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Evaluate the Efficacy of a Mixture of Peroxyacetic Acid and H2O2 Against the Survival and Cross-Contamination of the Salmonella Surrogate Enterococcus Faecium on Tomatoes during Triple-Wash

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Abstract

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Triple-wash with a mixture of peroxyacetic acid and H2O2 (SaniDate-5.0) during post-harvest processing of fresh produce has been recommended by West Virginia Small Farm Center to improve microbial safety. It has been well recognized that the washing of produce is more important for preventing cross-contamination than reducing foodborne pathogens. Furthermore, it may help improve public confidence in that the produce they obtain from locally grown farmers is safe for their consumption. determine the efficacy of SaniDate-5.0 for reducing the survival and preventing cross-contamination of the Salmonella surrogate Enterococcus faecium on tomatoes during triple-wash.

E. faecium ATCC-8459 (resistant to 100-ppm nalidixic-acid) was dip-inoculated onto 2-tomatoes and triple-washed with 4-un-inoculated-tomatoes following the procedure of water dip, water dip, and SaniDate-5.0 dip (0, 0.0064, 0.25, and 0.50%) with 45-s of each step. Each tomato was placed into sample bags with 150 ml of sterile tryptic soy broth for 2-min in a stomacher blender. The inoculated surrogate bacteria on tomatoes or in wash-waters were enumerated using a modified MPN-method in 8×6 deep-well micro-plates. The turbidity of each well after incubation (35oC, 24-h) was confirmed by adding 3-µl droplets of the incubated liquid arrayed onto bile esculin agars plus 100-ppm nalidixic-acid. The final MPN values of each treatment were determined by an online MPN-calculator followed by statistical analysis. We found that SaniDate-5.0 concentrations 0.25 and 0.50% prevented cross contamination in tomatoes after a

triple wash consisting of water dip + water dip + antimicrobial agent. This study provides evidence that SaniDate-5.0 is an effective antimicrobial agent that could be used by locally small produce growers in triple-wash process to improve microbial safety of locally grown tomatoes.

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Chapter 1
Introduction

Farmers markets contribute significantly to West Virginia's economy. In 2020, there are over 350 known farmers markets (FMs) in WV, which generated over $17 million in annual gross sales (WV Farmers Market Association, 2020) and accounted for a significant portion of farm household income. Very small produce growers (with annual sales ⩽ $10,000) comprised 76.6% of produce growers in WV (USDA, 2017), with the majority selling produce through direct-to-consumer channels such as FMs. Ensuring the safety of fresh produce sold at farmers markets from very small growers is essential for the continual growth of the local food sector in WV.

A recent WV FMs' microbial surveillance study, finding that Salmonella spp. was detected on 10.9% of tomatoes, 18.5% of peppers, and 56.3% of cantaloupes (Li et al., 2017). The presence of foodborne pathogens in such a high percentage of samples suggests a critical need to develop mitigation strategies to reduce foodborne pathogens of WV locally grown fresh produce. The triple-wash process (water rinse, water rinse, and final antimicrobial dip) can effectively inactivate pathogens from food surfaces and improve on-farm food safety (Strohbehn et al., 2013). The method is easy to implement and requires only a small initial investment. Our recent plant onsite validation study confirmed that applying SaniDate 5.0 Sanitizer Disinfectant [a mix of peroxyacetic acid (PAA) and H_2O_2] (U.S.-EPA, 2020) and Organic Materials Review Institute listed (OMRI, 2010) with triple-wash significantly reduced foodborne pathogens on butternut squash and extended their shelf life (Li et al., 2020a). SaniDate 5.0 Sanitizer Disinfectant was selected for the triple-wash study as it is required by the wholesale distribution company, Appalachian Harvest (Duffield, VA), which is a buyer of the stakeholder's produce. This buyer purchases from several farms across WV, including Turnrow Appalachian Farm Collective in southern WV, which aggregates produce from 75 very small local produce growers in WV. As

the awareness of food safety requirements has increased, more growers are interested in learning about new technologies for reducing surface bacteria on their products. Currently, the WVU-SFC also encourages local produce growers to apply the triple-wash method if their produce is eaten raw or grown close to the ground (Li et al., 2020a; b).

The U.S. Food Code suggested that fruits and vegetables may be washed by using chemicals as specified under§ 7-204.12 in the section of 3-302.15 (FDA, 2017). Our previous research found applying 0.25-0.50% of SaniDate 5.0 into triple-wash (water + water + antimicrobial) extended the shelf life of butternut squashes from 50 to 70 days in a local commercial squash processing plant validation study (Li et al., 2020a), in addition to multiple lab studies (Li et al., 2020a; 2020b; 2021). Cost-benefit analysis showed that 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 squash and an extra 5-220% cost will be added if the water is refreshed in each tank (Li et al., 2020a). Potable water is primarily used to remove soil and debris on commodities, water without or with an insufficient level of antimicrobials can serve as a vehicle of microbial cross-contamination when the contamination exists on the fresh commodity (Gombas et al., 2017). Recently, there is growing recognition that post-harvest washing reduces cross-contamination with no expectation of achieving log count reductions of pathogens on produce (Gombas et al., 2017). Therefore, the objective of this study was to evaluate the efficacy of SaniDate 5.0 to reduce and prevent-cross contamination of the *Salmonella* surrogate *E. facelium* on tomatoes using two different procedures of triple-wash process.

Chapter 2
Literature Review

2.1. Foodborne illness and outbreaks (Overview)

To meet the increasing demand for fresh produce, the global annual production of fruit and vegetables increased from more than 500 million tons to almost 3 billion tons each year from 1980 to 2004 (FAO & WHO, 2008). From 1970 to 2017, the supply of fresh produce in the U.S. increased drastically from 154.4 to 202.6 pounds per capita availability (USDA ERS, 2020). Fresh produce is susceptible to foodborne pathogen contamination since it is often consumed uncooked, even with modern precaution to reduce the chance of contamination from the farm, transportation, processing, food service, retailers, and consumers, foodborne illness remains a recurring problem, causing an estimated 9.4 million cases in the United States annually (Scallan et al., 2011). The Centers for Disease Control and Prevention (CDC) classifies a foodborne outbreak as when two or more people contracted the same disease by consuming the same product. From 1996 to 2010, the U.S. Food and Drug Administration (FDA) recorded 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 in 2014 (CDC, 2015a).

The number of farmers' markets has been increasing nationally over the last few decades with the increasing demand for fresh and locally grown produce to promote a healthier and sustainable lifestyle. The United States Department of Agricultural Marketing Service (USDA-AMS) showed the number of listed farmers' markets has increased from less than 2000 in 1994 to near 9000 in 2016 (USDA-ERS, 2017). The U.S. Department of Agriculture Food and Nutrition Service (USDA-FNS), a farmers' market is defined as "Two or more farmer-producers

that sell their agricultural products directly to the general public at a fixed location, which includes fruits and vegetables, meat, fish, poultry, dairy products, and grains"(USDA- FNS, 2016). As farmers' markets have become popular, there has been increasing concern regarding the microbial safety of produce being sold.

2.2. Microbial Safety in Farmers' Markets

The microbiological quality of produce varies significantly based on the commodity type. The methods used in microbiological quality studies though may not be directly comparable, but they show what types of commodities and tests have been conducted. The common evaluation method utilizes different microbiological measurements as an indicator of the microbial quality and cleanliness of the sample, including aerobic plate count, total/fecal coliforms, and common foodborne pathogens.

Foodborne outbreaks from farmers' markets can be difficult to track, due to the limited number of individuals that consumed a contaminated product, as well as the scale of local press coverage on localized outbreaks. Without extensive data to describe an outbreak, traceback to determine the cause of illness becomes more difficult.

A microbial survey involving 13 farmers' markets in Los Angeles and Seattle sampled 133 fresh herbs including basil, cilantro, and parsley. Of 133 fresh herbs sampled, the majority (n=112) were coliform positive. Thirty-two samples were generic *E. coli* positive, with up to 3.15 and 4.15 log CFU/g in coliform and E. coli, respectively. Based on guidelines for microbial quality of RTE foods established by the Public Health Laboratory Service, 16 samples (out of 133) contained more than 2 log CFU/g E. coli, and this level of contamination was unsatisfactory (Levy et al., 2015; RJ Gilbert et al., 2000).

In a similar study, Wood et al. (2015) sampled 68 Romaine lettuce from five farmers' markets for the level of aerobic bacteria, total coliforms, and E. coli. Isolated E. coli samples were tested to determine phylogenetic groupings and virulence genes using multiplex polymerase chain reaction (multiplex PCR) to detect virulence genes (eaeA, hlyA, stx1, and stx2). The mean aerobic plate count (APC) of lettuce samples was 6.3 log CFU/g and ranged from 4.8 to 7.8. While 72% (49) of samples contained coliforms at a mean of 1.9 logs CFU/g, 13% (9) contained approximately 0.7 log CFU/g E. coli (Wood et al., 2015), showcasing the potential concerns on microbial safety on farmers' market produce, and the importance of the role of the farmers' practices to protect consumers from potential foodborne illnesses.

2.3. Salmonella, E. Faecium, and recent outbreaks

The Interagency Food Safety Analytics Collaboration (IFSAC) consists of CDC, FDA, and USDA identified 3,981 outbreaks that were related to *Salmonella, E. coli* O157, *Listeria*, or *Campylobacter* through 1998 to 2018. Of which 1,459 outbreaks could be assigned to a single food category: 905 caused or suspected to be caused by *Salmonella,* 255 by *E. coli* O157, 44 by *Listeria*, and 255 by *Campylobacter*. They also reported *Salmonella* illnesses were the most evenly distributed out of the four pathogens observed (IFSAC, 2020).

2.3.1. *Salmonella*

The Centers for Disease Control and Prevention (CDC) estimated *Salmonella* spp. was responsible for a million foodborne illnesses every year in the United States (CDC, 2012), and was the leading death by foodborne illness in the United States (Scallan et al., 2011). Previous

outbreaks in the United States with produces as delivery vehicles include tomatoes, sprouts, cantaloupes, and more (James M. et al., 2006a).

Salmonella is a gram-negative, rod-shaped, motile, facultative anaerobe, with diameters around 0.7- 1.5 um and length 2-5um (Doyle & Buchanan, 2013; U.S. FDA, 2014a). The most common reservoirs for non-typhoidal salmonellae came from poultry and cattle, mainly chickens and turkeys, cows and pigs, as well as some wild animals. For typhoid and enteric fevers-inducing strains like *Salmonella enterica* serovar Typhimurium, there is no significant animal reservoir since their mode of spread mainly involves fecal-oral transmission, such as fecally 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 shown salmonellosis could be caused by less than ten vegetative cells (D'aoust et al., 1985). Hara-Kuda (2010) analyzed 11 *Salmonella* outbreaks and concluded the infectious dose could be as low as 363 MPN (Hara-Kudo & Takatori, 2011).

2.3.2. *Enterococcus (E. Faecium)*

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 as symbionts, as they make up a large portion of gut microflora (Bennett et al., 2015). They grow optimally at a temperature of 35 ^{o}C, although most species in the genus will grow at temperatures ranging from 10 to 45 ^{o}C (Franz et al., 1999).

With their tolerance to salts (40% bile salt) and wide range in pH (4.6 - 9.9), *Enterococcus* spp. can be adapted to the fermentation activity of cheese and dry sausages, as well as some food systems (Foulquié Moreno et al., 2006). When the meat is improperly processed, Enterococci including *E. faecalis* and *E. faecium* causes 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 as virulent as other foodborne pathogens, they displayed resistance to a variety of antibiotics caused concern. Vancomycin-resistant enterococci are one of the leading causes of hospital-acquired infections (Caballero et al., 2017; Khan et al., 2019). From 2009-2015, the CDC reported one instance of *Enterococcus faecalis* outbreak, causing 13 cases of illness (Dewey-Mattia et al., 2018).

Recent data reported by the IFSAC showed outbreaks and sporadic infections caused by the four priority pathogens were generally demographically similar, attributing illnesses to each of 17 food categories. They emphasized the need for interventions to reduce illnesses from these pathogens need to target a variety of food categories, including *Salmonella* in multiple food categories.

2.3.3 Roles of Surrogate 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 the alternative to their respective pathogens for intervention treatments studies determining their inactivation kinetics, while preventing the introduction of pathogen contamination in food processing facilities. Based on these requirements, a proper surrogate should be non-pathogenic, behaves similarly during

inactivation and susceptibility to injury, predictable kinetics when compared to target pathogen, simple to prepare, and genetically stable (FDA, 2018).

When selecting an appropriate surrogate strain for fresh produce studies, Busta *et al.* recommended an ideal strain should have stable and consistent growth patterns, easy to cultivate to high populations with stability until usage, inexpensive to enumerate, can be differentiated from background microflora, does not induce spoilage, has similar attachment characteristics, and have similar susceptibility to injury to that of the target pathogen (Busta *et al*., 2003).

Regression analysis can be used to validate the use of a surrogate bacteria, in a tested temperature range. Ceylan and Bautista evaluated *P. 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^{o}C. After enumeration, log-transformed plate counts were plotted against time for each temperature. The 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^{o}C (Ceylan & Bautista, 2015).

2.3.4. *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 for validation of thermal processing in almonds (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. The researchers reported that strain- and application-specific evaluations on *E. faecium* NRRL B-2354 as a conservative surrogate was needed. (Kopit et al., 2014)

Bianchini et al. 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^{o}C resulted in a 5-log reduction of the surrogate, 80.38^{o}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^{o}C, and below detection limits at 68.8^{o}C, showing the surrogate was more heat resistant than *Salmonella* spp., which suits as a safer, conservative alternative for these validation studies (Bianchini et al., 2014).

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 on thermal processing of dry pet food with moisture levels of 9.1, 17.9, and 27.0%, heated at 76.7 and 87.88^{o}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^{o}C, 2.66 and 4.08 min at 82.2^{o}C, and 1.07 and 1.69 min at 87.8^{o}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^{o}C to determine thermal death time. The researchers found no significant difference in thermal resistance when using plate-cultured or broth-cultured *Salmonella*, but found *E. faecium* to have greater heat resistance when cultured in broth when 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. 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 (Jeong et al., 2011).

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 are ones that 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.

2.4.1. Pathogen prevalence and survival on produce

Iturriaga, et al. (2007) studied the impact of relative humidity and storage temperature in *Salmonella* on the surface of tomatoes. Tomatoes were inoculated with 3.8 log 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 humidity (97%) promoted the growth of *Salmonella* as well as the formation of biofilm. *Salmonella* could survive for 10 days with around 2 log CFU increased population on the surface of tomatoes under favorable conditions. Even when humidity was low (60%), *Salmonella* maintained its population throughout the experimental period (Iturriaga, et al., 2007). This study showed storage conditions was a growth factor for pathogen and selecting an effective storage strategy alone cannot promise the microbial safety on fresh produce.

Another study tested effects below the critical temperature affected pathogen survival. Colás-Medà, et al., (2017) studied *Listeria monocytogenes* and *Salmonella* on fresh-cut pears for its survival against refrigerated conditions (consistent 4°C) and temperature abuse conditions (4°C for the first 3 days, 8°C for the rest of the study) for the total of 8 days. While comparing different storage conditions had no statistical differences on both pathogens, *Listeria monocytogenes* showed a 1.5-2.0 log CFU/g increase in population, and *Salmonella* showed a slight decline (0.5 log CFU/g). The experiment showed the psychrophilic properties of *Listeria monocytogenes* and the bacteria preserving nature in refrigerated environments (Colás-Medà, et al., 2017). Controlling storage temperature was shown to be ineffective in reducing microbial load on fresh produce, antimicrobials and their applications were examined.

Different applications of antimicrobial agents affect sanitizing efficiency and ease of use. A Beuchat et al. (1998) tested the efficiency of spraying as an application of chlorine to apples, tomatoes, and lettuce comparing to the traditional dipping method. *Escherichia coli* O157:H7, *Listeria monocytogenes,* and *Salmonella* were used to inoculate the produce samples. 200 and 2000ppm of sodium hypochlorite at room condition were applied at different times (0, 1, 3, 5, or 10 min) before being analyzed. Results showed using a higher concentration (2000ppm) chlorine yields a greater reduction, and inactivation by chlorine (0.35 – 2.30 log CFU/cm^2) takes effects within one minute after application. Results also suggested that spraying could be an alternative treatment to submersion as it yielded a similar level of reduction (Beuchat, et al., 1998).

The application of antimicrobials is not the only efficient way to decontaminate fresh produce by deactivating 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 *Escherichia 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, results found using ultrasound alone showed a similar reduction on both pathogens and produce (1.8 log CFU/cm^3 reduction). Bu combining ultrasound is with organic acid showed significantly higher reduction (2.1-3.0 log CFU/cm^3) (São José et al., 2014). The study results showed microbial safety could be enhanced by incorporating detachment by physical means.

2.4.2. Peroxyacetic acid

Peroxyacetic acid (PAA), also known as peracetic acid, is a colorless liquid with a low pH and a pungent odor similar to vinegar, oxidizer, and an effective sanitizer against both gram-positive and negative bacteria, as well as yeast and molds, by disrupting chemical bonds in

enzymes and cell membrane. It is formed from the reaction of acetic acid and hydrogen peroxide and is considered to be a stronger biocide than hydrogen peroxide (McDonnell & Russell, 1999).

PAA is synthesized from the reaction between acetic acid and hydrogen peroxide. The chemical reactions can produce up to 40% PAA in solution, with up to 25% hydrogen peroxide as residue and up to 40% acetic acid (USDA-AMS, 2016). There are multiple manufacturing processes for PAA recorded in the literature (USDA-AMS, 2016), and these products can be used for sanitizing produce, leafy greens, poultry, and meats. (FAO & Ma. Patricia V. Azanza, 2004)

PAA is stable in the 100-200 ppm range. Its sanitizing efficiency is reduced when above neutral pH. 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).

PAA is classified as generally recognized as safe (GRAS), and the U.S. FDA limits the use of PAA 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). For sanitizing food-processing equipment or food contact articles, the concentration range is permitted within 100-200ppm (U.S. FDA, 2011). It can be applied by a variety of methods including spray cabinet, dip tank, hand spray pump, and chiller. PAA is widely used in different industries including food and beverage, hospitals, and health care as an antimicrobial agent. It is also used in meat and poultry establishments, on carcasses, and products to reduce bacterial contamination (USDA- FSIS, 2021).

The efficacy of peroxyacetic acid has been studied with different food products. Applying 500ppm PAA to almonds without agitation caused up to a 1.93 log 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 log CFU/mL (Chang & Schneider, 2012). The significant difference in both studies may be caused by the combination of PAA and physical treatment.

2.5. Conclusion

If a foodborne disease were to orginate from a farmers' market, it could generate a negative perception towards the label of locally grown produce which would jeopardize economic viability for this sector of the food production industry. Such outcomes could be prevented when research-backed methods can be applied by farmers to help ensure the safety of their produce.

Chapter 3
Experimental Methods and Results

3.1. Materials and Methods

Tomato and Microbial Preparation.

Fresh red ripened, organic tomatoes were purchased from a local grocery store and stored overnight in a refrigerated cooler (3.4 Celsius). Bacteria species used in this study were nalidixic acid (NaL) resistant Salmonella surrogate Enterococcus faecium ATCC 8459 (NRRL B-2354). E. faecium was retrieved from frozen stock cultures and streak-plated onto tryptic soy agar (TSA; Hardy Diagnostics, Santa Maria, CA, USA) plus 200 ppm of NaL (Sigma-Aldrich, Darnstadt, Germany) and bile esculin agar (BEA; Hardy Diagnostics, Santa Maria, CA, USA) containing 100 ppm of NAL respectively. Plates were incubated at 35 oC for 48 h to generate pure colonies.

One day prior to each experiment, a single colony was picked from BEA-NaL (E. faecium) of each strain and grew in two 50 ml tubes with 10 ml tryptic soy broth (TSB; Hardy Diagnostics, Santa Maria, CA, USA) containing 200 ppm of NAL for E. faecium, and incubated at 35oC for 24 h. Then, individual bacterial suspension was centrifuged (5,000 × g) for 10 min (VWR Symphony 4417, VWR International, Radnor, PA) followed by TSB from both tubes was decanting the TSB and resuspending the pellets in a sterilized 10 ml of 0.1% buffered peptone water (BPW; Hardy Diagnostics®, Santa Maria, CA) and vortexed for 30 s. The duplicated E. faecium inoculum suspensions were combined and vortexed an additional 30 s before being added into the inoculum bath. The inoculum level of E. faecium was approximately 6 log CFU/ml.

Inoculation of Tomatoes.

Tomatoes were inoculated by placing 5 tomatoes into an autoclaved metal bowl containing 2 L of 0.1% BPW with aforementioned E. faecium inoculum. Temperatures of the inoculated tomatoes and inoculum bath were tested using a scan thermometer (Exergen Corporation, Watertown, MA, USA) by showing the temperatures were 7.3 and 7.5 oC, respectively. After checking temperatures, tomatoes were gently stirred in inoculum solution for 5 min, followed by placing on a foil paper in a biosafety cabinet for 10 min to facilitate bacterial attachment. Our preliminary studies indicated 10 min was enough for attachment as well as preventing premature microbial reduction (Stearns et al., 2022).

Preparation of SD in triple-wash solutions.

The SD solution was prepared in 3L of DI-water and included the following tested concentrations as 0.0064 (pH 6.25), 0.1 (pH 5.85), 0.25 (pH 5.52), 0.50 (pH 3.75), and 0.70% (pH 2.65) (BioSafe Sytems®, Oro Valley, AZ).

Triple-wash treatment of tomatoes.

After inoculation and drying, the tomatoes were either left untreated (control) or subjected the two triple-wash procedures in three autoclaved metal bowls with 3 L of prepared SD solutions. The first procedure was water dip + antimicrobial agent dip + water dip (WAW) and the second one was water dip + water dip + antimicrobial dip (WWA). The water dip + water dip + water dip (WWW) treatment was also included in this study. For either WAW or WWA, the 2 inoculated tomatoes were added to the wash solutions along with 4 uninoculated fresh ones. The 6 tomatoes were then washed concurrently in each of the three bowls and gently stirred by gloved hand for 45 s for each step. Gloves were refreshed after every wash step to avoid cross-

contamination. Immediately, following the last wash step and 10 min drying time, tomatoes were transferred into a sterile 55 oz WhirlPak® sample bag (Nasco, Modesto, CA) containing 150 ml of TSB with 0.1% sodium thiosulfate to neutralize the residual SD on tomato surfaces. The tomatoes were then vigorously shaken in the bag containing TSB solution for 30 s by hand to detach bacteria into solutions.

MPN Microbial Analysis and Enumeration.

After bacteria were detached from the tomato surface, 0.3 ml of rinse solution was added to the first column of the 6 × 8 sterilized most probable number (MPN) microplate, which was prefilled with 2.7 ml of TSB followed by 10-fold serially diluted consecutively along each of the 6 rows using a multiple channel pipettor (Thermo Fisher Scientific Inc. Pittsburgh, PA, USA). Microplates were then incubated at 35oC for 24 h. The turbidity of each well after incubation was pre-recorded and confirmed by pipetting 3 μL of droplets of the cultures onto BEA-NaL agar plates for E. faecium, followed by incubating at 35oC for 48 h. The confirmation plates were finally enumerated by counting positive colonies and transferred into an online MPN calculator to retrieve MPN per gram values before converting results to log10 MPN/g.

Data analysis.

In this study, experiments were repeated four times. Each repetition included 6 tomatoes per treatment with a total of 24 treated samples after 4 repeats. Experiments were conducted by 6 × 6 × 3 factorial design with 3 different wash methods (WWW, WWA, WAW) and 6 different concentrations of H2O2-PAA mixer (0, 0.0064, 0.1, 0.25, 0.50%, 0.70%).

Reductions were calculated as log10 (N0/N) per tomato, where N0 is the average plate counts of untreated controls and N is the count of individual treated tomatoes. Multiple comparisons of

reduction rates of inoculated tomatoes were analyzed using Mixed Model Analysis using JMP (version Pro 16.0, JMP Statistical Discovery LLC. Cary, NC). The means were compared with an $\alpha = 0.05$ significance level as determined by Tukey HSD. However, the Mixed Model Procedure of SAS (version 9.2, SAS Institute, Cary, NC) was used to analyze cross-contamination rates of tomatoes. Multiple comparisons were conducted for cross-contamination data using GAMA distribution and for water samples using followed by the Tukey-Kramer test (significant level of $\alpha = 0.05$).

3.2. Results and Conclusion

Table 1. Microbial population of Enterococcus faecium on tomatoes (log MPN/g ± SD) and in wash solutions (log MPN/ml ± SD) during triple wash process with water only for 45 sec of each step

Washing process	Water (Single wash)	Water + Water (Duplicate wash)	Water + Water + Water (Triple wash)
Reduction of inoculated tomatoes	1.21 ± 0.29a	1.02 ± 0.52a	1.76 ± 0.28b
Cross-contamination to uninoculated tomatoes	1.64 ± 0.71a	1.40 ± 0.12a	1.61 ± 0.29a
Survival in water solution	4.08 ± 0.39a	2.61 ± 0.67b	1.76 ± 0.29c

Note: *E. faecium* on inoculated unwashed tomatoes is 5.29 ± 0.26 log MPN/g. Different letters on the row indicate no significant differences ($P < 0.05$).

The initial E. faecium recovered on inoculated unwashed tomatoes is 5.29 ± 0.26 log MPN/g. Water wash only with single, duplicate and triple reduced the pathogen surrogate by 1.21, 1.02, and 1.76 log MPN/g ($P < 0.05$), respectively, which are mainly due to the physical removing ability of the attached pathogen surrogate by water. As expected, after single, duplicate, and triple wash with water only, the pathogen surrogate survivals in water solutions decreased ($P < 0.05$) from 4.08 to 1.76 log MPN/g. However, regardless of single, duplicate, or triple wash, the pathogen surrogate cross-contaminated from the inoculated tomatoes to fresh tomatoes ranging from 1.40 to 1.64 log MPN/g. Results emphasize that tomato wash waters

without antimicrobials are good vehicle for causing cross-contamination of bacterial cells. The new FDA-FSMA Produce Safety regulation defined any water used in direct contact with the harvestable portion of covered produce as agricultural water. FSMA also required that the microbial quality of agricultural water used during and after harvest needs to be strictly enforced to meet the standards that no detectable generic Escherichia coli in 100 mL of water since the presence of E.coli likely indicate the potential existing of pathogenic bacteria (U.S.-FDA, 2021).

Table 2. Survival and reduction of *Enterococcus faecium* on tomatoes (log MPN/g) by triple wash procedure water dip-antimicrobial dip-water dip (WAW) or water dip-water dip-antimicrobial dip (WWA) a PAA and hydrogen peroxide mixer (SD, SaniDate-5.0, with concentrations of 0.0064, 0.1, 0.25, and 0.50% by 45 sec of each step

	Survival		Reduction	
Treatment	WAW	WWA	WAW	WWA
Control	5.29 ± 0.26d	5.29 ± 0.26d	—*	—*
SD-0.0064%	2.52 ± 0.31c	2.95 ± 0.58c	2.61 ± 0.17bA	2.36 ± 0.54aA
SD-0.1%	2.07 ± 0.84b	1.95 ± 0.58b	3.22 ± 0.82aA	3.34 ± 0.39bA
SD-0.25%	1.67 ± 1.02a	1.48 ± 0.23a	3.28 ± 1.02aA	3.65 ± 0.23bA
SD-0.50%	1.85 ± 0.44a	1.39 ± 0.37a	3.25 ± 1.62aA	3.44 ± 0.44bA

—* 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 ($P < 0.05$)

The initial population of E. faecium on unwashed tomatoes was 5.29 log MPN/g after inoculation. Triple-wash with WAW or WWA procedure reduced the pathogen surrogate by the similar ($P > 0.05$) levels ranging from 2.61 to 3.28 and 2.36 to 3.44, respectively, as the tested SaniDate-5.0 concentrations increased from 0.0064 to 0.5%. It is noticed that applying 0.1% to 0.50% of SaniDate-5.0 for WWA process only slightly ($P > 0.05$) increased the reduction by 0.12

to 0.37 log MPN/g compared to the WAW procedure. These results are different from previous studies of Li et al. (2020a; 2020b; 2021), which reported that WWA procedure obtained better reduction than the WAW by increasing reductions of Salmonella and Listeria monocytogenes by 0.35–1.07 log CFU/g (Li et al., 2021), 1.09-1.48 log CFU/tomato (Li et al., 2020b), and 0.7-2.0 log CFU/squash (Li et al., 2020a) on spinaches, tomatoes and squashes, respectively. The discrepancy of the results might be explained by the reason that the 45 s washing time of each steps during triple-wash in this study compared to the 10 s dipping time in the previous ones. Currently, the stakeholder of this triple-wash project the Preston County Workshop in Reedsville WV are using 45 s washing time of each step. Extending the previous 10 s to 45 s might eliminate the difference of pathogen reduction efficacy between WAW and WWA procedures. Results of the current study also suggested that increasing SaniDate 5.0 concentrations from 0.1 to 0.5% did not enhance the antimicrobial effects of the surrogate E. faecium on tomatoes. Li et al (2020a) also found that no difference between the 0.0071% and 0.45% of SaniDate-5.0 was observed for inactivating aerobic plate counts, coliforms and lactic acid bacteria on squashes during an onsite plant shelf-life study. Both studies indicated that suggests simply adding a higher concentration of SaniDate-5.0 into wash waters does not necessary achieving a better control of microbial populations on fresh produce samples.

Table 3. Cross-contamination of *Enterococcus faecium* onto uninoculated tomatoes (log MPN/g) by triple wash procedure water dip-antimicrobial dip-water dip (WAW) or water dip-water dip-antimicrobial dip (WWA) in a PAA and hydrogen peroxide mixer (SD, SaniDate-5.0, with concentrations of 0.0064, 0.1, 0.25, and 0.50% by 45 sec of each step

Concentration	*E. faecium* on uninoculated tomatoes (log MPN/g)		Cross-contamination frequency (Positive/Total)	
	WAW	WWA	WAW	WWA
SD-0.0064%	0.61 ± 0.15	0.37 ± 0.90	8/8	8/8
SD-0.1%	0.83 ± 0.70	0.59 ± 1.12	4/8	5/8
SD-0.25%	-0.40 ± 0.0	N/A	2/16	0/16
SD-0.50%	N/A	N/A	0/12	0/8

Note: N/A indicates below detect limit of -0.40log MPN/g.

These results conclude that Sanidate-5.0 can be an effective antimicrobial agent for cross-contamination prevention of E. faecium in tomatoes. Concentrations ≥0.25% proved effective in preventing cross-contamination, however, increases above this amount do not appear to yield significantly greater results. In conclusion, a combination of PAA and hydrogen peroxide could be considered a viable organic antimicrobial agent that small farmers may utilize to ensure the safety of their produce prior to selling to the public.

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