2.5% treated samples (Figure 4.2).

4.3.4. Discussion

Results from the present study suggest that three-step wash procedure with WWA or WAW play an important role to determine the efficacy of inactivating *Salmonella* and *L. monocytogenes* on spinaches, which suggest that applying the WWA procedure in the three-step wash is more effective than the WAW for the control of the pathogens. This result agrees with the findings from our previous study reporting that WWA increased the reductions of *Salmonella* by 0.7 log₁₀CFU/product and *L. monocytogenes* by 1.6 log₁₀CFU/product on butternut squashes compared to the WAW process (Li et al., 2020b). Another related study also found that the three-step wash with WWA procedure is better than WAW in decreasing *Salmonella* on cucumber and tomatoes and reducing *L. monocytogenes* on tomatoes with the enhancement of the reductions by 0.4 to 1.5 log₁₀CFU/product (Li et al., 2020a). These results could be possibly explained by the residual antimicrobial agents applied through WWA continued to inactivate bacterial pathogens on the surface of the produce, since no further neutralization step was applied in these studies. The tap water-only treatment referred to as WWW in this study showed slight reductions of 0.2 to 0.5 log₁₀CFU/g against *Salmonella* and *L. monocytogenes* on spinach. A recent study found that immersion inoculated spinach leaves into sterile distilled water only slightly decreased *Salmonella* Saintpaul by 0.4 log₁₀CFU/cm² than the unwashed control (Ruengvisesh et al., 2019). These results suggest that physical removing only has a very limited impact on reducing pathogens on spinach and the antimicrobial efficacy of the three-step wash relied on the choice of antimicrobial agents.

Chlorine is widely used during the post-harvest washing process of fresh produce including spinach due to its ease to prepare, strong antimicrobial capability, and economic feasibility (Luo et al., 2012; Shen et al., 2013) with the maximum concentration of 200 ppm on

fresh produce and food contact surfaces (Nutrition, 2020). Chlorine water has very strong antimicrobial activity as shown in our previous study that 0.5 ppm of free chlorine with exposure time ≥ 30 s reduced three *Salmonella* strains by $> 4.5 \log_{10}$ CFU/ml (Shen et al., 2013). A recent study suggested that free chlorine at the concentration of 100 ppm reduced *Salmonella* by more than $4.5 \log_{10}$ CFU/per sample in a model flume water with a contact time of 30 s (Sreedharan et al., 2017). Although there are no locally small produce growers in WV that adjust the pH of sodium chlorite prepared water before washing their products (personal communication with Dr. Tom McConnell, Program Leader of the WV Small Farm Center), chlorine water is needed to adjusting pH to 6.8 with prepared 10% citric acid to ensure the most effective form of hypochlorite acid (HClO) dominate in the washing solution (Luo et al., 2012). Therefore, same to our recent three-step wash studies (Li et al., 2020a; 2020b), chlorine solution with a pH of 6.8 was included as an antimicrobial treatment in the current study. Results indicate that adding 100 ppm of chlorine water into a three-step wash process was effective against *Salmonella* and *L. monocytogenes* with the reductions of 1.1 to $1.4 \log_{10}$ CFU/g regardless of the WAW or WWA procedure. These results are the same as the previous study reported that the *Salmonella* reduction of minimally processed spinach was 1.2 to $1.4 \log_{10}$ after washing in 125 ppm of chlorine solution (pH 7.0) for 8 min with various water: produce ratios (Pirovani et al., 2000). A recent study reported that *L. monocytogenes* on chlorine (100 ppm, pH 7.3) treated spinach surfaces decreased by $2.14 \log_{10}$ CFU/cm² with a contact time of 15 min (Chhetri et al., 2019). These results verified that HClO as the most effective antimicrobial ingredient would be dominant in chlorine wash solutions with pH values close to 7.0 (Black & Veatch Corporation, 2009), therefore the training and practicing of correct preparation of chlorine water before triple-wash should be included in our future extension/outreach workshops in our WV local

community. In this study, it was also noticed that the anti-*Salmonella* effect of chlorine water can be improved when the WWA procedure was applied during triple-wash of spinach, as the reduction of *Salmonella* was greater in WWA than WAW process, which agrees with our previous triple-wash tests of *Salmonella* on cucumber and tomatoes (Li et al., 2020a). Three-step wash with chlorine water using the WWA process did not improve the reductions of *L. monocytogenes* on spinach samples, which is also similar to our previous study on cucumbers (Li et al., 2020a).

According to our recent personal communication with Dr. Tom McConnell, Program Leader of the WV Small Farm Center, WV small produce growers are not interested in including chlorine water in their three-step wash tanks due to the concern of certifying of organic processes of their products. Plus, studies have indicated that chlorine water is easy to lose the effective level of hypochlorous acid after reacting with organic matters in wash waters (López-Gálvez et al., 2012; Luo et al., 2012; Shen et al., 2013) and repeatedly replenish with fresh chlorine solution could form the carcinogenic halogenated disinfection by-products including trihalomethanes (Shen et al., 2016). There are growing interests among local small produce growers to apply other antimicrobial chemicals instead of chlorine. In 2009, a new chemical formula of the lactic and citric acid buffered solution, referred to as LCA, was generated by food chemists in Birko Corporation (Henderson, CO, USA) to prevent discoloration (red to yellow color) of poultry products caused by lactic acid (Laury et al., 2009). Previous studies showed that applying LCA during the post-harvest process of poultry products through dip solution (Li et al., 2017a), nozzle sprayer (Laury et al., 2009), or electronic static sprayer (Jiang et al., 2018), significantly reduce *Salmonella* or *Campylobacter* on poultry carcasses and eggs. Regarding fresh produce, a study of LCA on Jalapeno peppers conducted by microbiologists at Birko

corporation verified that 2.5% LCA decreased cell counts of natural background flora, *Salmonella*, and generic *Escherichia coli* by 0.8-1.3 log₁₀CFU/g through a commercial cabinet sprayer (Adler et al., 2016). The inactivation of LCA to bacterial cells is the synergistic effect of lactic and citric acid leads to acid accumulation in the cell cytoplasm to acidify cytoplasm, disrupt proton motive force, and prevent nutrient transport (Vasseur et al., 1999). Results of this study indicated that applying LCA in a three-step wash achieved approximately 1.0-1.9 log₁₀ CFU/g reductions of *Salmonella* and *L. monocytogenes* on spinach which are close (< 0.5 log₁₀ CFU/g) to the reductions of chlorine water. In our previous studies, triple-wash with LCA reduced *Salmonella* by 1.1-2.7, 1.8-2.1, and 0.9-3.3 log₁₀ CFU/product, and *L. monocytogenes* by 0.5-2.3, 0.8-1.2, and 0.9-2.3 log₁₀ CFU/product on butternut squashes (Li et al., 2020b), cucumber, and tomatoes (Li et al., 2020a), respectively, which also showed that similar reductions of *Salmonella* and *L. monocytogenes* were achieved by LCA compared to chlorine water.

The WV Small Farm Center recently recommended WV locally small growers to use SaniDate-5.0 in the triple-wash if their produce is grown directly from the soil because the largest WV wholesale buyer named Appalachian Harvest require it to be used during the post-harvest process especially for the organic farming process (Li et al., 2020a). SaniDate-5.0 is composed by the ingredients of 23% H₂O₂, 5.3% peroxyacetic acid (PAA), and 70% unknown components (likely water), which reported can oxidize cells of bacteria, denatures protein and disrupts cell wall structure to inactivate bacterial cells (McDonnell, et al. 2021). A study reported that the combination of H₂O₂ and PAA in a solution with high organic matter is still able to reduce bacteria cells including *Staphylococcus*, *Listeria*, and *E. coli* by more than 5 log₁₀CFU/ml with a contact time of 10-min (Briñez et al., 2006). Results of the current study suggested similar

($P > 0.05$) reductions (1.6 to 1.8 log₁₀CFU/g) of *Salmonella* and *L. monocytogenes* on spinach were achieved by SaniDate 5.0-0.25% and SaniDate 5.0-50% as compared to chlorine water and LCA. In this study, the better antimicrobial effect of SaniDate-5.0 than chlorine and acids could be explained by the combination of H₂O₂ and PAA in SaniDate-5.0 provide an additional synergistic impact for the oxidation of bacterial cells, although the detail of its antimicrobial mechanism remains unclear. Our previous study also found that applying SaniDate 5.0-0.25% and SaniDate 5.0-50% in three-step wash process reduced *Salmonella* by 2.7-2.9, 2.6, and 3.3 log₁₀CFU/product, and *L. monocytogenes* by 2.4-2.9, 1.1-1.3, and 2.5-2.7 log₁₀ CFU/product on butternut squashes (Li et al., 2020b), cucumber, and tomatoes (Li et al., 2020a), respectively, which were same or greater than the reductions of chlorine water or LCA treated samples. Results from the present and previous studies of triple-wash all suggested that SaniDate-5.0 is a good replacement for chlorine water to be used as an antimicrobial agent during the post-harvest washing process. Starting in 2019, the Preston Workshop, a small produce processor located in Reedsville, WV has already used the triple-wash with SaniDate-5.0 in their produce (butternut squashes, peppers, cucumbers, etc.) processing line, and their triple-washing facilities are expected to be updated by the end of 2021. However, for very small spinach growers, SaniDate-5.0 is needed to be verified of cost-effectiveness as a replacement of chlorine water by various agricultural economic models taking consideration of food safety risks, since the market price value for SaniDate-5.0 is \$330 (5-gallon pallet) compared to \$12 for Clorox® (chlorine water).

4.4. Conclusions

Results of this study suggested that applying the WWA procedure is better than the WAW in a three-step wash to inactivate *Salmonella* and *L. monocytogenes* on WV locally grown spinach. SaniDate-5.0, especially at the concentrations of 0.25% and 0.50%, indicates similar

or better antimicrobial efficacy than chlorine water and LCA on spinach. Results from this study provide validated academic laboratory results for WV locally small produce growers to adopt the three-step wash in their post-harvest process lines. Future studies are needed to evaluate whether SaniDate-5.0 is economically feasible for locally small produce growers and whether it will cause side-effect of sensory qualities of washed fresh produce. Future studies should also include a cross-contamination test of three-step wash with or without antimicrobials since it has now been well recognized that the washing of produce is more important for preventing cross-contamination than reducing foodborne pathogens (Gombas et al., 2017).

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Chapter 4 Tables and Figures

Table 4.1. Physical and chemical parameters of antimicrobial treatments in this study

Name	Concentration	pH	Temperature (°C)
Tap Water	~2 ppm of free chlorine	6.8	15.4
Sodium hypochlorite	100 ± 1 ppm ^a	6.8 ^b	14.4
Lactic/citric acid blend	2.5%	5.1	15.4
SaniDate-5.0	0.0064%	6.25	15.2
SaniDate-5.0	0.25%	5.25	15.1
SaniDate-5.0	0.50%	3.75	15.4

Note: ^aThe free chlorine concentration tested by DPD method using a chlorine photometer (CP-15, HF Scientific, Inc., Ft. Myers, FL);

^b adjusted by 10% citric acid

Figure 4.1. Reductions of *Salmonella* Typhimurium and Tennessee on spinaches (log₁₀ CFU/g) after three-step washing using the procedure of WAW (water dip + antimicrobial dip + water dip) or WWA (water dip + water dip + antimicrobial dip) with or without antimicrobials of sodium hypochlorite (SH-100 ppm), lactic/citric acid blend (LCA, 2.5%), and a mixture of hydrogen peroxide and peroxyacetic acid (SaniDate-5.0, 0.0064, 0.25 and 0.50%).

Note: Mean values with different letters on the standard deviation bar are statistically different ($P < 0.05$).

Figure 4.2. Reductions of *Listeria monocytogenes* on spinaches (log₁₀ CFU/g) after the three-step washing using the procedure of WAW (water dip + antimicrobial dip + water dip) or WWA (water dip + water dip + antimicrobial dip) with or without antimicrobials of sodium hypochlorite (SH-100 ppm), lactic/citric acid blend (LCA, 2.5%), and a mixture of hydrogen peroxide and peroxyacetic acid (SaniDate-5.0, 0.0064, 0.25 and 0.50%).

Note: Mean values with different letters on the standard deviation bar are statistically different (P < 0.05).

CHAPTER 5

Validation of Triple-Wash Procedure with A H₂O₂-Peroxyacetic Acid Mixer to Improve Microbial Safety and Quality of Butternut Squashes and Economic Feasibility Analysis

This study aims to i) evaluate two triple-wash procedures with an H₂O₂-peroxyacetic-acid mixer (SaniDate-5.0) to improve microbial safety and quality of butternut squashes and ii) determine the feasibility of the triple-wash application in a processing plant. In study I, fresh squashes were dip-inoculated with a mixture of *Salmonella* and *Listeria monocytogenes*, followed by two triple-wash steps including water dip-antimicrobial dip-water dip (WAW) or water dip-water dip-antimicrobial dip (WWA). Tested antimicrobials were i) lactic/citric acid blend (LCA; 2.5%); ii) sodium hypochlorite (SH; 100 ppm); and iii) SaniDate-5.0, 0.0064, 0.25 and 0.50%. Surviving bacteria were recovered using XLT-4 agar for *Salmonella* and MOX agar for *L. monocytogenes*. In study II, freshly harvested squashes were either left unwashed or triple-washed using WWA in water and SaniDate-5.0 (0.0071 and 0.45%) at a processing plant followed by storage at 9°C for 70 days. Aerobic Plate Counts (APCs), coliforms/*Escherichia coli*, lactic-acid-bacteria, and psychrotrophs on squashes were tested every seven days. Counts of *Salmonella* and *L. monocytogenes* on unwashed squash were 5.0-5.3 and 5.4-6.0 log₁₀ CFU/squash, respectively. WWA wash was more effective ($P < 0.05$) in reducing *Salmonella* (2.5 log₁₀ CFU/squash) and *L. monocytogenes* (2.3 log₁₀ CFU/squash) than WAW (1.8 log₁₀ CFU/squash for *Salmonella* and 0.3 log₁₀ CFU/squash for *L. monocytogenes*). Antimicrobials caused different degrees in reduction of *Salmonella* (1.7-2.6 log₁₀ CFU/squash) and *L. monocytogenes* (1.2-1.8 log₁₀ CFU/squash) on squashes, while the highest was achieved with SaniDate-5.0 (0.25 or 0.5%). During storage, SaniDate-5.0 treated squashes showed lower ($P < 0.05$) counts of APCs,

coliforms, lactic-acid-bacteria, and psychrotrophs than the unwashed and water-treated samples on days 50 to 70. The estimated annual operating cost of the triple-wash process with SaniDate-5.0 ranges from \$487.05 to \$1,977.33 for growers producing 1,000-5,000 squashes. The WWA procedure with SaniDate-5.0 appears to be an economically feasible way for local small producers to improve microbial safety and quality of squashes during postharvest processing.

Keywords: Triple-wash, Antimicrobials, Foodborne Pathogens, Surrogate, Butternut-Squash, Cost-effectiveness Analysis

5.1. Introduction

In West Virginia, there are 93 farmers' markets at over 112 locations (West Virginia Farmers' market Association, 2018), generating approximately \$9.1 million in annual sales with an average annual income of \$41,200 for full-time produce farmers and \$20,000 for part-time farmers (Li et al., 2018). As the farmers' markets have become more popular in WV, there has been increasing concern regarding the microbial safety and quality of the available produce. Our most recent study of farmers' markets in WV and Kentucky showed a high prevalence of *Salmonella* spp. (18.6% of spinach, 10.9% of tomatoes, 18.5% of peppers, and 56.3% of cantaloupes), and relatively high presence of *Listeria* spp. (3.78% of the samples) on locally grown produce surfaces (Li et al., 2017a). Ensuring the safety of fresh produce sold at farmers' markets is essential for the continuing growth of the burgeoning local foods sector in WV.

In 2017, the West Virginia Small Farm Center suggested produce growers use a three-step wash to remove pathogens from produce surfaces if their produce is eaten raw or grown close to the ground. The triple-washing process (water rinse, water rinse, and final antimicrobials dip) has been recognized as an effective tool for removing pathogens from food surfaces and improving on-farm food safety (Strohbehn et al., 2013). The effectiveness of this procedure largely depends on the types of antimicrobial solutions used. Commercial antimicrobials including chlorine water have been studied extensively as an effective and low-cost sanitizer (Shen et al., 2013); however, chlorinated water could be easily degraded by organic matters generated in a produce washing tank and create chlorine by-products (López-Gálvez et al., 2012; Shen et al., 2016). Therefore, produce growers are interested in learning the efficacy of new antimicrobial solutions. For example, SaniDate-5.0, a mix of peroxyacetic acid and H₂O₂, is considered by WV local produce processors as an organic sanitizer during post-harvest

processing. Validating this new antimicrobial solution during postharvest produce processing and storage, as well as the comparison of this strategy to other antimicrobial solutions, are important steps for providing scientific information toward improved food safety for locally grown produce in WV.

Butternut squashes, belonging to winter squashes (*Cucurbita spp.* L.), are very popular food and ornamental crops in WV. Butternut squashes are weighed from a few ounces to several hundred pounds and are high in vitamin A and fiber and low in calories (Jett, 2014). Although butternut squash has not been associated with any *Salmonella* outbreaks, in July 2019, Southern Specialties Inc. issued a recall notice for Marketside bagged fresh pre-cut butternut squash because of possible contamination with *Listeria spp.* (News Desk, 2019). Therefore, butternut squashes can be regarded as a previously unrecognized, under-researched, and emerging food vehicle possibly contaminated with foodborne pathogens.

Although the triple-wash is presumed to reduce foodborne pathogens effectively, the efficacy of antimicrobial solutions should be tested in the laboratory as well as in the real produce farm setting, where the dynamic processing conditions are more complex than the laboratory setting. In addition, an economic feasibility analysis with cost data from real plant settings is also needed to determine the potential of popularizing the proposed sanitizing procedure among small produce growers in WV. Therefore, the objectives of this study were to 1) evaluate two triple-wash procedures with antimicrobials to reduce *Salmonella* and *Listeria monocytogenes* on butternut squashes in the laboratory, 2) evaluate the shelf-life stability of washed squashes in a real processing plant, and 3) evaluate the economic feasibility of adopting the triple-wash process through cost-benefit analyses.

5.2. Materials and Methods

5.2.1. Preparation of inoculum and inoculation of squashes

In this study, two *Salmonella* strains (*Salmonella* Typhimurium ATCC 14028 and *Salmonella* Tennessee ATCC 10722), and two *L. monocytogenes* strains (L2624 and L2625-cantaloupe outbreak serotype 1/2b, donated by Dr. Joshua Gurtler, USDA-ARS, Wyndmoor, PA) were used in the laboratory triple-wash validation studies. Each *Salmonella* or *L. monocytogenes* strain was prepared separately. In each experiment, only one cocktail (*Salmonella* or *L. monocytogenes*) was used, and thus, no combination of bacteria inoculum occurred in this study.

Pure cultures of *Salmonella* and *L. monocytogenes* were generated by streak-plating frozen stock cultures onto Xylose-Lysine-Tergitol-4 (XLT-4, Hardy Diagnostics, Santa Maria, CA, USA) and Modified Oxford (MOX) agar (Hardy Diagnostics, Santa Maria, CA, USA), respectively, and then incubating at 35°C for 24 (XLT-4) or 48 h (MOX) to form single colonies. On the day of the experiment, two single colonies picked from XLT-4 (*Salmonella*) and MOX Agar (*L. monocytogenes*) of each strain were cultured in 10 mL of tryptic soy broth (TSB, Alpha Biosciences, Baltimore, MD, USA) and incubated at 35°C for 24 ± 2 h. The initial concentration was $7.41 \log_{10}$ CFU/mL for *Salmonella* Typhimurium, $7.96 \log_{10}$ CFU/mL for *Salmonella* Tennessee, $8.59 \log_{10}$ CFU/mL for *L. monocytogenes* 2624, and $8.77 \log_{10}$ CFU/mL for *L. monocytogenes* 2625. Each *Salmonella* and *L. monocytogenes* culture was centrifuged at a speed of $5,000 \times g$ for 15 min (VWR Symphony 4417, VWR International, Radnor, PA), and then triplicate washed in 0.1% buffered peptone water (BPW, Alpha Biosciences, Baltimore, MD, USA), followed by re-suspending in 10 mL of 0.1% BPW. After washing, the initial concentration of *Salmonella* and *L. monocytogenes* cells in the re-suspended solution was 7.6 and $8.7 \log_{10}$ CFU/mL, respectively. To make a similar initial inoculum level of *Salmonella* and

L. monocytogenes (difference $\leq 0.5 \log_{10}$ CFU/mL), each *Salmonella* strain was prepared in duplicate based on the preliminary studies. Inoculum of *Salmonella* and *L. monocytogenes* were made from the above-mentioned two-strain cocktail separately, where two *Salmonella* strains were combined, or two *L. monocytogenes* strains were combined. The volume of 40 mL (*Salmonella*) or 20 mL (*L. monocytogenes*) prepared cocktails were then added into 3 L 0.1% BPW solution to yield an inoculum solution with a target concentration of 6.2-6.7 $\log_{10}$ CFU/ml, which was used for the following dip inoculation process. Each butternut squash, obtained from Preston County Workshop Inc., was inoculated by placing the product in a plastic tub containing 3 L of the inoculum with manual stir for 5 min, followed by placement in a biosafety cabinet for 30 min to allow for pathogen attachment.

5.2.2. Triple wash squashes with antimicrobials in lab condition

Inoculated squashes were left unwashed (control) or triple washed in three large plastic tubs with 10 L solution in each. For each treatment, 6 squashes were randomly split into two groups (3 samples each), followed by two triple-wash procedures including a water dip-antimicrobial dip-water dip (WAW), or a water dip-water dip-antimicrobial dip (WWA), followed by draining for 2 min on fresh paper towel. Each wash step was 10 s of dip time with manual rotation. The triple-wash process was conducted by three workers, each carrying and washing one squash into each 10 L solution tub followed by WAW or WWA procedure at the same time. After washing three squashes, old solutions were added with 500 ppm chlorine for 30 min followed by autoclaving. The tubs were sanitized, refreshed with new water or new antimicrobial solutions to prevent cross-contamination from the previous treatment. Tested antimicrobials were i) sodium hypochlorite (SH; free available chlorine 100 ppm, pH=6.8 adjusted by 10% citric acid, 14.4°C; Birko, Henderson, CO, USA), ii) a lactic/citric acid blend

(LCA, Veggiexide[®], 2.5%, pH=5.1, 15.4°C, Birko); and iii) an H₂O₂-peroxyacetic-acid mixer (SaniDate-5.0, 15.2°C; Arbico Organics, Tucson, AZ, USA) with the concentrations of 0.0064 (pH 6.25), 0.25 (pH 5.52) and 0.50% (pH 3.75).

5.2.3. Triple wash squashes in a processing plant

The onsite plant shelf-life validation studies were conducted at Preston County Workshop, Reedsville, WV, from September to December 2018. Freshly harvested butternut squashes without any brown rust color (visible *Fusarium Wilt* contamination), animal bites, and large punctures on their skin were picked. The triple-wash was processed in three commercial washing tanks, each containing 175 gallons of water. Based on the laboratory test results, the WWA procedure was applied in this onsite plant study. The first step was dipping squashes into a water solution (pH 7.02, 13.3°C) and manually brushing with a produce brusher for 15 sec (Figure 5.1A); then the brushed samples were dipped into the second water tank (pH 7.05, 14.3°C) and with soft cloth towels scrubbed for 15 sec (Figure 5.1B). Finally, the samples were placed into a melt rinse container with mesh and completely immersed in the SaniDate-5.0 solutions for 45 sec (Figure 5.1C). The antimicrobial treatments tested in the third tank were 1) water, 2) SaniDate-5.0-0.0071% (pH 5.88, 15.1°C) and 3) SaniDate-5.0-0.45% (pH 3.79, 15.2°C). The unwashed squashes were included as the controls. After triple-washing, the squashes were dried in front of a fan for 30 min, followed by storage in a cooler at 9°C for 70 days. The temperatures ($9.1 \pm 0.59^{\circ}\text{C}$) and relative humidity values ($\text{RH } 72.04 \pm 2.68\%$) of the cooler were monitored and recorded during the whole experiment period. Squash samples were collected every 7 days and transferred to the Food Science Laboratory at West Virginia University to conduct microbial quality analysis.

5.2.4. Microbiological analysis

Each squash was placed into a sterile chicken-rinse bag (Nasco, Fort Atkinson, WI, USA) and rinsed in 200 ml of BPW, followed by vigorously shaking for 60 s to detach microorganisms from the surface. For laboratory studies, the squash rinse solutions were 10-fold serially diluted in 0.1% BPW and then spread-plated on XLT-4 and MOX agar to enumerate *Salmonella* and *L. monocytogenes* cells, respectively, followed by incubating agar plates at 35°C for 24 h (XLT-4) or 48 h (MOX). For onsite plant studies, squash rinse solutions for counting the total aerobic bacteria, coliform, and *Escherichia coli* populations were surface-plated onto 3M[®] APCs, *E. coli*/coliforms petrifilm (3M Microbiology Products, St. Paul, MN, USA) following manufacturer instructions and incubated at 35°C for 72 h (APCs) or 48 h (*E. coli*/coliforms). Samples for lactic acid bacteria (LAB) were spread-plated onto Man-Rogosa-Sharpe (MRS) agar (Hardy Diagnostics, MD, USA) and incubated in an anaerobic jar (Hardy Diagnostics) at 30°C for 3 days. Samples for psychrotrophs (PSY) were spread-plated onto tryptic soy agar and incubated at 4°C for 10 days. All agar plates and petrifilms were manually counted colonies after the incubation period.

5.2.5. Economic feasibility analysis

To examine the economic feasibility of the triple wash process, we calculated its operating cost for three types of growers, according to their production scale. In conducting the analysis, we assumed that (1) a 200-gallon water tank costs \$250 and lasts for 10 years; (2) scrub brush and microfiber towels are replaced annually at \$5 and \$1 each, respectively; (3) labor cost is \$10/hour; (4) water, including sewage, costs \$10 per 1,000 gallons (3,784 L); (5) each triple wash section takes three hours; (6) 500 squashes can be processed in each section with the help of four workers. The costs of antimicrobials (SaniDate-5.0 at 0.0071% and 0.45%) and the mixed

solutions are listed in Table 5.1. The market cost of SaniDate-5.0 is \$62.20/gallon. The amount needed for preparing a 175-gallon water solution with 0.0071% and 0.45% SaniDate-5.0 is 47.03 and 2981.01 mL, respectively. Therefore, the costs for 175-gallon mixed solution is \$2.52 (0.0071%) and \$50.73 (0.45%). For simplicity, we focus our discussion on the extra cost that would be incurred by farmers had they adopted the triple wash process, without considering the cost associated with other post-harvest procedures.

5.2.6. Data analysis

The laboratory study was triple-repeated with 3 squashes per treatment per repeat with a total of 9 samples per treatment. It was conducted with a completely randomized 2×5 factorial design for the 2 triple-wash procedures (WAW or WWA) and 5 antimicrobial treatments. The survival and reduction of *Salmonella* and *L. monocytogenes* were analyzed using the mixed model procedure of SAS (version 9.2, SAS Institute, Cary, NC), with the individual factors being the wash procedure, the antimicrobial treatment, and their interactions. The reduction data were determined by a reduction ratio of $\log_{10} (N_0/N)$, which includes N_0 , the average control plate counts, and N , the plate count of each antimicrobial treated sample (Adler et al., 2016). The means were compared at an $\alpha = 0.05$ significance level as determined by Tukey HSD. The plant onsite shelf-life studies were repeated twice, and for each replication, 3 individual samples per treatment at each sampling time were analyzed ($n = 6$). The Mixed Model Procedure in SAS® was used to analyze the counts of APCs, coliforms/*E. coli*, LAB, and PSY on squash surfaces, and the individual factors are the antimicrobial used, time period, and their interaction. A statistical significance level of 0.05 ($P < 0.05$) as determined by LSD was used.

5.3. Results and Discussion

5.3.1. Laboratory study: antimicrobial effect of treatments during the triple-wash process

The microbial population of unwashed and triple-washed inoculated squash was obtained on XLT-4 and MOX for *Salmonella* and *L. monocytogenes*, respectively. Initial levels of inoculated *Salmonella* and *L. monocytogenes* on unwashed squash surfaces were 5.0 to 5.3 log₁₀ CFU/squash and 5.4 to 6.0 log₁₀ CFU/squash, respectively. According to the mixed model procedure of SAS, for *Salmonella* the antimicrobial efficacy of triple wash was determined by the wash process ($P < 0.05$), and the type of antimicrobial agent ($P < 0.05$). For *L. monocytogenes*, the triple-wash process was also significant ($P < 0.05$), but the type of antimicrobial agent was not the main effect ($P > 0.05$). No interactions ($P > 0.05$) were significant for either *Salmonella* and *L. monocytogenes*.

Our internal survey during the produce wash short course at the 2018 WV Small Farm Conference showed that small produce growers applied two triple-wash processes. i.e., WAW and WWA, in WV. In this laboratory test, the least squares means (LS means) across the tested antimicrobials indicate that WWA is more effective ($P < 0.05$) than WAW in reducing *Salmonella* (2.54 versus 1.84 log₁₀ CFU/squash) and *L. monocytogenes* (2.30 versus 0.72 log₁₀ CFU/squash) on squash surfaces. No previous studies have been conducted to compare the reductions from these two triple wash procedures. For WWA, the first two water wash steps removed the organic matter from the squash surfaces and possibly lowered the initial population on squashes by physical removal which benefits the final antimicrobial wash.

Antimicrobials applied in triple-wash WAW procedure onto butternut squashes significantly reduced the *Salmonella* population (survival of 2.5 to 3.9 log₁₀ CFU/squash) compared to the unwashed control (5.0 log₁₀ CFU/squash), with the reductions ranging from 1.1

(SH) to 2.5 (SD-0.50%) log₁₀ CFU/squash (Table 5.2). Compared with the WAW, squashes washed through the WWA procedure in SH, LCA, SaniDate-5.0-0.0064%, and -0.25% achieved a significant additional reduction ($P < 0.05$) of *Salmonella* by 1.6, 0.8, 0.4, and 0.6 log₁₀ CFU/squash, respectively (Table 5.1). However, no significant difference ($P > 0.05$) between WAW and WWA was found for reductions in SaniDate-5.0-0.5% washed samples.

L. monocytogenes is a possible emerging and under-researched pathogen on butternut squashes. Similar to the results for *Salmonella*, triple-washing following a WAW process with antimicrobials significantly reduced *L. monocytogenes* ($P < 0.05$) on squashes, resulting in a surviving population of 4.8 (SH and LCA) to 5.5 log₁₀ CFU/squash (SaniDate-5.0-0.5%) compared to 6.0 log₁₀ CFU/squash of the unwashed control. Squashes washed in 0.5% of SaniDate-5.0 achieved the highest ($P < 0.05$) reduction (1.2 log₁₀ CFU/squash) among the five tested antimicrobial treatments, and no significant difference ($P > 0.05$) was observed among the reductions (0.5 to 0.8 log₁₀ CFU/squash) resulting from SH, LCA, SaniDate-5.0-0.0064% and SaniDate-5.0-0.25% (Table 5.2). Similarly, the WWA procedure significantly enhanced ($P < 0.05$) the reduction levels by 1.6 to 1.8 log₁₀ CFU/squash for all tested antimicrobial solutions as compared to the WAW (Table 5.2). However, there was no difference ($P > 0.05$) in reductions among these five treatments applied by the WWA procedure.

Chlorine water, primarily composed of hypochlorous acid (HOCl), is currently the most commonly used sanitizer during fresh produce processing due to its strong ability to kill microorganisms in solutions, minimal impact on produce quality, and economic feasibility (Shen et al., 2013). Chlorine was found to have significantly reduced the *Salmonella* population on cantaloupe (Fan, Annous, Keskinen, & Mattheis, 2009) and the antimicrobial effect was observed throughout the 6-day storage period at 4°C (Ukuku & Sapers, 2001). Results from this

study showed that reductions of 1.1 to 2.7 and 0.5 to 2.3 log₁₀ CFU/squash were achieved by SH to inactivate *Salmonella* and *L. monocytogenes*, respectively, on squashes, which are similar to previous results of SH on other fresh produce (Gil et al., 2009). Recently, fresh produce processors, including local small produce processors, have become more interested in evaluating the efficacy of alternative antimicrobials during the post-harvest washing process (Adler et al., 2016) due to certain disadvantages of chlorine, such as fast degradation and the generation of by-products (Shen, Luo, Nou, Wang, & Millner, 2013; Shen, Norris, Williams, Hagan, & Li, 2016).

LCA, a buffered mixture of the lactic and citric acid solution, can reduce the bacterial intracellular pH and disrupts the transmembrane proton motive force (Raybaudi-Massilia et al., 2009; Ricke, 2003). A recent study suggested that cabinet spraying 2.5% LCA onto Jalapeno peppers reduced the natural flora, generic *Escherichia coli*, and *Salmonella* counts by 1.3, 0.8, and 1.1 log₁₀ CFU/g, respectively (Adler et al., 2016). Results from the present study showed that LCA achieved very similar reductions compared to the SH for both *Salmonella* (1.1-2.7 vs. 1.3 to 2.1 log₁₀ CFU/squash) and *L. monocytogenes* (same at 0.5 to 2.3 log₁₀ CFU/squash), regardless of applying a WAW or WWA procedure. Hydrogen peroxide (H₂O₂) is another common sanitizer, which has been reported to effectively reduce the *Salmonella* population on produce surface in the postharvest washing process (Ukuku & Sapers, 2001; Ukuku & Fett, 2004). SaniDate-5.0, mainly composed of 23% H₂O₂ and 5.3% peroxyacetic acid, has been recommended by the WV Small Farm Center to treat fresh produce from small produce farms in WV (Li et al., 2017b). The mechanism of SaniDate-5.0 to inactivate pathogens is to oxidize bacterial cells, denature protein and further disrupt cell wall structure (Block, 2011). Results of the present study indicate a similar or greater ($P < 0.05$) reduction of *Salmonella* and *L. monocytogenes* by SaniDate-5.0 as compared to SH, especially for the 0.5% concentration level.

Therefore, SaniDate-5.0 could be potentially used by locally small produce processors as an alternative antimicrobial solution to SH.

5.3.2. Plant onsite study: evaluate the shelf-life stability of washed squashes

Validation of a new antimicrobial solution that can be readily adopted by the food processing industry often requires test conditions that match as closely as possible the commercial operation setting (Luo et al., 2012). Therefore, a plant onsite shelf life study was conducted in fall 2018 at our project stakeholder, Preston County Workshop, Reedsville, WV, a squash processing plant. Since the stakeholder is currently applying SaniDate-5.0 during its triple-wash process, this study only focussed on SaniDate-5.0 solutions with concentrations of 0.0071% (currently used by the stakeholder) and 0.45% as compared to the water-washed and unwashed samples.

Overall, no significant ($P > 0.05$) growth of the tested APCs, coliforms/ *E. coli*, PSY, and LAB counts on squash surfaces was detected during the storage period (70 days) due to the relatively low storage temperature of 9°C (Figure 5.2). The fluctuation of the microbial counts on squash surfaces during storage was attributed to inconsistent initial microbial loads among freshly harvested squashes. It is also noticed that a reduction of APCs, coliforms, PSY, and LAB counts started for almost all treated samples including the water and control group on day 49, which could be explained by the metabolic injury to bacteria at the low temperature. The death of bacteria slowly starts during extended storage at low temperature which begins with nonlethal physical or metabolic injury and further developed to progressively cold injury requiring additional nutrition to survive (Straka and Stokes, 1959). Squashes treated by SaniDate-5.0 with 0.0071% or 0.45% had lower ($P < 0.05$) counts of APCs, coliforms, LAB, and PSY than the unwashed and water-treated samples typically from days 49 to 70 (Figure 5.2) due to the use of

antimicrobials. In contrast to the laboratory tests, no difference ($P < 0.05$) between the 0.0071% and 0.45% of SaniDate-5.0 was observed in the onsite plant studies, which is possibly due to the dipping time of 10 s (laboratory test) versus 45 s (onsite plant studies) in each washing step. The dipping time was limited to 10 s in the laboratory test because this test was focused on the impact of antimicrobial treatments and the triple-wash processes (WAW vs. WWA). The 45s washing time used in the onsite plant test because the triple-wash process needs to be conducted the same as the stakeholder did for their commercial products. This result also suggests that 1) simply using a higher concentration of antimicrobials is not necessary to improve microbial quality of squashes; 2) the reduction efficacy is often lower than expected in commercial-scale processing plants because operational and storage conditions are much less controlled in the real commercial-scale implementation than under academic laboratory conditions.

Initial APCs on unwashed fresh raw butternut squashes were $7.3 \pm 0.2 \log_{10}$ CFU/squash (Figure 5.2). After the squashes were triple-washed and dried, APCs on squash decreased by 0.5, 0.3, and $0.7 \log_{10}$ CFU/squash to 4.5, 4.7, and $4.4 \log_{10}$ CFU/squash for water, SaniDate-5.0-0.0071% and -0.45% treatment samples, respectively, which were not significantly different ($P > 0.05$) (Figure 5.2). During storage, APCs remained between 6.6 to $7.9 \log_{10}$ CFU/squash and 6.2 to $8.0 \log_{10}$ CFU/squash on unwashed and water-washed samples, respectively (Figure 5.2). In the final 21 days of storage (days 49 to 70), compared to the unwashed control, APC declines ($P < 0.05$) of 0.5 to $1.2 \log_{10}$ CFU/squash were observed for the SaniDate-5.0-0.0071% and -0.45% washed squashes, which were greater ($P < 0.05$) than the water treatment reductions of 0.1 to $0.4 \log_{10}$ CFU/squash (Figure 5.2).

Although no studies have reported the APCs on butternut squashes, the APCs on similar crops such as cantaloupes and tomatoes treated by antimicrobials are well documented. In a

previous study (Fan, Annous, Keskinen, & Mattheis, 2009), the results suggested that 180 ppm chlorine and 80 ppm peroxyacetic acid (PAA) reduced APCs on cantaloupes by 0.8 and 0.7 log₁₀ CFU/cm²; however, the reduction effect was not statistically significant. Moreover, 80 ppm PAA treatment significantly reduced the aroma score at 4°C storage, which negatively affected the postharvest quality of cantaloupes. Another alternative sanitizer 5% acetic acid at 25°C also had no significant effect on APCs reduction on cantaloupe surfaces (Fouladkhah & Avens, 2010), a result similar to those found in Fan's study. Inconsistent results were reported for H₂O₂ as antimicrobials against APCs on cantaloupe. Although 2.5% H₂O₂ significantly reduced total mesophilic aerobes on cantaloupe and honeydew melon surfaces (Ukuku, Bari, Kawamoto, & Isshiki, 2005), 5% H₂O₂ at 50°C was reported to have no significant effect against APCs on shelf-life of fresh-cut cantaloupes (Sapers, Miller, Pilizota, & Mattrazzo, 2001). Heated sanitizer, even hot water, showed a better antimicrobial effect against APCs. APCs were significantly reduced on cantaloupe surfaces when water and 5% acetic acid were used at 95°C (Fouladkhah & Avens, 2010). As the surface of cantaloupes was highly uneven and rough, the antimicrobial effect of many sanitizers may be restricted without surfactants. For example, 150 ppm chlorine treatment on tomatoes was reported to have a significant effect against APCs (Sibomana, Ziena, Schmidt, & Workneh, 2017) while 180 ppm chlorine did not have a significant effect on cantaloupes (Fan, Annous, Keskinen, & Mattheis, 2009).

The initial population of PSY on unwashed squashes was 6.0 log₁₀ CFU/squash. Triple-washing squashes in water reduced ($P < 0.05$) the PSY counts by 0.9 log₁₀ CFU/squash on day 0, and the reductions increased to 1.2 ($P = 0.05$) and 1.5 log₁₀ CFU/g ($P < 0.05$) when SaniDate-5.0-0.0071% and 0.45% solution was applied, respectively (Figure 5.3). Between 14 and 49 days in storage, the levels of PSY among treatments did not change significantly ($P >$

0.05, Figure 5.2). Towards the end of the storage, from days 56 to 70, SaniDate-5.0-0.0071% (3.9 to 4.5 log₁₀ CFU/squash) and 0.45% (3.1 to 4.4 log₁₀ CFU/squash) treated samples showed significantly ($P < 0.05$) lower survivals of PSY, as compared to the unwashed control (5.1 to 5.4 log₁₀ CFU/squash) and water-treated samples (4.7 to 5.7 log₁₀ CFU/squash) (Figure 5.2).

Initial total coliform counts on butternut squashes from unwashed and water-treated samples were 5.1 and 4.7 log₁₀ CFU/squash, respectively (Figure 5.3). A significant lower ($P < 0.05$) count of coliforms of 3.0 log₁₀ CFU/squash was obtained on SaniDate-5.0-0.45% treated samples on day-0 and SaniDate-5.0-0.0071% treated samples on day 7, compared to the unwashed control and water treated samples (Figure 5.3). Similar to the APCs and PSYs, no significant ($P > 0.05$) difference in coliforms counts among all treatments were found on days 14-42 in storage. However, during the last three weeks of storage (days 49-70), coliforms ranged from 4.3 to 4.9 log₁₀ CFU/squash and 3.7 to 4.7 log₁₀ CFU/squash for samples washed in SaniDate-5.0-0.0071% and SaniDate-5.0-0.45% solutions, respectively, which were significantly lower than the samples of the unwashed control (5.1 to 6.0 log₁₀ CFU/squash) and those receiving water treatment only (5.3 to 5.8 log₁₀ CFU/squash) (Figure 5.2). For the *E. coli* counts, SaniDate-5.0-0.0071% and -0.45% solution did not cause significant reductions during the storage period except for days 56-63, with a very low survival of 0.0-0.3 log₁₀ CFU/squash (Data not shown in tabular form).

LAB is Gram-positive, acid-tolerant, and lactic acid-producing bacteria, which usually grows well in an environment rich in CO₂ and low in O₂ (Luo, 2007). In this study, the squashes were stored aerobically. Therefore the LAB counts on unwashed control samples ranged from 5.1 to 6.3 log₁₀ CFU/squash with no significant growth. On day 0, the SaniDate-5.0-0.45% washed samples resulted in lower ($P < 0.05$) LAB populations (5.2 log₁₀ CFU/g) than the

control (5.8 log₁₀ CFU/squash) and the water treated samples (6.2 log₁₀ CFU/squash) (Figure 5.2). From days 7 to 14, the washed samples showed lower ($P < 0.05$) survival of LABs than the unwashed control; however, no significant difference ($P < 0.05$) between SaniDate-5.0 and water treated samples were found. Similar to the aforementioned microbial quality results, from days 49 to 70, the SD washed samples indicated significantly lower ($P < 0.05$) LAB counts on squash surfaces than the control and water treated samples. For example, the LAB survival on SaniDate-5.0-0.45% remained as 3.9 to 4.4 log₁₀ CFU/squash, which is lower ($P < 0.05$) than the other three treatments.

The antimicrobial effects of chlorine and H₂O₂ were tested previously on LAB (Ukuku, 2006; Ukuku & Fett, 2004). Among the tested sanitizers, chlorine at 200 ppm or H₂O₂ at 2.5% and 5% were reported to be significantly effective at reducing the population of LAB when compared to unwashed or tap water-washed cantaloupe (Ukuku, 2006). As H₂O₂ is in the formula of SaniDate-5.0, the antimicrobial effect of SaniDate-5.0 was expected on squashes.

5.3.3. The operating cost of triple-wash on squashes

Our previous economic analysis calculated the cost of implementing triple wash for typical produce farmers in WV, finding only a modest increase to operating cost when applying the triple-wash procedure with chlorine and a slightly higher increase when using SaniDate-5.0 (Scarcioffololo et al., 2018). Even though earlier analysis showed that triple-wash could be considered a low-cost sanitizing procedure, more detailed analyses, in conjunction with the cost data obtained from real plant settings are needed to determine the economic feasibility of the triple-wash procedure for local small producers.

Table 5.4 lists the operating cost of triple-wash for squash growers of the small, medium, and large scales when the water and antimicrobial solution are re-used throughout each section.

The triple-wash process requires minimal initial set-up cost, and its fixed cost only includes the cost for the water tanks, hand brushes, microfiber, and annualized maintenance expenditure. Growers may choose to harvest squashes multiple times throughout the harvest window and conduct a triple-wash section when a total of 500 squashes have been harvested. Since the process is labor-intensive, it is not surprising to see in Table 5.2 that most of the operating cost comes from labor, especially for larger-scale growers. For SaniDate-5.0-0.0071%, labor cost accounts for 49.3%, 64.9%, and 80.3% of the added processing cost for growers producing 1,000, 2,000, and 5,000 squashes, respectively.

As shown in Table 5.4, when water and treatments are reused in the same section, the annual operating cost of the triple wash process ranges between \$487.05 and \$1,977.33 for growers producing 1,000-5,000 squashes using SaniDate-5.0 at two levels of concentration. As compared to the lower-concentration antimicrobial solution, it costs 20% extra to apply the solution with the higher SaniDate-5.0 concentration for two sections per year (1,000 squashes), which rises to 32% for 10 sections per year (5,000 squashes). Overall, it costs around 3 times more to apply 10 sections of triple-wash on squashes as compared to 2 sections.

Since the effectiveness of antimicrobials may decrease when the level of organic load increases, farmers may choose to process fewer squashes in each triple wash section. The shortened wash section also allows farmers more flexibility in deciding when to harvest squash during the harvest window as they can conduct the sections on different days. We compute the operating cost for the triple-wash process when 60 and 180 squashes are processed in each section, the results of which are presented in Table 5.5. Compared to the 500-squash section, it costs 20-220% more for the 60-squash section depending on the production scale and the antimicrobial concentration level (Table 5.5). For the 180-squash section, the cost would

increase by 5-55% compared to the baseline case of processing 500 squashes per section (Table 5.5). The increased operating cost is attributed to the extra cost required for more water and antimicrobial solutions which are replaced at the end of each section. Since the triple-wash procedure can be applied to a variety of produce, the fixed cost is expected to decrease when the system is operated more frequently.

In conclusion, applying the triple-wash process with the WWA procedure showed better bacterial pathogen reductions than the WAW procedure. SaniDate 5.0 indicates similar or better antimicrobial efficacy against *Salmonella* and *L. monocytogenes* compared to chlorine water and LCA solutions. SaniDate-5.0 treated squashes showed lower microbial counts than the unwashed and water-treated samples typically during the later storage period. The annual operating cost of the triple-wash using SaniDate-5.0 is approximately \$500 to \$2,000 for producing 1,000 to 5,000 squashes. An extra 5-220% cost will be added if the water is refreshed in each tank. Results from this study provide important information for local small produce growers who are interested in adopting the triple-wash procedure with SaniDate-5.0 during postharvest processing.

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Chapter 5 Tables and Figures

Table 5.1. Cost of SaniDate-5.0 at different concentrations

	SaniDate-5.0	
	0.0071%	0.45%
Gallon price (\$)	62.20	62.20
Amount needed for 175 Gallons of water (mL)	47.03	2981.01
Mixed solution cost, at 175 gallons (\$)	2.52	50.73

Table 5.2. Reduction of *Salmonella* Typhimurium and Tennessee on butternut squashes ($\log_{10}$ CFU/squash) by triple-wash procedure water dip-antimicrobial dip-water dip (WAW) or water dip-water dip-antimicrobial dip (WWA) in sodium hypochlorite (SH, 100 ppm), lactic and citric acid blend (LCA, Veggiexide®, 2.5%), a PAA and hydrogen peroxide mixer (SaniDate-5.0, 0.0064, 0.25 and 0.50%).

Treatment	WAW	WWA
Control	—*	—*
Sodium hypochlorite (SH-100 ppm)	$1.1 \pm 0.7cA$	$2.7 \pm 0.4aB$
Lactic and citric acid blend (LCA-2.5%)	$1.3 \pm 0.4cA$	$2.1 \pm 1.1cB$
SaniDate-5.0-0.0064%	$2.0 \pm 0.6baA$	$2.4 \pm 0.5bcB$
SaniDate-5.0-0.25%	$2.3 \pm 0.5aA$	$2.9 \pm 0.2aB$
SaniDate-5.0-0.50%	$2.5 \pm 0.4aA$	$2.7 \pm 0.4abA$

—* indicates reduction data are not available

Mean values with different letters within a column are significantly different ($P < 0.05$)

Mean values with different capital letters within a row are significantly different ($P < 0.05$)

Table 5.3. Reduction of *Listeria monocytogenes* on butternut squashes ($\log_{10}$ CFU/squash) by triple-wash procedure water dip-antimicrobial dip-water dip (WAW) or water dip-water dip-antimicrobial dip (WWA) in sodium hypochlorite (SH, 100 ppm), lactic and citric acid blend (LCA, Veggiexide®, 2.5%), a PAA and hydrogen peroxide mixer (SaniDate-5.0, 0.0064, 0.25 and 0.50%).

Treatment	WAW	WWA
Control	—*	—*
Sodium hypochlorite (SH-100 ppm)	$0.5 \pm 0.8aA$	$2.3 \pm 0.3aB$
Lactic and citric acid blend (LCA-2.5%)	$0.5 \pm 0.5aA$	$2.3 \pm 0.8aB$
SaniDate-5.0-0.0064%	$0.8 \pm 0.5aA$	$2.4 \pm 0.4aB$
SaniDate-5.0-0.25%	$0.6 \pm 0.5aA$	$2.4 \pm 0.4aB$
SaniDate-5.0-0.50%	$1.2 \pm 0.5bA$	$2.9 \pm 0.7bB$

—* indicates reduction data are not available

Mean values with different letters within a column are significantly different ($P < 0.05$)

Mean values with different capital letters within a row are significantly different ($P < 0.05$)

Table 5.4. Cost comparison of triple wash at different production scales. Total cost is calculated by assuming water and treatments are reused throughout the whole washing section.

	1,000 Squashes (2 sections)	2,000 Squashes (4 sections)	5,000 Squashes (10 sections)
Fixed cost			
Water tank (annualized)	\$ 75.00	\$ 75.00	\$ 75.00
Hand brush	\$ 50.00	\$ 50.00	\$ 50.00
Microfiber cloth	\$ 10.00	\$ 10.00	\$ 10.00
Maintenance (annualized)	\$ 100.00	\$ 100.00	\$ 100.00
Variable cost			
Labor	\$ 240.00	\$ 480.00	\$ 1,200.00
SaniDate-5.0			
0.0071%	\$ 1.55	\$ 3.09	\$ 7.73
0.45%	\$ 97.97	\$ 195.93	\$ 489.83
Water	\$ 10.50	\$ 21.00	\$ 52.50
Total cost			
SaniDate-5.0			
0.0071%	\$ 487.05	\$ 739.09	\$ 1,495.23
0.45%	\$ 583.47	\$ 931.93	\$ 1,977.33

Table 5.5. Cost comparison of triple wash at different production scales when water and antimicrobial treatments are refreshed every 60 or 180 squashes.

Refresh frequency	Total cost	1,000 Squashes	2,000 Squashes	5,000 Squashes
Every 60 squashes	SaniDate-5.0			
	0.0071%	\$ 583.41	\$ 931.82	\$ 1,977.06
	0.45%	\$ 1,451.19	\$ 2,667.37	\$ 6,315.93
Every 180 squashes	SaniDate-5.0			
	0.0071%	\$ 511.14	\$ 787.27	\$ 1,615.69
	0.45%	\$ 800.40	\$ 1,365.79	\$ 3,061.98

(A)

(B)

(C)

Figure 5.1. Triple-wash process including step 1-manually brush with produce brush (A), step 2-soft cloth towels scrub (B), step 3-melt rinse container with mesh and immerse into the SaniDate-5.0 solution (C).

Figure 5.2. Average and standard deviation of microbial populations (log₁₀ CFU/squash) measured as aerobic plate counts, psychrophiles, total coliforms, and lactic acid bacteria on butternut squashes after triple-wash in water and SaniDate-5.0 (SD, 0.0071% and 0.45%) solutions followed by storage at 9°C for 70 days.

CHAPTER 6

Assessing Farmers' market Produce Vendors' Handling of Containers and Evaluation of the Survival of *Salmonella* and *Listeria monocytogenes* on Plastic, Pressed-Card, and Wood Container Surfaces at Refrigerated and Room Temperature

This study aims to assess how small produce growers handle containers and evaluate the survival of *Salmonella* and *Listeria monocytogenes* on various produce container surfaces commonly used at farmers' markets, under storage conditions both at refrigerated and room temperature. In Study I, an anonymous survey was conducted to assess the practices of handling produce containers from 28 vendors at farmers' markets in Morgantown, WV, and 141 vendors from farmers' markets in Kentucky. In Study II, plastic, pressed-card, and wood containers were trimmed (25 cm²) and inoculated with *S. Typhimurium* and Tennessee, and two strains of *L. monocytogenes*, stored at 3.2°C (22.19% RH) and 22.5°C (50.40% RH), respectively, for 21 days and periodically analyzed for microbial populations on XLT-4 (*Salmonella*) and Modified-Oxford (*L. monocytogenes*) agars. The survey results showed that plastic, paper, and wood containers are the top three choices for small produce growers to transport and present produce at farmers' markets. The pathogens decreased slower ($P < 0.05$) at 3.2°C and on pressed-card and wood surfaces than at 22.5°C and a plastic surface. At 3.2°C, *Salmonella* counts decreased ($P < 0.05$) from 5.27-5.53 to 2.63-2.84 log₁₀ CFU/cm², and *L. monocytogenes* decreased ($P < 0.05$) from 6.39-6.93 to 4.89-5.46 log₁₀ CFU/cm² on the three material surfaces by the end of the storage period, with the lowest ($P < 0.05$) survival on a plastic surface. At 22.5°C, *Salmonella* populations decreased ($P < 0.05$) from 4.94-5.38 to <1.30 log₁₀ CFU/cm² (the detection limit) after 3, 9 and 12 days on plastic, pressed-card and wood surfaces, respectively. *L. monocytogenes* decreased ($P < 0.05$) from 6.39-6.93 to ≤1.30 log₁₀ CFU/cm² after 12, 12, and 21

days on plastic, wood, and pressed-card surfaces, respectively. These results were confirmed by different mathematical survival models for analyzing pathogen inactivation rates. Vendors at farmers' markets should choose plastic containers to store fresh produce and avoid storing containers at refrigerated temperature

Keywords: Container Surfaces, *Salmonella*, *Listeria monocytogenes*, Farmers' market

6.1. Introduction

The demand for “locally-grown” food in the United States has risen dramatically in the past two decades (Scheinberg et al., 2013; 2017). The surge in demand for locally produced food has created jobs and opportunities for small agribusinesses throughout the nation, especially through local farmers’ markets. According to the U.S. Department of Agriculture (USDA) Economic Research Service (ERS), the number of farmers’ markets in the U.S. has more than quadrupled since 1994 (USDA-Economic Research Service, 2017). Consumers often consider fresh produce sold at farmers’ markets to be healthier and safer than produce in conventional stores. Yet, information on whether these foods are indeed safer to consume is rather limited. Several outbreaks related to fresh produce from farmers’ markets were reported in the U.S. over the past decade, raising concerns on the safety of food sold at farmers’ markets (Food Safety News, 2011, 2015; Gardner et al., 2011; Levy et al., 2014).

Currently, there are 93 farmers’ markets at over 112 locations throughout West Virginia (West Virginia Farmers’ market Association, 2018). Farmers’ markets generated an estimated \$9.1 million in annual sales and served over half a million customers in WV in 2017. Farmers’ markets play an important role in West Virginia’s economy, creating an average annual revenue of \$41,200 for full-time produce farmers and approximately \$20,000 for part-time farmers (internal unpublished data, personal communication with Mr. Thomson Gross, GIS Analyst, WV FOODLINK). In Kentucky, there are more than 159 farmers’ markets providing locally grown fresh produce to consumers across the state (Kentucky Department of Agriculture, 2016). Our recent Good Agriculture Practices (GAP) survey of 160 small-scale produce growers in 21 KY counties indicated that participants failed to identify many sources of potential microbiological contamination, including soil, ice, and cooling and refrigeration (only 28-41% recognition,

Sinkel et al., 2018). Our most recent study of farmers' markets in WV and KY showed a high prevalence of *Salmonella* spp. (18.6% of spinach, 10.9% of tomatoes, 18.5% of peppers, and 56.3% of cantaloupes), and the relatively high presence of *Listeria* spp. (3.78% of the samples) on locally grown produce surfaces (Li et al., 2017). Levy et al. (2014) reported that 1 of 133 tested fresh herbs (basil, cilantro, and parsley) from Los Angeles and Seattle farmers' markets were positive for *Salmonella* spp. Scheinberg et al. (2017) found that 3.9% (6/152) of fresh produce samples contained *Listeria* spp., including 2% (1/54) of kale, 4% (2 of 52) of lettuce, and 7% (3 of 46) of spinach samples from 25 farmers' markets in Pennsylvania. As farmers' markets become increasingly popular over time, it is important to assess and understand the risks of foodborne pathogen contamination during the produce handling process and evaluate relevant strategies to mitigate such risks.

During post-harvest storage of produce, tree fruits such as apples, oranges, and peaches, in particular, are stored in unsanitized containers, which could serve as a potential reservoir for numerous plant pathogens (Cossentine et al., 2004; Higbee, et al., 2001; Randall, et al., 2011) and potentially harbor and transfer foodborne pathogens such as *Salmonella* spp. and *L. monocytogenes* (Killinger, 2014). One factor affecting post-harvest food safety risks is the material makeup of these storage containers, which can be wood, pressed-card, or plastic. Wood containers are subject to weathering. Pressed-card buckets with green molded pulp material often have rough surfaces that can harbor various pathogens. By contrast, plastic bins are easier to clean and sanitize due to their flat and smooth surfaces (Waelti, 1992).

Currently, little information exists regarding the role of storage container material as a potential source of pathogen contamination at local farmers' markets, especially the pathogen survival rate on various material surfaces. We aim to fill this data gap in the literature. The

objectives of this study were to 1) assess the knowledge and practices around the use of produce storage containers of farmers' market vendors in Kentucky and Morgantown, WV, and 2) evaluate and model the survival rate of *Salmonella* and *L. monocytogenes* on various storage material surfaces at refrigerated and room temperature.

6.2. Materials and Methods

6.2.1. Survey of handling produce containers at KY and WV farmers' markets

The survey questionnaire was developed by the authors after several group meetings and then distributed to selected industry professionals, extension agents, and academic faculty at Western Kentucky University (WKU) and West Virginia University (WVU) for review and comments. The wording of the survey questionnaire was reviewed and revised based on the comments from Dr. Jerry Daday, Professor of Sociology, WKU. The questionnaire was approved by WVU (IRB/IBC#15-04-07) and WKU's Institutional Review Board (IRB). Before the collection of formal data, the questionnaire was pre-tested at two farmers' markets in Nashville, TN (just across the KY state line) to ascertain whether the farmers clearly understood the survey questions contained on the survey instrument. The questionnaire was finalized after data analysis from the pre-test trials. The survey questionnaire consisted of 6 questions regarding the practices of handling produce storage containers: 1) the type of containers the vendor uses to present produce at farmers' markets; 2) the type of containers the vendor uses for storing/transporting produce; 3) whether the vendor washes the containers between visits to farmers' markets; 4) whether the vendor sanitizes the containers; 5) where the vendor stores the containers before selling at farmers' market; and 6) the vendor's awareness/knowledge of foodborne pathogens transmitted to fresh produce. The specific survey questions were included in the results of Tables 6.1 to 6.4.

The survey questionnaire was distributed by the face-to-face method at KY and WV farmers' markets. Produce growers who sold produce at farmers' markets during the data collection visits were recruited. A five-dollar gift card was provided as an incentive gift for the completion and return of the questionnaire. All survey participants were required to sign informed consent documents before answering the questionnaire. The consent document informed the respondents that the survey was voluntary and anonymous and that they are free to withdraw from the survey at any point in time. The recruiting of produce vendors at the Morgantown Farmers' market, Morgantown, WV, was coordinated with Ms. Lisa Jones, Program Coordinator, WVU Extension Service Small Farm Center. The survey questionnaire was sent to 600 farmers' market produce vendors in 21 counties in KY between April and August 2014. The counties in KY were selected to represent different regions of the state, including large cities such as Louisville, Lexington, and Bowling Green, and middle-sized cities such as Elizabethtown, Paducah, and Owensboro. There were a total of 141 (141/600, 23.5%) survey responses completed and returned, and the response rate of each question ranged from 19.5 to 23.5% (Tables 6.1 - 6.4). The same survey questionnaire was later distributed to 40 farmers' market produce vendors from July to Oct 2015 in Morgantown Farmers' Market, Morgantown WV, where WVU is located, with 28 (28/40, 70%) completed and returned questionnaires, and the response rate of each question ranged from 37.5 to 70.0% (Tables 6.1 – 6.4).

6.2.2. Preparation of inoculum

Salmonella Typhimurium ATCC 14028 and *Salmonella* Tennessee ATCC 10722, and two *L. monocytogenes* strains, L2624 and L2625 [cantaloupe outbreak serotype 1/2b], donated by Dr. Joshua Gurtler (USDA-ARS, Wyndmoor, PA), were used in this study. Frozen stock cultures

were streak-plated onto XLT-4 and Modified Oxford (MOX) agar followed by incubation at 35°C for 48 h to generate pure cultures. Single colonies from XLT-4 (*Salmonella*) and MOX Agar (*L. monocytogenes*) were cultured and sub-cultured in 10 ml of tryptic soy broth (TSB; Alpha Biosciences, Baltimore, MD, USA) at 35°C for 24 ± 2 h. Each *Salmonella* and *L. monocytogenes* culture was centrifuged (VWR Symphony 4417, VWR International, Radnor, PA; $5,000 \times g$, 15 min), triplicate washed in 0.1% buffered peptone water (BPW), and then re-suspended in 0.1% BPW. The two *Salmonella* and two *L. monocytogenes* strains were then combined (Li et al., 2017) to yield an inoculation level of 4-6 log₁₀ CFU/cm² when 0.2 ml of inoculum was applied to material surfaces.

6.2.3. Storage material inoculation and microbiological analysis

Plastic, pressed-card, and wood containers obtained were the same as what is used at the farmers' markets in Morgantown, WV. The plastic container is made of polyethylene board (donated by a produce grower at Morgantown Farmers' Market, Morgantown, WV, USA), the pressed-card container was the green molded pulp produce basket (donated by Whole Foods Market, Pittsburgh, PA, USA), and the wood container (purchased from Texas Basket Co., Inc., Jacksonville, TX, USA) was manufactured by a mixture of sweet gum, elm, and cottonwood. The inner bottom surfaces of each container were manually trimmed and cut into 5 cm × 5 cm square areas (25 cm²) and inoculated with the aforementioned four-strain mixture of *Salmonella* and *L. monocytogenes* in a biosafety hood. Before inoculation, each trimmed material surface was exposed to UV light in a biosafety hood for 1 h to sanitize the surfaces. The inoculum (0.2 ml) was spread uniformly onto the material surfaces using a sterilized spreader, and the inoculated surfaces were left for 30 min under the hood for attachment. All inoculated materials were then stored at 3.2°C (22.9% RH) and 22.5°C (50.4% RH) for 21 days. On days 0, 0.13,

0.25, 0.5, 1, 3, 6, 9, 12, 15, 18, and 21, individual samples were placed in a sterile Whirl-Pak[®] bag (Whirl-Pak, Nasco, Modesto, CA, USA) containing 50 ml of 0.1% BPW, shaken and massaged for 30 s, and stomached for 5 min in a masticator (IUL Instruments, Barcelona, Spain). Samples were then 10-fold serially diluted in 0.1% BPW, followed by spread-plating onto XLT-4 (*Salmonella*) and MOX agar (*L. monocytogenes*). Microbial colonies of *Salmonella* and *L. monocytogenes* were counted manually after incubation at 35°C for 24 h and 48 h, respectively. Samples below the detectable limit ($1.3 \log_{10}$ CFU/cm²) by spread-plating were enriched in BPW at 35°C for 24 h and then streak-plated onto XLT-4 (*Salmonella*) and MOX agar (*L. monocytogenes*) to verify the presence/absence of pathogens on surfaces.

6.2.4. Data analysis

Survey answers were analyzed using the Chi-Square test on SAS (version 9.2, SAS Institute, Cary, NC) to examine the bivariate relationships between categorical variables, with statistical significance set at an alpha level of 0.05. The studies for testing the microbial survival on various material surfaces during storage were repeated twice, and for each replication, 4 individual samples per material surface (plastic, pressed-card, or wood) at each sampling time were analyzed ($n = 8$). The Mixed Model Procedure in SAS was used to analyze the survival population of *Salmonella* and *L. monocytogenes* on container surfaces, with the individual factors of storage material, temperature, time period, and the interaction terms between them using a statistical significance level of 0.05 ($P < 0.05$). GInaFit software (Geeraerd et al., 2006) was used to estimate pathogen survival kinetic parameters on container surfaces. The root means square error (RMSE) and R^2 were used to determine the best survival model that fit the data. The lower the RMSE is, the better the model (Ratkowsky, 2002).

6.3. Results and Discussion

6.3.1. Assessing vendor's handling of produce containers

All of our survey results from Tables 6.1 to 6.4 reflect self-reported behaviors of farmers' market vendors from Morgantown WV and KY. According to our survey results shown in Table 6.1, plastic containers were the most ($P<0.05$) common choice by 22 of 27 respondents (81%) in Morgantown, WV, and by 111 of 140 (79%) respondents in KY to present produce at local farmers' markets, followed by paper (43-52%) and wood containers (33-48%). At least 80% of paper containers were green molded pulp produce baskets (data not shown in tabular form). Only 15-24% of vendors interviewed used metal containers. Out of 28 respondents, three used containers made of other materials, including mesh bags, natural fiber baskets, and wicker baskets. Instead of allowing consumers to take the containers, as is common in wholesale markets, almost all farmers' market vendors reused the containers to save cost. This practice of reusing produce display containers raises the concern of microbial cross-contamination between products. Similarly, as shown in Table 6.1, the vast majority ($P<0.05$) of respondents in Morgantown, WV (96%), and KY (89%) used plastic containers to transport produce to local farmers' markets. Approximately 25-30% and 21-40% of respondents used paper and wood containers, respectively, followed by only 4-16% who used metal containers. Two out of the 28 respondents in Morgantown, WV used containers made of other materials such as cardboard and plastic-lined disposable bags. According to Siroli et al. (2017), cardboard material is better at reducing microbial cross-contamination than plastic.

In order to assist West Virginia locally small produce growers and farmers' market produce vendors in preventing cross-contamination of pathogens from food contact surfaces (i.e., storage containers) to produce, the three-step washing process (water rinse, antimicrobial

application, final water rinse) has been suggested by the WV Small Farm Center as an effective tool for removing pathogens from food surfaces (Strohbehn et al., 2013). When asked whether the farmers' wash/sanitize produce containers between visits from farmers' markets, 85% and 93% of vendors surveyed in Morgantown, WV, and KY farmers' markets, respectively, responded with a "Yes" (Table 6.2). However, in the follow-up question, approximately 30-40% of them did not apply any sanitizers in the wash water (Table 6.2). Among the respondents who replied that they use sanitizers, chlorine water, and Lysol® disinfection wipes were indicated as the top choices (data not shown in tabular form).

Effective temperature control is a critical component of the post-harvest practice of produce storage. Sixty-two (62%) percent (KY) to 67% (Morgantown, WV) of respondents indicated that they stored produce containers at refrigerated temperature, and 63% (Morgantown, WV) to 84% (KY) stored containers at room temperature. This suggests that some vendors store containers at both refrigerated and room temperatures (Table 6.3). As expected, no vendors store containers in freezers (Table 6.3). At the end of the survey questionnaire, vendors were given a list of four microbiological foodborne pathogens and asked to select what they believe were food safety risks for fresh produce; 15 of 28 (Morgantown, WV) and 117 of 140 (KY) vendors surveyed answered this question. The results suggest that Shiga toxin-producing *Escherichia coli* (83-87%), *Salmonella* (80-91%), and *Listeria* (71-87%) were well recognized by respondents as potential foodborne pathogens contaminated on produce. However, 46% of respondents failed to identify *Staphylococcus aureus* as a pathogen on produce.

6.3.2. *Salmonella* and *L. monocytogenes* survival on various container surfaces

The microbial survival curves of *Salmonella* and *L. monocytogenes* on plastic, pressed-card, and wood surfaces during storage at 3.2 and 22.5°C are shown in Figure 6.1. As expected,

both *Salmonella* and *L. monocytogenes* populations on the three material surfaces decreased ($P < 0.05$) during storage due to drying and desiccation of the surface condition and the limited nutrition available for bacteria. The decrease in pathogen populations during the storage period was significantly affected by the bacterial strain ($P < 0.05$), storage temperatures ($P < 0.05$), and surface materials ($P < 0.05$). In general, the decrease in pathogen populations was faster ($P < 0.05$) at 22.5°C than 3.2°C and, in most cases, faster ($P < 0.05$) on plastic surfaces than pressed-card or wood. In addition, *L. monocytogenes* was more resistant ($P < 0.05$) to environmental conditions on the surfaces tested than *Salmonella*.

The initial populations of *Salmonella* on plastic, pressed-card and wood surfaces were 5.27-5.53 log₁₀ CFU/cm² and 4.94-5.38 log₁₀ CFU/cm² for storage at 3.2 and 22.5°C, respectively (Figure 6.1). For samples stored at 3.2°C, *Salmonella* counts on surfaces decreased linearly by 1.11, 1.25, and 2.60 log₁₀ CFU/cm² on plastic, pressed-card, and wood container surfaces, respectively, on the first day (Figure 6.1). From day one to day 21 (the end of the storage period), *Salmonella* counts declined gradually, though larger fluctuations were observed on several sampling days. The surviving population of *Salmonella* ranged from 2.63 to 3.03 log₁₀ CFU/cm² across three material surfaces by the end of the 21-day storage period (Figure 6.1). Compared to 3.2°C, samples stored at 22.5°C were more detrimental to *Salmonella* on all three material surfaces. At 22.5°C, *Salmonella* levels decreased from approximately 5.0 log₁₀ CFU/cm² to below the detection limit (1.30 log₁₀ CFU/cm²) at days 3, 9, and 12 on plastic, wood, and pressed-card surfaces, respectively. The previous study by McEldowney and Fletcher (1988) reported that bacterial survival on glass surfaces was shorter at 22.5°C than at 3.2°C. However, the pathogen was still present on all three material surfaces by the end of storage (day 21) as detected by the enrichment for recovering bacterial populations below detectable limits. These

results indicate that *Salmonella* on dry produce container surfaces could remain viable and present a contamination hazard for a considerably long time.

The initial population of *L. monocytogenes* recovered from the three material surfaces was higher than *Salmonella*, ranging from 6.39 to 6.93 log₁₀ CFU/cm² for 3.2 and 22.5°C (Figure 6.1). At 3.2°C, *L. monocytogenes* levels declined significantly by 1.09, 0.52, and 2.26 log₁₀ CFU/cm² on pressed-card, wood, and plastic surfaces, respectively, within the first day (Figure 6.1). After that, the pathogen populations maintained a constant level or declined only slightly during the 21-day storage period. The *L. monocytogenes* population remaining on pressed-card, wood, and plastic surfaces were as high as 4.89 to 5.73 log₁₀ CFU/cm², respectively, by the end of storage. Similar to *Salmonella*, the survival rates of *L. monocytogenes* decreased dramatically at 22.5°C. It took 21 days for *L. monocytogenes* counts to decrease below the detectable limit on wood and plastic surfaces (still present on surfaces after enrichment), whereas there were 2.03 log₁₀ CFU/cm² of the pathogen remaining on pressed-card surfaces by the end of storage. The reduction rate of *L. monocytogenes* was slower and its residual pathogen population was higher than *Salmonella* regardless of the surface material and the storage temperature. Our results are consistent with the previous study by Siroli et al. (2017), who found that *Salmonella* Enteritidis (inoculation level ~5 log₁₀ CFU/cm²) decreased to below detectable limits after 8 and 48 h on cardboard and plastic surfaces (22.5°C), whereas 1-3 log₁₀ CFU/cm² of *L. monocytogenes* remained on both surfaces by the end of the 48-h storage. In this study, the relative humidity (RH) level was 22.9% at 3.2°C and 50.4% at 22.5°C; therefore, our results could be explained by the fact that Gram-positive bacteria (*L. monocytogenes*) are more resistant to external dry and desiccation conditions than Gram-negative bacteria (*Salmonella*) (Siroli et al., 2017). The heavy

layers (peptidoglycan) in the cell wall of Gram-positive bacteria compared to that of Gram-negative bacteria could protect the former against desiccation (Milling et al., 2005).

According to our previous survey results, more than 60% of surveyed produce growers store produce containers with produce at refrigerated temperatures. However, the results of this study indicated that the survival of foodborne pathogens decreased faster at room temperature than at refrigerated temperature, which suggested that small local produce growers should avoid putting the containers with produce at cooler or refrigerated temperatures. This important information should be mentioned in future extension/outreach short courses regarding post-harvest practices for produce to improve the microbial safety of locally grown produce at farmers' markets.

6.3.3. Modeling of pathogen survival during storage

The seven bacterial survival models in the GInaFit software were used to predict the pathogen-inactivating kinetics of cells on the three material surfaces and the two storage temperatures. The appropriateness of each model was evaluated using the R^2 and RMSE values. Except for *Salmonella* at 3.2°C, the estimated parameters from various models were consistent with the visual patterns in the survival curves (Figure 6.1).

For *Salmonella* at 3.2°C, the kinetic survival parameters can be determined by the linear model with tail ($R^2=0.79-0.92$, RMSE=0.32-0.43), Weibull ($R^2=0.75-0.92$, RMSE=0.17-0.56), and biphasic models ($R^2=0.80-0.98$, RMSE=0.11-0.38) (Table 6.5). According to the linear with tail and biphasic model, the inactivation rate of *Salmonella* on pressed-card was 14.68-14.19, which was greater ($P < 0.05$) than on plastic (5.95) and wood (3.76-12.79) surfaces. According to Peleg (2006), the Weibull model can indicate the failure of tested microorganisms to resist the harsh conditions with the smaller k value, suggesting a greater inactivation rate. In this study, the

k values for plastic (0.04) and pressed-card (0.01) samples were similar ($P > 0.05$) and less ($P < 0.05$) than the wood samples (0.33), suggesting that *Salmonella* was more sensitive (vulnerable) on plastic and pressed-card than on wood surfaces, a result that differs from the linear with tail and biphasic models. The discrepancies between the results could be explained by the fluctuation of the pathogen survival on surfaces during the storage period (day one to 21). For example, on plastic surfaces, the *Salmonella* population decreased from 2.67 to 2.33 log₁₀ CFU/cm² from day one to three but increased to 3.25 log₁₀ CFU/cm² on day six and declined to 2.52 log₁₀ CFU/cm² on day nine. Similarly, *Salmonella* counts on wood surfaces decreased from 3.42 to 3.12 log₁₀ CFU/cm² from day one to three but returned to 3.38 and 3.32 log₁₀ CFU/cm² from day three to day six and nine (Figure 6.1). The α value of the Weibull model indicated that the shape of the survival curves with $\alpha > 1$ showed downward concavity, $\alpha=1$ showed a linear curve, and $\alpha < 1$ showed an upward concavity with residual cells adapting to stresses (Geeraerd et al., 2006). The α values of the three material surfaces ranged from 0.11-0.21, indicating that the inactivated curves show a similar upward concavity and that the remaining cells on all three material surfaces were resistant to dry, desiccated, and poor nutrition conditions at 3.2°C (Lopez-Galvez et al., 2012).

For *Salmonella* at 22.5°C, the linear ($R^2=0.50-0.69$, RMSE=0.61-0.92) model indicated a greater ($P < 0.05$) inactivation rate on plastic surfaces (Kmax=2.47) than the pressed-card (Kmax=0.55) and wood surfaces (Kmax=0.45) (Table 6.6). This result is consistent with the pattern observed in Figure 6.1 in which the pathogen population declined faster on plastic surfaces than pressed-card or wood samples.

As shown in Tables 6.7 and 6.8, both the linear with tail and biphasic models provide a good fit for *L. monocytogenes* survival on three material surfaces at 3.2 and 22.5°C, and the

Weibull model fits the pathogen survival at 22.5°C best. At 3.2°C, the inactivation rate of samples on plastic surfaces ($K_{max} = 7.10$, $K_{max1} = 6.04$) was greater ($P < 0.05$) than on pressed-card ($K_{max} = 2.16$, $K_{max1}=2.44$) and wood surfaces ($K_{max}=0.83$, $K_{max1}=2.24$) as indicated by the linear with tail (K_{max} value) and biphasic models (K_{max1} value), respectively (Table 6.7).

The biphasic model has been developed as an alternative to the linear model to explain whether there is a subpopulation of the initial population that is resistant (less sensitive) to thermal or non-thermal stress conditions (Geeraerd et al., 2006). The f value of the biphasic model explains the fraction of the initial population in a major subpopulation, and $(1-f)$ is the fraction of the initial population in a minor subpopulation, and k_{max1} and k_{max2} each represent the specific inactivation rates of the two subpopulations (Geeraerd et al., 2006). In this study, the f value for the pressed card was 0.9062 and 0.7168 for wood (Table 6.7), suggesting that approximately 10% ($1-0.9062$) and 29% of ($1-0.7168$) the subpopulation of *L. monocytogenes* on pressed-card and wood surfaces were adapted and resistant to the surface conditions at 3.2°C. The K_{max2} values of pressed-card and wood surfaces were close to zero. These results were visually confirmed by the pathogen survival curve in Figure 6.1, which shows a long “tail” of *L. monocytogenes* ($5-6 \log_{10}$ CFU/cm²) surviving on pressed-card and wood surfaces from day one to 21. The f value on a plastic surface was 0.9889 and the K_{max2} value was zero, suggesting that only 1.2% ($1-0.9889$) of the subpopulation from the initial population was resistant to the dry and desiccated surface conditions. This result was also consistent with Figure 6.1 in that the “tail” of *L. monocytogenes* remaining on plastic surfaces was $4-5 \log_{10}$ CFU/cm², down from the $6.93 \log_{10}$ CFU/cm² of the initial population.

At 22.5°C, the K_{max} value of the linear with tail model and the K_{max1} value of the biphasic model both showed a larger ($P < 0.05$) inactivation rate of *L. monocytogenes* on plastic surfaces ($K_{max}=6.71$, $K_{max1}=17.94$) than on pressed card ($K_{max}=0.86$, $K_{max1}=3.70$) and wood surfaces ($K_{max}=2.24$, $K_{max1}=3.50$, Table 6.8). The K_{max2} value (0.29-0.41) of the biphasic model was similar ($P > 0.05$) between plastic, pressed-card, and wood surfaces, which was again consistent with the survival curve observed in Figure 6.1. As shown in Figure 6.1, the pathogen level for samples on all three material surfaces dramatically declined within day one, followed by a slow reduction afterward. According to the Weibull model, the lowest ($P < 0.05$) k value was from plastic samples (0.01), followed by wood (0.23) and pressed-card ones (0.97) (Table 6.8), again suggesting that *L. monocytogenes* was easier to be inactivated on plastic than on wood and pressed-card surfaces at room temperature. The results agree with those from the linear with tail and biphasic models.

In this study, both microbial survival curves and data modeling indicate that the pathogen counts decreased faster on plastic surfaces compared to pressed-card and wood surfaces during storage. Plastic surfaces used in this study were fresh, new, clean, and have smooth, plane, and homogeneous surfaces without holes and pores to entrap bacteria. As the water dried out in the first 30 min after inoculation, the only available nutrient source for the attached bacterial cells on plastic surfaces was the absorbed organic macromolecules, which can be exhausted rapidly during storage. In contrast, the microstructure of pressed-card and wood surfaces contains pores and niches that can absorb water for bacterial use and favor bacteria forming biofilms over time during storage. The bacteria matrix on pressed-card and wood surfaces could obtain fresh nutrients from the cell lysis of neighboring cells. The detailed mechanism would need to be

examined using scanning or transmitting electronic microscopy to determine the entrapment of pathogens on various material surfaces during different storage conditions.

The results of this study indicated that plastic containers could potentially reduce the cross-contamination of foodborne pathogens to produce during storage, transport, and display at farmers' markets due to a faster viability loss of pathogenic microorganisms compared to pressed-card or wood ones. At farmers' markets, storage containers are typically owned by the vendors, who are viewed as having the primary responsibility for cleaning and sanitation practices. Plastic containers with smooth surfaces are easier cleaned and sanitized than pressed-card and wood containers, which contain hard-to-reach areas and usually are damaged after exposure to water solutions. In agreement with the aforementioned survey results, plastic containers should be the choice for small local produce growers to improve the microbial safety of produce.

6.4. Conclusions

In conclusion, based on the results from this study, it is suggested that local, small, fresh produce farmers should choose plastic containers to present fresh produce at farmers' markets and avoid putting containers directly into a cooler with unsold produce. Future studies are needed to validate the efficacy of the three-step wash with sanitizers to decontaminate foodborne pathogens on various container surfaces. The results of this study will be of interest to the states included in the study, West Virginia and Kentucky, as well as the county extension specialists/agents who are developing and implementing produce safety training programs, such as the new "Writing Your Food Safety Plan" short course for produce growers offered by the WVU Extension Service Small Farm Center in coordination with the WV Food Safety Training

Team. These research results can also contribute to the development of training materials for farmers' market managers and vendors by local farmers' market associations.

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Chapter 6 Tables and Figures

Table 6.1. Type of containers for presenting and transporting produce at farmers' market

Survey questions	What kind(s) of containers do you use to present your produce at the market? (check all that apply)		What kind(s) of containers do you use for storage/transport of your produce? (check all that apply)	
	1. Wood 2. Plastic 3. Metal 4. Paper 5. Other (please specify)		1. Wood 2. Plastic 3. Metal 4. Paper 5. Other (please specify)	
	Containers for presenting produce		Containers for transporting produce	
Type ^a	Morgantown, WV (N=27, 67.5% ^b)	KY (N=140, 23.3%)	Morgantown, WV (N=28, 70.0%)	KY (N=138, 23.0%)
	n (%)	n (%)	n (%)	n (%)
Wood	9 (33)a	67 (48)a	6 (21)a	55 (40)a
Plastic	22 (81)b	111 (79)b	27 (96)b	123 (89)b
Metal	4 (15)c	33 (24)c	1 (4)c	22 (16)c
Paper	14 (52)d	60 (43)a	7 (25)a	44 (32)a
Others ^c	3 (11)c	N/A	2 (7)c	N/A

^a Respondents were allowed to indicate more than one choice.

^b Response rate of total distributed questionnaires (WV: 40; KY: 600)

^c Others include basket-natural fiber, mesh bags, wicker bags, cardboard, plastic-lined with disposable bag. Mean values with different letters within each column are significantly different ($P < 0.05$, LSD test)

Table 6.2. Wash and/or sanitize produce containers between farmers' market visits

Survey questions	Do you wash your storage containers between visits to the farmers' market? 1. No 2. Yes		If yes, do you sanitize your containers? 1. No 2. Yes (If yes, please specify the type(s) of sanitizer you use)	
Answers	Wash containers between visits		If yes, do you sanitize ^a	
	Morgantown, WV (N=27, 67.5% ^b)	KY (N=137, 22.8%)	Morgantown, WV (N=22, 95.6% ^c)	KY (N=121, 94.5%)
	n (%)	n (%)	n (%)	n (%)
No	4 (15)a	9 (7)a	9 (41)a	35 (29)a
Yes	23 (85)b	128 (93)b	13 (59)a	86 (71)b

^a Sanitizer includes chlorine, soapy water, Lysol, and vinegar water

^b Response rate of total distributed questionnaires (WV: 40; KY: 600)

^c Response rate among those who answered "yes" (WV: 23; KY: 128)

Mean values with different letters within each column are significantly different ($P < 0.05$, LSD test)

Table 6.3. Temperature of storing containers with produce before transport to farmers' market

Survey question	How do you store your produce containers before transporting them to the farmers' market? (check all that apply)	1. I freeze my produce containers 2. I store my produce containers at refrigerated temperatures 3. I store my produce containers at room temperature
Temperature ^a	Morgantown, WV (N=27, 67.5% ^b)	KY (N=141, 23.5%)
	n (%)	n (%)
Freeze	0 (0)a	0 (0)a
Refrigerated temperature	18 (67)b	87 (62)b
Room temperature	17 (63)b	119 (84)c

^a Respondents were allowed to indicate more than one response.

^b Response rate of total distributed questionnaires (WV: 40; KY: 600)

Mean values with different letters within each column are significantly different ($P < 0.05$, LSD test)

Table 6.4. Awareness of foodborne pathogens contaminated on fresh produce

Survey question	Are the following bacteria transmitted from fresh fruits and vegetables?		1. Shiga Toxin Producing <i>E. coli</i> (No, Yes) 2. <i>Salmonella</i> (No, Yes) 3. <i>Listeria monocytogenes</i> (No, Yes) 4. <i>Staphylococcus aureus</i> (No, Yes)	
Pathogens	Morgantown, WV (N=15, 37.5% ^a)		KY (N=117, 19.5%)	
	Yes	No	Yes	No
	n (%)	n (%)	n (%)	n (%)
Shiga-Toxin Producing <i>E. coli</i>	13 (87)a	2 (13)b	97 (83)a	20 (17)b
<i>Salmonella</i>	12 (80)a	3 (20)b	107 (91)a	10 (9)b
<i>Listeria monocytogenes</i>	13 (87)a	2 (13)b	83 (71)a	34 (29)b
<i>Staphylococcus aureus</i>	8 (53)a	7 (47)a	63 (54)a	54 (46)a

^a Response rate of total distributed questionnaires (WV: 40; KY: 600)

Mean values with different letters within each row are significantly different ($P < 0.05$, LSD test)

Table 6.5. Parameters (mean $\pm$ standard error) of models estimated for the survival of *Salmonella* on plastic, pressed-card, and wood surfaces after storage at 3.2°C (22.9% RH) for 21 days.

Model	Parameters	Plastic	Pressed-card	Wood
Linear + Tail	RMSE	0.3259	0.4331	0.1762
	R ²	0.9203	0.7903	0.9454
	Kmax	5.95 $\pm$ 1.48a	14.19 $\pm$ 5.93b	3.76 $\pm$ 1.64a
Weibull	RMSE	0.5690	0.3275	0.1762
	R ²	0.7572	0.8801	0.9454
	k	0.04 $\pm$ 0.12a	0.01 $\pm$ 0.01a	0.33 $\pm$ 0.30b
	α	0.18 $\pm$ 0.07	0.11 $\pm$ 0.03	0.21 $\pm$ 0.03
Biphasic	RMSE	0.3456	0.3757	0.1173
	R ²	0.9203	0.8072	0.9824
	f	0.9972 $\pm$ 0.002a	0.9839 $\pm$ 0.014a	0.9557 $\pm$ 0.012a
	Kmax1	5.95 $\pm$ 1.62a	14.68 $\pm$ 6.46b	12.79 $\pm$ 2.34b
	Kmax2	0.00 $\pm$ 0.05a	0.12 $\pm$ 0.04b	0.13 $\pm$ 0.01b

Mean values with different letters within each row are significantly different ($P < 0.05$, LSD test)

Table 6.6. Parameters (mean $\pm$ standard error) of models estimated for the survival of *Salmonella* on plastic, pressed-card, and wood surfaces after storage at 22.5°C (50.4% RH) for 21 days.

Model	Parameters	Plastic	Pressed-card	Wood
Linear	RMSE	0.9215	0.7341	0.6190
	R ²	0.6837	0.5040	0.6947
	Kmax	2.47 $\pm$ 0.84a	0.55 $\pm$ 0.19b	0.45 $\pm$ 0.11b

Mean values with different letters within each row are significantly different ($P < 0.05$, LSD test)

Table 6.7. Parameters (mean $\pm$ standard error) of models estimated for the survival of *Listeria monocytogenes* on plastic, pressed-card, and wood surfaces after storage at 3.2°C (22.9% RH) for 21 days.

Model	Parameters	Plastic	Pressed-card	Wood
Linear + Tail	RMSE	0.2268	0.2677	0.2624
	R ²	0.9286	0.7879	0.4999
	Kmax	7.10 $\pm$ 1.68a	2.16 $\pm$ 1.13b	0.83 $\pm$ 0.96b
Biphasic	RMSE	0.2572	0.2790	0.2783
	R ²	0.9183	0.7952	0.4999
	f	0.9889 $\pm$ 0.007a	0.9062 $\pm$ 0.064b	0.7168 $\pm$ 0.224c
	Kmax1	6.04 $\pm$ 1.75a	2.44 $\pm$ 1.47b	0.83 $\pm$ 1.22b
	Kmax2	0.00 $\pm$ 0.03a	0.02 $\pm$ 0.04a	0.00 $\pm$ 0.05a

Mean values with different letters within each row are significantly different ($P < 0.05$, LSD test)

Table 6.8. Parameters (mean $\pm$ standard error) of models estimated for the survival of *Listeria monocytogenes* on plastic, pressed-card, and wood surfaces after storage at 22.5°C (50.4% RH) for 21 days.

Model	Parameters	Plastic	Pressed-card	Wood
Linear + Tail	RMSE	0.6623	0.5040	0.5414
	R ²	0.8992	0.9370	0.9218
	Kmax	6.71 $\pm$ 2.04a	0.86 $\pm$ 0.15b	2.24 $\pm$ 0.59c
Weibull	RMSE	0.5962	0.3599	0.4118
	R ²	0.9183	0.9655	0.9579
	k	0.01 $\pm$ 0.01a	0.23 $\pm$ 0.20b	0.97 $\pm$ 0.66c
	α	0.22 $\pm$ 0.06	0.39 $\pm$ 0.08	0.52 $\pm$ 0.11
Biphasic	RMSE	0.4843	0.3734	0.3702
	R ²	0.9551	0.9639	0.9695
	f	0.9996 $\pm$ 0.004a	0.9943 $\pm$ 0.005a	0.9928 $\pm$ 0.008a
	Kmax1	17.94 $\pm$ 1.75a	3.70 $\pm$ 1.21b	3.50 $\pm$ 1.20b
	Kmax2	0.34 $\pm$ 0.12a	0.29 $\pm$ 0.05a	0.41 $\pm$ 0.13b

Mean values with different letters within each row are significantly different ($P < 0.05$, LSD test)

Figure 6.1. Survival of *Salmonella* and *Listeria monocytogenes* on plastic, pressed-card, and wood surfaces during storage at 3.2°C (22.9% RH) and 22.5°C (50.4% RH) for 21 day

CHAPTER 7

Summary and Future Directions

Summary

The microbial quality and safety of local produce can be improved with the triple wash process. **Study one** assessed the two triple-wash procedures in inactivating foodborne pathogens and surrogate bacteria on cucumbers and tomatoes. Results showed the WWA procedure better at inactivating *Salmonella*, *L. monocytogenes*, and *E. faecium* on cucumbers and tomatoes. SaniDate-5.0 at the concentration of 0.25% and 0.50% had similar or better antimicrobial efficacy than chlorine water without or with pH adjustment. *E. faecium* also showed potential for *Salmonella* surrogate used for validation studies of antimicrobial treatments during post-harvest washing process on produce. **Study two** assessed the two triple-wash procedures on spinach. Results are similar to the first study. A procedure was the better performer to inactivate *Salmonella* and *L. monocytogenes* on locally grown spinaches. SaniDate-5.0 at the concentrations of 0.25% and 0.50%, indicates similar or better antimicrobial efficacy than chlorine water and LCA on spinach. **Study three** evaluated the two triple-wash procedures with SaniDate-5.0 to improve microbial safety and quality of butternut squashes, results indicate the WWA procedure performs better pathogen reductions than the WAW procedure on squashes against *Salmonella* and *L. monocytogenes*. Squashes treated with SaniDate-5.0 showed lower microbial counts than the unwashed and water-treated samples typically during the later storage period. This study also determined the feasibility of the triple-wash application in a processing plant. The annual operating cost of the triple-wash using SaniDate-5.0 is approximately \$500 to \$2,000 for producing 1,000 to 5,000 squashes. Refreshing water in each tank adds an extra cost of 5-220%. **Study four** evaluated the survival of *Salmonella* and *Listeria monocytogenes* on

various produce container surfaces commonly used at farmers' markets, under storage conditions both at refrigerated and room temperature. Results indicate produce farmers should choose plastic containers to present fresh produce at farmers' markets and avoid storing unsold produce in containers inside a cooler.

Future Direction

Further studies are needed to investigate cross-contamination of the triple-wash process in three washing tanks with or without antimicrobials. An antibiotic marker should be introduced into *E. faecium* to filter out background microflora. In addition, the residual effects on sanitizers applied on produce samples need to be compared, to determine if a neutralization step is needed after the WWA process. Sensory evaluations are needed to investigate the side-effect of sensory qualities of washed fresh produce. Finally, the efficacy of the triple-wash process with sanitizers to decontaminate foodborne pathogens on various container surfaces needs to be validated.