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The efficacy of preharvest application of electrolyzed water and chemical sanitizers against foodborne pathogen surrogates on leafy green vegetables

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Abstract

Preharvest control strategies, to reduce or eliminate pathogenic bacteria in leafy vegetables that may be consumed raw, may provide additional food safety protection and shelf life quality extension beyond what is possible to achieve with postharvest sanitation alone. The aim of this study was to characterize the efficacy and effect of contact time of electrolyzed water (e-water), 1-bromo-3-chloro-5-dimethylhydantoin (BCDMH), and peracetic acid (PAA) at 80 and 150 ppm against pathogen surrogates *Escherichia coli* M23 (*E. coli* M23)and *Listeria innocua* ATCC 33090 (*L. innocua*), and a representative spoilage microorganism *Pseudomonas fluorescens* (*P. fluorescens*) on leafy green vegetables (LGV) mizuna, rocket (arugula), and red chard. Each of the leafy vegetables has a distinctly different leaf architectures that could alter the effectiveness of preharvest sanitation treatments. e-Water, BCDMH and PAA were equally effective in inactivating plant total viable count, *E. coli* M23, *L. innocua* and *P. fluorescens* (reduction compared to water control—0.5–4.0 log CFU/g). On average an additional 0.8 (0.4–1.1) log CFU/g inactivation was obtained by increasing sanitizer contact time from 30 min to 2 h, whereas increasing sanitizer concentrations produced, at maximum, an extra 0.5 log CFU/g inactivation. These findings suggest that e-water, BCDMH, and PAA are all useful for in-field preharvest application on a wide range of plants and increasing contact time rather than concentration improves sanitation efficacy.

1 | INTRODUCTION

Leafy green vegetables (LGV) are recommended as good sources of nutrients, vitamins (especially A and C) and fiber for the human diet and provide health benefits (Adebawo et al., 2006; Boeing et al., 2012; WHO/FAO, 2003; Slavin & Lloyd, 2012). There is an increasing demand for LGV alongside an increasing consumer awareness of the need and benefits of healthy eating (Mercanoglu Taban & Halkman, 2011). LGV are identified as a commodity group connected with microbiological safety concerns. These crops are consumed raw or minimally processed and are often grown in open fields. They are more vulnerable to contamination or colonization by pathogenic bacteria from irrigation water, soil, and organic fertilizers, especially where there has been contact with feces of livestock and wildlife (FAO/WHO,

2008). Exposure to contaminated food results in 600 million cases of foodborne diseases and 420,000 deaths worldwide in 2010 (WHO, 2015). Pathogenic bacteria, in particular *Escherichia coli* (*E. coli*), *Listeria monocytogenes* (*L. monocytogenes*), and various *Salmonella enterica* serotypes are responsible for most foodborne disease outbreaks associated with the consumption of LGV (Berger et al., 2010; Eng et al., 2015; Hammons & Oliver, 2014). Other than foodborne pathogens, the presence of spoilage microorganism such as *Pseudomonas fluorescens* (*P. fluorescens*) can also pose food shelf life issues (King Jr. et al., 1991; Nguyen-the & Carlin, 1994) by hastening deterioration of fresh produce, resulting in loss and wastage (Porter et al., 2016).

There are many postharvest preventive measures and intervention options to control microbial risks and hazards on LGV. The use of chemical sanitizers is the most common method for killing bacteria on LGV. Numerous types of sanitizers and disinfectants are available on the market, including chlorine, chlorine dioxide, hydrogen peroxide, peracetic acid, organic acids, and ozone (Koseki et al., 2001; Park et al., 2001). However, some of these sanitation methods have certain drawbacks during their production and application, such as high cost, ineffectiveness, chemical residues, environmental harm, and adverse effects on the organoleptic quality of produce (Al-Haq, Sugiyama & Isobe, 2005). Relying solely on conventional sanitizers during postharvest treatment may not provide the required food safety protection for consumers because of the limitations in their efficacies that lead to a risk of residual pathogens after treatment. Therefore, developing effective in-field preharvest sanitation of LGV will provide knowledge that can subsequently improve preharvest and postharvest safety and quality of LGV.

Electrolyzed water (e-water) has been regarded as a broad-spectrum sanitizer within the food industry of many countries over the last few decades (Rahman, Khan & Oh, 2016). It also has been studied as an alternative to conventional chemical sanitizers in the food industry (Gil, Gómez-López & Hung, 2015). e-Water is produced by the electrolysis of water and sodium chloride (NaCl) in an electrolytic cell that contains a diaphragm that is used to separate the anode and cathode to produce acidic (pH 2–3, oxidation–reduction potential [ORP] > 1,100 mV) and alkaline (pH 10–13, ORP -800 to −900 mV) anolytes (Al-Haq, Sugiyama & Isobe, 2005; Hricova, Stephan & Zweifel, 2008; Premier, 2013; Rahman, Khan & Oh, 2016). Neutral electrolyzed water (NEW, pH 6–7, ORP 800 to 900 mV) is generated by the electrolysis of NaCl in a single-chamber electrolytic cell without the diaphragm or by mixing the anodic solution with OH^- ions (Hricova, Stephan & Zweifel, 2008). The predominate species of NEW is hypochlorous acid (>95%), which is the most effective element of chlorine in killing microbial cells (Guentzel et al., 2008). Due to its neutral pH, chlorine off-gassing is greatly reduced thus limiting corrosion of surfaces and plant phytotoxic effects while at the same time maximizing the availability of hypochlorous acid species (Guentzel et al., 2008; Rahman, Khan & Oh, 2016). There are numerous studies showing that e-water as a surface sanitizer has broad antimicrobial activity against different types of pathogens on food processing surfaces (Liu & Su, 2006; Monnin, Lee & Pascall, 2012), barley grains (Rood et al., 2018), meat (Al-Holy & Rasco, 2015; Mansur et al., 2015; Rahman et al., 2012b), seafood (Al-Holy & Rasco, 2015; Mansur & Oh, 2015; Ratana-Arporn & Jommark, 2014), and on a range of fresh fruits and vegetables (Ding et al., 2015; Graça et al., 2011; Hao et al., 2015; Mansur & Oh, 2015). The inactivation (killing) efficacy of e-water against bacteria may range from having no effect to achieving at least six log reduction of viable counts (Huang et al., 2008; Rahman, Khan & Oh, 2016). Therefore, e-water has the potential for application in preharvest safety application but requires evaluation in terms of its realistic capability in activating plant adhered bacteria.

Peracetic acid or peroxyacetic acid (PAA) is commercially available under various brands and can be formulated into mixtures. An example is Tsunami on Farm (Ecolab, Inc., Saint Paul, MN, USA), which is a mixture of acetic acid, PAA, and hydrogen peroxide. It is a strong oxidant and has been widely applied in the food industry (Joshi et al., 2013) including application onto fresh produce without a rinsing aid (Premier, 2013). It can also be used to avoid cross-contamination between contaminated and noncontaminated produce (Lopez-Galvez et al., 2009). Unlike chlorine, PAA does not create harmful disinfection by-products since PAA decomposes quickly into acetic acid, water, and oxygen (Joshi et al., 2013; Kitis, 2004). In addition, efficacy of PAA is not strongly influenced by the presence of organic matter (Chen et al. 2020). PAA is generally used at 50–150 ppm and is highly effective in inactivating a wide spectrum of pathogens, and is especially effective against *L. monocytogenes* (Premier, 2013). The major disadvantage of PAA is that the presence of excessive concentrations of PAA can cause leaf damage, adverse effects on nutritional quality and also shorten the shelf life of the produce (Premier, 2013). Evaluation of the PAA concentrations able to inactivate food-borne pathogens from LGV surfaces needs to be defined more clearly.

1-Bromo-3-chloro-5, 5-dimethylhydantoin (BCDMH), is sold under many brand names has full usage approval from the Food Standards Authority Australia and New Zealand and the Australian Pesticides and Veterinary Medicines Authority (Premier, 2013). BCDMH is a chemically stable sanitizer, active at both neutral to acid pH, and has been used to control plant and human pathogens on fruit and vegetables without rinsing (Premier, 2013). Many industrial applications use BCDMH at 5–10 ppm, which is approximately 10 times lower in concentration than chlorine (Premier, 2013). Most studies that have focused on the antimicrobial activities of PAA and BCDMH at the postharvest stage (Hilgren & Salverda, 2000; Neo et al., 2013; Premier, 2013). However, knowledge about BCDMH efficacy at the preharvest stage is still not well documented. This information is important as on-farm sanitation of LGV in the field could provide additional levels of food safety protection.

The aim of this study was to determine the effectiveness of the preharvest use of e-water, PAA, and BCDMH at different concentrations and contact times for inactivating proxy pathogens and potential spoilage bacteria. Tests were performed on mizuna, rocket and red chard, plants with different leaf architectures with a view to developing a broadly applicable preharvest control strategy for field application.

2 | MATERIALS AND METHODS

2.1 | Preparation of plants

Mizuna (*Brassica rapa* var. nipposinica), arugula/rocket (*Eruca vesicaria* subsp. *sativa*) and red chard (*Beta vulgaris* subsp. *vulgaris*) seeds were

TABLE 1 Strains used in this study.

Strain	Features, strain designations	Source
E. coli EC1604	Nonpathogenic laboratory strain	CSIRO
E. coli EC1605	Nonpathogenic laboratory strain	CSIRO
E. coli EC1606	Nonpathogenic laboratory strain	CSIRO
E. coli M23	Acid tolerant, nonpathogenic laboratory strain	University of Tasmania culture collection
E. coli O111:H-	VTEC	Calf (abattoir) feces
E. coli O157:H-	VTEC	Calf (abattoir) feces
E. coli O157:H7 (ATCC 43895)	VTEC	Cattle (feedlot) hide
E. coli O157:H7 Sakai	VTEC	Radish sprouts
E. coli O157:H12 (ATCC 43889)	VTEC	Calf (feedlot) feces
E. coli R31	VTEC	University of Tasmania culture collection
Listeria innocua	Type strain ATCC 33090	Dairy cow brain
L. monocytogenes	DS_81	Food (processed meat)
L. monocytogenes	MDU-FW06-22	Human
L. monocytogenes	102-195-S1	Food (chilled meat product)
L. monocytogenes	80-4904	Sheep
L. monocytogenes	Liver	Liver tissue
L. monocytogenes	LO28	Human
L. monocytogenes	FW035-0032	Food (salad product)
L. monocytogenes	87-0707	Bird (finch)
L. monocytogenes	83-1804	Sheep
L. monocytogenes	83-0159	Sheep
L. monocytogenes	69-1793	Cattle
L. monocytogenes	70-0387	Cattle
L. monocytogenes	84-1886	Cattle
L. monocytogenes	FW035-0035	Food (salad product)
L. monocytogenes	89-1931	Dog
L. monocytogenes	80-4798	Sheep
L. monocytogenes	79-1994	Sheep
L. monocytogenes	76-2120-1	Sheep
L. monocytogenes	EGD Δ*luxS*	Guinea pig. Poor adhesion, Δ*luxS* deletion (Sela et al., 2006)

Abbreviations: CSIRO, Commonwealth Scientific and Industrial Research Organization, Australia; VTEC, verotoxigenic *Escherichia coli*.

sourced from a local commercial farm (OneHarvest, Richmond, Tasmania, Australia). Four seeds of each variety were sown and grown in 15 cm round plastic pots containing standard potting mix (Horticultural and Landscape supplies, Brighton, Tasmania, Australia) in a glasshouse with a constant temperature set to 24°C. Plants were irrigated with an automatic sprinkler system for 5 min (approximately 1.6 mm), four times a day. Supplementary spray irrigation was supplied as required based on weather, soil moisture or evapotranspiration. Plants reached the growth stage suitable for harvest 3–4 weeks after sowing.

2.2 | Preparation of sanitizer solutions

e-Water was produced by electrolysis of a dilute salt (0.05%–1.0% NaCl) brine solution using a Model ELA-400 Envirolyte instrument (Envirolyte Industries International, Ltd., Tallinn, Estonia). The initial pH, and oxidation–reduction potential (ORP), and free chlorine concentration of e-water were 7.5, 890 mV, and 720 ppm, respectively. The stock solution of e-water was diluted with distilled water to obtain the desired free chlorine concentrations of 5, 20, and 100 ppm. The active chlorine concentration (ACC) was measured using a Compact ClO_2^+ meter (Palintest, Peakhurst, NSW, Australia). Tsunami on Farm solution (Ecolab, Inc., Saint Paul, MN, USA) containing a mixture of PAA (16% v/v) and hydrogen peroxide (11% v/v) was obtained by diluting the original stock solution with sterile distilled water to 80 and 150 ppm. The desired BCDMH (YM-FAB Nylate, Wobelea Pty, Ltd., Pakenham, Victoria, Australia) concentrations of 5, 20, and 50 ppm were obtained by dissolving the Nylate powder in sterile distilled water. All pH and ORP readings for this work were measured with an Accumet AB150 pH meter (Thermo Fisher scientific,

Waltham, MA, USA) and a MW500 ORP meter (Milwaukee Instruments, Rocky Mount, NC, USA), respectively.

2.3 | Preparation of bacterial inocula

The strains of *E. coli* and *Listeria* used in this study are listed in Table 1. These strains were stored as glycerol (30% v/v) stocks at −80°C. Each bacterial strain was cultured from glycerol stock by streaking onto tryptic soy agar (TSA) plus 0.6% (w/v) yeast extract (TSAY, Oxoid, Ltd., Basingstoke, Hampshire, UK). A single colony from each culture was picked and suspended in 10 mL of tryptic soy broth plus 0.6% (w/v) yeast extract (TSBY, Oxoid, Ltd., Basingstoke, Hampshire, UK) and incubated at 37°C for 24 h. Following this, 1 mL of each suspension was added in a 15 mL centrifuge tube (Corning, NY, USA) containing 9 mL of sterile water, 100 ppm e-water, 50 ppm BCDMH, and 80 ppm Tsunami on Farm. Each centrifuge tube was incubated at 25°C for 30 min. Tenfold serial dilutions in 0.1% (w/v) peptone water (Oxoid, Ltd., Basingstoke, Hampshire, UK) were prepared, and Diluted samples (0.1 mL) were surface plated onto TSAY. After incubation at 25°C for 72 h, bacterial colonies were enumerated. Nonpathogenic *Escherichia coli* M23 (*E. coli* M23, genome WGS project accession code AWQI01), *Listeria innocua* (*L. innocua*, ATCC 33090) and *P. fluorescens* were selected for this study based on the results from suspension experiment. *E. coli* M23 and *L. innocua* ATCC 33090 were obtained from cryopreserved (30% v/v glycerol) strain stocks stored at −80°C. *P. fluorescens* was isolated directly from the surface of plants used in this study and identified by 16S rRNA gene sequencing. *E. coli* M23, *L. innocua*, and *P. fluorescens* inocula were prepared as stated above. Following this, 9 mL *E. coli* M23, *L. innocua*, and *P. fluorescens* suspensions were transferred into a sterile 500 mL spray bottle. Each bacterial solution was then diluted with 0.1% (w/v) peptone water at a 1:5 ratio to achieve a cell density of approximately 1×10^8 colony forming units (CFU/mL) for trials to determine the antimicrobial efficacy of sanitizers.

2.4 | Inoculation of plants with bacteria and treatment with sanitizers

A randomized block design was used to determine the antimicrobial efficacy of PAA, BCDMH, and e-water against *E. coli* M23, *L. innocua*, and *P. fluorescens* on mizuna, rocket, and red chard. Plants were grown for 3–4 weeks to maturity before application of each bacterial inoculum. The control had no sanitizer treatment applied to the plant. For each application of inoculum, 7.5 mL of bacterial suspensions was spray-inoculated manually using a 500 mL spray bottle. The spray bottle was sprayed from a distance of approximately 15 cm from the leaves and sprayed in multidirections to ensure full and even coverage. Plants were then allowed to air-dry for 30 min before the applications of sanitizers. Approximately 45 mL of Tsunami on Farm (80 and 150 ppm), BCDMH solution (5 and 20 ppm) or e-water (5 and 20 ppm) was applied evenly with a 500 mL spray bottle on both control and inoculated plants. Plants were left for 30 and 120 min before collection of samples. All treatments were replicated five times.

2.5 | Microbial enumeration

After sanitizer treatment, 10 g of leaves were harvested and placed in a stomacher bag (Whirl-Pak, Madison, WI, USA) containing 90 mL of 0.1% (w/v) peptone water (Oxoid, Ltd., Basingstoke, Hampshire, UK) and homogenized in a Stomacher Lab Blender (Colworth Stomacher 400, Seward Medical, London, UK) for 1 min. Tenfold serial dilutions in 0.1% (w/v) peptone water (Oxoid, Ltd., Basingstoke, Hampshire, UK) were prepared, and 0.1 mL of diluted samples were surface plated onto TSA (Oxoid, Ltd., Basingstoke, Hampshire, UK) for the enumeration of total viable counts (TVC), onto PALCAM (Oxoid, Ltd., Basingstoke, Hampshire, UK) for *Listeria* spp., and onto King Agar B (Merck & Co, Kenilworth, NJ, USA) for pyoverdine-forming *Pseudomonas* spp. One milliliter of diluted samples was added on *E. coli* Petrifilm (3 M, St Paul, MN, USA) for *E. coli* counts. Petrifilm samples were incubated at 37°C for 24 h. PALCAM plates were incubated at 37°C for 48 h. All other plates were incubated at 25°C for 72 h.

2.6 | Statistical analysis

Microbial populations were expressed as log CFU/mL or log CFU/g (wet weight plant leaf biomass). The different species of LGV samples were analyzed separately to avoid any systematic plant-based variation. Data were analyzed using the R statistic package (RStudio version 1.2.5019, Vienna, Austria). The response variables were TVC, *Listeria*, *E. coli*, and *Pseudomonas* counts. The antimicrobial effect of sanitizer concentrations and contact times were analyzed by using the restricted maximum likelihood (REML) linear mixed model algorithm. Sanitizer treatments, LGV types, concentrations and contact times were analyzed independently and in combination to examine LGV × sanitizer × concertation × time interactions. Tukey's honestly significant difference (HSD) test were established at a confidence level $\alpha = .05$.

3 | RESULTS

3.1 | Inactivation of bacterial suspension with different sanitation treatments

3.1.1 | Antimicrobial efficacy of e-water, PAA, and BCDMH against *E. coli* strains

The purpose of the initial experiment was to assess the degree of sanitizer resistance the nonpathogenic proxy pathogen strain *E. coli* M23 possessed. The efficacy of e-water, BCDMH, and PAA against 10 different *E. coli* strains was performed using fully suspended

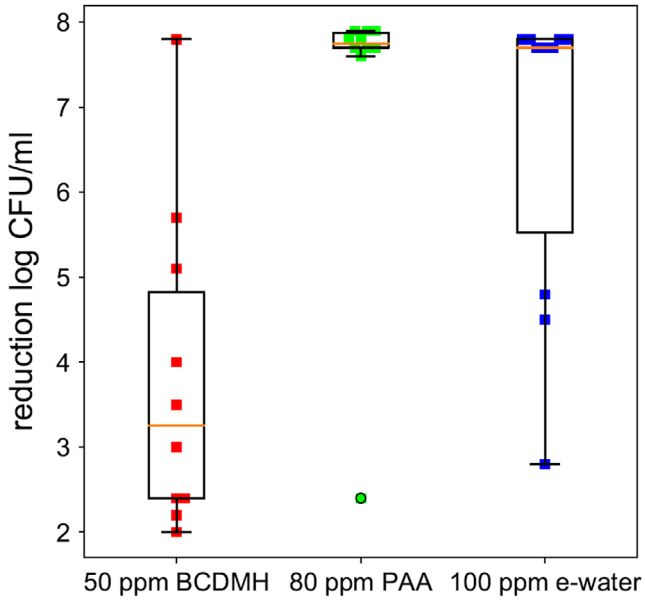

FIGURE 1 Efficacy of e-water, BCDMH, and PAA against 10 strains of *E. coli* (indicated in Table 1) in suspended cultures. The average initial level of the strain was on average 9.0 (8.8–9.5) log CFU/g (mL). Inactivation responses by pathogen proxy strain M23 used in subsequent plant inoculation experiments are indicated on the graph.

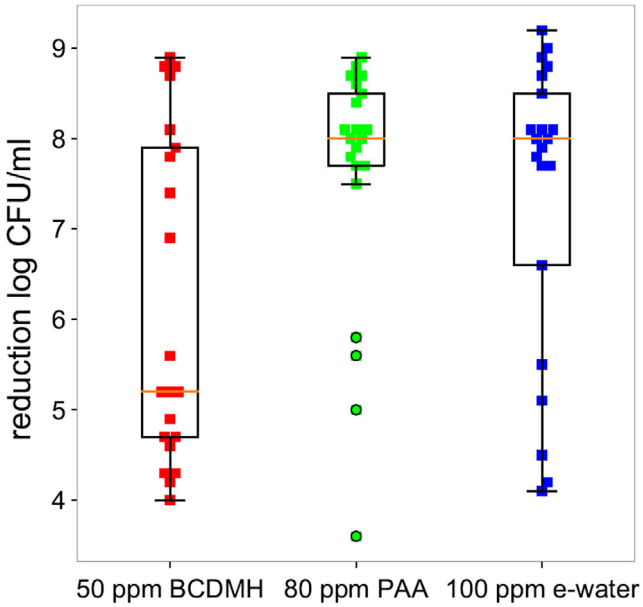

FIGURE 2 Efficacy of 100 ppm e-water, 50 ppm BCDMH, and 80 ppm PAA against 21 strains of *Listeria* in suspended cultures. The initial population densities of the strains was 7.7–9.2 CFU log/mL. Inactivation responses by pathogen proxy strain *L. innocua* ATCC 33090, used in subsequent plant inoculation experiments are indicated on the graph.

cultures are shown in Figure 1. The initial population of the *E. coli* strains was approximately 9.0 (8.8–9.5) log CFU/mL. The populations of *E. coli* strains were reduced by 6.6 (standard deviation ±1.9) log CFU/mL after being treated with 100 ppm e-water for 30 min at 25°C. Similarly, PAA was able to reduce *E. coli* strains by 7.2 (± 1.7) log CFU/mL. By comparison, 50 ppm BCDMH reduced by 3.8 (± 1.9) log CFU/mL. The inactivation responses of *E. coli* M23, indicated the resistance of this strain compared well with that of the other O157 and O111 strains and, in relative terms, showed close to average responses to all sanitizers, in particular PAA and e-water (reduction 7.7 log CFU/mL for both). Strain M23 was less resistant to BCDMH compared to the average strain response but this was close to the estimated range of variation (5.7 log CFU/mL reduction).

3.1.2 | Antimicrobial efficacy of e-water, PAA, and BCDMH against *Listeria* strains

Similarly, the estimated resistance to sanitizers was assessed for *L. innocua* ATCC 33090 by comparing it to a set of Listeria monocytogenes strains from a range of clinical, food and farm animal sources. The efficacy of e-water, BCDMH, and PAA against 21 different *Listeria* strains is shown in Figure 2. The initial cell density of strains ranged from 7.7 to 9.2 log CFU/mL. Among 21 strains of *Listeria*, a reductions of 6.2 (± 1.8), 7.6 (± 1.4), and 7.4 (± 1.7) log CFU/mL was observed from 50 ppm BCDMH, 80 ppm PAA, and 100 ppm e-water, respectively. The inactivation responses of *L. innocua* ATCC 33090 suggested it was comparatively resistant to e-water and BCDMH (4.2 and 4.3 log CFU/mL reduction). ATCC 33090 had a PAA sensitivity matching the average response of *L. monocytogenes* strains (reduction 7.8 log CFU/mL).

3.2 | Recovery of proxy bacteria from LGV plant surfaces

The cultivable populations of native microbiota on untreated mizuna, rocket and red chard were 3.9–5.3 log CFU/g, respectively. The initial inoculum levels of *E. coli* M23, *L. innocua*, and *P. fluorescens* applied to plants were between 8.2 and 8.4 log CFU/mL, respectively. The recovery of sprayed inocula on untreated mizuna, rocket, and red chard ranged from 0.18% to 0.64% of the original sprayed populations. Inactivation levels are reported against the recovered populations and are based on the average of five replicates per given treatment. The experiments were performed as separate trials with the inactivation responses of plant-derived TVC, *E. coli* M23, *L. innocua*, and *P. fluorescens* reported in separate sections.

3.3 | Reduction of TVC on mizuna, rocket, and red chard using different sanitizer treatments

The efficacies of different sanitizers in reducing TVC on different LGV are summarized in Figure 3. A significant combined effect of LGV × sanitizer × concentration interaction was observed for TVC. There was no significant effect when contact time was included into

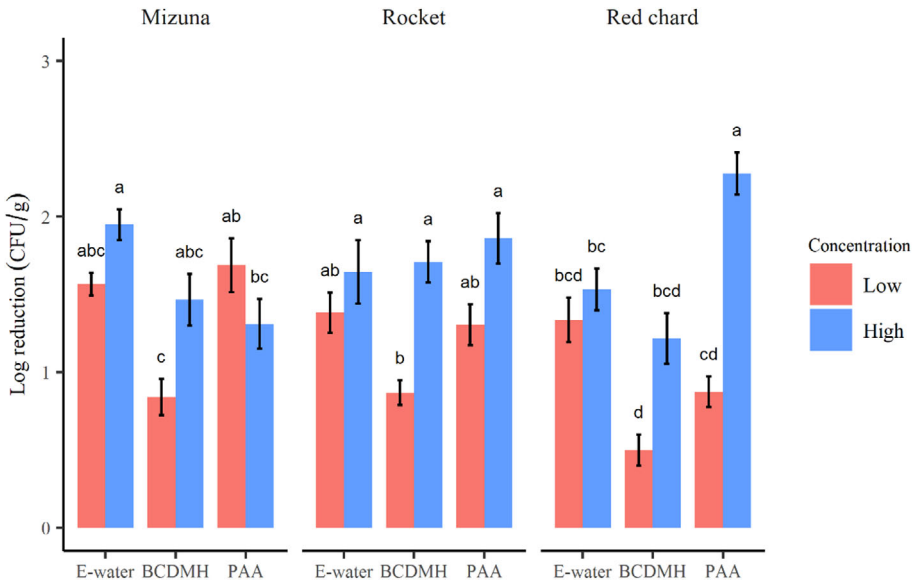

FIGURE 3 Efficacy of 5 and 20 ppm e-water, 5 and 20 ppm BCDMH, 80 and 150 ppm PAA against TVC on mizuna, rocket, and red chard. Error bars shows standard error ($n = 10$). Letters above bars indicate whether the inactivation differed (different letter combination) or did not differ statistically (shared letter combination) (REML, $p < .05$).

the interaction. Treatment of mizuna, rocket and red chard with 5 and 20 ppm e-water, 5 and 20 ppm BCDMH, and 80 and 150 ppm PAA reduced TVC populations by 0.8–1.9, 0.9–1.9 and 0.5–2.3 log CFU/g, respectively. It was observed that the levels of TVC reduction were similar on mizuna and rocket, while red chard showed a slightly different pattern with a lower minimum and higher maximum TVC reduction compared to mizuna and rocket. More specifically, mizuna treated with 20 ppm e-water resulted in 1.95 log CFU/g reduction of TVC, which was significantly higher than 5 ppm BCDMH and 150 ppm PAA (0.8 log and 1.3 log CFU/g, respectively). While all other treatments were equally effective against TVC on mizuna as 20 ppm e-water. Treating rocket with 20 ppm e-water, 20 ppm BCDMH, and 150 ppm PAA (1.6–1.9 log CFU/g) achieved a significantly higher TVC reduction than 5 ppm BCDMH (0.9 log CFU/g), but was statistically equal to 5 ppm e-water and 80 ppm PAA (1.4 and 1.3 log CFU/g, respectively) in reducing TVC populations. For red chard trial, 150 ppm PAA reduced TVC populations by 2.3 log CFU/g, and showed a significantly higher disinfection effect than all other treatments (0.5–1.5 log CFU/g). Also, e-water at 20 ppm exhibited a greater TVC reduction (1.5 log CFU/g) compared to 5 ppm BCDMH (0.5 log CFU/g). E-water at 5 and 20 ppm, BCDMH at 20 ppm, and PAA at 80 ppm achieved similar inactivation levels of TVC reduction, ranging from 0.9 to 1.5 log CFU/g.

3.4 | Reduction of *E. coli* M23, *L. innocua*, and *P. fluorescens* on mizuna, rocket, and red chard using different sanitizer treatments

Based on REML analysis there were significant combined effects of LGV × sanitizer × concentration, LGV × sanitizer, LGV × concentration, sanitizer × concentration for *E. coli* M23, *L. innocua*, and *P. fluorescens* counts. No significant effect was noted when contact time was included into the interaction.

3.4.1 | Efficacy of different sanitizer for inactivation of *E. coli* M23 on LGV

The efficacies of different sanitizers in reducing *E. coli* M23 on different LGV are summarized in Figure 4. Overall, all treatments achieved relatively similar *E. coli* M23 reduction (1.7–3.0 log CFU/g) on mizuna, rocket, and red chard, except for 5 ppm BCDMH, which caused a significantly lower *E. coli* M23 reduction on mizuna (1.7 log CFU/g) and rocket (1.1 log CFU/g) compared to the other treatments. PAA at 150 ppm produced the greatest level of inactivation with the majority treatments having a marked inactivating effect, of up to 3.0 log CFU/g. The overall results showed *E. coli* M23 was reduced to a greater relative extent than TVC for the three LGV (medians 0.6–0.7 log CFU/g). This tolerance difference was similar across LGV and for sanitizers. Specific reduction of *E. coli* M23 varied to an extent between LGV and treatment concentrations, but the efficacy of sanitizers against *E. coli* M23 had approximately similar effects when compared overall.

3.4.2 | Efficacy of different sanitizers for inactivation of *L. innocua* on LGV

The efficacies of different sanitizers in reducing *L. innocua* on different LGV are summarized in Figure 5. After mizuna, rocket, and red chard was treated with 5 and 20 ppm e-water, 5 and 20 ppm BCDMH, and 80 and 150 ppm PAA, the levels of *L. innocua* was reduced by 0.9–2.9 log CFU/g. More specifically, treatment of mizuna with 20 ppm e-water achieved the best observed reduction of *L. innocua* (2.9 log CFU/g), which was significantly higher than 5 ppm e-water (1.5 log CFU/g). While other treatments exhibited similar inactivation levels to 20 ppm e-water, resulting in 2.2–2.7 log CFU/g reductions. For rocket, all sanitizer treatments showed equivalent disinfection effect for *L. innocua* reduction. Red chard treated with 80 and 150 ppm PAA

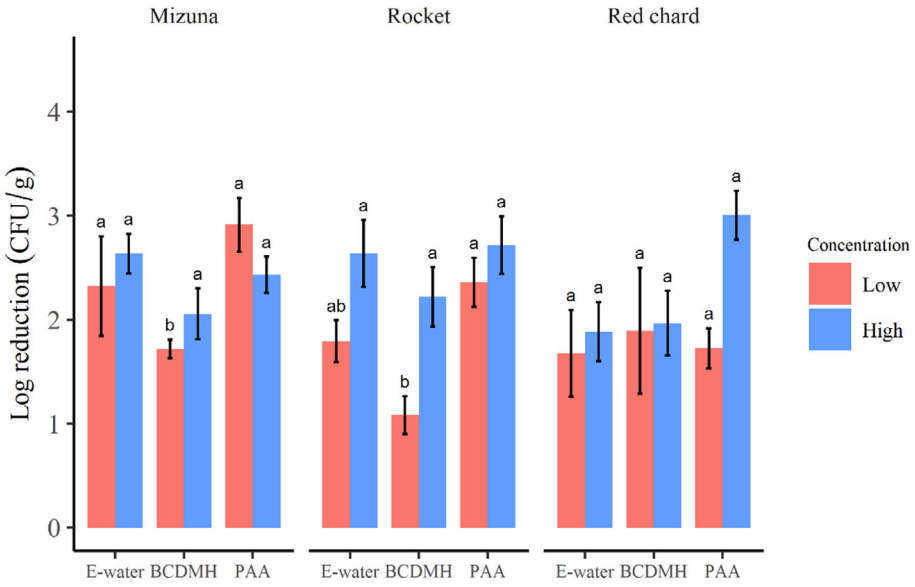

FIGURE 4 Efficacy of 5 and 20 ppm e-water, 5 and 20 ppm BCDMH, 80 and 150 ppm PAA against *E. coli* M23 on mizuna, rocket, and red chard. Error bars shows standard error ($n = 10$). Letters above bars indicate whether the inactivation differed (different letter combination) or did not differ statistically (shared letter combination) (REML, $p < .05$).

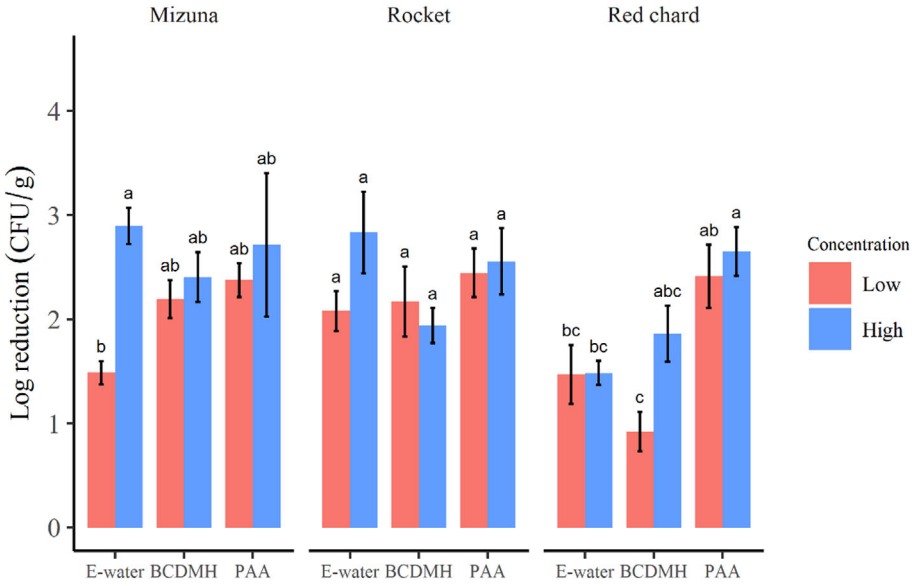

FIGURE 5 Efficacy of 5 and 20 ppm e-water, 5 and 20 ppm BCDMH, 80 and 150 ppm PAA against *L. innocua* on mizuna, rocket, and red chard. Error bars shows standard error ($n = 10$). Letters above bars indicate whether the inactivation differed (different letter combination) or did not differ statistically (shared letter combination) (REML, $p < .05$).

achieved 2.4 and 2.6 log CFU/g *L. innocua* reductions, respectively. Other treatments generated reductions of a similar degree (0.9–1.5 log CFU/g reductions). It was observed that *L. innocua*, like *E. coli* M23 was more sensitive to the sanitizer treatments when compared with TVC data for the same treatments. The relative difference was found to be independent of the LGV type but was affected by the sanitizer type.

3.4.3 | Efficacy of different sanitizer for inactivation of *P. fluorescens* on LGV

The efficacies of different sanitizers in reducing *P. fluorescens* on different LGV are summarized in Figure 6. The populations of *P. fluorescens* was reduced by 0.8–4.0 log CFU/g on mizuna, rocket, and red chard after treated with 5 and 20 ppm e-water, 5 and 20 ppm BCDMH, and 80 and 150 ppm PAA. The highest *P. fluorescens* reductions were obtained from PAA treatments, which produced 3.1, 3.5, and 4.0 log CFU/g on mizuna, rocket, and red chard, respectively. e-Water and BCDMH treatments had similar inactivation levels of *P. fluorescens* on three LGV regardless of concentration, ranging from 0.8 to 2.1 log CFU/g. *P. fluorescens* was found to be more sensitive to PAA treatments, but was more resistant to e-water and BCDMH treatments compared to *E. coli* M23 and *L. innocua*.

3.5 | The microbial inactivation of TVC, *E. coli* M23, *L. innocua*, and *P. fluorescens* using different contact times of sanitizer

The efficacy of sanitizer treatments was significantly improved by prolonging contact time though this difference varied between the LGV types. On average, the extension in contact time reduced the populations of TVC, *E. coli* M23, *L. innocua*, and *P. fluorescens* by an

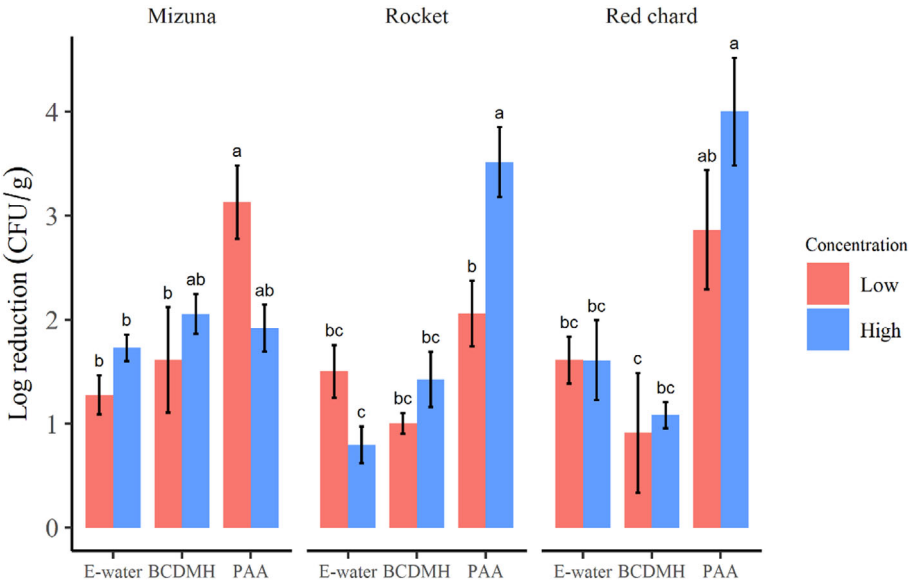

FIGURE 6 Efficacy of 5 and 20 ppm e-water, 5 and 20 ppm BCDMH, 80 and 150 ppm PAA against *P. fluorescens* on mizuna, rocket, and red chard. Error bars shows standard error ($n = 10$). Letters above bars indicate whether the inactivation differed (different letter combination) or did not differ statistically (shared letter combination) (REML, $p < .05$).

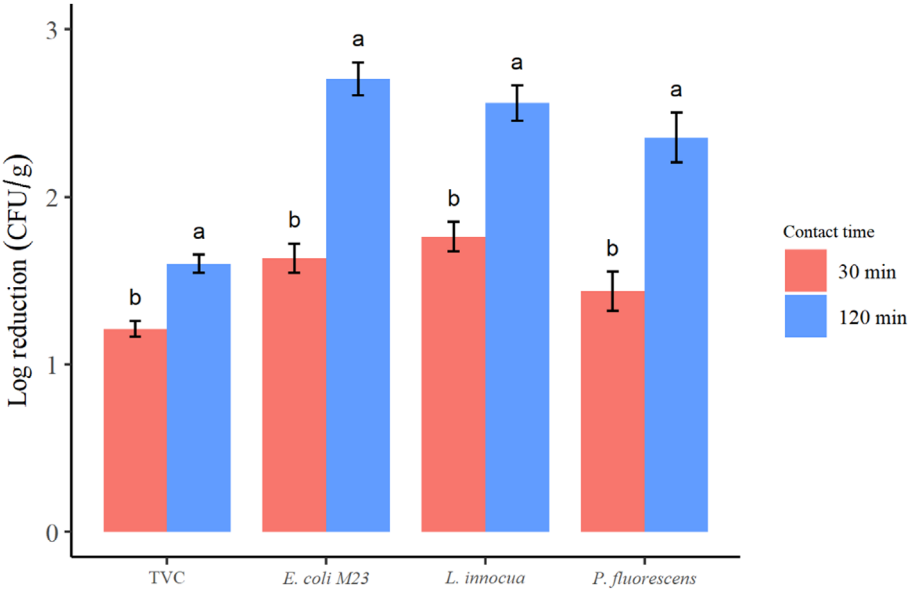

FIGURE 7 Reduction of TVC, *E. coli* M23, *L. innocua*, and *P. fluorescens* on LGV using 30- and 120-min contact time of sanitizer treatments. Error bars shows standard error, $n = 270$ samples for TVC, and $n = 90$ samples for *E. coli* M23, *L. innocua*, and *P. fluorescens*. Letters above bars indicate whether the inactivation differed (different letter combination) or did not differ statistically (shared letter combination) (REML, $p < .05$).

additional 0.4, 1.1, 0.8, and 0.9 log CFU/g on LGV, respectively (Figure 7). The reduction of *E. coli* M23, *L. innocua*, and *P. fluorescens* was substantially higher than TVC when using a longer contact time. The overall results showed that an increase in concentration and contact time enhanced the overall efficacy of the sanitizer treatments in reducing TVC, *E. coli* M23 and *L. innocua* on LGV, while the reduction of *P. fluorescens* was significantly increased by contact time but not concentration.

3.6 | The microbial inactivation of TVC, *E. coli* M23, *L. innocua*, and *P. fluorescens* using different concentrations of sanitizer

The reduction of TVC, *E. coli* M23, *L. innocua*, and *P. fluorescens* using different concentrations and contact times of sanitizer treatments is summarized in Figure 8. For TVC, low-concentration sanitizer treatments produced 1.1 log CFU/g reductions on LGV, and the efficacy was significantly enhanced with increasing concentration, which led to an average of an additional 0.5 log CFU/g reductions of TVC. The reduction levels of *E. coli* M23 and *L. innocua* on LGV were also significantly increased with an increase in sanitizer concentrations, resulting in 0.4–0.5 CFU/g differences. A total of 1.8–2.0 log CFU/g reduction of *P. fluorescens* was obtained with sanitizer treatments. However, increasing the concentration of sanitizers did not promote a significantly higher reduction of *P. fluorescens* on LGV.

4 | DISCUSSION

The suspension inactivation experiments (Figure 1) support the justification to use *E. coli* M23 as surrogate organism for pathogenic *E. coli* strains for application for LGV sanitation as has been shown by

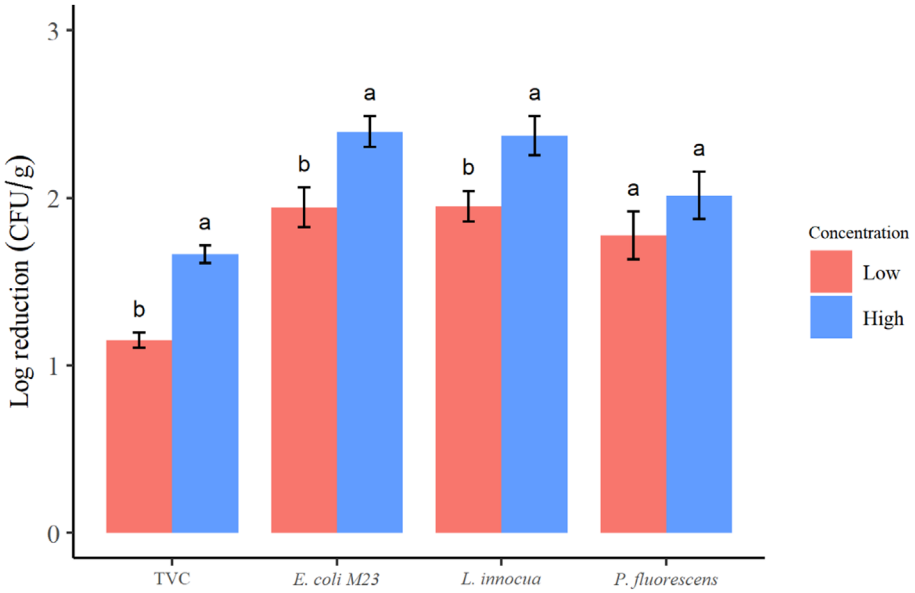

FIGURE 8 Reduction of TVC, *E. coli* M23, *L. innocua*, and *P. fluorescens* on LGV using low and high concentrations of sanitizers. See Section 2 for concentrations applied. Error bars shows standard error, $n = 270$ samples for TVC, and $n = 90$ samples for *E. coli* M23, *L. innocua*, and *P. fluorescens*. Letters above bars indicate whether the inactivation differed (different letter combination) or did not differ statistically (shared letter combination) (REML, $p < .05$).

previous studies. Salter, Ross and McMeekin (1998) found that only a little difference was observed in the growth rate and response to salt and temperature between *E. coli* M23 and pathogenic *E. coli* O157:H7 under growth permitting conditions. In addition, the acid tolerance of *E. coli* M23 appeared similar to the virulent strains of pathogenic *E. coli* O157:H- (Brown et al., 1997). Similarly, the inactivation responses of *L. innocua* ATCC 33090 suggested it was comparatively resistant to e-water and BCDMH with a reduction of approximately 4 log CFU/mL, while 14 and 9 of the 20 *L. monocytogenes* strains tested were sensitive to e-water and BCDMH, respectively, producing approximately 8 log CFU/mL reduction (Figure 2). Moreover, *L. innocua* and 15 L. *monocytogenes* strains displayed equal inactivation responses to PAA, which resulted in a nearly complete, to complete, loss of viability. These results imply that *L. innocua* had similar levels of sensitivity to sanitizer exposure, suggesting it is also suitable as a surrogate for *L. monocytogenes* for further plant-based trials. Previous studies indicated that *L. innocua* and *L. monocytogenes* share ecological co-habitation and have similar genomic and phenotypic characteristics, hence *L. innocua* is ideal for predicting *L. monocytogenes* behaviors in farm and food processing environments (Aarestrup, Knochel & Hasman, 2007; Milillo et al., 2012). Silva-Angulo et al. (2015) indicated *L. innocua* could represent a "worst-case scenario" in relation to *L. monocytogenes* contamination. The presence of *L. innocua* is also indicative of the likely occurrence of *L. monocytogenes* (Encinas et al., 1999; Silva-Angulo et al., 2015).

e-Water, BCDMH, and PAA treatments were about equally effective against TVC on the three LGV tested with the inactivation levels from 0.5 to 2.3 log CFU/g (Figure 3). Similar results were found in the previous studies, where Vandekinderen et al. (2009) reported that 80 and 150 ppm PAA treatments resulted in a reduction of 0.8–2.5 log CFU/g for native microflora on carrot, cabbage, iceberg lettuce, and leek. Hilgren and Salverda (2000) also showed that the 80 ppm PAA reduced the total aerobic bacteria by 0.8–1.5 log CFU/g on celery, cabbage and potatoes. In addition, a 1.0–1.5 log CFU/g reduction for total bacteria counts occurred on Chinese cabbage, lettuce, sesame leaf and spinach using 21–22 ppm of slightly acidic electrolyzed water (SAEW) when left for about 3 min (Forghani & Oh, 2013). The treatment of Chinese celery, lettuce, and daikon sprouts with 21.4 ppm SAEW for 5 min reduced total aerobic bacteria by 2.7– 2.8 log CFU/g (Issa-Zacharia et al., 2011). The reduction of total bacteria populations on spinach leaves was approximately 1.9 log CFU/g after treatment with 5 ppm e-water for 3 min (Rahman, Ding & Oh, 2010b). These results demonstrated that e-water is an effective sanitizer for disinfection of LGV with most of the effect occurring rapidly.

e-Water was equally as effective as PAA and BCDMH in inactivating *E. coli* M23, *L. innocua*, and *P. fluorescens*, resulting in 1.1–3.0, 0.9–2.9, and 0.8–4.0 log CFU/g reductions on all three LGV (Figures 4–6). This study also showed that the best reduction of *P. fluorescens*, the proxy used for microbial spoilage during chilled storage, was obtained with PAA treatments and was equally effective on all three LGV tested (Figure 6). The results are therefore comparable with other findings and suggest the treatments used in this study could be useful in reduction of microbial loads on LGV plant surfaces both preharvest and postharvest. Neo et al. (2013) found that 70 ppm PAA for 180 s reduced 2.3 and 1.8 log CFU/g for *E. coli* O157:H7 and *L. monocytogenes* on mung bean sprouts, respectively. Shredded lettuce treated with 80 ppm PAA for 5 min yielded 4.3 and 4.6 log CFU/g reductions of *E. coli* O157:H7 and L. monocytogenes, respectively (Rodgers et al., 2004). Applying 500 ppm PAA on ungerminated barley malt resulted in a 3.79 log reduction of *Pseudomonas* spp. (Rood et al., 2018). Furthermore, treatment of spinach leaves with 5 ppm e-water for 3 min reduced inoculated *E. coli* O157:H7 and *L. monocytogenes* by 2.3 and 2.8 log CFU/g, respectively (Rahman, Ding & Oh, 2010b). Microbial reduction of inoculated *E. coli* O157:H7 and L. were 0.4 to 2.9 log CFU/g monocytogenes on spinach and 0.1–3.0 log CFU/mL on lettuce after treatment with 4 and 20 ppm NEW for 10 min (Guentzel et al., 2008). In addition, Vasquez (2020)

reported that the populations of *E. coli* and *L. innocua* were reduced by 0.5–4.0 and 0.5–4.1 log CFU/g as a result of treatment with 5 ppm BCDMH at pH of 4.5, 6.0, 7.0, and 8.0 for 120 min.

A consistent lower reduction of TVC populations compared to the bacterial and spoilage microorganism counts was observed in this study when mizuna, rocket, and red chard were treated with e-water, BCDMH, and PAA (Figures 3–6). The results indicate that native microflora, combined with inoculated pathogens, are collectively more resistant to sanitizer treatments compared to the inoculated pathogens alone. The antimicrobial effect of PAA was evaluated in a study by Neo et al. (2013), who reported that PAA treatments only resulted in approximately 1 log CFU/g reduction of natural microflora, while the same treatments reduced inoculated *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp. by up to 2.3 log CFU/g on mung bean sprouts, showing PAA produced a higher reduction in the number of inoculated pathogens than natural microflora. Such observation might be due to the inability of sanitizer treatments to access strongly attached bacteria on the surface of plants. With sufficient time since germination, the native microflora may be present within recesses of the plant epidermis and protected from topical sanitizers (Franz & van Bruggen, 2008; Neo et al., 2013). Another explanation could be the presence of biofilm on the surface of plants. Bacteria within the biofilm can be more resistant to the sanitizer treatments as the biofilm can protect bacteria from disinfection and allows them to recover from disinfection injures (Bridier et al., 2011). Furthermore, sanitizer treatments may not be able to efficiently penetrate plant tissue and reach bacteria that reside within the internal tissues (Bridier et al., 2011; Izumi, 1999).

Preharvest sanitizer application may require extended contact times to overcome these barriers to be more effective in reducing bacterial contaminants. It was determined that the antimicrobial efficacy of e-water, BCDMH, and PAA was influenced by contact time and concentration, primarily the former (Figures 7 and 8). Increased contact times increased efficacy in obtaining up to 1 log unit extra inactivation (Figure 7). Similar results have also been reported by Sharma (2003), who found that increasing contact time of e-water from 2 to 64 min reduced the *E. coli* O157:H7 by an additional 1.6 log CFU/g on alfalfa sprouts. Furthermore, Rahman et al. (2012a) showed e-water achieved an average of an additional 0.8 log CFU/g reductions on pure cultures of *E. coli* O157:H7 and *L. monocytogenes* when increasing the contact time from 0.5 to 1.5 min. For PAA treatments, Vandekinderen et al. (2009) observed that an increase in contact time had a greater antimicrobial effect on the microbial reduction. When increasing the contact time from 1 to 10 min, PAA at 50 and 200 ppm increased the microbial reduction by an additional 0.5 and 2.0 log CFU/g on fresh produce, respectively (Vandekinderen et al., 2009). The finding of this study clearly shows increased contact time has a significant effect (Figure 7) and this has significance for practical application at the preharvest level. Owing to the time needed for spraying and subsequent harvest of crops short-term applications of sanitizers provide less-safety risk reduction due to inferior inactivation levels. Furthermore, as no adverse effect of the sanitizers on plant leaf appearance and health was noted in this study multiple sanitizer applications may be beneficial in reducing microbial pathogen loads. Repeated sprays of sanitizers, however, may cause plant tissue damage (Gómez-López et al., 2007), but in the application envisaged at field scale here even a single sanitizer application appears effective. Alternatively, sanitizer applications could be spaced out to allow plant recovery and the regrowth of innocuous native flora. In addition, since improved inactivation was observed with a longer contact time, this allows more of the crop to be harvested and enables better coordination between spraying and harvesting if a rapid sanitation intervention is needed, for example, owing to a possible contamination event (i.e., dust deposition, rain just before harvest). Most studies have used the strategy of rapid application of sanitizers, typically less than 5 min for determining postharvest sanitization efficacy (Issa-Zacharia et al., 2011; Neo et al., 2013; Rahman, Ding & Oh, 2010a). This is because the inactivation of bacteria by the sanitizers follows a classic Weibull type response (Weibull & Sweden, 1951), where rapid inactivation occurs followed by a slower die off rate (Wang, Feng & Luo, 2006). This suggests a protected (internalized via stomata) and/or physiologically reliant subpopulation can survive on the leaf surfaces (Bridier et al., 2011; Franz & van Bruggen, 2008), but the timely application of sanitizer treatments could eliminate the majority of the pathogenic population and that extended contact time may also help reduce the more resilient contaminating subpopulations.

Generally, increasing the concentration of e-water, BCDMH and PAA led to better inactivation of TVC, *E. coli* and *L. innocua* on three LGV, achieving an extra 0.5 log CFU/g reductions (Figure 8). Similarly, Guentzel et al. (2008) reported the efficacy of e-water against inoculated pathogens on lettuce and spinach was improved when increasing the concentration from 4 to 20 ppm. Rahman et al. (2012a) also found that the concentration of e-water increased from 5 to 10 ppm resulted in an average of an additional 0.6 log CFU/g reductions of *E. coli* O157:H7 and *L. monocytogenes*. Moreover, both Vandekinderen et al. (2009) and Neo et al. (2013) indicated that the microbial reduction on fresh produce was influenced by the concentration of PAA. However, this study observed that a higher concentration of sanitizers had no additional effect on the reduction of *P. fluorescens* on three LGV. The efficacy of sanitizer treatments might be affected by differences between bacterial species, which for example have different attachment capabilities. *P. fluorescens* preferentially attaches to leaf surface, due to an ability to produce peptidolipid biosurfactants and form biofilms on hydrophobic surfaces (i.e., intact leaf surface), a characteristic that would tend to lend resistance to disinfection treatments (Takeuchi et al., 2000). Increasing the concentration of sanitizers does not appear to improve inactivation of bacteria internalized within biofilms or tissue structures (Franz & van Bruggen, 2008). In addition, microbial distribution and attachment on surface of produce can be influenced by surface hydrophobicity, where the microbial reductions were negatively correlated to hydrophobicity of the decontamination surface (Park & Kang, 2017). LGV plant surfaces, compared to cereal grains, are considerably less hydrophobic and not as structurally complex as cereal seed. This difference may allow for inactivation via sanitizer to be inherently more effective and predictable, especially for sanitizers like e-water that can be rapidly quenched by organic material

reactions (Hricova, Stephan & Zweifel, 2008; Koseki et al., 2001; Rahman, Khan & Oh, 2016). Indeed, Rood et al. (2018) found that barley grain treated with 5–500 ppm e-water did not produce any substantial antimicrobial effects on *Pseudomonas* spp., however, it was still effective against the native fungi present. Although antimicrobial efficacy of e-water can be influenced by the surface area, anatomy, and microstructure of each produce (Izumi, 1999), it was found that the LGV-specific experiments gave comparably similar responses as suggested by the TVC data (Figure 3). It is worth mentioning that increased concentrations of sanitizers appear to result in diminishing returns, which also works against the concept of optimizing the cost: benefit ratio. The increase in sanitizer concentration does not linearly correlate to improved microbial inactivation. Rood et al. (2018) who applied 500 ppm PAA to barley grain obtained up to 3.8 log CFU/g reduction of *Pseudomonas* spp. Given the protection, the plant surface gives to contaminating bacteria the highest levels of inactivation are thus not expected to be realistically more than 3–4 log units.

Based on the findings from this study, it must be highlighted that a high level of variability is expected for field-based applications. Since inocula physiology and the plant microbiota plus other environmental variables such as spray applications, water quality, wettability of the plants, solar insolation, and recovery of bacteria from plant surfaces may contribute to the estimates of inactivation. For instance, the recovery of bacteria from plant material via stomaching itself likely contributes to approximately 0.5 log unit variation in this study.

It is also worth noting that although BCDMH, registered in Australia and New Zealand for the postharvest control of plant and human pathogens on fruits and vegetables (Gupta et al., 2018; Premier, 2013), has limited data available in terms of field-based application and even with postharvest application, its efficacy is still not well documented in the current literature. In this study, BCDMH behaves somewhat like e-water in terms of effectiveness and in terms of increased inactivation following extended contact time (Figures 3–7). This is likely due to the fact the antimicrobial activity is based on hypobromous acid and hypochlorous acid generated through BCDMH decomposition (Simons & Sanguansri, 1997). Neutral pH BCDMH application likely could improve the impact on microbial cells, much like neutral pH e-water, which has the benefit of having a less deleterious impact on fresh produce quality.

5 | CONCLUSION

The results of this study revealed that between the LGV trialed e-water and BCDMH at 20 ppm and PAA at either 80 or 150 ppm exhibited similar levels of inactivation against plant microbiota and proxies for foodborne pathogen contamination. Extended contact times provided improvements to the inactivation suggesting preharvest field application is feasible and that measuring TVC and/or suitable proxies would result in microbial load reduction and food-born pathogen reduction. Results from this study are thus potentially useful to the fresh produce industry and LGV growers as the development of preharvest control strategy may help reduce the risk of foodborne illness and improve fresh produce safety along the supply chain to protect public health. Future studies are ideal for further validating the efficacy of sanitizers under agricultural field conditions owing to the variables that can impede effective inactivation of contaminating microbes on plant surfaces, including internalization, strong attachment and biofilm formation. Downstream effects such as effect of LGV quality and shelf-life and sensorial impact also need to be determined to prove the cost–benefit of preharvest safety interventions using chemical sprays.

AUTHOR CONTRIBUTIONS

Hongshan Shang, Rosalind Deaker, Roger Stanley, and John Bowman participated in conceiving and planning the research. Hongshan Shang performed the laboratory and green house work, analyzed the data and wrote the manuscript. Linxi Huang contributed to laboratory work, Roger Stanley provided key support and advice during the implementation of the research. Rosalind Deaker and John Bowman helped analyze the data. Rosalind Deaker and John Bowman, participated in critical evaluation and writing of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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