AIMS Agriculture and Food

AIMS Agriculture and Food, 4(2): 320–339.
DOI: 10.3934/agrfood.2019.2.320
Received: 29 December 2018
Accepted: 03 April 2019
Published: 22 April 2019

http://www.aimspress.com/journal/agriculture

Research article

Evaluating the efficacy of three sanitizing agents for extending the shelf life of fresh-cut baby spinach: food safety and quality aspects

Thi-Van Nguyen[1,2,*], Tom Ross[2] and Hoang Van Chuyen[1,3]

[1] Faculty of Agriculture and Forestry, Tay Nguyen University, 567 Le Duan, Buon Ma Thuot, Daklak 63000, Vietnam
[2] Tasmanian Institute of Agriculture, School of Land and Food, University of Tasmania, Sandy Bay Campus, Hobart, Tasmania 7005, Australia
[3] Institute of Research and Development, Duy Tan University, Da Nang 550000, Vietnam

* **Correspondence:** Email: ntvan@ttn.edu.vn; baobien35@gmail.com; Tel: +84977083316.

Abstract: The aims of this project were to: 1) compare the antimicrobial efficacy and feasibility of three sanitizers including chlorine dioxide (ClO_2), neutral electrolysed water (EW) and peroxyacetic acid (PAA) for the treatment of fresh baby spinach leaves, and 2) to investigate the shelf life and quality attributes of spinach treated with these sanitizers. In the experiment for food safety, spinach leaves were inoculated with *Salmonella* Typhimurium and then treated by immerging in cold solutions of PAA, ClO_2, EW at different concentrations. *Salmonella* Typhimurium and total viable count (TVC) were determined immediately before and after the treatment. The treatment with 20 mg/L ClO_2 solution resulted in the highest reductions of *S.* Typhimurium and TVC (by 1.6 ± 0.1 log CFU/g) compared to other treatments. In the experiment for the shelf life of spinach, the samples were stored at 4 °C for 13 days after being treated with 75 mg/L PAA, 60 mg/L free chlorine (FCh) EW and 20 mg/L ClO_2 solutions. The results showed that TVC was significantly influenced by the treatments and storage time. At day 0, TVC of all treated samples was significantly lower than this of the control and the TVC of sample treated with ClO_2 was the lowest. However, by day 10, the TVC was not significantly different among the treated samples and the control. For the sensory qualities and physio-chemical properties of the spinach leaves, the treatment with ClO_2 showed significant reduction in quality of the treated sample since day 7 of the storage while other treatments did not show any significant effect on those parameters during the storage. In summary, although the treatment with 20 mg/L ClO_2 solution resulted in the highest antimicrobial efficacy against *S.* Typhimurium and TVC of spinach leaves, it also caused negative effects to the quality of spinach during the storage.

Keywords: fresh-cut baby spinach; *Salmonella* Typhimurium; total viable count; sanitizing treatment; storage quality

1. Introduction

Post-harvest treatments can significantly contribute to the prevention or reduction of microbial contamination and the shelf-life extension of fresh food products. The current trend in processing fresh-cut produce is to reduce the time of preparation, storage, transportation and the cost for minimal processing. The reduction of microbial cross-contaminations in processing and storage practices of the fresh products is also very important. Thus, sufficient and effective standardized methods should be established in industry practice. Minimal processing of fresh produce includes steps such as harvesting, cooling, storage, trimming, shredding, washing, rinsing, draining, packing, cold storage and distribution. Processors make efforts to maintain high sensory quality, nutritional values as well as ensure food safety for consumption [1]. Fresh-cut fruits and vegetables are alive and will continue to respire during storage. The production does not include any critical control point such as heat treatment, sterilization and freezing to reduce and control the microbial load [2]. Thus, the washing step plays an important role for the prevention of pathogenic contamination in fresh cut produce.

Conventionally, washing is conducted after the cutting or shredding steps and carried out in the water tanks with or without sanitizing agents [3,4]. The effectiveness of sanitizers used in washing step depends on several factors such as their characteristics, concentration, contact time and treating temperature [5,6]. However, many chemical sanitizers such as chlorine, trisodium phosphate and bromine used in food processing may cause negative effects for human health and environment like excess of residues and formation of by-products [1]. In addition, some sanitizers are corrosive which may cause damages to processing equipment [7].

To date, the most used sanitizer in fresh produce washing is chlorine [2,8]. Chlorine application offers a number of advantages including a good oxidizing effect, affordable cost, relatively simple operation and widely commercial availability [8–10]. Despite the popularity of chlorine as a sanitizer, chlorine has shown a range of negative effects. For example, the formation of chlorinated by-products such as trihalomethanes, halo acetic acids and haloketones which may cause negative effects on human health and environment [4,11]. Therefore, more eco-friendly and inovative sanitizers have been developed to minimize the negative effects [12,13]. Several innovative disinfection agents have been applied in fresh cut industry including chlorine dioxide (ClO_2), peroxyacetic acid (PAA), electrolyte water (EW) [10,14–17]. The treatments with these sanitizers are expected to prolong the shelf-life without compromising the quality of the produce [9,18,19].

Chlorine dioxide (ClO_2) has been developed as a promising alternative sanitizer for chlorine in food processing. ClO_2 application has been approved by FDA for the use in fruits and vegetables processing since 1998 [1,20]. There are two forms of ClO_2 (liquid and gas) have been applied in food industry. While the gas can possibly give antimicrobial effect within a wide range of pH values, the aqueous form, on the other hand, is advantageous in term of portability, stability, storage and operation [10]. In comparison with conventional methods, the treatment with ClO_2 produces fewer by-products than hypochlorite forms. This does not form the chloramines because there is no reaction with ammonia compounds [1]. In addition, the treatment with ClO_2 forms less halogens and provides higher oxidizing power than chlorine treatment [1,21]. ClO_2 treatment causes bacterial

membrane damages thus it is effective in the inactivation of various microorganisms like yeast, mold and virus [21]. The antimicrobial activity of ClO_2 has been shown in many studies. For examples, the significant reductions in *Escherichia coli, Listeria monocytogenes, Salmonella* spp., *Bacillus subtilis*, rotavirus populations in fresh-cut fruits and vegetables by applying this sanitizer were reported [9,22–26]. Thus, this agent could be considered as a promising replacement for controversy chemical sanitizing agents like sodium hypochlorite using in processing of fresh fruits and vegetables [27,28].

Peroxyacetic acid (PAA) is considered as Generally Recognized as Safe (GRAS) for using as a disinfectant and a sterilizing agent in food processing and drinking water treatment [2]. It is a mixture of acetic acid and hydrogen peroxide (H_2O_2) which generates cytotoxic oxidizing effect or hydroxyl radicals [29]. It results in microbial inactivation by damaging microbial membrane, DNA and biomolecules [2]. Unlike ClO_2, PAA is broken down into O_2, water and acetic acid so this does not generate critical residue halogens in produce [23]. Its efficacy in reducing microorganism load in fresh produce such as cucumber, bell peppers and sprout has been well documented [16,30]. Treatment with this agent for romaine and iceberg lettuces was more effective in the inactivation of *L. monocytogenes* than the treatment with chlorine at the same contact time [23]. *S.* Typhimurium load in lettuce treated with 40 mg/L (aerosolized) PAA for 10–40 minutes was reduced by 0.3–3.3 log CFU/g [31]. Baert et al. (2009) confirmed that 250 mg/L PAA used in washing solution for shredded iceberg lettuce could reduce *L. monocytogenes* by 1.03 log CFU/g and *E. coli* O157:H7 by 1.30 log CFU/g [32]. In another study, PAA solutions with concentration ranging from 0 to 250 mg/L were investigated and it was confirmed that all treated fresh produce samples were safe for consumption [67].

Electrolyte water (EW) has recently been introduced into the food industry as a novel disinfectant agent [33]. EW is an effective antibacterial sanitizer which is relatively inexpensive, easy to use and has been widely used in the healthcare environment [12,34]. This sanitizer is generated in an electrolyte cell by electrolyzing sodium chloride (NaCl) to produce either acidic (pH 2–3) or alkaline (pH 11–13) EW solutions which are drained from anode and cathode sites, respectively [33]. At the electrode surface, NaCl (electrolyte) is transformed into activated form called metastable state when applying a direct current. This results in the elevation of the chemical reactivity which causes ionic structure modification of the molecule [35]. The microbiocidal mechanism of EW is varied among studies. Some studies stated that its antimicrobial effect is owing to the leaking cellular components accompanied with the increase in cell membrane permeability and the inactivation of some certain enzymes [36]. This allows unstable electrons penetrate into cell cytoplasm which impair regular cellular mechanism [37]. The oxidation-reduction mechanism involved in inactivation of microorganism was also described by Liao et al. (2007) [36]. According to these authors, oxidizing-reduction potential (ORP) can result in the interference of glutathione disulphide—glutathione couple (cellular redox signaling pathways). The formation of oxidative intra-cellular disulphide bridges may disrupt membrane functions. Consequently, protein structure is modified and thereby cell lysis occurs [36]. For the growth of bacteria based on the metabolites via the osmotic barrier on the cell membrane, substances are transported into the cell via protein channels. The excess of ions in the electrolyzed solution causes an oxidation-reduction gradient and therefore promotes water diffusion. Consequently, transmembrane potential occurs due to the irreversible modification and leads to the out-flowing of bacterial cell contents which compromises or even kills bacterial viable cells [7]. Furthermore, it is also postulated that the involvement of multiple target sites in antibacterial action could lead to the less resistant of pathogens to the agent [38,39].

There have been critical pathogen outbreaks associated with fresh-cut produce occurring

recently in Australia so the reevaluation of the efficacy of sanitizers used in fresh produce industry is necessary [68]. Although many studies dealing with the issue have been conducted, the results were varied among studies and between experimental conditions and industrial conditions (industry officer, personal communication, March 2016). Thus the investigation of the use of novel sanitizers is necessary to provide recommendations for the application of alternative sanitizers in fresh produce practice. To exploit the advantages of the agents for sanitizing and extending the shelf-life of fresh-cut spinach, the efficacy of two novel sanitizing agents (ClO_2 and EW) was compared with PAA, the currently used sanitizer in the fresh produce industry, in terms of food safety and quality aspects. Effects of these sanitizing agents on the *S.* Typhimurium load and TVC of fresh-cut spinach, sensory quality and physio-chemical properties of the treated samples during the storage were examined.

2. Materials and Methods

2.1. Materials

Media including Trypticase Soy Agar (TSA), Trypticase Soy Broth (TSB), Xylose Lysine Deoxycholate Agar (XLD) were purchased from Oxoid Microbiology Products (Basingstoke, UK). PAA was purchased from Ecolab Inc. (St. Paul, MN, USA) and Zydox product was purchased from Zychem Technologies (Bergen, Norway). Packaging film (O_2 permeability of 450 cc/100 sq.in/24 h) was provided by a fresh produce producer in Australia. *S.* Typhimurium strain M48 was kindly provided by Microbiology lab, University of Tasmania, Australia.

2.2. Sample preparation

Fresh spinach was supplied by a farm located in Tasmania, Australia. Samples were cooled to about 5 ℃ and then transported in an Esky cooler box (EvaKool, Queensland, Australia) to the laboratory. They were then stored in a refrigerator at 4 ℃ and used within 3 days. Leaves showing clear signs of defect or decay were excluded from the experiments.

2.3 Preparation of sanitizing solutions

All sanitizing solutions were prepared using sterile tap water which was obtained by autoclaving tap water at 121 ℃ for 15 minutes. Working PAA solution was prepared from an appropriate amount of initial product in 2 L sterile tap water to achieve the final concentration of 50 mg/L or 75 mg/L and used within 5 days. For preparation of ClO_2 solution, 1000 mg/L ClO_2 stock solution was prepared from the commercial Zydox product according to manufacturer's instructions. Working solutions of 2 mg/L and 20 mg/L ClO_2 solutions were prepared using tap sterilized water and activated by 54.6% citric acid from the stock solution. For the preparation of EW, working solutions of 20 mg/L and 60 mg/L FCh concentrations were obtained by diluting with sterilized tap water from EW that had an initial FCh concentration of approximately 500 mg/L. These sanitizing solutions were prepared at low temperature (less than 10 ℃) owing to being diluted in cold tap water or stored at 4 ℃ in a refrigerator about more than 2 hours before being used (Table 1).

Table 1. ORP and pH values of sanitizing solutions.

Sanitizing solutions	ORP, (mV)	pH	Suppliers
50 mg/L PAA	587	3.29	Tsunami 100, Ecolab, St. Paul, Minn.
75 mg/L PAA	596	3.25	Tsunami 100, Ecolab, St. Paul, Minn.
2 mg/L ClO_2	710	3.4	Zydox AD-05, Zychem Technologies, Bergen, Norway
20 mg/L ClO_2	705	2.89	Technologies, Bergen, Norway
20 mg/L FCh EW	858	6.33	Prof Roger Stanley, University of Tasmania, Launceston, Australia
60 mg/L FCh EW	890	6.5	Prof Roger Stanley, University of Tasmania, Launceston, Australia

2.4. Preparation of bacterial inoculum (Salmonella Typhimurium)

S. Typhimurium was resuscitated from isolate, streaked by using the 16-streak method on TSA media and incubated overnight (20–24 hours) at 37 ℃ in an incubator. Stock culture was prepared by transferring a single colony grown on the TSA plate into 10 mL Trypticase Soy Broth (TSB) and incubated at 37 ℃ for 24 hours. Working culture was freshly prepared one day before the experiment by adding 10 µL stock culture into 10 mL TSB and incubated at 37 ℃ for 18–24 hours to obtain a concentration of 10^7–10^9 cells/mL. To harvest the cells for inoculation, working cultures were centrifuged at 4500 rpm for 10 minutes and washed once with 5 mL of 0.1% Peptone Water (PW). This centrifugation step was repeated one more time. The cell pellets were then resuspended in 1mL of 0.1% PW (Peptone Water) to obtain a concentration of 10^8–10^{10} cells/mL.

2.5. Experiment 1 (food safety): antimicrobial efficacy of sanitizers in the treatment of inoculated spinach

The first experiment was designed to evaluate the potential for using sanitizing agents (ClO_2, PAA and EW) to eliminate enteric pathogens, *S.* Typhimurium, on the inoculated spinach. PAA has been used in fresh-cut vegetables in the industry plants with the concentration ranging from 50 to 75 ppm (industry officer, personal communication, March 2016). Within this range, the lowest concentration was selected to investigate its efficacy in terms of food safety aspects and the highest concentration was selected to evaluate the effects of this sanitizer on shelf life and quality properties of treated spinach during storage. The antimicrobial efficacy of the treatment with 50 mg/L PAA was compared with the treatments with two different concentrations of either ClO_2 (2 mg/L and 20 mg/L) or neutral EW (20 mg/L and 60 mg/L FCh). Spinach samples were rinsed with cold sterilized tap water in a bulk container for 1 minute at room temperature with the ratio of 1:30 (material weight/volume of water). This was followed by a draining step using a kitchen salad spinner to remove free water on the surface of samples. To attach the viable cells to spinach, spinach samples were inoculated with *S.* Typhimurium prior to being treated with the sanitizing solutions. Inoculation solutions were prepared by diluting 3 mL resuspended cells solution (10^8 –10^{10} cells/mL) into 3 L sterile tap water. These solutions (10^5–10^7 cells/mL) were then mixed with 100 grams spinach in 5 minutes. Thereafter, free surface water was removed from the inoculated spinach using a sterilized kitchen salad spinner. About 10 grams of the inoculated sample were weighed and placed into a plastic container containing sanitizing solutions with a sample-solution ratio of 1:30 (w/v). The sample was agitated gently using a stainless-steel spoon for 1 minute and then drained in a salad spinner. Three repetitions were performed for each assay. The kitchen salad spinners were sanitized by washing with 70% ethanol and then with sterilized tap water between treatments.

2.6. Experiment 2 (shelf life): the effects of the sanitisers on the shelf life and quality properties of treated spinach

In the experiment for the shelf life of spinach, the effects of these sanitizers on quality properties and shelf life of treated spinach were examined for 13 days of the storage at 4 ℃. After the treatments, all samples were packed by a packaging film (O_2 permeability of 450 cc/100 sq.in/24 h) supplied by a fresh produce producer with the same ratio of packing size to material as the ratio used for their commercial products.

The treatment procedure for this experiment was similar to the treatment for the food safety experiment without *S.* Typhimurium inoculation step. Sanitizing solutions including 75 mg/L PAA, 60 mg/L FCh EW and 20 mg/L ClO_2 were prepared in cold tap water and kept in the refrigerator at 4 ℃ before use. In this experiment, unsterile tap water was used so the microbial quality of 100 mL tap water was analyzed and the result was expressed as the number of CFU/100 mL tap water.

From the results of experiments for food safety, the higher concentrations (20 ppm ClO_2 and 60 ppm EW) have selected to investigate the effect of sanitizers on quality properties and shelf life of the stored spinach. The lower concentrations were not considered for the shelf life experiments because those did not showed any significant reduction in the microbial loads of the treated spinach.

Spinach samples were rinsed with cold unsterile tap water for 1 minute, free water was removed using a kitchen salad spinner and then the samples were treated with sanitizing solutions for 1 minute with a ratio of product to solution of 1:30 (w/v). The treated samples were drained and each 40 grams of spinach was packed in a plastic bag with the same product to volume ratio as the ratio for commercial products before being sealed by a heat sealer (CJ-4, Premium Balloon Accessories, Taiwan). The bags were then stored at 4℃ in a refrigerator for the analyses during the storage. Samples were collected at pre-rinsed, pre-sanitized and at day 0, 3, 7, 10 and 13 during the storage to examine the TVC, sensory quality, texture, chlorophylls, carotenoids and total soluble contents.

2.7. Microbiological analysis

All samples treated with sanitizers and one untreated control were weighed with the same amount (5 grams) for microbial analysis of *Salmonella* and total microbial viable count (TVC). All samples were diluted tenfold with 0.1% peptone water maintained at 4 ℃ for 1 hour in a refrigerator. The homogenization was conducted using a food stomacher (Model 400, Colworth, London, UK). The microbial loads were determined by the enumeration method (plate-count) and expressed as $\log_{10}$ CFU/g. Each 0.1 mL of the diluted sample was spread onto a plate and incubated at 37 ℃ for 24 ± 2 hours. TSA medium was used to enumerate TVC and XLD medium was selected to determine *S.* Typhimurium population. The membrane-filtration method was used to analyze microbial quality of the unsterile tap water during the day of treatment. 100 mL of tap water was collected immediately before and after the experiment and kept at 4 ℃ in a refrigerator before using for the determination of the microbial quality. The tap water sample was filtered through a sterilized Envirocheck filter (Metricel GA-8, Gelman Instrument Co., Ann Arbor, Mich.) by a vacuum pump using a 0.45 μm, 47 mm diameter filter disc with grid lines. After filtering, the disc was aseptically transferred onto a TSA plate and incubated at 37 ℃ for 24 hours to determine the TVC. The results are presented as number of TVC colonies present in 100 mL of tap water.

2.8. Free chlorine and ClO_2 measurement

Concentrations of free chlorine and ClO_2 were measured by N, N-diethyl-r-phenylene-diamine (DPD) method using a colorimeter (Palintest PTH046, NSW, Australia) [40]. pH of solutions was determined by a pH meter (PH 915600, Orion, Boston, USA) and the oxidizing-reduction potential (ORP) was measured using a portable ORP meter (MW500, Milwaukee Instruments, Rocky Mount, NC, USA).

2.9. Texture measurement

The texture of spinach was measured using a texture analyzer (Instron 4302 Universal Testing Machine, Canton MA, USA) at day 0 and day 10 of the storage. A representative piece of a spinach leaf (1.8 cm × 4 cm diameter), avoiding the main mid-rib, was prepared for the texture assessment. The sample was placed between two bars with a 10 mm gap. The texture was presented as the firmness which was measured by the maximum force (N) used to shear the leaf piece. Three replicates per a sample were carried out and the results are presented as averages of three values.

2.10. Chlorophyll and carotenoid analysis

Chlorophyll and carotenoid contents were spectrophotometrically determined [41] using an UV-vis spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific Inc., Waltham, MA, USA). An amount of sample (about 0.1–0.2 grams) was weighed and placed in a stone mortar. Liquid nitrogen, magnesium carbonate ($MgCO_3$) and sand were then added into the mortar and the sample was ground with a pestle. After that 2 mL of acetone was added and the liquid was transferred into a Falcon vial. The vial was then centrifuged using a Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) at 4500 rpm for 5 minutes at 4 ℃. The supernatant was collected and the residual was ground again. This step was repeated three times and the liquids were mixed and filled up to a total volume of 6 mL by acetone. The absorbance of the extract was measured at four wavelengths (470, 645, 662, 710 nm). Chlorophyll (Chl) and carotenoid concentrations were calculated as mg/g based on the following equations:

$$\text{Chl A} = [11.2 \times (A_{662} - A_{710}) - 2.04 \times (A_{645} - A_{710})] \times \text{dilution factor} \quad (1)$$

$$\text{Chl B} = [20.13 \times (A_{645} - A_{710}) - 4.19 \times (A_{662} - A_{710})] \times \text{dilution factor} \quad (2)$$

$$\text{Bulk carotenoids} = [(1000 \times (A_{470} - A_{710}) - 1.90 \times \text{ChlA} - 63.14 \times \text{Chl B})/214] \times \text{dilution factor} \quad (3)$$

2.11. Sensory quality assessment

Effect of sanitizing treatments on sensory quality was evaluated using freshness indicators which commonly used in industry (Mr. Tesh Sharma, personal communication, March 2016). A panel of five people was asked to assess tangible attributes including yellowing, bruising, browning, odor, wilting, sliming and physical damage based on a 5-score rating system (Barrett, 2010, Rico et al., 2008). The separated samples were placed in plastic containers and coded by letters before providing to the assessors. The assessors independently evaluated each sample and recorded their scores on a provided worksheet. The result of the sensory quality assessment is present as overall visual

score which is the average of freshness indicators. According to industry's recommendation, the product has the highest score shows the best quality and the score less than 3 is considered as 'not fresh' by consumers.

2.12. Statistical analysis

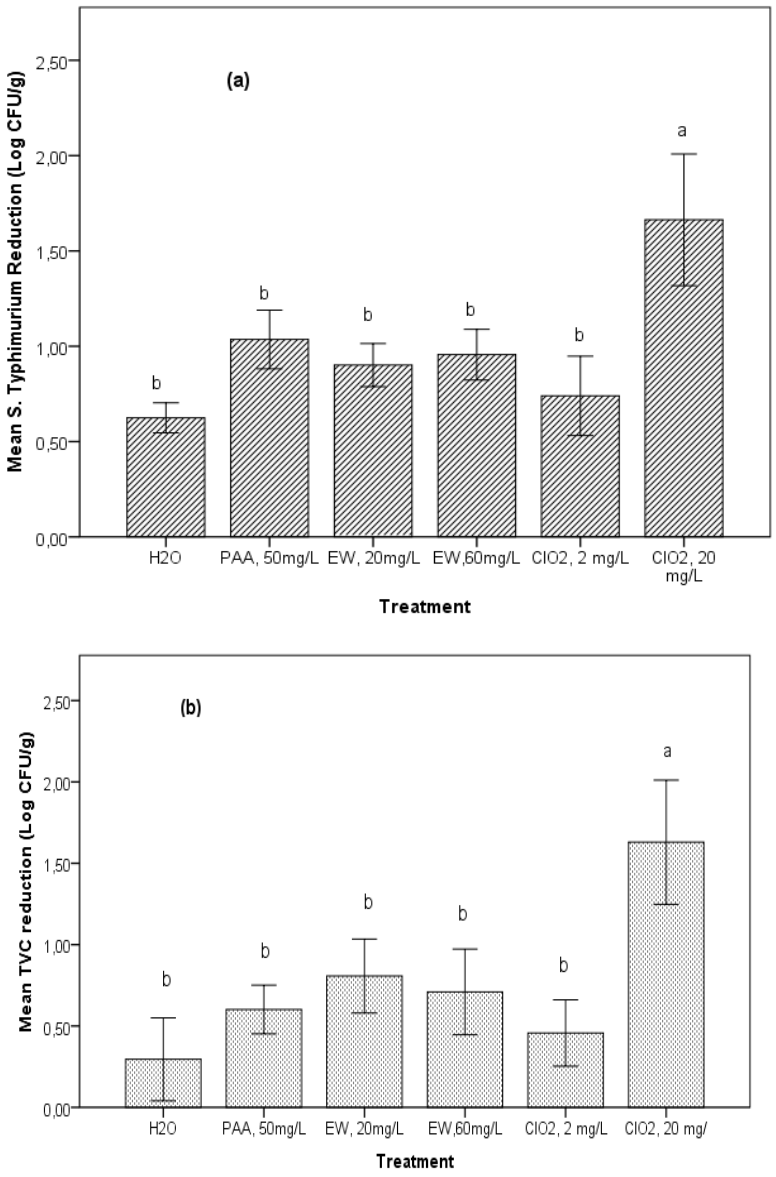
One-way ANOVA test coupled with Fisher's Least Significant Difference (LSD) test was used to analyze variance of the mean of the TVC and *S.* Typhimurium reduction among the treatments in the experiment for food safety. Two-way ANOVA test with two factors (day and treatment) was used to analyze the significant difference in the mean of TVC of samples during storage. Nonparametric tests were also employed to analyze the variance among the treatments for Max load force (Kruskal-Wallis test), chlorophyll and carotenoid content and sensory quality scores (Friedman test) in the shelf life experiment. All tests were performed with three replicates and analyzed with $\alpha = 0.05$.

3. Results

3.1. Efficacy of sanitizing treatments to reduce S. Typhimurium and TVC on the inoculated spinach

Both *S.* Typhimurium and TVC were significantly reduced by the sanitizing treatments ($F_{5,9} = 4.385$; $p < 0.05$) (Figure 1a). The initial *S.* Typhimurium load after inoculation of the untreated sample was 4.9 ± 0.1 log CFU/g. The treatment with 20 mg/L ClO_2 resulted in the highest decontamination efficacy with a reduction in *S.* Typhimurium of 1.6 ± 0.1 log CFU/g. This figure was significantly different ($p < 0.05$) from other treatments and the control. In contrast, the treatments with 2 mg/L ClO_2 and sterile tap water gave the lowest *S.* Typhimurium reductions (0.7 ± 0.1 and 0.6 ± 0.1 log CFU/g, respectively). The treatments with EW at two concentrations (20 mg/L and 60mg/L FCh) and 50 mg/L PAA reduced *S.* Typhimurium inoculated on spinach samples by 0.9–1.0 log CFU/g, which was not significant different from that in the control treatment.

The same pattern of TVC reduction, as for *S.* Typhimurium, caused by the sanitizing treatments was also observed (Figure 1b). The native microbial load of the pre-inoculated samples was 6.3 ± 0.1 log CFU/g. There was a significant difference ($F_{5,11} = 3.183$; $p < 0.05$) in the reduction of TVC load among different treatments. While the TVC count of the control decreased by only 0.3 log CFU/g, the reduction of TVC count resulted by the treatment with 20 mg/L ClO_2 was 1.6 log CFU/g which was the highest among the treatments. The antimicrobial efficacy of treatments with 50 mg/L PAA and 20 mg/L and 60 mg/L FCh of EW were not significantly different ($p < 0.05$). In addition, ClO_2 at low concentration (2 mg/L) was not effective in reducing microbial loads with only 0.4 ± 0.2 log CFU/g of the reduction of TVC count.



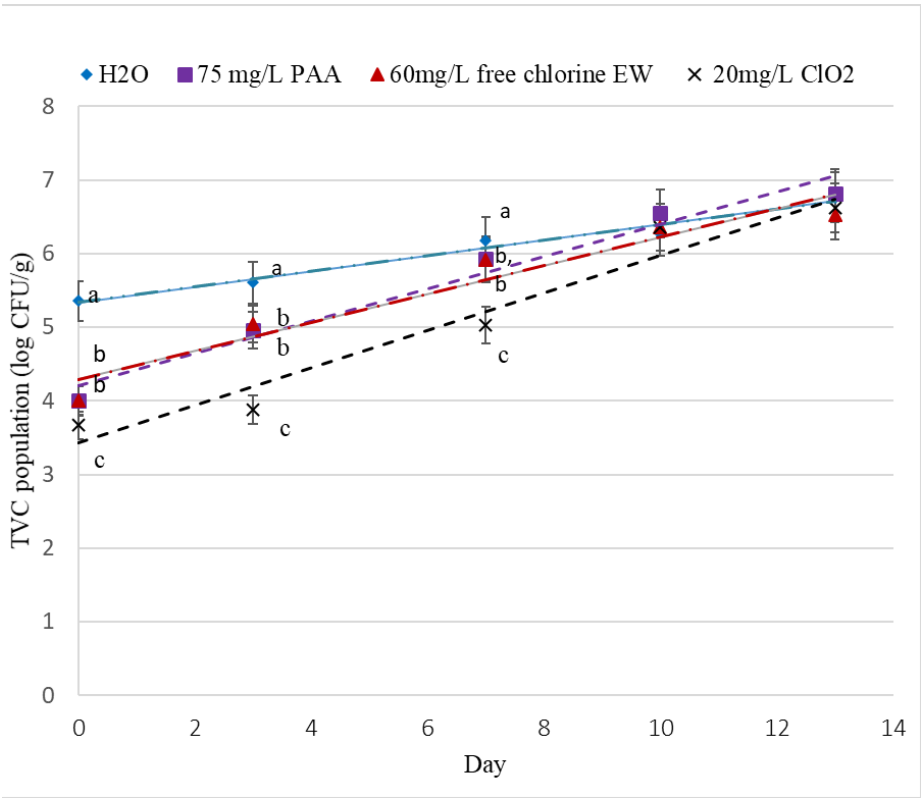
Values are means of three replicates ± standard deviations (SD).
Different letters denote significant difference between treatments.

Figure 1. Effects of sanitizing treatments on the reductions of *S.* Typhimurium (a) and TVC (b) in spinach.

3.2. Shelf life and changes in quality properties of treated spinach during the storage

3.2.1 Microbial quality (TVC)

TVC was significantly influenced by sanitizing treatments ($F_{3,40}$ = 93.337, $p < 0.05$) and storage times ($F_{4,40}$ = 535.359; $p < 0.05$). Immediately after the sanitizing step (Day 0), there was a significant difference in TVC counts among the treatments ($F_{3,8}$ = 104.734; $p < 0.05$). However, the TVC counts of the samples increased at different rates and reached the same level after 10 days of storage ($F_{3,8}$ = 1.354; $p > 0.05$) (Figure 2).



Values are means of three replicates ± SD.

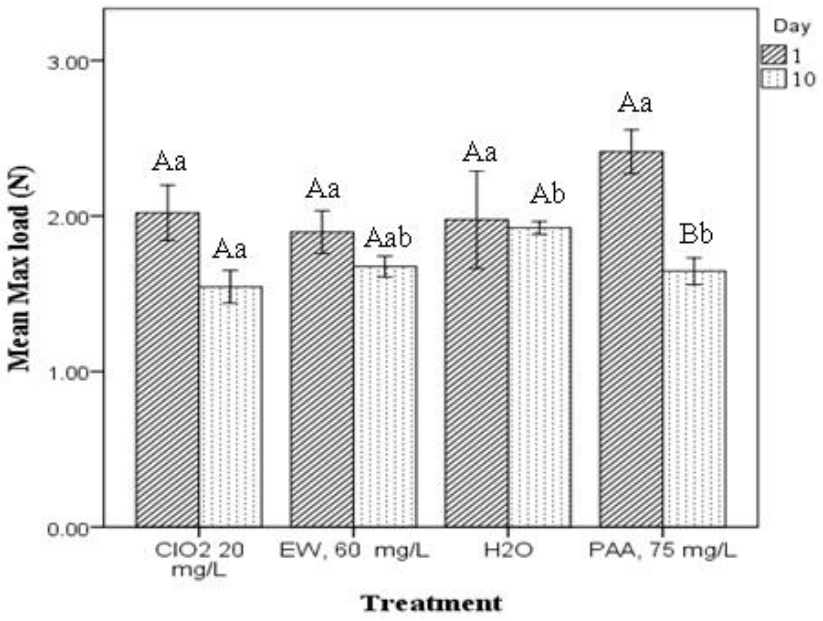
Different letters denote significant differences between treatments at each day.

Figure 2. Effect of sanitizing treatments on TVC during the storage.

More specifically, TVC load of the sample treated with 20 mg/L ClO_2 was the lowest (3.6 ± 0.1 log CFU/g) at the beginning of the storage ($p < 0.05$). However, this population showed the highest growth rate (r) compared to other treatments (r = 0.0545; $R^2 = 0.96$). The treatments with 75 mg/L PAA or 60 mg/L EW solutions reduced the native TVC to similar levels (about 4.0 log CFU/g) and the same growth rate was also observed in samples from these treatments (r = 0.042, $R^2 = 0.91$ and r = 0.038, $R^2 = 0.86$, respectively). The control sample had the highest initial microbial population (immediately after treatment) but this population grew with the lowest rate (r = 0.019; $R^2 = 0.97$) compared to the treated samples. The TVC of unsterile tap water was also analyzed during the treatment which showed less than 10 CFU/100 mL of tap water.

3.2.2. Texture

There was a significant decrease ($p < 0.01$) in the firmness (mean max load force, N) of the spinach between day 0 and day 10 but no significant difference ($p < 0.05$) was observed among the treatments (Figure 3). The general firmness of spinach in the 20 mg/L ClO_2 treatment was 2.02 ± 0.31 (N) at day 0, however, this declined to 1.54 ± 0.18 N at day 10 of the storage.



Values are means of three replicates ± SD.

Different lowercase letters show significant differences among values of different treatments ($p < 0.05$).

Different uppercase letters show significant differences between values of day 1 and day 10 ($p < 0.05$).

Figure 3. Max load force (N) of spinach pieces (1.8 cm × 4 cm) of different treatments at day 0 and day 10.

3.2.3. Chlorophyll and carotenoid concentrations

Regardless different treatments, there was a general decrease in content of chlorophyll and carotenoid of the samples during the storage. Although a significant difference in total chlorophyll among treatments and different storage time ($p < 0.05$), no significant differences in chlorophyll content were found among treated samples at day 13 of the storage (Table 2).

Table 2. Changes in total chlorophyll content of spinach from different treatments during storage.

Treatment	Storage time				
	Day 1	Day 3	Day 7	Day 10	Day 13
20 mg/L ClO_2	0.985 ± 0.106^{Bc}	0.893 ± 0.038^{Abc}	0.599 ± 0.072^{Aa}	0.811 ± 0.047^{Bb}	0.611 ± 0.075^{Aa}
60 mg/L Fch EW	0.753 ± 0.046^{Ac}	0.952 ± 0.028^{Bd}	0.612 ± 0.055^{Aa}	0.709 ± 0.037^{Bbc}	0.638 ± 0.050^{Aab}
75 mg/L PAA	1.037 ± 0.065^{Bd}	0.930 ± 0.026^{ABc}	0.732 ± 0.062^{ABab}	0.809 ± 0.076^{Ab}	0.687 ± 0.050^{Aa}
H_2O	1.011 ± 0.037^{Bc}	1.069 ± 0.017^{Cc}	0.754 ± 0.096^{Bb}	0.655 ± 0.001^{Aab}	0.586 ± 0.128^{Aa}

Values are the means of three replicates ± standard deviation

Different lowercase letters show significant differences among values within a row ($p < 0.05$).

Different uppercase letters show significant differences among values within a column ($p < 0.05$).

As regards to types of chlorophyll, the same pattern was found in the change of chlorophyll A and chlorophyll B with an overall decreasing tendency (Table 3 and Table 4). However, the ratio of these types of chlorophyll was unchanged over the storage period.

Table 3. Changes in chlorophyll A content of spinach (mg/g) from different treatments during storage.

Treatment	Storage time				
	Day 1	Day 3	Day 7	Day 10	Day 13
20 mg/L ClO_2	0.759 ± 0.070^{Bc}	0.678 ± 0.033^{Abc}	0.460 ± 0.057^{Aa}	0.611 ± 0.035^{Cb}	0.468 ± 0.057^{Aa}
60 mg/L Fch EW	0.580 ± 0.026^{Ab}	0.729 ± 0.027^{Bc}	0.479 ± 0.033^{ABa}	0.551 ± 0.023^{ABb}	0.490 ± 0.028^{Aa}
75 mg/L PAA	0.768 ± 0.060^{Bc}	0.730 ± 0.020^{Bc}	0.558 ± 0.036^{ABab}	0.627 ± 0.067^{BCb}	0.516 ± 0.052^{Aa}
H_2O	0.754 ± 0.030^{Bc}	0.836 ± 0.007^{Cc}	0.575 ± 0.077^{Bb}	0.499 ± 0.003^{Aab}	0.460 ± 0.106^{Aa}

Values are the means of three replicates ± standard deviation.

Different lowercase letters show significant differences among values within a row ($p < 0.05$).

Different uppercase letters show significant differences among values within a column ($p < 0.05$).

Table 4. Changes in chlorophyll B content of spinach from different treatments during storage.

Treatment	Storage time				
	Day 1	Day 3	Day 7	Day 10	Day 13
20 mg/L ClO_2	0.226 ± 0.040^{Bb}	0.214 ± 0.013^{ABa}	0.139 ± 0.016^{ABa}	0.120 ± 0.012^{Cb}	0.142 ± 0.022^{ABb}
60 mg/L Fch EW	0.173 ± 0.024^{Ab}	0.226 ± 0.009^{Bc}	0.134 ± 0.023^{Aa}	0.157 ± 0.018^{ABab}	0.148 ± 0.023^{ABab}
75 mg/L PAA	0.269 ± 0.010^{Bc}	0.201 ± 0.007^{Ab}	0.174 ± 0.025^{ABa}	0.182 ± 0.013^{BCab}	0.170 ± 0.004^{Ba}
H_2O	0.257 ± 0.013^{Bc}	0.233 ± 0.016^{Bc}	0.179 ± 0.020^{Cb}	0.156 ± 0.005^{Aab}	0.127 ± 0.025^{Aa}

Values are the means of three replicates ± standard deviation.

Different lowercase letters show significant differences among values within a row ($p < 0.05$).

Different uppercase letters show significant differences among values within a column ($p < 0.05$).

Similarly, there were no significant differences found in carotenoid level among the treatments but there is a significant change in carotenoid concentration of the samples over the storage period (Table 5).

Table 5. Changes in total carotenoid content of spinach from different treatments during storage.

Treatment	Storage time				
	Day 1	Day 3	Day 7	Day 10	Day 13
20 mg/L ClO_2	0.229 ± 0.017^{Bc}	0.194 ± 0.007^{Ab}	0.144 ± 0.015^{Aa}	0.197 ± 0.019^{Bb}	0.157 ± 0.023^{Aa}
60 mg/L Fch EW	0.177 ± 0.012^{Ac}	0.213 ± 0.014^{Bd}	0.152 ± 0.002^{ABa}	0.174 ± 0.009^{Abc}	0.160 ± 0.004^{Aab}
75 mg/L PAA	0.227 ± 0.021^{Bc}	0.228 ± 0.010^{Bc}	0.173 ± 0.009^{BCa}	0.199 ± 0.007^{Bb}	0.164 ± 0.011^{Aa}
H_2O	0.229 ± 0.013^{Bb}	0.261 ± 0.009^{Cb}	0.179 ± 0.021^{Ca}	0.156 ± 0.001^{Aa}	0.147 ± 0.034^{Aa}

Values are the means of three replicates ± standard deviation

Different lowercase letters show significant differences among values within a row ($p < 0.05$).

Different uppercase letters show significant differences among values within a column ($p < 0.05$).

3.2.4 Sensory quality

Overall, there was a significant difference in the visual quality of spinach among different treatments ($\chi 2$ (3, n = 300) = 13.735, $p < 0.05$) with a general decline observed over the storage period. While the treatment with 20 mg/L ClO_2 resulted in a dramatically decrease of visual scores, slight decreases were observed in samples treated with other sanitizers and tap water (Table 6). All

samples, except the sample treated with 20 mg/L ClO_2, remained above acceptable levels (score of 3 or greater) over 13 days of storage.

From day 7 of the storage, severe bruising and physical damage were observed in the sample treated with 20 mg/L ClO_2. Severe sliming also occurred on the samples from this treatment since day 10 and caused a significant decrease in visual quality. In addition, sweating occurred inside the bags containing spinach from all treatments since day 7 of the storage.

Table 6. Overall visual score of samples treated with different sanitizers and tap water over 13 days of the storage**.**

Treatment	Storage time				
	Day 1	Day 3	Day 7	Day 10	Day 13
20 mg/L ClO_2	5.00 ± 0.00^{Ac}	4.94 ± 0.09^{Ac}	4.09 ± 0.04^{Ab}	1.90 ± 0.16^{Aa}	1.62 ± 0.54^{Aa}
60 mg/L Fch EW	5.00 ± 0.00^{Ac}	4.95 ± 0.04^{Ac}	4.86 ± 0.07^{Bbc}	4.59 ± 0.32^{Cb}	3.26 ± 0.08^{Ba}
75 mg/L PAA	4.95 ± 0.04^{Ac}	4.97 ± 0.04^{Ac}	4.97 ± 0.04^{Cc}	4.04 ± 0.14^{Bb}	3.33 ± 0.48^{Ba}
H_2O	5.00 ± 0.00^{Ab}	4.95 ± 0.04^{Ab}	4.97 ± 0.04^{Cb}	4.00 ± 0.39^{BCb}	3.50 ± 0.50^{Ba}

Values are the means of three replicates ± standard deviation. The highest score denotes the best visual quality.

Different lowercase letters show significant differences among values within a row ($p < 0.05$).

Different uppercase letters show significant differences among values within a column ($p < 0.05$).

4. Discussion

4.1. The antimicrobial efficacy of sanitizers used for washing inoculated spinach

In this study, the treatment with 20 mg/L aqueous ClO_2 for 1 minute of contact time reduced *S.* Typhimurium on inoculated spinach by 1.6 ± 0.1 log CFU/g from the initial amount of 5 log CFU/g. This reduction was higher than that reported in some other studies. For examples, Keskinen et al. (2009) found 1.04 and 0.76 log CFU/g of *E. coli* O157H7 reductions on inoculated Iceberg and Romaine lettuce, which had the initial load of 6–7 log CFU/g, washed with 20 mg/L ClO_2 solution (pH = 2.6) for 2 minutes, respectively [9]. However, the inoculated lettuce used in their study was incubated for 24 hours at 4 ℃ before the treatment. Microorganisms were incubated for 24 hours on lettuce may become more resistant to sanitizing treatments. Wu and Kim (2007) reported that blueberries (5 log CFU/g on contaminated sample) treated with 15 mg/L aqueous ClO_2 for 1 minute at 21 ℃, reduced *S.* Typhimurium by only 0.23 log CFU/g [40]. The difference in the findings between such studies could result from higher concentration (20 mg/L) of ClO_2 used in this study than that used in the study of Wu and Kim (2007) [40]. Also, the difference between blueberry and spinach surface in this study could result in different antimicrobial efficacy. The inhibitory efficacy may be affected by types of fresh produce and their physical surface characteristics such as hydrophobic tendency and texture like surface crevices and cracks [42].

In the present study, the sanitizing treatments were conducted immediately after the inoculation step. Maintaining inoculated samples for longer (e.g. several hours) before conducting sanitation could enhance the attachment of the cells. For example, the sanitizing treatment was less effective against inoculated samples which were kept at 4 ℃ for 12 hours before treating [43]. Additional contact time may allow introduced strains to attach better to samples and behave as naturally attached cells. In other words, native microorganisms are more resistant to sanitizers probably

because the attachment of viable cells in the natural conditions would be stronger than that in the artificial inoculation conditions [2].

With regard to the other treatments, the results of this study for antimicrobial efficacy against inoculated strains on leafy vegetables were consistent with previous published studies. Neal et al. (2012) reported that a 0.7 log CFU/g of *Salmonella* reduction was achieved by washing the inoculated spinach (about 6 log CFU/g) with tap water and a *S.* Typhimurium reduction of 0.62 log CFU/g was also obtained [44]. They also found that the treatment with 80 mg/L PAA could reduce *Salmonella* spp. by 0.8 log CFU/g on inoculated spinach (5.1 log CFU/g). Additionally, Buchholz (2010) revealed that *S.* Stanley on alfalfa seeds was reduced by 1 log CFU/g when the samples were treated with 0.1–0.3% PAA solution for 15 minutes [45]. The reductions attained in those studies were not statistically significantly different to that in water-only treatments. Regarding EW treatment, Pinto, Ippolito and Baruzzi (2015) reported that the treatment with 200 mg/L FCh EW at 4^{o}C for 5 minutes reduced *Pseudomonas* spp. by about 1 log CFU/g on the inoculated lettuce leaves (initial load was 7.5 log CFU/g) [46]. These findings are similar to the *S.* Typhimurium CFU/g reduction ranges of the treatments with EW (20 mg/L and 60 mg/L FCh) and the 50 mg/L PAA (0.9–1.0 log CFU/g) in this study.

However, there are some inconsistencies between this study and relevant studies with regards to the antimicrobial efficacy of the sanitizers. Significant microbial reductions on spinach treated with neutral EW solutions (20 mg/L and 50 mg/L FCh; pH = 6.3–6.5) for 10 minutes at 25 ℃ were reported by Guentzel et al. (2008) [47]. In this study, results did not confirm those significant reductions ($p < 0.05$). However, there were differences in study conditions that could explain the differences in the findings. This includes that their treatment time (10 minutes) was longer than the treatment time (1 minute) in the current study.

Although the inoculated *S.* Typhimurium may lead to a higher number in TVC population of the treatments in the present study, there are some similarities among findings obtained between the current research and previous studies. Lopez-Galvez (2013) [19] observed 0.9 log CFU/g mesophilic bacterial reduction by washing fresh-cut lettuce (initial microbial population of 5–7 log CFU/g) with 80 mg/L PAA solution for 1 minute. That reduction is similar to the results in this study in which the 50 mg/L PAA treatment reduced TVC on spinach by 1 log CFU/g. Izumi (1999) [48] and Tomás-Callejas et al. (2011) [49] have found a significant disinfectant effect when spinach leaves (5–6 log CFU/g) were washed with neutral EW solution (20 mg/L FCh) for 3 minutes followed by 1 minute rinsing with tap water reduced TVC by 0.7–1.1 log CFU/g.

On the other hand, it is necessary to taken into account that strong acidic solutions can reduce the sensory quality of leafy vegetables [1,13]. For example, Neal et al. (2012) [44] addressed the effects of sanitation treatments at low pH (pH 2.6) on spinach sensory quality. The decrease in pH level may result in the increase of chlorophyll and other pigment degradations of leafy vegetable thereby decreasing the visual quality [44,50].

4.2. Effect of sanitiser treatments on the microbial quality of spinach in the shelf life trial

Similar microbial population changes on fresh produce treated with sanitizers were reported in previous studies. Chen et al. (2010) [51] found 6.7±0.5 log TVC CFU/g were present in lettuce samples washed with 100 mg/L ClO_2 solution in 20 minutes with ratio 1:5 (w/v) at Day 14 of storage. Those findings are similar to current study's results although a lower concentration of ClO_2 solution (20 mg/L) was used. However, from microbial quality point of view, unacceptable TVC

population levels (>8 log CFU/g) of control samples were reported in study of Chen et al. (2010) [51]. This difference could be due to the difference in surface and texture characteristics of spinach and lettuce.

This study has highlighted trends in population kinetics of microbes on leafy produce washed with sanitizers. Antimicrobial treatments, as many authors have reported, can reduce initial native or inoculated microbial populations of treated produce [6,45,52]. However, these counts eventually increased and even exceeded the level of corresponding untreated or control samples over the storage period [53–55]. The growth rates in sanitized samples were higher than untreated or control samples. This phenomenon could possibly be explained by more space or 'room' for microorganism to grow when initial populations are reduced [56], though simple calculations of the size of bacteria and space available suggest that this idea is not credible. Similarly, Nicholl et al. (2004) [57] suggested that the surviving microorganisms could grow more rapidly due to less competitors for nutrients though, again, simple calculations of stoichiometry of growth suggest this is unlikely until very high cell densities (e.g., $\gg 10^7$ CFU/g) are again achieved. More credibly, it is postulated that treatments could cause physical damages to tissues which provide easily accessed nutrient sources. Severe physical damages were visually observed on spinach in the treatments of the currents study from Day 7. Consequently, the microorganisms could grow faster owing to such conditions [58].

Some authors hold the view that surface produce treatments are not effective in reducing microbial loads on leafy vegetables [6,43,59]. For instance, Allende et al. (2008) [43] found that there was no significant difference in the microbial reduction between washed and unwashed fresh-cut escarole. However, this is not consistent with the findings of this study since the 20 mg/L ClO_2 treatment did reduce microbial loads on spinach by a significant quantity. Also, it is necessary to note that agitation of the leaves in the sanitizing solutions as occurs in industrial conditions to intensify the contacting between samples and sanitizing solution did not occur in our experiments. Thus, there may be differences between present experimental findings and industrial experience.

4.3. The effects of sanitizing treatments on chlorophyll and carotenoid contents

There was an overall decrease in chlorophyll and carotenoid content across all sanitizing treatments and the tap water control. The degradation of chlorophyll and carotenoids of spinach untreated or treated with different sanitizers were reported in some studies [60,61]. As pH of washing solution was reduced, the chlorophyll degradation during storage of samples was increased [44]. Similar findings were presented by Martínez-Sánchez et al.(2006) [61] when rocket leaves were treated with 300 mg/L PAA, 100 mg/L chlorine and 20 mg/L lactic acid solution. These degradations of chlorophyll and carotenoids in spinach samples throughout the storage could be influenced by other factors under storage conditions [62]. However, our results did not show a significant difference between treatments in chlorophyll and carotenoid concentrations of spinach washed with these sanitizing solutions.

4.4. Effects of sanitation treatments on the sensory quality and physio-chemical properties of spinach after the treatment

With the exception of the 20 mg/L ClO_2 treatment, the sensory evaluation rated all other treatments as being above acceptable levels (scores of 3) at the end of the 13-day shelf life trial. These results are similar to previous findings for EW and PAA sanitizers used for fresh-cut produce samples. For examples, Tomás-Callejas et al. (2011) [49] reported that baby mizuna leaves treated

with neutral and acidified EW solutions retained acceptable levels of sensory attributes after 11 days of storage. According to Izumi (1999) [48] and Koseki et al. (2001) [56], acidic EW solution significantly reduced the occurrence of browning in fresh lettuce. The high ORP of EW is suggested to inhibit or slow down the activities of browning enzymes [56]. Another hypothesis is that NaCl present in EW may inhibit polyphenol oxidase due to the formation of complex between copper in the enzyme and halide ions [63]. Chen et al. (2010) [51] conducted experiments with asparagus washed with 100 mg/L ClO_2 treatment (1:5 (w/v)) at 20–24 ℃ for 1 minute, re-rinsed and stored at 4 ℃ for 14 days. Their findings showed browning enzymes were inhibited.

As regards the 20 mg/L ClO_2 treatment, the organoleptic assessment revealed a significant reduction in sensory quality, particularly physical damage and bruising, after 7 days of the storage. Very few studies have reported this phenomenon possibly because most studies with ClO_2 treatment include a rinsing step after sanitizing treatment [27,51,64]. The ClO_2 residue left after draining may be responsible for compromising the sensory quality of the product. A rinsing step after sanitation was not included in the study as this step is generally not practical by industry (Mr. Tesh Sharma, personal communication, March 2016). As noted in previous studies, the efficacy of a sanitizer may be influenced by the differences between laboratory and industrial conditions or commercial processing conditions [5,23]. Thus, although the 20 mg/L ClO_2 solution could be effective in reducing microbial load, it was not able to extend the shelf life of fresh-cut spinach products under these conditions. If this sanitizer is applied at this level without the rinsing step for fresh-cut leafy vegetables, it will be more important to quantify the amount of ClO_2 residue on the produce before storage and distribution [65]. According to Food and Drug Administration (FDA) [66], the free ClO_2 residue should not exceed 3 mg/L in the final product**.**

5. Conclusion

Despite the fact that sanitizers are selected based on the decontamination efficacy, it is vital to consider the effect of them on fresh produce's shelf life and sensory quality and customer's expectations in the reality. In this study, a significant microbial load reduction was observed on spinach treated with 20 mg/L ClO_2 solution but this treatment was detrimental to sensory quality of spinach throughout the storage. The PAA and EW treatments also showed similar antimicrobial efficacy but were not effective against *S.* Typhimurium. Thus there is a need to optimize experimental conditions and develop alternative sanitation methods to improve the food safety, shelf-life and quality of fresh-cut spinach during storage. To optimize research methodology, inoculated samples should be incubated longer before sanitation treatment in order to ensure the attachment more similar to that of the natural microbiota. Potable water used in experiments of this study may differ from water used under industrial conditions so further studies evaluating the efficacy of sanitizers should take into account the ratio between water and waste water used in industry practice. In addition, it would be better if samples for analysis were collected in-situ on the line of processing in factories.

Acknowledgments

The authors are grateful to Houston's Farm Cambridge, Tasmania, Australia and their staff, who kindly provided fresh spinach and packaging materials, especial thanks to Prof Roger Stanley, University of Tasmania, Launceston, Australia for kindly providing EW solution.

Conflict of interest

There is no conflict of interest for this journal article.

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