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A pH-neutral electrolyzed oxidizing water significantly reduces microbial contamination of fresh spinach leaves

Abiodun D. Ogunniyi [a,*,1], Sangay Tenzin [a,1], Sergio Ferro [b], Henrietta Venter [c], Hongfei Pi [a], Tony Amorico [b], Permal Deo [c,**,2], Darren J. Trott [a,2]

[a] Australian Centre for Antimicrobial Resistance Ecology, School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, 5371, Australia
[b] Ecas4 Australia, Unit 8 / 1 London Road, Mile End South, 5031, Australia
[c] Health and Biomedical Innovation, Clinical and Health Sciences, University of South Australia, Adelaide, 5000, Australia

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ABSTRACT

There are growing demands globally to use safe, efficacious and environmentally friendly sanitizers for post-harvest treatment of fresh produce to reduce or eliminate spoilage and foodborne pathogens. Here, we compared the efficacy of a pH-neutral electrolyzed oxidizing water (Ecas4 Anolyte; ECAS) with that of an approved peroxyacetic acid-based sanitizer (Ecolab Tsunami® 100) in reducing the total microbial load and inoculated *Escherichia coli*, *Salmonella* Enteritidis and *Listeria innocua* populations on post-harvest baby spinach leaves over 10 days. The impact of both sanitizers on the overall quality of the spinach leaves during storage was also assessed by shelf life and vitamin C content measurements. ECAS at 50 ppm and 85 ppm significantly reduced the bacterial load compared to tap water-treated or untreated (control) leaves, and at similar levels (approx. 10-fold reduction) to those achieved using 50 ppm of Ecolab Tsunami® 100. While there were no obvious deleterious effects of treatment with 50 ppm Tsunami® 100 or ECAS at 50 ppm and 85 ppm on plant leaf appearance, tap water-treated and untreated leaves showed some yellowing, bruising and sliming. Given its safety, efficacy and environmentally-friendly characteristics, ECAS could be a viable alternative to chemical-based sanitizers for post-harvest treatment of fresh produce.

1. Introduction

There is a growing demand worldwide for the production and consumption of low-risk, fresh minimally processed fruits and vegetables as an integral part of a 'one health' approach to achieving better public health outcomes (WHO, 2015). Areas of particular interest include the implementation of safe, environmentally and economically sustainable food safety practices, the prevention of zoonotic diseases and the fight against the rise of antibiotic resistance in human and animal populations. In the context of food safety, microbial contamination in irrigation water (pre-harvest) or in washing water (post-harvest) are the dominant sources of fresh produce contamination by opportunistic human pathogens (FSANZ, 2011). For example, outbreaks of *Salmonella* spp. and *Listeria monocytogenes* have been associated with cantaloupes, pre-packaged baby spinach and lettuce leaves, leading to a major recall of these products (FSANZ, 2016; Zhu et al., 2017).

From the above, it is apparent that the quality of irrigation water directly affects the safety of edible fresh produce, and there is sufficient evidence to suggest that contaminated irrigation water acts as a conduit for transferring pathogens onto the leaf surface (De Keuckelaere et al., 2015; Jongman and Korsten, 2018; Markland et al., 2017; Uyttendaele et al., 2015). Microbial pathogens found in irrigation water and most commonly associated with disease outbreaks in fresh produce include toxin-producing *Escherichia coli*, *Salmonella* spp., *Yersinia enterocolitica*, and *L. monocytogenes*. However, to date, farm management practices have mainly focused on the post-harvest treatment of fresh produce (Mahajan et al., 2014), although effective removal of bacteria from leaf surfaces through post-harvest washing is difficult once the bacteria are firmly attached (Banach et al., 2017).

A number of post-harvest treatments of fresh produce have been

* Corresponding author.
** Corresponding author.
E-mail addresses: david.ogunniyi@adelaide.edu.au (A.D. Ogunniyi), Permal.Deo@unisa.edu.au (P. Deo).
[1] Co-first author.
[2] Co-senior author.

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described in the literature (see e.g. the review by (Mahajan et al., 2014)). These include physical treatments (e.g. heat, gamma irradiation), gaseous treatments (e.g. ozone, modified atmosphere packaging), chemical sanitizers (e.g. chlorine-based solutions, peroxyacetic acid (PAA), organic acids, hydrogen peroxide and electrolyzed oxidizing (EO) water). A report (Premier, 2013) on the various post-harvest chemical treatments used for commercial vegetables in Australia concluded that PAA-based sanitizers are more effective for treating post-harvest leafy vegetables than organic-based sanitizers (such as Citrox, Aussan and CitroFresh) but are more expensive and result in lower shelf-life of the vegetables. Noteworthy, the report pointed out that emerging technologies such as EO water are safe, economical and could offer superior efficacy compared to other sanitization methods, while also leading to an increase in shelf life of the fresh produce. Cheng and colleagues (Cheng et al., 2012) also reported that EO water is highly effective in reducing the levels of major human pathogens such as *E. coli* O157:H7, *L. monocytogenes* and *S.* Enteritidis in fruit and vegetable products.

In 2017, the US Department of Agriculture, Food Safety and Inspection Services (FSIS) approved a specific type of EO water, "electrolytically generated hypochlorous acid", also known as neutral electrolyzed oxidizing water (NEW), as an antimicrobial product for sanitizing and disinfecting surfaces (USDA-FSIS, 2016). In Europe, Ecas4 supplies the same product under the name "Electro-Chemically Activated Solution" (ECAS or Ecas4 Anolyte), which is mainly used in the healthcare industry to control *Legionella* in water supplies (Migliarina and Ferro, 2014). The Ecas4 solution is also available on the Australian market, and we have shown that it significantly increases the shelf life of Southern Australian King George Whiting and Tasmanian Atlantic Salmon fillets (Khazandi et al., 2017). The pH-neutral Ecas4 Anolyte is synthesized through the electrolysis of a dilute solution of NaCl in a patented electrochemical reactor comprising 4 chambers (Ferro, 2015; Migliarina and Ferro, 2014). It is a non-hazardous, certified "organic" solution that contains active chlorine mainly in the form of hypochlorous acid.

Published studies have demonstrated the efficacy of Ecas4 Anolyte in the healthcare and seafood industries. However, there has been no report on its efficacy in the decontamination of known pathogens of fresh produce. In this study, we investigated the effects of Ecas4 Anolyte on total organoleptic properties of minimally processed baby spinach leaves and examined its efficacy in eliminating known, non-pathogenic microorganisms and its effects on the overall reduction of total microbial load using a currently approved PAA-based sanitizer for fresh produce (Tsunami® 100), as a comparator.

2. Methods

2.1. Reagents, solutions and instruments

Freshly prepared Ecas4 Anolyte (ECAS) containing approx. 350 ppm of free chlorine was supplied by Ecas4 Australia, stored at 4 ± 1 °C and used within a week from manufacture. Ecolab Tsunami® 100 (15.2% peroxyacetic acid, 11.2% H_2O_2 and 73.6% inert ingredients, including 30–60% acetic acid) was purchased from Ecolab USA Inc.; it is largely used as a post-harvest sanitizer in the fresh produce industry.

2.2. Fresh produce

Untreated, freshly cut baby spinach leaves were supplied by a Tasmanian commercial horticulture farm in 2-kg consignments and shipped at 4 ± 1 °C. The leaves were received within 48 h of harvest and used within 24–48 h on receipt for inoculation experiments.

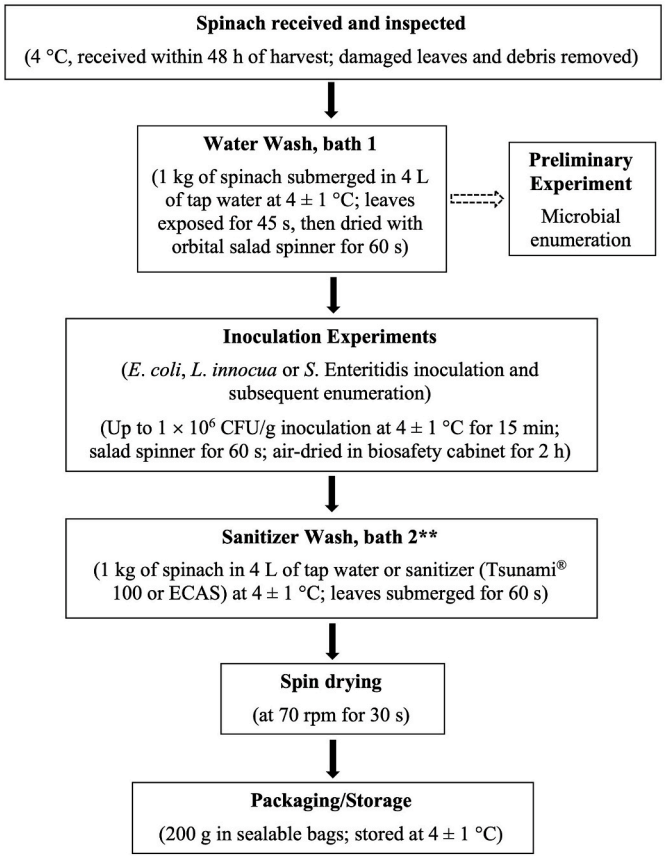

Fig. 1. Schematic of the processing steps and conditions used for washing minimally processed vegetables. ** Tap water; Tsunami® 100 (50 ppm); ECAS (50 or 85 ppm).

2.3. Temperature, pH, oxidation-reduction potential (ORP), turbidity and chlorine level measurements

The temperature, pH and ORP of ECAS, Ecolab Tsunami® 100 and tap water were measured using a model MC-80 handheld meter (TPS Pty Ltd, Australia). Turbidity measurements of the solutions were carried out on a Jenway 6320D spectrophotometer (Cole-Parmer, UK). The amounts of free and total chlorine in ECAS were measured using a Free Chlorine Checker® HC-HI701 and a Total Chlorine Checker® HC-HI711 (Hanna Instruments). The amount of active agent in the Tsunami® 100 solution was determined using a PAA titration kit (Ecolab).

2.4. Bacterial strains and growth conditions

The bacterial strains used in this study were *E. coli* (ATCC 25922), *L. innocua* 6a (ATCC 33090) and *S.* Enteritidis 11RX (Ogunniyi et al., 1994; Ushiba et al., 1959). *L. innocua* is commonly used as a surrogate of *L. monocytogenes*, a pathogen of fresh produce, since it displays similar behaviour but does not require biosafety level 2 containment (Rasch, 2004). Glycerol stocks were maintained at −80 °C and streaked onto Luria Bertani (LB) agar (Oxoid) to obtain isolated colonies. Single colonies were streaked onto the following selective agar plates (ThermoFisher Scientific) to confirm purity: Eosin Methylene Blue (EMB) agar (PP2169) for *E. coli*; *Listeria* Selective Agar Oxford (OXF) agar (PP2141) for *L. innocua* 6a, and Xylose Lysine Deoxycholate (XLD) agar (PP 2004) for *S.* Enteritidis 11RX.

For the experiments, single colonies from selective agar plates were inoculated into LB broth and grown overnight at 37 °C with aeration at 150 rpm on a digital platform mixer (Ratek Instruments). Subsequently, the bacteria were sub-cultured at a 1:10 dilution in fresh LB broth and

Table 1
Quality assessment scheme for baby spinach leaves used in this study.

Scoring criteria	Score/Rating				
	5	4	3	2	1
Yellowing	No yellowing	slight yellowing	just acceptable	bad unacceptable yellowing	very severe yellowing
Bruising	No bruising	slight bruising	just acceptable	bad unacceptable bruising	very severe bruising
Wilting	No wilting	slight wilting	just acceptable	bad unacceptable wilting	very severe wilting
Sliming	No Sliming	No rating	sliming evident	bad sliming	very severe sliming

further incubated at 180 rpm for 2–3 h until $A_{600} = 1.0$ (for *E. coli* and *S.* Enteritidis 11RX) or $A_{600} = 0.5$ (for *L. innocua* 6a) was reached (equivalent to approx. 1×10^9 colony-forming units (CFU)/mL for each strain). The bacteria were then harvested and washed extensively (3×) in autoclave-sterilized Milli-Q water (Milli-Q Academic A10, MILLIPORE) to remove residual culture medium and suspended in sterile Milli-Q water to approx. 1×10^6 CFU/mL for each strain.

2.5. Preliminary efficacy assessments and bacterial inoculation experiments

In a preliminary assessment of the efficacy of ECAS and Ecolab Tsunami® 100 (Figure 1), the damaged spinach leaves and debris were removed followed by treatment with tap water, Tsunami® 100 (50 ppm), ECAS 15% (50 ppm) or ECAS 25% (85 ppm) for 60 s. Subsequently, excess liquid was removed from the spinach leaves using an orbital salad spinner at 70 rpm for 30 s; 200 g samples were placed in sealable bags and stored at 4 ± 1 °C for post-treatment sampling and analysis. For the bacterial inoculation experiments, leaves were briefly washed with tap water at 4 ± 1 °C (1 kg of spinach in 4 L of water) for approx. 45 s, and the excess liquid removed as described above. The leaves were then submerged in *E. coli*, *S.* Enteritidis or L. *innocua* suspension at a concentration of between 5×10^5 and 1×10^6 CFU/g of sample weight and mixed intermittently by swirling in a sterile plastic container. After a contact time of 15 min, excess liquid was removed using the salad spinner for 60 s. The inoculated samples were placed in open containers and air-dried for 2 h in a biosafety level 2 cabinet to allow complete attachment of bacteria onto the spinach leaves. Subsequently, a sub-sample of the inoculated leaves was analyzed for initial count of *Salmonella*, *Listeria* or *E. coli*. The rest of the inoculated leaves were divided into 4 groups and submerged in tap water, Tsunami® 100 (50 ppm) or ECAS (either at 50 ppm or 85 ppm) for 60 s, with intermittent mixing. Samples were then placed in sealable bags and stored at 4 ± 1 °C for post-treatment sampling and analysis, as described above for the uninoculated leaves.

2.6. Post-treatment sampling and analysis

2.6.1. Microbiological analysis

On days 0, 5 and 10 post-treatment, 25 g samples (3–5 replicates for each treatment) were homogenized in 225 mL of 0.1% peptone water (ThermoFisher Scientific) in a Seward BA6021 stomacher (Seward Limited, Worthing, UK) for 60 s. Ten-fold serial dilutions of each sample were carried out and duplicates of each dilution were plated for bacterial enumeration using the following media: Plate Count Agar for total viable counts; EMB agar and Brilliance™ *E. coli*/coliform Selective Agar for *E. coli* counts; Oxford agar for *Listeria* counts; XLD Agar for *Salmonella* counts and Compact Dry YM plate for Yeast & mold counts. Plates were incubated aerobically for 24–72 h at 35 ± 1 °C except for the Compact Dry YM plates that were incubated at 25 ± 1 °C for 72–96 h.

2.6.2. Sensory evaluation of spinach leaves

For each treatment, 5 individual leaves were packed in separate sealable plastic bags. All bags with leaves were stored in a container with ice or ice packs at 4 ± 1 °C. On day 0, 5 and 10 post-treatment, the samples were independently assessed by three trained sensory panelists to arrive at an average score. For sensory evaluation, a previously optimized shelf-life assessment sheet was used (Table 1). The sensory index score was calculated as described previously (Khazandi et al., 2017). The evaluated merit points were summed and averaged to give overall sensory scores between a minimum of 1 and a maximum of 5, where a higher score represented better quality. The cut-off SI score was fixed at score ≥3.0.

2.6.3. Determination of ascorbic acid content

The Vitamin C content in the spinach leaves was determined using a previously described iodometric titration technique (Spinola et al., 2012) with a slight modification. Briefly, 1 mL of 10 mg/mL starch solution and 1 mL of 100 mg/mL potassium iodide solution were mixed with accurately weighed spinach extract. The mixture was homogenized for 30 s using a magnetic stirrer before titrating with a previously standardized 0.005M potassium iodate solution until the mixture turned dark blue and the color persisted for at least 60 s. All solutions were prepared and standardized with standard ascorbic acid and sample analysis was done in triplicates. The results were expressed as mg of Vitamin C/100g sample.

2.7. In vitro comparison of the antibacterial action of ECAS and PAA

In order to compare the antibacterial action of ECAS and PAA, we measured the metabolic activity of bioluminescent *E. coli* Xen14 (PerkinElmer Inc, MA, USA) after treatment with various concentrations of freshly prepared sanitizers over a period of 10 min. For this assay, approx. 5×10^7 CFU of Xen14 were washed and resuspended in sterile Milli-Q water and then added to ECAS or PAA solutions containing 1, 2, 5, 10, 20 and 50 ppm of active agent. The reaction was stopped after 2, 5 or 10 min using 0.05% (v/v) sodium thiosulfate. Untreated bacteria resuspended in sterile Milli-Q water were used as control. Thereafter, samples were serially diluted in PBS and plated on LB agar for bacterial enumeration. To measure bioluminescence, approx. 1×10^6 CFU of Xen14 from each treatment (after the addition of sodium thiosulfate) was added to 200 μL of sterile LB broth in a Nunc™ F96 MicroWell™ Black plate (ThermoFisher Scientific, 237,105) which was then incubated at 37 °C in a Cytation 5 Cell Imaging Multi-Mode Reader (BioTek; Winooski, VT, USA). Absorbance at $OD_{600\ nm}$ and total luminescent signals were measured over a 48-h incubation period.

2.8. Statistical analyses

In all experiments, differences in microbial load between treatments were determined using unpaired *t*-test (two tailed). A P value of <0.05 was considered statistically significant.

3. Results

To evaluate the efficacy of ECAS in decontaminating known pathogens of fresh produce and assess its effects on the total organoleptic properties of post-harvest baby spinach leaves, we compared the outcomes of washing with two different concentrations of ECAS to those of tap water and a PAA-based sanitizer approved for fresh produce (Ecolab Tsunami® 100).

3.1. Preliminary efficacy assessments

In a preliminary investigation, the pH, temperature, ORP, turbidity

Table 2
Measured pH, temperature, ORP, turbidity and active agent content in tap water, 50 ppm of Tsunami® 100, 50 ppm ECAS and 85 ppm ECAS before and immediately after washing of spinach leaves.

Treatment	Before/After spinach wash				
	pH	Temperature (°C)	ORP (mV)	Turbidity (NTU)[a]	Active agent (ppm)
Tap water	7.4/7.4	4.9/5.0	287/290	0.0/0.0	0.38/0.35[b]
Tsunami® 100	4.2/4.0	5.6/6.4	427/426	0.0/0.0	50/50[c]
50 ppm ECAS	7.0/7.0	4.5/4.4	857/820	0.0/0.0	56/56[b]
85 ppm ECAS	7.4/7.0	4.8/4.5	868/867	0.0/0.0	89/89[b]

[a] NTU = Nephelometric Turbidity Units.
[b] = Available chlorine.
[c] = Peroxyacetic acid.

and active agent levels were measured for each treatment solution (tap water, Tsunami® 100 and ECAS) before and after washing of the spinach leaves. We found that the parameters measured for each treatment were essentially similar before and after washing (Table 2). We also ascertained that the free available chlorine levels on homogenized spinach leaves treated with 50 ppm and 85 ppm ECAS had reduced to <5 ppm after 5 min contact time and activity was also quenched in 0.1% peptone water after 5-min contact time (not shown).

Next, we carried out bacterial enumeration and sensory analysis for the preliminary experiment at days 0, 5 and 10 post-treatment. The total plate, coliform and yeast/mold counts for tap water, 50 ppm ECAS, 85 ppm ECAS and Tsunami® 100 and the corresponding sensory attributes are presented in Figure 2 and Fig. S1, respectively.

On day 0, the total bacterial load was significantly reduced in all treated groups (tap water, 50 ppm ECAS, 85 ppm ECAS and Tsunami® 100) compared to the control (untreated) group. On day 5, the total bacterial counts for 85 ppm ECAS treatment was significantly lower compared to Tsunami® 100 and tap water treatments. On day 10, the total bacterial and coliform counts for spinach leaves treated with tap water, 50 ppm ECAS, 85 ppm ECAS and Tsunami® 100 were significantly lower, being reduced by approximately 10 times, compared to the control (untreated leaves). Notably, no *Listeria* spp., *Salmonella* spp. or *E. coli* were isolated from the uninoculated spinach leaves either before or after treatment with tap water, 50 ppm ECAS, 85 ppm ECAS or Tsunami® 100. For the yeast and mold counts, spinach leaves treated with tap water, 50 ppm ECAS, 85 ppm ECAS and Tsunami® 100 had significantly lower counts in comparison to the control (untreated leaves) at day 0. On day 5, the yeast and mold count of spinach leaves treated with tap water, 50 ppm ECAS and Tsunami® 100 were significantly lower compared to untreated leaves. Unexpectedly, the yeast and mold count for spinach leaves treated with 85 ppm ECAS was not

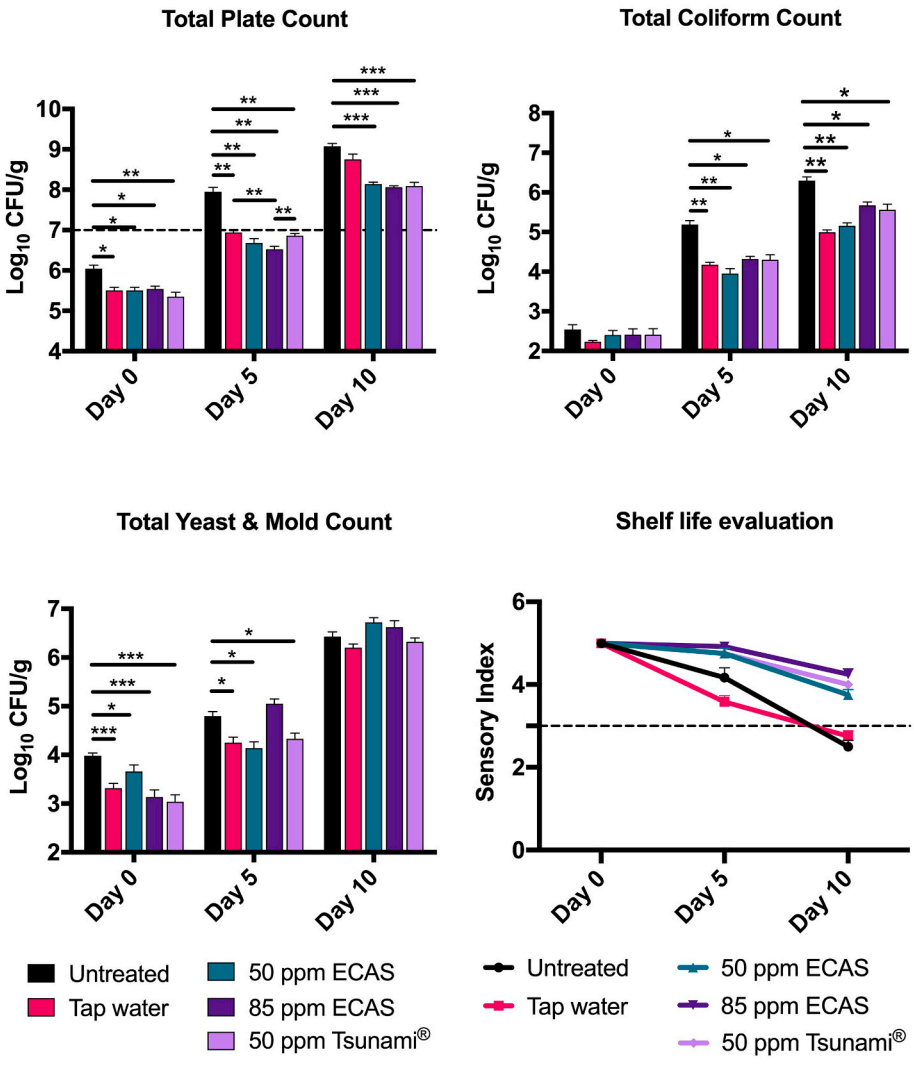

Fig. 2. Effect of treatment with tap water, 50 ppm ECAS, 85 ppm ECAS and 50 ppm of Tsunami® 100 on the microbial load of spinach leaves in the preliminary experiment. Baby spinach leaves were treated with tap water, 50 ppm ECAS, 85 ppm ECAS or 50 ppm of Tsunami® 100 as described in Methods. At days 0, 5 and 10, 25 g of leaves from each treatment ($n = 5$) were assessed for total bacterial, coliform, yeast/mold counts and shelf life quality. Differences in microbial load between treatments were determined using unpaired *t*-test (two tailed). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

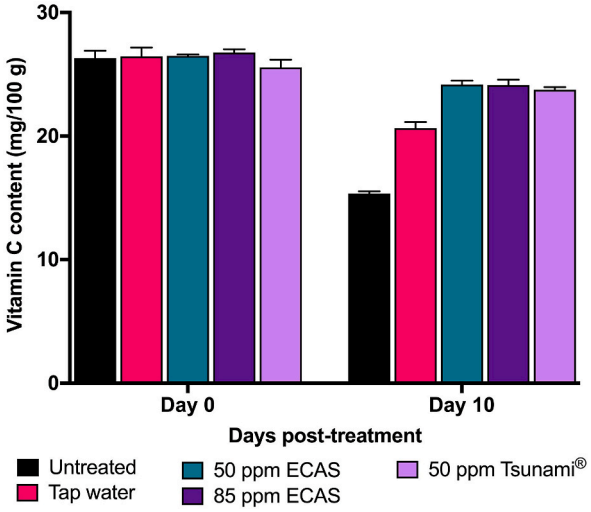

Fig. 3. Vitamin C content in spinach leaves treated with tap water, 50 ppm ECAS, 85 ppm ECAS or 50 ppm of Tsunami® 100, in comparison to that in untreated leaves on day 0 and day 10. For statistical analysis, please see Table S2.

different from those for the untreated leaves. The reason for the spike in the yeast and mold counts on spinach leaves treated with 85 ppm ECAS on day 5 of storage is unclear.

To ascertain that sanitizer treatment does not diminish the nutritional value of the baby spinach leaves during storage, the vitamin C content of the leaves was measured. The vitamin C content in the leaves treated with 50 ppm ECAS, 85 ppm ECAS and Tsunami® 100 was more stable during storage and significantly higher than that of untreated leaves or those washed with tap water (Figure 3; Fig. S1; Table S1; Table S2). Together, these results suggest that treatment of the leaves with the sanitizers significantly reduced the total bacterial counts and improved the shelf life of leaves, and indicate that ECAS at either 50 ppm or 85 ppm was as effective as a 50 ppm Tsunami® 100 treatment of leaves.

3.2. ECAS is as effective as Tsunami® 100 in reducing E. coli populations on spinach leaves

On account of the promising results obtained for ECAS in preliminary investigations, we proceeded to examine its efficacy in sanitizing leaves deliberately spiked with approx. 5×10^5 CFU *E. coli* per g of sample, as illustrated earlier in Figure 1. The average measured pH, temperature, ORP, turbidity and active agent content in tap water, 50 ppm of Tsunami® 100, 50 ppm ECAS and 85 ppm ECAS before and after washing the spinach leaves in the *E. coli* inoculation experiment were similar to those shown in Table 2.

The analysis of bacterial counts on day 0 showed that 85 ppm ECAS and Tsunami® 100 significantly reduced levels of *E. coli* on spinach leaves by 0.3 and 1.5 log CFU/g, respectively, compared to tap water treatment (Figure 4). On day 5, there were no significant differences in the *E. coli* counts between tap water wash and 50 ppm ECAS or 85 ppm ECAS, but surprisingly the number of *E. coli* increased significantly for the leaves treated with Tsunami® 100. In addition, on day 10, the total *E. coli* count was significantly lower for the 85 ppm ECAS treatment compared to washing with Tsunami® 100 and tap water (Figure 4), suggesting that ECAS is at least as effective as Tsunami® 100 in reducing *E. coli* populations on spinach leaves.

3.3. ECAS is more effective than Tsunami® 100 in reducing L. innocua populations on spinach leaves

Following the *E. coli* inoculation experiment, we compared the efficacy of tap water, 50 ppm ECAS, 85 ppm ECAS or 50 ppm of Tsunami® 100 in sanitizing spinach leaves spiked with approx. 5×10^5 CFU of *L. innocua* per g of sample. On day 0, Tsunami® 100 significantly reduced *L. innocua* counts compared to treatments with 50 ppm and 85 ppm ECAS (Figure 5). On day 5, no significant difference in *L. innocua* counts between treatments was found. However, on day 10, the *L. innocua* counts were significantly lower in spinach leaves treated with either 50 ppm or 85 ppm ECAS compared to leaves treated with 50 ppm Tsunami® 100 (Figure 5), an indication of better sanitizing efficacy of ECAS over Tsunami® 100 at the concentrations used.

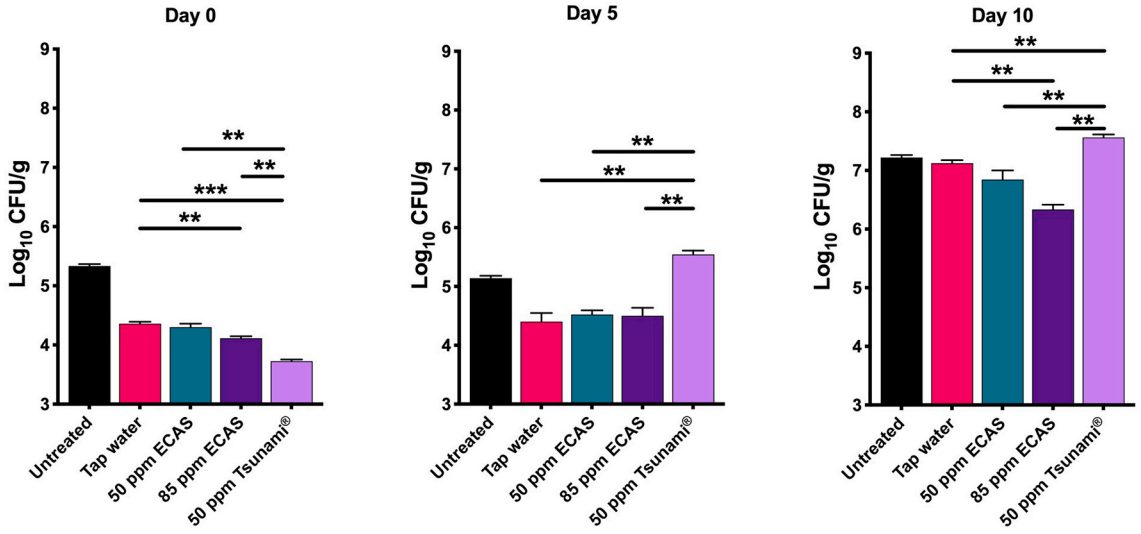

Fig. 4. Effect of treatment with tap water, 50 ppm ECAS, 85 ppm ECAS and 50 ppm of Tsunami® 100 on the microbial load of spinach leaves after inoculation with *E. coli* at approx. 5×10^5 CFU/g of sample. Baby spinach leaves were treated with tap water, 50 ppm ECAS, 85 ppm ECAS, or 50 ppm of Tsunami® 100 as described in Methods. At days 0, 5 and 10, 25 g of leaves from each treatment ($n = 3$) were assessed for *E. coli* counts. Differences in microbial load between treatments were determined using unpaired *t*-test (two tailed). $^{**}P < 0.01$; $^{***}P < 0.001$.

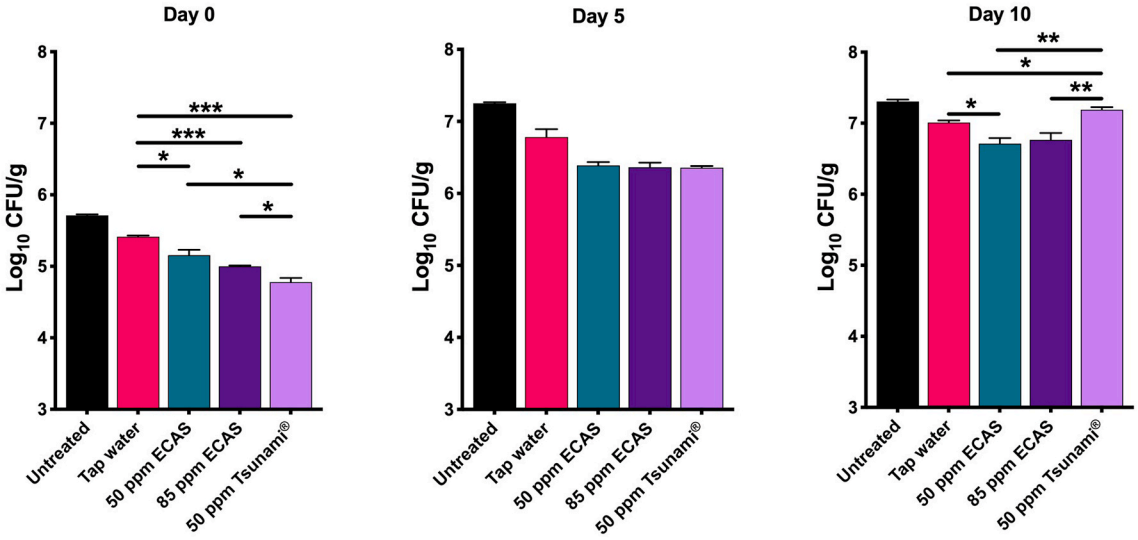

Fig. 5. Effect of treatment with tap water, 50 ppm ECAS, 85 ppm ECAS and 50 ppm of Tsunami® 100 on the microbial load of spinach leaves after inoculation with *L. innocua* at approx. 5×10^5 CFU/g of sample. Baby spinach leaves were treated with tap water, 50 ppm ECAS, 85 ppm ECAS or 50 ppm of Tsunami® 100 as described in Methods. At days 0, 5 and 10, 25 g of leaves from each treatment ($n = 3$) were assessed for *L. innocua* counts. Differences in microbial load between treatments were determined using unpaired *t*-test (two tailed). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

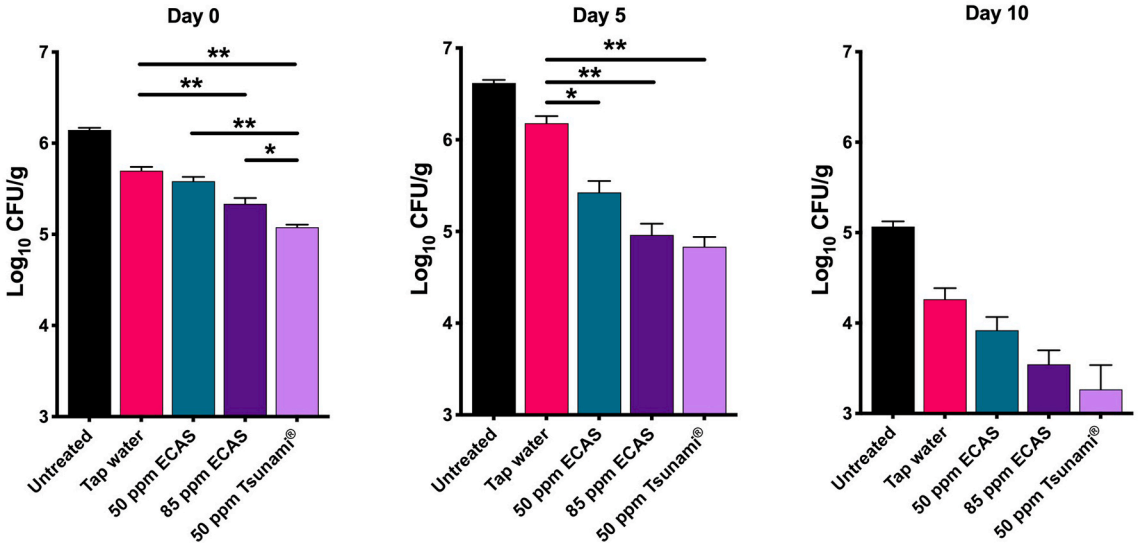

Fig. 6. Effect of treatment with tap water, 50 ppm ECAS, 85 ppm ECAS and 50 ppm of Tsunami® 100 on the microbial load of spinach leaves after inoculation with *S.* Enteritidis 11RX at approx. 1×10^6 CFU/g of sample. Baby spinach leaves were treated with tap water, 50 ppm ECAS, 85 ppm ECAS or 50 ppm of Tsunami® 100 as described in Methods. At days 0, 5 and 10, 25 g of leaves from each treatment ($n = 3$) were assessed for *S.* Enteritidis 11RX counts. Differences in microbial load between treatments were determined using unpaired *t*-test (two tailed). $^*P < 0.05$; $^{**}P < 0.01$.

3.4. ECAS and Tsunami® 100 are equally effective at reducing S. Enteritidis 11RX contamination of spinach leaves

In a third spiking experiment, we compared the efficacy of tap water, 50 ppm ECAS, 85 ppm ECAS or 50 ppm of Tsunami® 100 in sanitizing spinach leaves inoculated with approx. 1×10^6 CFU of *S.* Enteritidis 11RX per g of sample. On day 0, 85 ppm ECAS and Tsunami® 100 significantly reduced *S.* Enteritidis 11RX counts when compared to tap water (Figure 6). On day 5, the *S.* Enteritidis 11RX counts in spinach leaves treated with 50 ppm ECAS, 85 ppm ECAS and 50 ppm of Tsunami® 100 were significantly lower compared to leaves washed with tap water. Although there was further reduction in *S.* Enteritidis 11RX counts on the treated spinach leaves compared to the untreated control by day 10, these differences did not reach statistical significance (Figure 6).

3.5. ECAS is more effective than Tsunami® 100 at killing bacteria at low concentrations in vitro

Given the promising results obtained with the treatment of spiked spinach leaves with ECAS, we sought to determine its effective antibacterial concentration by comparing its efficacy with that of Tsunami® 100 at different concentrations over a 10-min period. For this assay, we used bioluminescent *E. coli* Xen14 to measure metabolic activity (Ogunniyi et al., 2019). We found that both sanitizers were bactericidal after 2-, 5- and 10-min contact time, and no metabolic activity was observed in the range of 10–50 ppm of active agent content over the 48-h incubation period (Figure 7). Interestingly, growth and detectable metabolic activity were observed for Xen14 treated with Tsunami® 100 at 1, 2 and 5 ppm of PAA, and this was consistent through the 48-h incubation period (Figure 7). However, under the same conditions, no

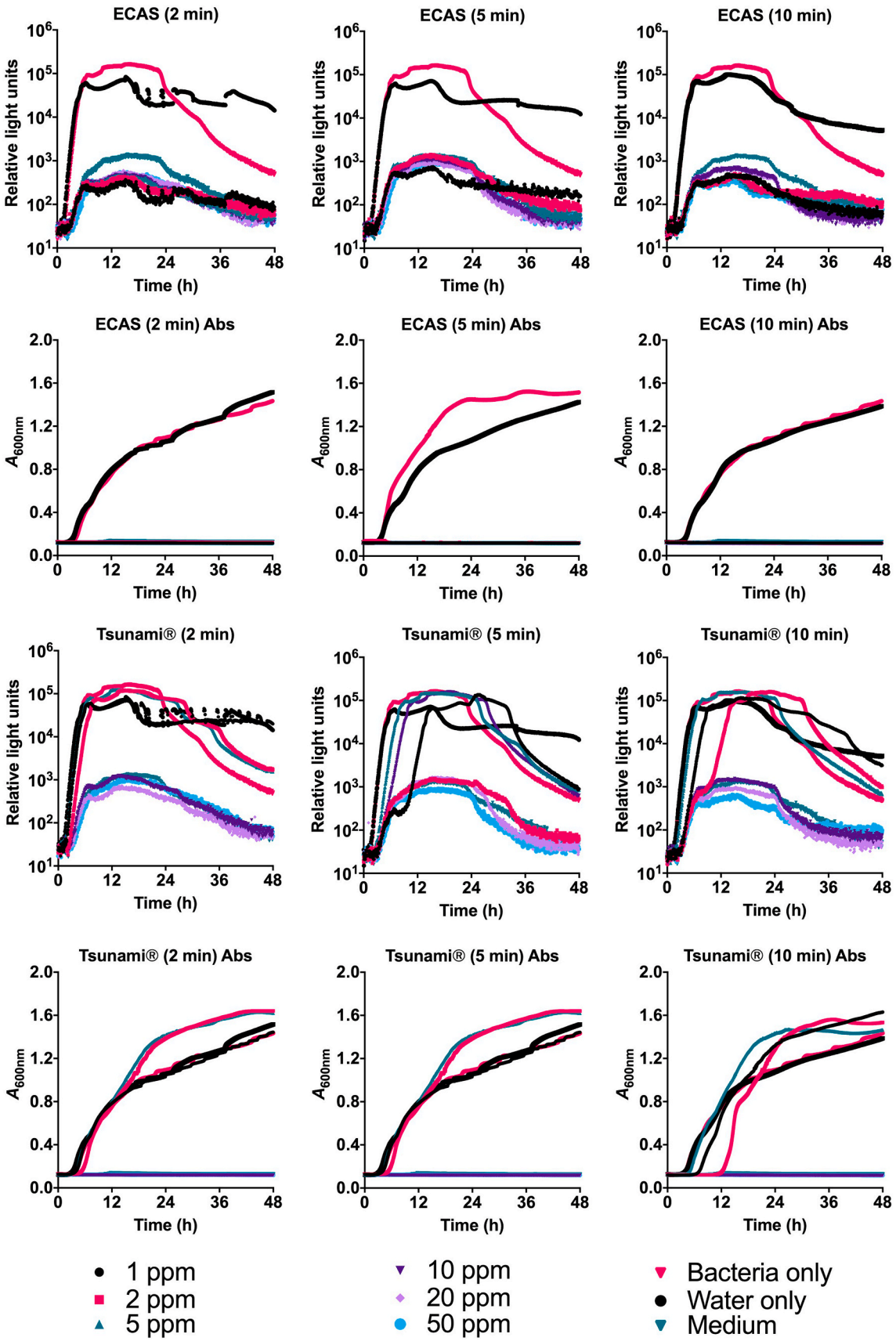

Fig. 7. Bioluminescence and absorbance measurements of *E. coli* Xen14 after treatment with various concentrations of freshly prepared ECAS and Tsunami® 100 over a 48-h period. Results show growth and detectable metabolic activity for Xen14 treated with Tsunami® 100 at 1, 2 and 5 ppm, but not at 10, 20 or 50 ppm, whereas no growth or detectable metabolic activity for Xen14 treated with ECAS could be observed at any of the concentrations tested.

metabolic activity was observed for Xen14 treated with ECAS at 1, 2 or 5 ppm of free available chlorine.

4. Discussion

In this study, we have examined the potential of a pH-neutral electrolyzed oxidizing water (Ecas4 Anolyte or ECAS) in reducing the bacterial load and increasing the shelf life of post-harvest baby spinach leaves inoculated with three bacterial species, and compared its effectiveness with that of a widely used peroxyacetic acid-based sanitizer (Tsunami® 100). The study was carried out in response to the global need to use safe, effective and environmentally friendly sanitizers for the post-harvest treatment of minimally processed fruits and vegetables to reduce or eliminate spoilage and foodborne pathogens and increase the nutritional value and overall quality of fresh produce.

Tsunami® 100 has proven to be highly effective in the post-harvest treatment of fresh produce to reduce pathogen and spoilage load (Mahajan et al., 2014; Premier, 2013), and readily decomposes into harmless byproducts such as acetic acid (CH_3COOH), O_2 and H_2O (Kitis, 2004; Koivunen and Heinonen-Tanski, 2005; Sigge et al., 2016). However, the increase of the organic content in the treated water due to the presence of CH_3COOH in the mixture, the safety concerns with handling the stock solutions, the potential microbial regrowth after peracetic acid decomposition and the high initial purchase cost have somewhat limited its widespread use. Given that previous studies have shown the efficacy of ECAS in controlling *Legionella* in water supplies (Migliarina and Ferro, 2014) and in increasing significantly the shelf life of seafood while remaining safe at high concentrations (up to 150 ppm) (Khazandi et al., 2017), we compared its effectiveness at 50 ppm and 85 ppm with that of 50 ppm of the widely used Tsunami® 100.

Our examination of the effects of ECAS on spinach leaves indicated that treatment with 85 ppm ECAS is better than the use of 50 ppm ECAS. We also found that treatment with 85 ppm ECAS compared favorably (in terms of bacterial load reduction) with treatment using 50 ppm of Tsunami® 100, particularly over the 10 days of storage. In previous studies, neutral electrolyzed water at $\geq$100 ppm free chlorine was required to reduce the microbial load in fresh vegetables (Navarro-Rico et al., 2014; Rico et al., 2008) whereas 85 ppm of ECAS proved to be effective in our studies. In a recent study, a slightly acidic electrolyzed water (4 ppm) combined with levulinic acid (3% v/v) showed bactericidal efficacy against natural microbial load and reduced survival population of *E. coli* and *L. innocua* compared to acidic electrolyzed water (4 ppm) alone (Zhao et al., 2019). Furthermore, we observed that the treatment of spinach leaves with 50 ppm of Tsunami® 100 or ECAS at 50 ppm and 85 ppm did not show any apparent negative effects on leaf appearance, whereas tap water-treated or untreated (control) leaves showed some yellowing, bruising and sliming during storage. It is also noteworthy that ECAS performed consistently well against all pathogens that are most likely to be found as contaminants in fresh produce, while Tsunami® 100 did not work as well against *E. coli* in this study. Other studies have suggested the potential formation of viable but non-culturable (VBNC) state and subsequent microbial regrowth after PAA treatment of foods at up to 50 ppm (Gu et al., 2020; Purevdorj-Gage et al., 2018). Our *in vitro* bioluminescence (metabolic activity measurement) data with *E. coli* Xen14 using PAA also provides corroborating evidence that the VBNC state could be responsible for the regrowth seen with the *E. coli* cells. Additionally, our finding of a potent *in vitro* bactericidal effect of ECAS at low concentrations in the bioluminescence assay could be important in the context of using sanitizers that have the ability to potentially eliminate the induction of the VBNC state (Ferro et al., 2018; Ogunniyi et al., 2019), although further detailed investigations will be required to verify this proposition.

5. Conclusions

The overall desirable effects (safety, efficacy, environmentally friendly characteristics and potentially low costs of use) described here for ECAS on the post-harvest sanitization of baby spinach leaves are encouraging for the horticulture industry, especially in consideration of the drive to move away from the use of chemical-based sanitizers. Further studies extending the use of ECAS to treat other minimally processed fresh produce such as lettuce, broccoli, tomato and capsicum and assess the overall quality and shelf life of such products are therefore welcome, as they will shed light on its wide applicability. Experiments examining the combination of ECAS treatment of fresh produce with appropriate storage practices such as temperature control and modified atmosphere packaging to increase the physical, nutritional, sensory attributes and shelf life of such products are also desirable.

It is important to note that the bacterial strains used in this study were not pathogenic and were used to test the efficacy of ECAS. Therefore, it would be essential to replicate the assays using known human pathogens, either singly or in a mixture. Moreover, it would be critical to perform on-field (pre-harvest) sanitization of leafy greens and compare the results with post-harvest treatment to assess the best practices that allow the most relevant effects on the overall safety, nutritional value and shelf life of the products.

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Declaration of competing interest

Sergio Ferro and Tony Amorico are technical manager and managing director, respectively, of Ecas4 Australia.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2020.103614.

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