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Comparison of Antimicrobials and Delivery Methods on the Inactivation of Listeria monocytogenes on Apples

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ABSTRACT

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The United States Department of Agriculture-National Institute of Food and Agriculture recognizes *Listeria Monocytogenes* as an emerging and under researched pathogen. Due to this recognition for more research and the numerous multistate listeriosis outbreaks originating from apples (2014-2015,2017, and 2019), experiments in this paper were focused on inactivating *Listeria Monocytogenes* from apples. Contamination of Listeria on apples can occur at the harvest and/or packing process of apple production. Some strategies that producers utilize to reduce this risk are by applying antimicrobials via garden spray, electrostatic spray, and dipping methods. There are also several antimicrobials claimed to kill microbes on produce surfaces that are on the market for farmers and apple producers. However, the wholesale distributor Appalachian Harvest requires their local West Virginia suppliers to only utilize a H_2O_2-PAA mixer known as SaniDate-5.0®.

Therefore, as described in chapter two, experiments were conducted to analyze which delivery method and concentration of SaniDate-5.0® is the most effective at reducing *Listeria monocytogenes* on fresh apple surfaces. In order to compare these processes, three organic apple types (*Honey Crisp, Pink Lady,* and *Fuji*) were dip inoculated with *Listeria monocytogenes* (2-strain, serotype 1/2b). Apples were either not treated (control) or treated with water only, or with the H_2O_2-PAA mixer (0.0064, 0.1, 0.25 and 0.50%) for 20 s via garden spray, electrostatic spray, or dip. The initial microbial load of the control groups was 6.80-6.90 log/CFU. Overall, the dip method (3.33 log/CFU) was more effective than garden spray (2.5 log/CFU) and electrostatic spray methods (2.0 log/ CFU) at inactivating *Listeria monocytogenes,* regardless of the concentration of antimicrobial ($P<0.05$). On average, the garden spray methods were more effective than the electrostatic spray methods for most SaniDate-5.0® concentrations ($P<0.05$). Reductions of *Listeria monocytogenes* were greatest ($P<0.05$) when 0.25% and 0.5% of the antimicrobial was applied to the apple by all of the delivery methods.

To confirm results, the method of action of SaniDate 5.0® on Listeria monocytogenes inactivation was analyzed using atomic force microscopy. Samples were sent to the University of Connecticut where Dr. Luo performed Atomic Force Microscopy analysis. The topographical observation under atomic force microscopy revealed changes in membrane permeability that led to extracellular leakage and eventually cytolysis when introduced to 0.5% of the antimicrobial solution. There was not a significant change in cell morphology until 0.1% of SaniDate 5.0 was added and more drastic changes of the cell were noticed when Listeria cells were subjected to 0.25 and 0.5% antimicrobial.

Overall findings of this research indicated that 0.5% delivered through the dip method was most effective in inactivating *Listeria monocytogenes.* Atomic force microscopy revealed the possible mechanism of action of this concentration. That is to say that 0.5% SaniDate 5.0® causes oxidation and cytolysis of the bacterial cell, thus leading to cell death. It was hypothesized that the reason the dip method was more effective than either spray method at inactivating Listeria, despite not having the most residual water on the apples, was due to the

apple being fully submerged in the washing solution. Although there are other aspects to consider such as cost of treatment and cross-contamination occurrences in wash methods, this research can be utilized as a tool to help local apple farmers prevent the spread of *Listeria monocytogenes* to consumers.

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER TWO

ABBREVIATION KEY

Chapter 1:

1. *Listeria monocytogenes-L. monocytogenes*
2. Food and Drug Administration-FDA
3. United States Department of Agriculture -National Institute of Food and Agriculture-USDA-NIFA
4. SaniDate 5.0-SD
5. Ready-to-eat-RTE
6. West Virginia-WV
7. Peroxyacetic Acid -PAA
8. Hydrogen Peroxide H_2O_2
9. Garden Spray- GS
10. Electrostatic Spray -ES
11. Water Activity -Aw
12. Granny Smith Apples -GSA
13. Colony Forming Units -CFU
14. Room temperature-RT
15. Controlled Atmosphere-CA
16. Carbon Dioxide -CO_2
17. Oxygen-O_2
18. Kentucky-KY
19. Parts Per Million-ppm
20. Lactic/Citric Acid Blend -LCA
21. Sodium Hypochlorite-SH
22. Water, Water, Antimicrobial-WWA
23. Water, Antimicrobial, Water-WAW
24. Escherichia coli -E. coli
25. Species-spp.
26. N-acetyl-l-cysteine -NAC
27. Lauric Arginate-LAE
28. Sodium Dodecyl Sulfate-SDS
29. Garden Spray-GS
30. Electrostatic Spray-ES
31. Air-Assisted, Induction Charged -AAIC
32. Malic Acid -MA
33. Grape Seed Extract -GSE

Chapter 2:

1. *Listeria monocytogenes-L. monocytogenes*
2. Food and Drug Administration-FDA
3. United States Department of Agriculture -National Institute of Food and Agriculture-USDA-NIFA

4. Ready-to-eat-RTE
5. Garden Spray-GS
6. Electrostatic Spray-ES
7. Peroxyacetic Acid -PAA
8. Hydrogen Peroxide- H_2O_2
9. Virginia-VA
10. Honey Crisp-HC
11. Pink Lady- PL
12. Fuji-FJ
13. Colony Forming Units -CFU
14. Modified Oxford agar -MOX agar
15. Tryptic Soy Broth-TSB
16. Buffered Peptone Water-BPW
17. Atomic Force Microscopy -AFM
18. Root-Mean-Square -RMS
19. Least Square Means- LS means
20. Red Delicious-RD
21. Statistical Analysis System – SAS

Chapter 3:

1. Atomic Force Microscopy-AFM
2. Peroxyacetic Acid -PAA
3. Hydrogen Peroxide H_2O_2
4. Buffered Peptone Water- BPW
5. CFU-Colony forming units

CHAPTER ONE
LITERATURE REVIEW

I. Introduction

Listeria monocytogenes (L. monocytogenes) are gram-positive, rod-shaped, facultative anaerobes responsible for the infection listeriosis. These microbes are prevalent in moist environments such as soil, water, as well as decaying vegetation and animals, which make contamination of foods and produce probable *(18)*. Early symptoms of listeriosis include fever, nausea, diarrhea, and chills. If not treated quickly with antibiotics, *Listeria* infection can spread to the nervous system and cause headaches, convulsions, confusion, and death. Particularly, the elderly, pregnant women, and fetuses are at higher risk for adverse infection outcomes *(18).* To date, *Listeria* is one of the most infectious microbes in relation to food outbreaks. Though not the deadliest bacteria, it can be fatal to those more susceptible to infection *(21)*. According to the U.S.-Food and Drug Administration (FDA), *Listeria* has a mortality rate between 20-30%. Annually, 1600 people are infected, and 260 people die from listeriosis *(18)*. Recently, *L. monocytogenes* has been recognized by the United States Department of Agriculture -National Institute of Food and Agriculture (USDA-NIFA) as an emerging, under researched pathogen on produce *(21)*.

There is growing concern regarding *L. monocytogenes*, stemming from its presence on fruits such as apples. One of the latest apple recalls occurred in 2019, where 2,297 variety cases of *McIntosh, Honeycrisp*, *Jonathan*, *Fuji, Jonamac,* and *Red Delicious* apples were contaminated with *L. monocytogene*s *(19)*. Earlier recalls on plain apples and caramel apples occurred in 2014-2015 and 2017, sparking concerns among farmers and consumers alike (*16*, *3*). These outbreaks of *L. monocytogenes* occur because of the microbes' ability to grow in a wide range of

temperatures (0-45°C), its strict adherence to surfaces, and its resistance against disinfectants. In most cases, the bacteria can be destroyed by heating common ready-to-eat (RTE) foods such as deli meats to ~74°C, however, in fruits that are often consumed raw, other precautions must be addressed *(18).* Contamination can happen at the harvest and/or packing process of apple production. To eliminate contamination, safeguards need to be taken at both steps.

There are several organic acids and inorganic chemicals utilized to eradicate microorganisms from produce. One of the most common agents used in West Virginia (WV) among smallholdings is SaniDate-5.0® (SD) *(22)*. This peryoxy compound is an organic formula composed of peroxyacetic acid (PAA) and hydrogen peroxide (H_2O_2). The company claims SD is effective at killing gram negative and gram positive bacteria without having to rinse the product *(22)*. It is also versatile in how it can be administered to products.

In order to control foodborne pathogens on produce/fruits surfaces, antimicrobials such as SD, are widely used and delivered by garden spray (GS), electrostatic spray (ES), and dipping treatments. The ES functions by applying a positive charge to the solution. These positively charged droplets that are diffused from the nozzle are attracted to negatively charged surfaces which creates an even coating *(25)*. On the other hand, the GS, also known as compression sprayers, have no charge or ability for the solution to be attracted to the food surface. The GS is pumped with air manually and the compressed air forces the liquid to be sprayed from the nozzle. The GS often needs to be continually pumped of air to ensure a heavy and constant mist is expelled. The ES also differs from the GS because it has a flow rate of 0.97 mL/s, whereas the GS measured 7.23mL/S *(8)*. Both methods have been utilized by farmers, but there are questions as to which delivery method is the best and if SD is better than other antimicrobials for produce.

Therefore, this review aims to evaluate the anti-listeria efficacy of a mixer composed of PAA and H_2O_2 by the application processes GS, ES, and dip methods. Currently, no study has focused on using the organic chemical SD in combination with three different treatment methods on apples. Determining the most effective antimicrobial and delivery method to use on apples to inactivate *L. monocytogenes* could help apple farmers prevent the spread of the microbes to their other crops as well as their consumers.

Apple Physiology and Storage

Though *L. monocytogenes* is widely recognized as a pathogen that can reside on ready-to-eat (RTE) foods, its survival on apples is not expected because the fruit does not have a hospitable environment for *Listeria (6)*. Therefore, Glass et al., 2015 questioned the unexpected listeriosis outbreak from apples and conducted an experiment to find how *L. monocytogenes* were able to grow on caramel apples, as conditions are not suitable for the microorganism. Though Listeria can grow in various temperatures, it is not as favorable toward extremes of pH or water activity (Aw). Caramel has a low Aw (<.80), and apples have a high inner acidity of ≤ 4.0, which indicate that *L. monocytogenes* should not be present on such a food source. Glass et al., 2015 inoculated Granny Smith apples (GSA) with 4 $\log^{10}$ colony forming units (CFU) of *L. monocytogenes*. In this study, a stick was placed in half the apples and then all apples were dipped in caramel and stored for 11-28 days. There was a significant increase in bacteria at room temperature in the apples with inserted sticks compared to that of the caramel apples with no sticks. This led to the conclusion that apples nor caramel are ideal for *Listeria* and it should not grow on the food alone, but the sticks inserted to the caramel apples possibly created a more favorable environment for the bacteria by releasing the acidic juices normally found in a whole apple *(6)*.

Macarisin et al., 2019 proposed another possible mechanism in which *Listeria* can thrive on plain apples. A 160-day storage study using waxed and unwaxed apples, recorded a 1.1 log higher reduction in apples that had less sinuses. There was also a higher reduction in *L. monocytogenes* in those cultivars that contained a thicker wax covering. The author inferred that the sinuses leave the bacteria vulnerable to antimicrobials and the waxing helped prevent bacteria from reaching the nutrients on the apple that it needs to survive. It was also stated that epiphytic yeasts tend to live in the stem and calyxes of the apples during post-harvest. These yeasts secrete extracellular hydrolytic enzymes that can degrade the apple cuticles and pectin polymers. Through this degradation, the microbiota can access the nutrients necessary to live on apple surfaces that are normally poor in nutrients *(15)*.

However, the degradation of apples is impacted by how the fruit is stored as well. Not only does the type of apple seem to contribute to the survival of *Listeria* on its surface, but the method in which it is stored post-harvest is also a significant contributor. There is dissimilarity when it comes to commercial and small-scale apple processing. Large industries are able to keep apples year-round in grocery stores because of refrigeration and controlled atmosphere (CA) environments where oxygen (O_2) is lowered to 1-3% and carbon dioxide (CO_2) is maintained at 3% by flushing the gasses out with nitrogen gas. This method is effective for delivering excellent quality fruits by reducing cellular respiration thus preventing decay and bacterial growth *(26)*. However, this is not an affordable method for smallholdings in rural areas. For local-small farming in states like WV and KY, refrigeration is the only method of fruit preservation, or they are held at room temperature (RT).

There have been a few studies that analyzed the growth of pathogenic microbes in various temperatures and conditions. Refrigeration is shown to have a minimal effect on *Listeria*

reduction in most cases and does not eradicate the pathogen after 12 weeks or 160 days *(15, 24)*. At RT, *L. monocytogenes* survival rates were higher than those stored in refrigeration in the long term. On the contrary, one study indicated that RT storage for the short term (1-4 days) had less *L. monocytogenes* survival than those held at 10°C. It was suggested that this was due to RT apples having a faster cellular respiration and are able to produce more volatile compounds such as alcohols, ketones, esters, and aldehydes that have antimicrobials effects that normally would be hindered by colder storage. Although, this study did conclude that prolonged storage showed optimal reduction at 1°C compared to RT and 10°C *(24)*.

Nevertheless, apples have been a source for listeriosis outbreaks despite its unfavorable environment for bacteria and post-harvest storage methods of suppliers. Since small-scale producers do not have the technology capable of CA, studies showing how different cultivars are more susceptible to contamination offer important insight for local farmers. These studies indicate a possible need for specific treatments based on the individual apple topology if CA is not an option *(6, 15).* Perhaps a different antimicrobial concentration is needed depending on the number of sinuses or waxing of the specific apple type.

II. Antimicrobial Treatments on Produce

One of the most common antimicrobials used by farmers is an organic chemical called SaniDate 5.0® (SD). Though it has been studied on many types of fruits and vegetables, it has not been analyzed for its efficacy on apples. Sanidate-5.0® is composed of 5.3% peroxyacetic acid (PAA) and 23% hydrogen peroxide (H_20_2) *(22).* BioSafe Systems® claims the product is effective at instantly killing fungi, viruses, gram positive and gram-negative bacteria without having to rinse the product. The post-harvest concentration recommendation for this product is 0.0512%-0.1816% (512ppm-1817ppm) and the product should remain in contact with the

produce surface for a minimum of 45 seconds for maximum effectiveness *(22)*. This chemical has gained popularity with local smallholdings in West Virginia (WV) because of the recommendations made by WV Small Farm Center. There was also a requirement by the whole sale buyer Appalachian Harvest, which stated farmers who sell to the company must utilize SD post-harvest (*12*). It is recommended that if produce is grown directly in soil then SD should be used during post-harvest of the produce to mitigate pathogen growth, which has been and ongoing issue at WV and KY Farmers' markets *(13).*

To distinguish the efficacy of SD against other antimicrobials, Li et al., 2021 used a triple wash method on spinach to compare sodium hypochlorite (SH;100 ppm, pH-6.8), lactic/citric acid blend (LCA; 2.5%), and a H_2O_2-peroxyacetic-acid mixer (SD, 0.0064%, 0.25%, and 0.50%). Spinach was inoculated with a 2-strain mixture of *L. monocytogenes.* The two *L. monocytogenes* strains used were L2624 and L2625, which were also serotype 1/2 b isolated from the previous *Listeria* outbreaks. Spinach was contaminated by the strains, then washed in either two procedures: water, water, antimicrobial (WWA) or water, antimicrobial, water (WAW). This study indicated that the WWA method had greater bacterial reduction than the WAW method (0.35–1.07 log10 CFU/g). The study showed there was no difference between 0.25% and 0.5% concentration of SD on spinach and SD (0.25%-0.5%) was more effective ($P<0.05$) at removing bacteria than SH-100ppm and LCA (2.5%) (*12*). The study did show that SD is more effective than other antimicrobials at reducing bacteria on spinach, but the physiology of spinach differs from that of apples. Previous studies show how concentrations of SD varied for vegetables with tougher exteriors, similar to the apple, such as cucumbers and butternut squash *(10, 11)*.

Earlier studies concluded that concentrations of SD at 0.25% and 0.5% showed a significant *L. monocytogenes* reduction on butternut squash compared to lower concentrations. Furthermore, 0.5% SD was more effective at reducing *L. monocytogenes* than 0.25% SD. Based on the least square means test, there was a significant difference between 0.25% vs 0.5% SD, 0.25% vs 0.1%, and 64ppm vs 0.1% treatments of SD. Concentrations of SD at 0.25% and 0.5% showed a significant *L.* monocytogenes reduction on butternut squash compared to the lower concentrations *(11).*

In contrast, Li et al., (2020) showed that 0.25% SD was more effective than 0.5% SD at reducing *L. monocytogenes* on cucumbers. In this study, a triple wash experiment was also performed using SD in comparison to various chemicals. It was indicated that the triple wash dipping method, WWA was more effective at reducing bacteria compared to the WAW method. SD proved to be as or more effective than a lactic-citric acid blend, sodium hypochlorite, and acidified sodium hypochlorite. Results indicated that 0.25% and 0.5% SD reduced bacteria by 1.75 and 1.56 log/cfu respectively on cucumbers *(10).*These results show that there is inconsistency among studies regarding which concentration of SD is the most effective antimicrobial *(6,7,8).*

Based on the studies reviewed, 0.5% concentration of SD was effective at killing *Listeria* in vegetables such as spinach and butternut squash, but not cucumbers. This suggests the effectiveness of SD in different produce is varying and the exterior of the cultivar could be a factor. This is important because apples have unique properties that results in more resistance to antimicrobials. However, SD has not yet been tested on apples. Rather, research has only focused on studying antimicrobials such as chlorine, chlorine dioxide, peracetic acid, Carvacrol, Citrox,

vanillin, hydrogen peroxide, and N-acetyl-l-cysteine and their effects on microbes, including *Listeria* on apples *(2,3,20).*

III. Antimicrobial Application on Apples

One of the earliest studies analyzing the effects of antimicrobials on apples utilized spray applications of 200 or 2000ppm of chlorine. Beuchat et al., 1998 examined the efficacy of chlorine for killing *Salmonella*, *Escherichia coli (E. coli) OI57:H7*, and *L. monocytogenes* on whole apples. Inoculated apples were treated (sprayed and then soaked) with water (control) or solutions containing 200 or 2,000 ppm of chlorine for 0, 1, 3, 5, or 10 min, rinsed with sterile water, and enumerated (CFU/cm2). Apples were placed under a laminar hood and sprayed 6 times with treatments and then rubbed with a gloved hand for 20 s each followed by rinsing, incubation, and enumeration. Chlorine was observed to be effective within 1 minute of application. Also, 2000 ppm was more effective at bacterial inactivation than 200 ppm and water treatments. *Salmonella* was significantly decreased when treated with 2000ppm (3.98log/CFU) when compared to water treatments (2.80-3.01 log CFU) regardless of soak/rinse time. *E. coli 0157:H7* on the other hand was affected by soak/ rinse times, meaning the longer the apple was soaked, the more cells of *E. coli 0157:H7* was released from the apple surface. There was not a significant difference in the effectiveness of 200ppm and 2000ppm on reducing *E. coli*. Moreover, only 2000ppm was effective at killing *L. monocytogenes* on apples as the bacteria seemed to have more resistance to chlorine *(2)*.

In a later study, Abadias et al., 2011 tested the efficacy of the antimicrobials Carvacrol (a monoterpene phenol) , Citrox (a combination of organic acids, and bioflavonoids derived from

bitter oranges), PAA (80 and 120 mg L−1), vanillin (a phenolic aldehyde)(12 g L−1), H_2O_2 (5, 10, 20 mL L−1) and N-acetyl-l-cysteine (NAC) (5 and 10 g L−1) on reducing *E.coli O157:H7, Salmonella species* (*spp*.) and *Listeria spp*. populations on fresh-cut apple. Treatment reductions were compared to water and standard sodium hypochlorite (SH) 100 mg L−1, pH 6.5). No pathogens were detected in the PAA, H_2O_2, Citrox and SH washing solutions after apple treatment. *E. coli* inoculated apples were more effected by H_2O_2 solutions. H_2O_2, NAC 10 g L−1, and PAA 80 and 120 mg L−1 had reductions between 0.8 and 2.0 log higher than SH. NAC 10 g L−1, and H_20_2 also resulted in the highest reduction of *Salmonella spp*. on apples. Finally, *Listeria spp*. had the greatest reduction when apples were treated with PAA (2.3 log CFU) and H_20_2 (1.6 log CFU) *(1).*

The most recent study examining the effects of antimicrobials on *Listeria* on apples did not only treat apples with antimicrobials. The author made note of the difference in cultivar morphology and applied surfactants to the antimicrobials being studied. *Listeria Innocua* (An *L. monocytogenes* surrogate) was used to inoculate whole apples. Pietrysiak et al. 2019 applied surfactants: cationic lauric arginate [LAE], anionic sodium dodecyl sulfate [SDS], and nonionic Tween 20 [T20] alone and combined with peracetic acid (PAA) to evaluate the inactivation rates of *L. Innocua* (7 log CFU/ml) on organic *Gala* apples. After inoculation, apples were either treated via a plastic spray bottle method (treatment 1) or dip method (treatment 2). Treatment 1 required apples be sprayed with ~ 4.2ml of one of the eight cleaning solutions mixed with surfactants (six apples for each treatment): water, 0.1% LAE, 0.1% SDS, 0.1% T20, 80 ppm of PAA, 0.1% LAE with 80 ppm of PAA, 0.1% SDS with 80 ppm of PAA, and 0.1% T20 with 80 ppm of PAA. The second method of treatment for inoculated apples included a 10s dip in 250ml in one of the chemical treatments not combined with surfactants. In both methods the apples

were rubbed with gloved hands for 1 minute to stimulate the apples being cleaned on a commercial brush bed. Enumeration and scanning electron microscopy were used to analyze bacterial reductions and the superficial effects of the antimicrobials. It was reported that treatment 2 was more effective than treatment 1, but all treatments with surfactants were better than water or PAA alone. The maximum reduction occurred when treatment PAA-T20 was applied and resulted in a ~2.2 log reduction in *L. innocua*. Based on the enumeration results and the images from the scanning electron microscopy, surfactants (LAE, SDS, and T20) helped downgrade surface tension of water and liquid-slid interface which aided in the reduction of bacteria in the most harbored areas of the apple (stem bowl, calyx cavities, and microcracks) *(20)*.

As shown by these studies, PAA in addition to a surfactant, and high concentrations of chlorine could be viable options to use as sanitizers on apples to help mitigate *Listeria* growth. However, none of these studies showed a complete elimination of *Listeria* from any of these treatments and there is a lack of research showing the effectiveness of other antimicrobials like SD on pathogen prevention on apples. Along with finding what antimicrobial is most effective at eliminating microbial growth on apples, the most effective delivery method of antimicrobials also needs to be considered to enhance efficiency. The only delivery methods of antimicrobials tested on apples thus far has been the dip and spray bottle methods and none of these experiments involved using SD as a treatment option. As seen previously SD has been effective for other produce at removing bacteria when applied via the triple wash methods *(10, 11)*. Other studies have applied SD using methods such as electrostatic spray and conventional garden sprays to various inoculated products and have shown inconclusive results *(8, 14, 17, 23)*.

IV. Methods of Antimicrobial Delivery

Though the concentrations of antimicrobials play an imperative role in the inhibition of bacterial growth, the method in which the antimicrobial is delivered on to food surfaces could be equally important. Antimicrobials, to include SD, can be used in a variety of methods to include GS, ES, and dipping. Factors that a farmer must consider before choosing a delivery method are cost and efficiency.

According to Jiang et al., 2018, the ES has a flow rate of 0.97 mL/s, whereas the GS measured 7.23mL/S. This effected the cost analysis of both products. An economic analysis indicated that although the initial purchase of the ES ($3000) is more expensive than the GS ($60), ES can save money over time because of its efficiency in antimicrobial waste, water usage, and manual labor. When using the ES, antimicrobials were 20-30% cheaper because less was needed. However, the ES had higher maintenance cost than that of the GS of roughly $500 per year and $120 per year, respectively. Jiang et al., 2018 concluded that the ES may be more cost effective over time but the initial purchasing cost of the ES is too expensive for small-scale producers *(8).*

In the same study, GS and ES delivery systems were used to deliver organic acids such as peroxyacetic acid (PAA; 0.1%), lactic acid (5.0%), lactic and citric acid blend (2.5%), sodium hypochlorite (SH; 50 ppm), and SD 0.25% to inoculated eggs. In this study eggs were inoculated with *L. monocytogenes*, and then treated with the various sanitizers. Jiang et al., 2018 showed that ES was more effective at reducing bacterial colonies on eggs, while being more cost effective in the process. ES was more effective ($P < 0.05$) than GS at reducing *L. monocytogenes* on eggs by 2.53 log/CFU and 1.11 log/CFU, respectively *(8)*.

The ES also showed similar results in a study by Lyons et al., 2011. In this study, the difference between the conventional sprayer with no charge, the full rate hydraulic-atomizing nozzle, and the air-assisted, induction charged (AAIC) sprayer was studied based on its efficacy of removing antimicrobials. However, food contact surfaces were inoculated, rather than food. These surfaces included types of packaging such as stainless steel, cardboard, and a poly-vinyl chloride (PVC) conveyor belt. The surfaces were contaminated with *Salmonella* and then sprayed with peracetic acid from perpendicular and parallel angles on the front and back of each surface. This study concluded that the AAIC sprayer achieved equal to or more than the other two methods while dispensing only 1/6th of the volume. The study noted that AAIC had the greatest impact or bacterial reduction on the waxed cardboard surface of 4.64 log/CFU than the PVC conveyor belt which had a reduction of 3.60 log/CFU, suggesting that the surface being subjected to antimicrobials should be uniquely treated *(14)*.

Similarly, ES was more effective than the GS at reducing microbes on spinach. Ganesh et al. 2010 conducted a study using the organic acids: malic acid (MA) (1%), tartaric acid (2%), lactic acid (LA) (3%), and grapeseed extract (GSE) (3%) alone and in combination with one another were sprayed via ES or GS on *Salmonella Typhimurium* inoculated spinach. It was concluded that all treatments showed reductions independently or being combined with one another except for grape seed extract which was not beneficial at removing bacteria. MA and MA + GSE treatments showed inhibitory effects that were statistically significant from other treatments on each day of plating. Concurrently, EC spray showed a significant reduction in bacteria (2.6-33. log/cfu) while conventional spraying had minimal effects on reductions (0.0-0.3 log/cfu) *(5)*.

As recognized with the efficacy of SD concentration, a single method might not be the best for every situation. McCarty et al., 2016 reported that dip and GS methods with 380 ppm of peroxyacetic acid was more effective at reducing *Escherichia coli (E.coli)* on beef subprimals than the ES treatments *(17)*.

Another study conducted by Shen et al., 2019 used a meat subject as well and showed similar results, stating that ES may not be the most effective method of antimicrobial delivery. Results showed no difference ($P > 0.05$) in the inactivation of *Campylobacter jejuni* cells on chicken wings between dip and ES methods. ES treatments had less reduction of the pathogen than either GS or dip treatments. The author concluded that the results could be attributed to less volume of the SD solution delivered onto chicken wings surfaces compared to the GS or dip treatment *(23)*.

In agreement with the previous studies, Youssef et al., 2012 found that at least 0.1 ml/cm2 of antimicrobial solution should be applied to receive a significant log reduction of *E. coli* on beef trimming surfaces. The study also reported that ES delivered a significantly smaller volume rate than the conventional spray system (0.045 vs 0.26 ml/cm2) which could contribute to less bacterial reduction on the meat products. In this study different concentrations of lactic acid were administered to the beef carcasses rather than SD. It was concluded in this study, much like the apple studies, that bacteria are able to survive better in certain areas of the food than others. The authors suggested that *E.coli* entrapped by muscle and fat were more resistant to treatment than the membrane layer of the carcass, providing further evidence that treatment should differ based on the food being treated *(27)*.

In contrast, Hudson et al., 2015 reported that ES is more effective at killing bacteria on the beef surface and lactic acid was better at reducing bacteria than tap water, cetylpyridinium

chloride, peroxyacetic acid, and 3% lauric arginate. The study showed that lactic acid was more effective at killing *E. coli* on beef carcasses when delivered through ES *(7)*.

Similar to the inconsistent results regarding SD concentration efficacy, the method food manufacturers and farmers use to deliver antimicrobials to surfaces appeared to be dependent on the object it was being applied. The studies that test antimicrobials on meat showed that ES was not as effective as GS and dip methods, however studies that utilized produce concluded that ES was more efficient than GS at killing bacteria. Apples were not among the produce tested. Therefore, the inclusivity of these results still yield unanswered questions of how an apple would be impacted by SD if delivered through ES, GS, or dipping methods.

V. Conclusion

In conclusion, previous studies indicated that SD is effective at reducing bacteria such as *L. monocytogenes* at concentrations of 0.25% and 0.5% although studies differ in suggesting which concentration is better 0.25% or 0.5%. However, the effectiveness of SD has not been tested on apples. Previous amounts of antimicrobials applied to apples, such as peracetic acid, lactic acid, chlorine and hydrogen peroxide *(2, 9, 20)*, although reduced microbes significantly on contaminated fruits, were never enough to fully eradicate the bacteria from the apple surfaces. Based on previous research using SD, it can be concluded that the produce can be impacted by the antimicrobial but because produce vary in their physiology, the concentration needed to eliminate certain bacteria on different produce types are specific. Similarly, the method of antimicrobial delivery appears to be specific.

The studies in this review are inconsistent over which delivery method was more effective, ES, GS, or dipping. Foods e.g., eggs, spinach, and surfaces inoculated with bacteria

had the greatest reduction when treated with antimicrobials via the ES method *(5, 8, 14)*. The same was not indicated for inoculated chicken wings and beef subprimals, which resulted in a greater reduction of bacteria from the GS method *(17, 23, 27)*. The results from these studies further show that the spray method utilized should be determined by the product being subjected to treatment rather than using the same method for every product. For example, apples have differing physiology components that may warrant different application methods than that of spinach and eggs.

Due to the recent outbreaks of *Listeria* on apples, it is imperative that further studies examine these differences in the effectiveness of SD on removing *L. monocytogenes* on different cultivars with varying physiologies and discover which method and concentration is most effective to deliver to the contaminated apple surface and prevent additional listeriosis outbreaks.

VI. Future Research

Future studies are needed to compare *Listeria* reductions on apples when treated with different concentrations of the antimicrobial SaniDate 5.0® applied through various delivery methods. First, no study has yet conducted this comparison on apples, which were associated with the multi-state listeriosis outbreak. Additionally, an atomic force microscopy test analysis needs to be conducted to observe the interaction of this particular antimicrobial and *L. monocytogenes*. Lastly, waxing and physiology of various organic apples need to be taken into account after antimicrobial treatment applications to determine differences in efficacy among cultivars. Future research in these areas could help local farmers in states such as WV and KY improve their harvesting methods and ensure protection from food borne illness on their produce. It is increasingly important that farmers understand the best practices and concentrations for

antimicrobials since it is currently recommended that they use it in practice to prevent further outbreaks.

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CHAPTER TWO

The Efficacy of Conventional Spray, Electrostatic Spray, and Dip with a Combination of Hydrogen Peroxide and Peroxyacetic Acid to Inactivate *Listeria monocytogenes* on Apples

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Primary Audience: Smallholdings, Researchers, Microbiologists

HIGHLIGHTS

- GS, ES and Dip method were tested for reducing *L. monocytogenes* on apples.
- Dip is better than GS and ES for reducing *L. monocytogenes*.
- H_2O_2-PAA mixer (0.25 and 0.5%) decreased *L. monocytogenes* on apples.
- Atomic microscopy analysis indicated H_2O_2-PAA mixer disrupt cell outer membrane.

ABSTRACT

This study aimed to evaluate the efficacy of a hydrogen peroxide (H_2O_2) and peroxyacetic acid (PAA) mixer delivered by conventional garden spray (GS), electrostatic spray (ES), and dip methods to inactivate *Listeria monocytogenes* on apples. Organic *Honeycrisp*, *Fuji*, and *Pink Lady* apples were dip inoculated with *L. monocytogenes* (two strains, serotype 1/2b), which were then kept untreated (control), sprayed with water only, or treated with the H_2O_2-PAA mixer (0.0064, 0.1, 0.25, and 0.50%) for 20 s via GS, ES, or dip, followed by draining (for 2 min) on aluminum foil. Surviving bacteria were recovered on modified Oxford agar. Atomic force microscopy was used to detect the structural changes of inactivation of *L. monocytogenes* in broth medium by the H_2O_2-PAA mixer solution. Data (two replicates, with six samples per replicate) were analyzed using the mixed model procedure of SAS ($P < 0.05$). Initial counts of *L. monocytogenes* on untreated apples were 6.80 to 6.90 log CFU per apple. The dip method was the most effective treatment ($P < 0.05$) for pathogen reductions (2.31 to 2.41 log CFU per apple), followed by GS (1.44 to 1.70 log CFU per apple) and then ES (0.84 to 1.20 log CFU per apple). Reductions of *L. monocytogenes* were greatest ($P < 0.05$) when apples were treated with H_2O_2-PAA mixer 0.25 and0.50%. Atomic force microscopy analyses indicated that inactivation of *L. monocytogenes* cells in H2O2-PAA mixer solutions resulted from disruption of the outer membrane. The H_2O_2-PAA mixer–treated cells had increased width and height and decreased roughness compared with the untreated cells. Results suggested that applying a H_2O_2-PAA mixer by dip or GS methods is better for pathogen reduction than ES on apples

INTRODUCTION

Listeria monocytogenes are prevalent in moist environments such as soil, water, as well as decaying vegetation and decaying animals, also endemic within processing facilities which make contamination of foods and produce possible *(23).* To date, *Listeria* is a deadly microorganism in relation to food outbreaks *(16).* The U.S.-Food and Drug Administration (FDA) state that *L.* monocytogenes has a mortality rate between 20-30% and 1600 people are infected from *listeriosis* annually, which indicated that *L. monocytogenes* is not the deadliest but has high mortality rates especially in highly susceptible persons *(23)*. Recently, *L. monocytogenes* has been recognized by the United States Department of Agriculture -National Institute of Food and Agriculture (USDA-NIFA) as an emerging, under researched pathogen on produce *(21).*

There is growing concern regarding *L. monocytogenes*, stemming from its presence on fruits such as apples. One of the latest multistate apple recalls occurred in 2019, where 2,297 variety cases of McIntosh, Honeycrisp, Jonathan, Fuji, Jonamac, and Red Delicious apples were contaminated with *L. monocytogenes (15).* Earlier recalls on plain apples and caramel apples occurred in 2014-2015 and 2017, and cases of the 2014-2015 outbreak initiated by the field contaminated apples and then *Listeria* spread in the dunk tank, which sparks concerns among farmers and consumers alike *(13, 20).* These outbreaks of *L. monocytogenes* occur because of the microbes' ability to grow in a wide range of temperatures (0-45°C), its strict adherence to surfaces, and its resistance against disinfectants *(5).* In most cases, the bacteria can be mitigated by cooking common ready-to-eat (RTE) foods to around 74°C, however, in fruits that are often consumed raw, other precautions must be addressed. Contamination can occur at the harvest and/or packing process of apple production. To eliminate contamination, safeguards need to be taken at both steps.

A strategy to control foodborne pathogens on fruit and vegetable surfaces, includes the use of commercial antimicrobials such as sodium hypochlorite, lactic acid, citric acid, and peroxyacetic acid, delivered by garden spray (GS), electrostatic spray (ES), and dipping treatments *(5, 6, 8)*. Electrostatic Sprayers function by applying a positive charge to the sanitizing solution as it passes through the nozzle, whereas as compression spraying produces a simple drench. The positively charged droplets that are diffused from the nozzle are attracted to negatively charged surfaces which creates an even coating *(5, 22).* On the other hand, the GS, also known as compression sprayers, have no charge or ability for the solution to be attracted to the food surface. They are pumped with air manually and the compressed air forces the liquid to be sprayed from the nozzle. The GS often needs to be continually pumped with air to ensure a heavy and constant mist is expelled. Both methods have been utilized by farmers, but there are questions as to which method is better for pathogen reduction and how much of the antimicrobial should be administered on produce to inactivate bacteria.

There are several organic acids and inorganic chemicals used to remove contamination or prevent cross-contamination on produce. A H_2O_2-PAA mixer was selected in this study, because it is required by the wholesale distribution company Appalachian Harvest (Duffield, VA), which is a buyer of 75 very small produce farms in WV *(1).* This peroxyl compound is an organic formula composed of 23% H_2O_2 and 5.3% PAA. Previous studies in our lab showed that the water + water + antimicrobial dip application process with the H_2O_2-PAA mixer reduced *Salmonella and L. monocytogenes*, on butternut squashes, cucumbers, tomatoes and spinaches *(6,8)* and extended the shelf life of butternut squashes *(6).*

Therefore, this study aims to evaluate the anti-listeria efficacy of a H_2O_2-PAA mixture using GS, ES, and dip processing method. Currently, there are not reported studies that focused on

using the organic chemical in combination with three different applications on apples. Determining the most effective delivery method and concentration of a H_2O_2-PAA mixer to use on apples to inactivate *L. monocytogenes* could help apple farmers prevent the spread of the microbes on apples as well as consumers.

MATERIALS AND METHODS

Preparation of Apples. In this study, three types of organic apples (containing only natural wax on surfaces) including *HC*, *FJ*, and *PL* were purchased from local grocery stores. The average diameter of each apple was 5.72 ± 0.25 cm according to the retailer and had an average weight of 148.93, 140.37, and 156.95 g for *HC*, *FJ*, and *PL* apples, respectively. Apples without visible bruising or scars were selected and stored in a walk-in cooler (5ºC) and testing was conducted within 1-week.

Preparation of inoculum. Two *L. monocytogenes* 2624 and 2625, serotype 1/2b, isolates from cantaloupe were used in this study. Both strains were donated by Dr. Joshua Gurtler (USDA-ARS, Wyndmoor, Pennsylvania) *(20)*, and have been used in our previous studies of the H_2O_2-PAA mixer on produce *(5, 6, 7, 8, 9)*. The 2-strain cocktail cultures used in this study were prepared individually before combining them. Each individual *L. monocytogenes* strain was activated by picking smears from a frozen stock culture and streak-plating onto Modified Oxford agar (MOX, Hardy Diagnostics, Santa Maria, CA) to isolate a pure culture (single colonies) after incubating at 35°C for 48 h. Then two isolated colonies were picked from each of the *L. monocytogenes* strains on MOX agars and transferred into a sterilized 15 ml tube containing 10

ml of tryptic soy broth (TSB, Hardy Diagnostics, MD) followed by incubating in a shaker incubator (New Brunswick Scientific® Co. Edison, NJ) at 35ºC for 24 h.

Inoculation of Apples. After 24h incubation, the two individual strains growing in 10 ml TSB solutions were pelleted by centrifugation at 5000 rpm (Eppendorf® centrifuge 5430, Darmstadt, Germany) for 7 min at 4°C. TSB was decanted and pellet was resuspended in 10 ml of 0.1% fresh buffered peptone water (BPW) (Hardy Diagnostics®, Santa Maria, CA) by vortexing for 30 sec. The cell density of the inoculum suspension was 8.7 log CFU/ml by spread plating onto MOX agars. The two individual strain washed solution were then combined and added to a sterile metal bowl pre-filled with 2 liters of 0.1% BPW. Six apples were added to the contaminated bowl and swirled continuously by a gloved hand for 5 min. The temperature of apple inoculum solution was 3.0ºC and temperature of apple surface was 3.4ºC. The apples were then placed under a biological safety cabinet, Class 2 Type A/B3 (Nuaire®, Plymouth, MN) to dry for 10 min to allow bacterial attachment on surfaces. Our preliminary study indicated 10 mins was the maximum time allotment before *L. monocytogenes* cells declined. The residual liquid left on apples ranged from 0.11 to 0.24 ml.

Concentration of H_2O_2-PAA mixer and Methods of delivery. A H_2O_2-PAA mixer (BioSafe Sytems®, Oro Valley, AZ) containing 23% of H_2O_2 and 5.3% of PAA, with the concentrations of 0.0064 (pH 6.25), 0.1 (pH 5.85), 0.25 (pH 5.52) and 0.50% (pH 3.75) prepared in tap water (Morgantown city municipal tap water, 15.2°C) were used in this study. Antimicrobials and water were delivered via three different methods: conventional spray using a garden sprayer (GS, 1-gal [3.8L] plastic tank sprayer, Chapin, Batavia, NY), electrostatic spray

using a portable electrostatic sprayer (ES, BP2, Electrostatic Spraying Systems, Watkinsville, GA) and dipping in commercial metal bowls (8 Qt.=7.57 liter, Dip). For GS, ES or dip treatment, six apples were sampled per treatment with 6 untreated inoculated apples as controls. Each type of apple was subjected to the individual spray or dip methods with prepared H_2O_2-PAA mixer concentration of 0 (water), 0.0064%, 0.1%, 0.25%, and 0.5% into sprayer bottles (GS and ES) or metal bowls (Dip). The antimicrobial solutions were prepared using 3 liters of tap water with the appropriate concentration of theH_2O_2-PAA mixer for the previously mentioned concentration levels. For GS and ES treated samples, each was sprayed for 10 sec on each side of the apples for a total of 20s. The distances of the manually hold-on sprayer nozzles for both GS and ES appliance onto apple surfaces were kept at 25 cm from the sprayer to 6 apples with a 45^o angle. The apples receiving the dip treatment were immersed in bowls and gently stirred by a gloved hand for 20 sec. After treatment (GS, ES or dip), apples were set to dry under a biological safety cabinet (Class 2 Type A/B3, Nuaire®, Plymouth, MN) for 5 min. The 20s application time in addition to the 5min drying time in total exceeded the manufacturer's recommended time (45s) that the sanitizer needs to be in contact with bacteria to be effective *(1)*. The residue of solution on apples after 5 min drying were determined by weighing samples before sanitizer treatments and reweighing them immediately after drying and calculated as ($Weight_{residual} = Weight_{after} - Weight_{before}$).

Enumeration of *L. monocytogenes* on Apples. Followed by 5 min of drying, apples were then placed into a sterile WhirlPak® sample bag (Nasco, Modesto, CA) with 100 ml TSB with 0.1% sodium thiosulfate to neutralize the residual sanitizer on apples. The apples were shaken vigorously in the bag for 30 sec by hand before being plated. The sample solutions were

then 10- or 100-fold serial diluted in 0.1% buffered peptone water (BPW) followed by spread-plating on MOX agar plates by pipetting 0.1 ml and spreading uniformly using a sterile L-shaped spreader. Plates were incubated for 48 h at 37°C before enumeration.

Atomic Force Microscopy Analysis. Atomic Force Microscopy (AFM) was used to determine the mechanism in which the H_2O_2-PAA mixer was able to inactivate *L. monocytogenes*. Samples were sent out for analysis to Dr. Yangchao Luo (University of Connecticut). A single colony of *L. monocytogenes* was grown in 10 ml of TSB for 24 h at 35°C. The bacterial solution was then centrifuged at 7000 g for 15 min and resuspended in 2 ml of 0.1% BPW to create the stock solution. Five tubes were prepared with 2 ml of 0.1% BPW, distilled water, 0.1%, 0.25%, and 0.5% sanitizer solution. The 0.1 ml of the stock bacterial solution was added into each of the five tubes and vortexed for 30 sec. In order to neutralize the reaction, 18 ml of 0.1% BPW with 0.1% sodium thiosulfate was also added to each tube with constant agitation. The reaction solution was centrifuged at 7000 g for 10 min and the supernatant was removed and discarded. The bacteria pellet was then redispersed in the remaining supernatant. Then 10 µl of the concentrated solutions were added on the top of a clean Si-glass chip and dried in a fume hood for 20 h. The morphology of air-dried samples was conducted using an AFM (Tosca 200, Anton Paar, Graz, Austria) under tapping mode with an AP-ARROW-NCR silicon cantilever (a force constant of 42 N/m and a tip radius less than 10 nm). The height, width, and root-mean-square (RMS) roughness of the bacteria were analyzed using Tosca Analysis Software. For RMS roughness, a 250 × 250 nm^2 central region on the cell surface was used and at least 16 measurements were carried out for each treatment.

Data analysis. In this study, experiments were repeated twice. Each repetition included 6 apples per treatment with a total of 12 samples/treatment after 2 repeats. Experiments were conducted by 3 × 5 × 3 factorial design with 3 antimicrobial delivery methods (GS, ES, and dip) and 5 different concentrations of H_2O_2-PAA mixer (0, 0.0064, 0.1, 0.25, and 0.50%) for 3 different apple cultivars (*HC*, *FJ*, and *PL*) The Mixed Model Procedure of SAS (version 9.2, SAS Institute, Cary, NC) was used to analyze the reduction of *L. monocytogenes* of each apple cultivar under different delivery methods, concentrations, and their interactions. Reductions were calculated as $\log_{10}(N_0/N)$ per apple, where N_0 is the average plate counts of untreated controls and N is the plate count of each individual antimicrobial treated sample *(3)* with the significant level of $\alpha = 0.05$ as determined by Tukey HSD.

RESULTS

Preliminary study to test the residual water volume left on apples after GS, ES, and Dip methods followed by 5 min drying. After first taking the average dry weight of each apple cultivar *HC*, *FJ*, and *PL*, they were subjected to treatment via the GS, ES, and dip method followed by 5 min drying as previously described in the material and methods section. The highest ($P < 0.05$) residual water volume left on the *HC* apple was a result of the GS (1.04 g) followed by dip (0.74 g) and ES (0.61 g). In contrast, *FJ* indicated the highest ($P < 0.05$) volume when subjected to the dip (1.05 g) following GS (0.94 g) and ES (0.84 g). *PL* was similar to *HC* in that its residual volume decreased ($P < 0.05$) as GS (1.44 g) ≥ dip (1.38 g) > ES (0.84 g) (Data not shown in tabular format).

Comparison of Antimicrobial Delivery Methods. The Least square (LS) means of reductions of *L. monocytogenes* achieved by GS, ES, and dip across all concentrations of H_2O_2-PAA mixer on three apple cultivars were calculated and analyzed. According to the mixed model analysis, the anti-listeria efficacy was determined by the processing method ($P < 0.05$) and the concentration of the treatment ($P < 0.05$), and their interactions were also significant ($P < 0.05$). Results indicate that the dipping method was the most effective antimicrobial delivery method compared to GS and ES, as shown by the reductions which decreased ($P < 0.05$) from 2.41 (dip) to 1.54 (GS) and to 1.06 (ES), from 2.31 (dip) to 1.79 (GS) and to 1.20 (ES), and from 2.41(dip) to 1.44 (GS) and to 0.84 log CFU/apple (ES) on *HC*, *FJ*, and *PL* apples, respectively (Data not shown in tabular format).

Efficacy of the H_2O_2-PAA mixer against *L. monocytogenes* on Apples by GS, ES, and Dip. In this study, the reductions of *L. monocytogenes* on *HC*, *FJ*, and *PL* apples by GS, ES, and dip methods were first tested with water only before applying any concentrations of sanitizer solutions. Reduction levels of the pathogen on apples by water declined ($P < 0.05$) from 1.34 (*HC*) to 1.50 (*PL*), followed by 0.61 (*HC*) to 0.82 log CFU per apple (*FJ*), and to 0.17 (*FJ*) to 0.76 log CFU per apple (*HC*) for dip, GS, and ES methods, respectively ($P < 0.05$, Table 1).

Reductions of *L. monocytogenes* on apples are shown in Table 1. Conventional spraying the H_2O_2-PAA mixer onto *HC* apples significantly reduced ($P < 0.05$) *L. monocytogenes* population compared to the untreated control (6.83 log CFU/apple), with the reductions increased from 0.81 to 2.58 log CFU/apple as concentrations of sanitizer increased from 0.0064 to 0.50% (Table 1), which were greater ($P < 0.05$) than the reduction of water treatment except for the 0.0064% treated samples (Table 1). Compared to the water treated samples, conventional

spraying 0.10 to 0.50% of the H_2O_2-PAA mixer increased ($P < 0.05$) the reductions to 0.84 to 1.97 log CFU/apple. Compared to GS, the sanitizer sprayed by ES decreased ($P < 0.05$) reduction levels of *L. monocytogenes* by 0.26 (0.0064%) to 1.0 log CFU per apple (0.25%) (Table 2). However, dip *HC* apples into 0.0064, 0.1, 0.25, and 0.50% of sanitizer solutions achieved the reduction levels of 1.63, 2.54, 3.52, and 3.01 log CFU/apple, respectively, which were greater ($P < 0.05$) than those of GS apples ranging from 0.81 to 2.58 log CFU/apple (Table 1).

For *FJ* apples, delivery of 0.10 to 0.50% of H_2O_2-PAA mixer solutions by GS significantly ($P < 0.05$) reduced *L. monocytogenes* on *FJ* apples with the reductions ranging from 1.76 to 2.52 log CFU/apple which were greater than GS-water samples (0.82 log CFU/apple, Table 1). No difference ($P > 0.05$) of reductions was found between 0.0064% of H_2O_2-PAA mixer (1.00 log CFU/apple) and GS-water samples (0.82 log CFU/apple). Similar to *HC* apples, ES decreased ($P < 0.05$) the reduction levels by 0.65, 0.6, 1.33 log CFU/apple for 0, 0.0064,0.1%, 0.25% of H_2O_2-PAA mixer solutions, respectively (Table 1). No difference ($P > 0.05$) of the pathogen reduction levels on *FJ* apples was observed in 0.25 and 0.50% of H_2O_2-PAA mixer between GS and ES. Dipping *FJ* apples into 0.0064 to 0.50% of H_2O_2-PAA mixer solutions increased ($P < 0.05$) the reductions to 1.48 to 3.28 log CFU/apple (Table 1) with no difference ($P > 0.05$) between 0.25 and 0.50% treated samples.

Similar to *HC* and *FJ* apples, dipping *PL* apples into 0.10 to 0.50% H_2O_2-PAA mixer solutions reduced *L. monocytogenes* counts by 1.49 to 2.45 log CFU/apple, which were greater ($P < 0.05$) than reductions of apples dipped in water (0.69 log CFU/apple) and 0.0064% H_2O_2-PAA mixer solutions (0.79 log CFU/apple) (Table 1). Applying ES with 0.0064 to 0.50% of H_2O_2-PAA mixer solutions onto *FJ* apples resulted lower ($P < 0.05$) reductions ranging from

0.24 to 1.57 log CFU/apple than those from the GS treated samples (Table 1). Again, it is obviously noticed that dipping *FJ* apples into H_2O_2-PAA mixer solutions suggested greater reductions ranging from 1.48 to 3.28 log CFU/g than the samples sprayed by GS (Table 1).

Topographical observation under AFM. In order to observe the reaction between H_2O_2-PAA mixer and *L. monocytogenes*, the topographic structures of bacteria with or without sanitizer treatment were visualized by an AFM. When 0.1% BPW alone was added to the *L. monocytogenes* cells, the original rod shape of the bacteria was clearly apparent with smooth surfaces, which were covered with a layer of extracellular polysaccharide matrix (Figure 1). The original width, height, and roughness when bacteria were subjected to 0.1% BPW was 0.49, 212.5, and 16.68 nm respectively (Figure 1). Distilled water treatment had the similar results as 0.1% BPW under the examination of the AFM. The width (0.49 nm) of the bacteria did not significantly change when distilled water was applied to the cell, but the height increased to 225.10 nm and the roughness decreased to 15.02 nm (Figure 1), which may be due to the slight change of osmolarity during treatment. *L. monocytogenes* was not largely affected until it was subjected to 0.1% of H_2O_2-PAA mixer solution, which increased the cellular width to 0.56 nm, the height to 277.5 nm, the extracellular matrix to thin, and the intracellular compounds to visibly leak out of the cell (Figure 1). An even more transformative shift was seen from 0.25% and 0.5% of H_2O_2-PAA mixer concentrations as shown in Figure 1. Under a 0.25% concentration, the cell continued to have intracellular leakage, but the height increased to 343.06 nm, which was caused by membrane permeability (Figure 1). Unlike the 0.25% of the sanitizer, 0.5% concentrations morphed the rod shape of the bacteria resulting that it was no longer identifiable, and cytolysis was evident (Figure 1). The width of 0.5% of H_2O_2-PAA mixer was

not detectable due to the conglomeration of cells, and the height increased to 300.5 nm compared to the BPW and distilled water treated samples (Figure 1). The roughness of the cell started to decrease from 0.1% (15.59 nm) and more so to 0.25% (11.70 nm) and 0.5% (8.90 nm) (Figure 1), perhaps due to the cells leaking out and transforming into an undistinguishable, flat pattern.

DISCUSSION

In this study, ES treatments generated less reduction of the pathogen than either GS or dip treatments, which could be attributed to less volume of the H_2O_2-PAA mixer solution delivered onto apple surfaces compared to the GS or dip treatment according to the preliminary study results. This is because the flow rate of ES used in this study is 0.97 ml/s compared to the 7.23 ml/s of the GS *(5)*. This explanation is also supported by the previous study reported by Youssef et al. *(24)*, who found that at least 0.1 ml/cm2 of antimicrobial solution should be applied to receive a significant log reduction of *Escherichia coli* on beef trimming surfaces. They also reported that ES delivered a significantly smaller volume rate than the conventional spray system (0.045 vs 0.26 ml/cm^2). Results of comparisons between various antimicrobial delivery methods are mixed in previous studies. As reported by Ganesh et al. (2012), ES was more effective than the conventional sprayer (2.6-3.3 vs. 0.0-0.3 log CFU/g) for reducing *Salmonella* Typhimurium on spinaches *(3)*. Our recent study also showed that ES of a H_2O_2-PAA mixer achieved additional reductions of *L. monocytogenes* of 1.19 to 3.05 log CFU per apple than those of GS *(5).* However, a different study reported that dip and conventional spray methods with 380 ppm of PAA is more effective at reducing *Escherichia coli* on beef sub-primals than the ES treatments *(14)*. Our previous study showed no difference ($P > 0.05$) in the inactivation of *Campylobacter jejuni* cells on chicken wings between dip and ES methods *(17)*.

Though, the dip and GS methods were close in volume administration, GS on average had the most volume change. It was originally hypothesized that dipping would leave more volume on the apple surface because dipping had the greatest reduction of *L. monocytogenes* on apples overall. Rather than the bacterial reduction being indicative of the volume increase, it can be inferred that because dipping allows for full submersion of each individual apple then there is better overall coverage of the apple surface and exposure of bacteria to the treatment. Results from studies indicate an economic benefit for WV (water cost is low) small local apple processors by applying GS or dip treatments rather ES since the initial purchasing cost of the ES ($3000) is much more expensive than the GS ($60). In addition, the ES has greater a maintenance cost than that of the GS ($500 vs $120 per year) *(5, 17)*.

The H_2O_2-PAA mixer solution applied to inoculated apples is claimed to be effective at killing fungi, viruses, gram positive and gram-negative bacteria without having to rinse the product *(1).* The post-harvest concentration recommendation for this product is 0.0512%-0.1816% (512-1817 ppm) and the product should remain in contact with the produce surface for a minimum of 45 sec. for max effectiveness (*1*). Contamination of produce is sometimes an issue for small holdings producers in rural areas such as West Virginia (WV) and Kentucky (KY); therefore WV Small Farm Center has recommended the use of the H_2O_2-PAA mixer during post-harvest for produce grown in or directly contact with soil *(9).*

Our recent study found that triple-washing cucumbers using a H_2O_2-PAA mixer was equally or more effective than a lactic-citric acid blend, sodium hypochlorite, and acidified sodium hypochlorite to inactivate *L. monocytogenes* on cucumbers *(6)*. Applying triple-wash process (water, antimicrobial, water), 0.25% and 0.5% of the H_2O_2-PAA mixer reduced *L. monocytogenes* by 1.75 and 1.56 log CFU/cucumber on cucumbers *(6)*. In a related study,

various antimicrobials were compared to a H_2O_2-PAA mixer using the triple wash method on spinach. The study concluded that 0.25 to 0.50% of the H_2O_2-PAA mixer were more effective ($P < 0.05\%$) at removing bacteria than 100 pm of sodium hypochlorite and 2.5% of lactic/citric acid blend with the reductions of 1.76–1.81 (0.25 to 0.50% of H_2O_2-PAA mixer) verse 1.44 $\log_{10}$ CFU/g (sodium hypochlorite and lactic/citric acid blend) *(8)*.

Overall, in this current study, 0.25% and 0.5% of the H_2O_2-PAA mixer were the most effective at inactivating *L. monocytogenes* from all cultivars of apples studied. Based on the LS means, there was a significant difference between 0.25% vs 0.5% H_2O_2-PAA mixer, 0.25% vs 0.1%, and 64ppm vs 0.1%. There was a significant decrease in *L. monocytogenes* on apples that were treated with 0.1%, 0.25%, and 0.5% of H_2O_2-PAA mixer. However, water and 64 ppm of the H_2O_2-PAA mixer had no significant difference of anti-listeria effect on apples for any delivery method ($P > 0.05\%$). Similarly, Li et al (2020b) showed that compared to water dip method, a H_2O_2-PAA mixer solution was far more effective at removing various bacterial strains to include *Listeria* on WV locally grown squashes. Concentrations of the H_2O_2-PAA mixer at 0.25% and 0.5% showed a *L. monocytogenes* reduction on butternut squash of 2.4 and 2.9 log CFU/squash, respectively *(8).*

Applying AFM technology to image the bacterial cell surface structure and to study the characteristic morphology of microorganisms are used to elucidate the mechanism of antimicrobials, including oxidizing reagents against foodborne pathogens in liquid solution or on food products *(2, 4, 10, 25).* In this study, the difference in concentrations of sanitizer solution and their effect on *L. monocytogenes* cells can be observed using the AFM. The AFM confirmed our results by showing *L. monocytogenes* cells with decreased length, width and height but enhanced roughness when treated with 0.25% and 0.5% of the H_2O_2-PAA mixer, whereas the

water treatment caused little to no changes on the cell morphology. Peroxyl acetic acid, the unstable organic peracid, acts as an antimicrobial agent mainly through the production of reactive oxygen species (ROS). These ROS can disrupt the lipopolysaccharide layer of the cell. Furthermore, the permeability of membranes can also be impaired by the oxidation of sulfur and sulfhydryl bonds of the proteins on the cell walls *(19)*. The appearance of a layer of intracellular leakage that occurred outside the cell and the gradual disappearance of the cell boundary may indicate the reaction between the microbe cell and chemicals. As the antimicrobials levels increased, so did size of the cell and the rate of cytolysis. Results from this study agree with other studies *(11)*, that reported that sodium chloride combined with sodium bicarbonate solution generated electrolyzed water as a strong oxidizing antimicrobial agent caused wrinkled bundles and lesions of *Listeria inoculant* forming a rougher microbial surface. The oxidative stress from the combination of PAA and H_2O_2 resulted more irregular cells with shrinking morphology which could further alter cell membrane permeability and cause the unbalanced osmotic pressure of the pathogen cells *(9).* However, since AFM can only measure surface properties of cells and cannot directly determine the intracellular changes, more experiments such as transmission electron microscope, lipidomics, proteomics, genomics are needed to investigate the structural changes within the cells and identify the content of the leakages.

The total reduction average for all methods were 1.56,1.74,1.67 log CFU/apple for *HC*, *FJ*, and *PL*, respectively. *FJ* apples had the highest total reduction of all apple cultivars, although it was not significant in all tests. Similarly, *FJ* apples had slightly higher reductions of bacteria than GS when stored at room temperature according to Sheng et al., 2017 *(18)*. Though not significant, the difference in antimicrobial efficacy and the type of apples could be due to a few factors such as natural waxing on the apple or the number of sinuses that the apple contains.

Macarisin et al. 2019 showed opposing results in microbial reductions between *FJ*, *GS* and *RD* apples. In this study, 200 fruits per cultivar were inoculated on the stem and calyx end with a 6-strain cocktail of listeria to obtain 10^6 log CFU/ml. Unwaxed *FJ* apples had approximately 1.1 log CFU/apple more than that of *Granny Smith* or *Red Delicious apples*. The study suggested that the reason for the lower microbe reduction in *FJ* apples could be due to the number of open sinuses in *FJ* (less than or equal to 63%) compared to *GS* (less than or equal to 1%) *(12)*.

Results from this study indicate that the H_2O_2-PAA mixer is effective at reducing *L. monocytogenes* on apples at levels of 0.25 to 0.5% concentrations. The dip method delivered the greatest reduction of bacteria in every concentration level applied, followed by the GS. The ES was not as effective at inactivating bacteria than dip and GS methods, which is in contradiction with previous studies using other tested food products. Our study is not conclusive with results of previous studies that indicated that the electrostatic sprayer is more effective than the conventional garden sprayer *(5).* This is likely due to the lack of optimization of operational parameters of GS and ES, and difference in the produce or product being tested, suggesting a need for different antimicrobial approaches to various types of food products. However, dip washes have the disadvantage of increasing the risk of cross-contamination along with high water usage, whereas ES uses less sanitizer, less water and reduced risk of cross-contamination. Therefore, future studies are needed to compare the GS, ES, and dip process related to the prevention of cross-contamination on apples. Furthermore, this study indicated that even apples are not all created equal and may require different treatment methods or times of antimicrobial subjection. Future studies are needed to assess wax and physiology differences in organic apples and how it impacts the survival of *L. monocytogenes* even in various storage temperatures.

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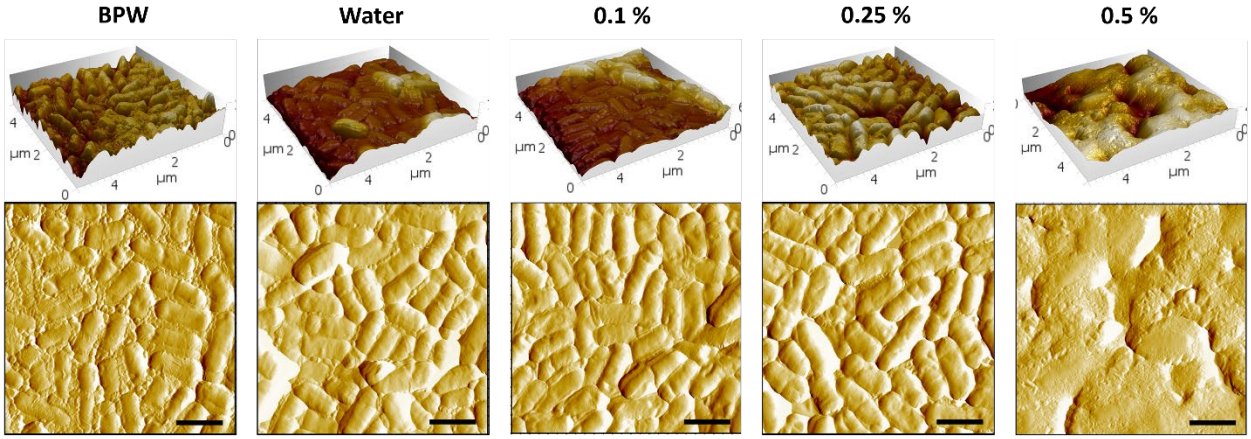
FIGURE LEGEND

FIGURE 1. Atomic force microscopy (AFM) images of *Listeria monocytogene*s cells in buffered peptone water solution with water, 0.1, 0.25, and 0.5% of a mixer of hydrogen peroxide (H_2O_2) and peroxyacetic-acid (PAA).



	BPW	Water	0.1 %	0.25 %	0.5 %
Width (nm)	0.49 ± 0.047	0.50 ± 0.01	0.56 ± 0.11	0.56 ± 0.026	-
Height (nm)	212.5	225.1	277.5	343.6	300.5
Roughness (nm)	16.68 ± 4.39	15.02 ± 3.35	15.59 ± 2.89	11.70 ± 3.37	8.90 ± 2.18

TABLE 1. Reduction of *Listeria monocytogenes* (log/CFU apple) on *Honey Crisp*, *Fuji*, and *Pink Lady* apples by conventional garden spray (GS), electrostatic spray (ES), and dip methods with 0 to 0.5% of a mixer of peroxyacetic acid and hydrogen peroxide (H_2O_2-PAA).

Treatment (H_2O_2-PAA mixer, %)	*Honey Crisp*			*Fuji*			*Pink Lady*		
	GS	ES	Dip	GS	ES	Dip	GS	ES	Dip
Water	0.61±0.16Aa	0.76±0.12Aa	1.34±0.38Ab	0.82±0.23Ab	0.17±0.10Aa	1.42±0.09Ac	0.69±0.13Ab	0.23±0.15Aa	1.50±0.13Ac
0.0064	0.81±0.23Aa	0.55±0.51Aa	1.63±0.12Ab	1.00±0.23Ab	0.40±0.23Aa	1.48±0.51Ac	0.79±0.25Ab	0.24±0.10Aa	1.49±0.50Ac
0.1	1.45±0.43Bb	0.92±0.10Ba	2.54±0.54Bc	1.76±0.28Bb	0.43±0.09Aa	2.21±0.29Bc	1.49±0.24Bb	0.79±0.16Ba	2.23±0.27Bc
0.25	2.25±0.45Cb	1.25±0.39Ca	3.52±0.41Dc	2.38±0.41Ca	2.20±0.60Ba	3.28±0.72Cb	1.77±0.37Ba	1.57±0.38Ca	3.04±0.35Cb
0.5	2.58±0.32Cb	1.80±0.47Da	3.01±0.72Cc	2.52±0.42Ca	2.82±0.60Ca	3.17±0.33Cb	2.45±0.61Cb	1.38±0.64Ca	3.81±0.35Dc

Note: Mean values with different capital letters within a column are significantly different ($P < 0.05$); Mean values with different letters within a row are significantly different ($P < 0.05$).

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Rebecca Stearns[1], Jingyi Xue[2], Nettie Freshour[1], Kristen Matak[1], Yangchao Luo[2], and Cangliang Shen[1]* The Efficacy of Conventional Spray, Electrostatic Spray, and Dip with a Combination of Hydrogen Peroxide and Peroxyacetic Acid to Inactivate Listeria monocytogenes on Apples. (Published in Journal of Food Protection).

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