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Effectiveness of a peracetic acid solution on *Escherichia coli* reduction during fresh-cut lettuce processing at the laboratory and industrial scales

J.L. Banach[a,*], H. van Bokhorst-van de Veen[b], L.S. van Overbeek[c], P.S. van der Zouwen[c], M.H. Zwietering[d], H.J. van der Fels-Klerx[a]

[a] Wageningen Food Safety Research, P.O. Box 230, 6700 AE Wageningen, the Netherlands
[b] Wageningen Food & Biobased Research, P.O. Box 17, 6700 AA Wageningen, the Netherlands
[c] Wageningen Plant Research, P.O. Box 16, 6700 AA Wageningen, the Netherlands
[d] Food Microbiology, Wageningen University, P.O. Box 17, 6700 AA Wageningen, the Netherlands

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ABSTRACT

Fresh leafy greens like lettuce can be consumed raw and are susceptible to foodborne pathogens if they become contaminated. Recently, the number of reported pathogenic foodborne outbreaks related to leafy greens has increased. Therefore, it is important to try to alleviate the human health burden associated with these outbreaks. Processing of fresh-cut lettuce, including washing, is a step in the supply chain that needs to be well controlled to avoid cross-contamination. Current measures to control the quality of lettuce during washing include the use of chemicals like chlorine; however, questions regarding the safety of chlorine have prompted research for alternative solutions with peracetic acid (PAA). This study evaluates the effectiveness of a PAA (c.a. 75 mg/L) solution on the reduction of a commensal *E. coli* strain during the washing of fresh-cut lettuce. Experiments were performed at the laboratory scale and validated at the industrial scale. We observed that the use of PAA was not adversely affected by the organic load in the water. The contact time and dose of the PAA showed to be relevant factors, as observed by the approximately 5-log reduction of *E. coli* in the water. Results showed that once introduced during washing, *E. coli* remained attached to the lettuce, thus supporting the need to control for pathogenic bacteria earlier in the supply chain (*e.g.*, during primary production) as well as during washing. Moreover, our results showed that the use of PAA during washing did not have an apparent effect on the levels of fluorescent pseudomonads (FP) and total heterotrophic bacteria (THB) in lettuce. Overall, our results at the laboratory and industrial scales confirmed that during the processing of fresh-cut produce, where the accumulation of soil, debris, and other plant exudates can negatively affect washing, the use of a PAA (c.a. 75 mg/L) solution was an effective and safe wash water disinfectant that can potentially be used at the industrial scale.

1. Introduction

Fresh leafy greens like lettuce can be consumed raw and are susceptible to food borne pathogens if they become contaminated. Several outbreaks of *Escherichia coli* O157:H7 related to leafy greens have been reported recently in the United States (CDC, 2018a, 2018b, 2018c). Therefore, it is important to try to alleviate the human health burden associated with these outbreaks. During fresh-cut vegetable processing, the cutting and washing steps can encourage the opportunity for (pathogenic) cross-contamination. Therefore, these steps are critical to control in order to ensure food safety and quality during processing. Since washing can bring the possibility for pathogen survival and cross-contamination, additional measures to control the water quality, such as disinfecting the wash water, would then be needed.

One commonly applied disinfectant used during fresh(-cut) vegetable processing is chlorine. There is an ongoing discussion surrounding the use of chlorine, given the potentially harmful by-products that may form (*e.g.*, trihalomethanes and haloacetic acids) and its effect on public health. This discussion has prompted research for alternative wash water disinfectants to chlorine (Allende et al., 2008; Banach et al., 2015; Fatica and Schneider, 2009; Meireles et al., 2016; Ölmez and Kretzschmar, 2009) such as peracetic acid (PAA) which has a similar effectivity and is less controversial in terms of its effect on public health. In comparison to other chemical disinfectants, PAA is an oxidizing agent that can dissolve in water to hydrogen peroxide and acetic acid, which can further break down into water, oxygen, and carbon

* Corresponding author.
E-mail address: jen.banach@wur.nl (J.L. Banach).

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dioxide. By-products of PAA are non-toxic, while only negligible or low levels of aldehydes (Banach et al., 2015; Van Haute et al., 2015) and modest levels of carboxylic acids have been reported to form (Dominguez Henao et al., 2018). PAA disinfection has been attributed to the denaturation of proteins and enzymes and increased cell wall permeability due to the disruption of sulfhydryl (-SH) and sulfur (S-S) bonds (McDonnell and Russell, 1999; Muñío and Poyatos, 2011). In brief, PAA is an alternative water disinfectant to consider for use during food processing.

PAA disinfection in other domains, like that for wastewater treatment, has been studied and reported in the scientific literature. The attributes of PAA such as its ease of implementation also considering costs, broad-spectrum of activity given organic matter presence, short contact time, limited dependence on pH, and absence of toxic or mutagenic residual by-products, make it advantageous to use for wastewater disinfection (Fatica and Schneider, 2009; Kitis, 2004). Nonetheless, the application of PAA to improve the microbial quality of water effluent needs further research to ascertain its efficacy (Bonetta et al., 2017). Similarly, additional research is needed in the food (safety) domain. The use of PAA for treating water has been suggested for agricultural practices (Van Haute et al., 2015). Research has shown the effect of PAA on foodborne pathogens like *E. coli* O157:H7 in the residual water after lettuce processing, demonstrating that PAA may help prevent cross-contamination (Baert et al., 2009). However, experiments at the laboratory scale and validation of industrial-scale processing of (fresh-cut) produce are needed (Banach et al., 2015).

The objective of this study is to evaluate the effectiveness of PAA to disinfect the water used during the washing of fresh-cut lettuce at the laboratory and industrial scales. We compared the results of the treated water, namely the effect of a PAA solution on the reduction of *E. coli* in the water, by evaluating the physicochemical and microbial quality of the water and the microbial quality of the fresh-cut lettuce.

2. Materials and methods

This study was conducted at both laboratory and industrial scales. Experiments used the same bacterial strain (Section 2.1), PAA solution (Section 2.2), and statistical analyses (Section 2.5); preparation differences are specified. The experimental design, subsequent materials and processing, and analyses are described for laboratory (Section 2.3) and industrial-scale experiments (Section 2.4).

2.1. Bacterial strain and inoculum preparation

A commensal *E. coli* strain (meaning without any selectable makers like extended-spectrum beta-lactamase (ESBL)) isolated from surface water, previously reported as 12–123.2 (Banach et al., 2018), had been stored at -80 °C in Luria Broth (LB; L1704 LB Broth High Salt, Duchefa Biochemie B.V., Haarlem, the Netherlands), supplemented with 25% (v/v) glycerol, before use. The strain was streaked on Brilliance *E. coli* coliform selective agar (BECSA; CM1046, Oxoid Ltd., part of Thermo Fisher Scientific, Basingstoke, United Kingdom). Cultures were prepared by transferring a single colony from a BESCA plate to 25 mL LB followed by incubation at 37 °C in a 200 rpm shaking incubator until either the exponential phase (4–8 h) or the stationary phase (16–18 h), respectively, for laboratory and industrial scale experiments.

For laboratory experiments, the cells were transferred into sterile Eppendorf tubes (1.5 mL volume) with 1 mL LB media and centrifuged at 9391 ×g (Eppendorf Microcentrifuge 5415, VWR International B.V., Amsterdam, the Netherlands) for 1 min at room temperature. After centrifugation and decanting, the cells were resuspended in sterile Ringer's solution (BR0052; Oxoid Ltd., part of Thermo Fisher Scientific, Breda, the Netherlands). Centrifugation, decanting, and resuspension in Ringer's solution proceeded twice more; however, before the final resuspension, cells were measured at an optical density of 600 nm (OD_{600}; Ultrospec 10 Cell Density Meter, Amersham Biosciences) and then were diluted with sterile Ringer's solution to an OD_{600} of 0.1 (given a start suspension of about 10^9 CFU/mL).

For industrial-scale experiments, cells were not washed as described above for the laboratory experiments; instead they were further prepared as described by Banach et al. (2018) of which about 4.5 L of the liquid cultures (c.a. 10^9 CFU/mL) was added to the wash tank (3.5 m^3), resulting in a final concentration of about 10^6 CFU/mL.

2.2. Peracetic acid solution

A commercial solution with a concentration of 15.2% PAA and 17.1% hydrogen peroxide (H_2O_2) was used (Tsunami 100, Ecolab B.V., Nieuwegein, the Netherlands). The following procedure was used to obtain the desired concentrations. A two-step iodometric titration procedure, based on Greenspan and MacKellar (1948) and Sully and Williams (1962), was used to determine PAA and H_2O_2 concentrations. First, H_2O_2 was consumed from the addition of potassium permanganate solutions, and then, PAA was titrated with iodide/thiosulfate. Here, 25 mL of 25% sulfuric acid (diluted from 98% sulfuric acid, k47573680, Merck) was added to a 50 mL sample mixed with 50 mL tap water at 20–25 °C. The solution was then mixed and titrated with 0.1 N potassium permanganate (Fixanal, 38136-1EA, Fluka) until a stable, faint pink color appeared. The amount (mL) of consumed potassium permanganate was multiplied by 17 to calculate the concentration of H_2O_2 (ppm) of the solution. Then, 1–2 g of potassium iodide (6227.1000, J.T. Baker A.C.S.) was added and mixed, followed by 20 mL of 25% sulfuric acid (diluted from 98% sulfuric acid, k47573680, Merck). The solution was then titrated with a 0.1 N sodium thiosulfate solution (Titrisol, 1.09961.0001, Merck) using a starch indicator (3 drops of a 1% solution, Zulkowsky, 1.01257.0250, Merck) to show the end of the titration. The amount (mL) of consumed potassium permanganate was multiplied by 34 to calculate the available PAA concentration (ppm) of the solution. For laboratory experiments, concentrations of 0, 20, 40, 60, and 80 mg/L PAA were used.

For industrial-scale experiments, the target concentration was about 75 mg/L of PAA and was continuously supplied to the washing tank throughout processing up until the input of the lettuce stopped (*i.e.*, after 90 min of processing). The PAA and H_2O_2 concentrations were determined, as indicated above, and manually adjusted during the first 90 min of processing to obtain the target concentration (ELADOS® EMP II, E10 or 60 Diaphragm Metering Pump, Ecolab B.V., Nieuwegein, the Netherlands). PAA and H_2O_2 measurements during processing were performed about every 20 min and repeated for verification as required.

2.3. Laboratory experiments

Laboratory experiments evaluated the effectiveness of the PAA solution in laboratory-made washing water and on 'Batavia' lettuce washed with laboratory-made washing water. PAA was tested with and without the addition of *E. coli*. Experiments with tap water and non-supplemented *E. coli* served as controls. Water and lettuce were quantitatively examined for the presence of *E. coli*.

2.3.1. Laboratory-made wash water

Laboratory wash water was made from whole endive, which was purchased from a local supermarket (Wageningen, the Netherlands) and transported within 15 min to the laboratory. The endive was used to make the wash water as previous analyses of endive washing waters had shown the highest concentrations of total organic carbon (TOC) (data not shown). The outer leaves of the endive were manually removed and discarded, while the internal leaves were cut by hand and washed with 2 L of potable (tap) water. Cutting and washing were repeated twice with the same endive, each time using the same wash water. The endive was cut into 1 cm pieces and then into about 0.5 cm pieces. After aliquoting, the wash water was stored at −20 °C until further use during experiments.

2.3.2. Experiments with laboratory-made wash water

The laboratory-made wash water was defrosted and then diluted with cold tap water to obtain TOC concentrations of about 500 mg/L and 750 mg/L, reflecting high and very high organically loaded waters, respectively. The TOC was determined before experiments with PAA at 0, 20, and 40 mg/L (Shimadzu 5050A). These PAA concentrations were chosen to evaluate the effect of lowered PAA concentrations on *E. coli*.

The efficacy of the PAA solution at 0, 20, and 40 mg/L on *E. coli* (initially about $5 \cdot 10^6$ CFU/mL) in tap water, and laboratory-made wash water with TOCs of 500 and 750 mg/L were used. During these experiments, the *E. coli* culture was periodically swirled and maintained at a temperature of about 4–5 °C to reflect industrial conditions. The pH was measured before and after the experiments. The PAA and H_2O_2 concentrations of the stocks were measured using iodometric titration, as previously described. The PAA solution was freshly prepared before each experiment. At regular time intervals, 1 mL samples were taken and serially diluted into a peptone physiological salt solution (PPS; Tritium Microbiologie B.V., Eindhoven, the Netherlands); neutralizing agents were not applied. After 1, 3, and 5 min of treatment with the PAA solution, 100 μL of the appropriate dilutions were plated on BECSA and incubated at 37 °C for 2 d (*i.e.,* until no additional colonies appeared). The number of culturable cells was determined at 0, 1, 3, and 5 min to compare the efficacy over time. Also, a control with no disinfectant was included and determined at 0 and 12 min.

2.3.3. Lettuce

Batavia lettuce (*Lactuca sativa* L. *var. capitata*) was purchased from a local farm (De Hoge Born, Wageningen, the Netherlands). All lettuce was stored at 4 °C and used within 14 d of delivery. Circular punches of the lettuce were made (c.a. 1.5 g) and pre-treated as described by Banach et al. (2017) with slight variations as follows. Lettuce leaf punches ($n = 2$) were placed into each Petri dish and inoculated by pipetting 10 μL of a 100× diluted *E. coli* starting suspension (which was c.a. $10^{5.9}$ CFU/mL). After 1 h incubation at room temperature, *E. coli* liquid drops were removed with sterile filter paper.

2.3.4. Experiments with lettuce

The lettuce leaf punches were cut and exposed to *E. coli* as previously described. The laboratory-made wash water was defrosted and then diluted with cold tap water to obtain TOC concentrations of about 500 mg/L and 750 mg/L. The TOCs were verified directly before experiments with PAA at 0, 60, and 80 mg/L (Skalar SFA, model SAN++ in accordance with NEN-EN 1484). These PAA concentrations were chosen to correlate with the industrial-scale experiments.

Lettuce leaf punches were quickly prewashed with 40–50 mL of tap water. Then, lettuce leaf punches were gently shaken and treated for 2 min at room temperature with 20 mL of water at 4–5 °C (*i.e.*, with either tap water or laboratory-made washing waters with TOCs of 500 mg/L or 750 mg/L). Treatments were with and without 60 and 80 mg/L PAA, after which lettuce punches were rinsed with 50 mL tap water to remove possible residues before further analysis; neutralizing agents were not applied. Afterward, the lettuce punches were transferred to BioReba bags (BioReba AG, Reinach, Switzerland) containing 1 mL sterile Ringer's solution (BR0052; Oxoid, part of Thermo Fisher Scientific, Breda, the Netherlands) and gently homogenized. Tenfold serial dilutions in Ringer's solution of the homogenized lettuce leaf punches were plated on BESCA and incubated for 18–24 h at 37 °C for the recovery of *E. coli* CFUs. Independent experiments on lettuce were carried out in duplicate ($n = 2$) each time by using four leaf punches from two separate plants.

2.4. Industrial-scale experiments

Industrial-scale experiments assessed the efficacy of a PAA solution during 'Lollo Rossa' lettuce processing (800 kg), with and without the addition of *E. coli*. A target concentration of about 75 mg/L of PAA was assessed in the wash tank (3.5 m^3 flotation washer, Remie, build year 1997). Experiments with disinfectant-free (tap) water and non-supplemented *E. coli* served as controls. Two independent runs of the washing operations were conducted for both experiments with and without *E. coli* additions. Lettuce processing took about 90 min, after which the PAA supply stopped and, when applicable, *E. coli* was added directly to the water of the wash tank.

Before each experiment, the processing line was swabbed to verify hygiene (*i.e.*, to check for the absence of background *E. coli)* with swab rinse kits (SRK; 922C,CR, SRK 10 mL TRIPLE PACKED, Copan Italia SpA, Brescia, Italy) as described by Banach et al. (2018). Water and lettuce samples collected during the experimental runs were quantitatively examined for *E. coli*. Furthermore, water samples were analyzed for several physicochemical parameters: pH, T, ammonium-N (NH_4 –N), nitrate-N ((NO_3 + NO_2) –N), phosphate-P (PO_4 –P), TOC, and chemical oxygen demand (COD). Stored lettuce samples were quantitatively examined for *E. coli* as well as microbial communities: fluorescent pseudomonads (FP) and total heterotrophic bacteria (THB).

2.4.1. Lettuce and processing line

Lolla Rossa lettuce (*Lactuca sativa* var. crispa 'Lollo Rossa') cultivated in Spain was delivered (< 7 °C) to a Dutch processor who stored it at 4 °C and used it for experiments within 3 d of delivery. Lettuce was cored and pre-trimmed onsite by hand before further processing. A small-scale commercial lettuce processing line consisting of a lettuce shredder, step conveyor, infeed vibrator, washer — with output trill band and produce chute, and centrifuge was used for processing as described by Banach et al. (2018). PAA and potable (tap) water were supplied *via* inlets on the washer furthest from the product inflow and *E. coli* supply. PAA and lettuce were supplied for 90 min after which the inflow of each stopped and, when included in the treatment, the *E. coli* were added to the wash tank. The processing line continued running for an additional 12 min (*i.e.*, 102 min after the initial start).

2.4.2. Sample collection and analyses

Sample collection of the process wash water (PWW) and lettuce was performed as described by Banach et al. (2018). A diagram of the commercial lettuce processing line with PWW, lettuce, and swab sampling points is depicted in Fig. 1.

The PWW samples (~2 L) were collected from the wash tank after 80, 91, 93, 96, and 102 min of processing, corresponding to the first, second, third, fourth, and fifth-time points for water samples. These times were chosen as once the inflow of lettuce stops (at 90 min), the outflow of lettuce is minimal after 96 min. For microbiological analyses, 100 μL was directly plated on BECSA, and 1 mL was serially diluted into PPS, of which 100 μL of the appropriate dilutions were subsequently plated; all plating took place on-site. Plates were transported the same day to the laboratory. Plates were then incubated at 37 °C with daily inspection of colonies for up to one week. In addition to *E. coli* quantification, the concentration of the PAA and H_2O_2, as well as the temperature and pH of the PWW, were periodically determined during processing by collecting 50 mL of PWW (in duplicate) and analyzed with iodometric titration as described earlier (Section 2.2). For chemical analyses, PWW samples were stored in sealed containers and transported under refrigerated conditions to the laboratory before analyses for pH, ammonium-N (NH_4 –N), nitrate-N ((NO_3 + NO_2) –N), phosphate-P (PO_4 –P), TOC, and COD.

Lettuce samples were collected after 80, 91, 93, and 95 min of processing (*i.e.*, 10 min before and 1, 3, and 5 min after the PAA and lettuce inflow stopped). Samples were collected from the outflow of the line before being centrifuged (Zyliss Smart Touch Salad Spinner, Farnborough, United Kingdom) and processed on-site. A sample of the lettuce from the crate was also taken. Lettuce (10 g) was rinsed with potable water, twice, transferred to BioReba bags (Bioreba AG, Reinach, Switzerland) to which about 10 mL sterile Ringer's solution were added before being gently homogenized. Subsequently, tenfold serial dilutions

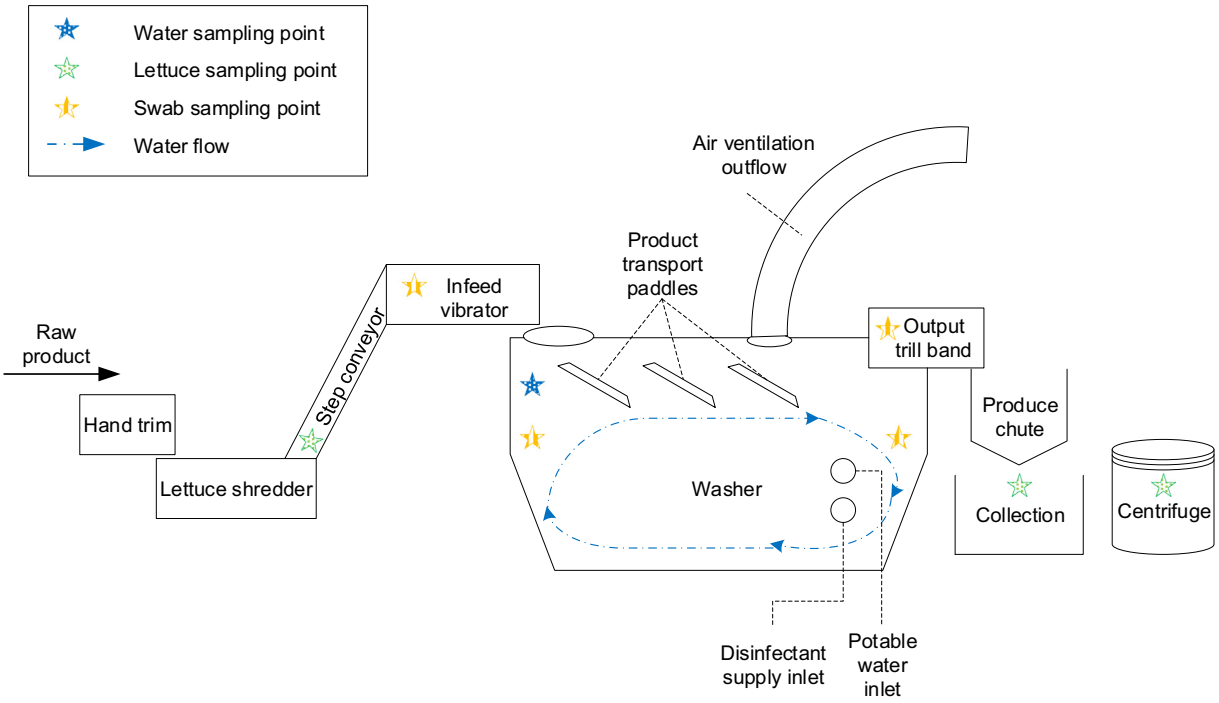

Fig. 1. Diagram of industrial-scale fresh-cut lettuce processing. Water, lettuce, and swab sampling points are starred, while the water flow in the washer is indicated with arrows.

of the lettuce homogenates were made in Ringer's solution, of which 100 µL of undiluted and diluted homogenates were spread plated onto BECSA, King's B Agar (KB; K5165 KB Medium, Duchefa Biochemie B.V., Haarlem, the Netherlands) and R2A Agar (218262 Difco™ R2A Agar, BD Diagnostics, Breda, the Netherlands), to determine *E. coli*, FP, and THB, respectively. Plates were then transferred the same day to the laboratory. BECSA plates were then incubated at 37 °C for 24 h with daily inspection of colonies for up to one week, while KB and R2A plates were incubated at 27 °C for 24–48 h.

Furthermore, lettuce samples were collected from the step conveyor at 2–3 min and from the product chute at 92–93 min to elucidate the effects of storage on *E. coli*, FP, and THB. Samples were immediately rinsed with potable water and centrifuged before packaging on-site and then were transported under refrigerated conditions to the laboratory for further analyses. Packaged lettuce samples were stored for 5 d at 4 °C before microbiological analyses, as previously described for *E. coli*, FP, and THB. No chemical analyses were performed on stored lettuce samples.

Swab samples of the equipment (c.a. 9 cm^2) were taken at (i) the infeed vibrator, (ii) the front wall of the washer, (iii) the rear wall of the washer, and (iv) the output trill band of the washer and analyzed for *E. coli* as described by Banach et al. (2018).

2.5. Statistical analyses

The effect that PAA had on *E. coli*, FP, and/or THB CFUs in lettuce washing water (laboratory), lettuce leaf punches (laboratory), and lettuce samples (industrial) were averaged for each independent experiment before being log-transformed to achieve a normal distribution. To visualize the data as Log_{10} (N/N_0), the log reduction of each experiment was determined by subtracting the log CFUs before treatment from the log CFUs after treatment, *i.e.,* log reduction = log_{10} (CFUs after treatment) − log_{10} (CFUs before treatment). Data were used for statistical comparison using a two-way analysis of variance (ANOVA) with a Bonferroni *post hoc* test in GraphPad Prism (version 5.02).

3. Results

3.1. Physiochemical properties of the wash water

During laboratory experiments with 20 and 40 mg/L of PAA, the pH decreased during washing. The pH before and after treatment was, respectively, 7.0 and 6.1 during 20 mg/L PAA experiments with 500 mg/L of TOC; 7.2 and 5.9 during 20 mg/L PAA experiments with 750 mg/L of TOC; and 7.2 and 5.3 during 40 mg/L PAA experiments with 750 mg/L of TOC. During laboratory experiments with 0, 60, and 80 mg/L of PAA, the concentrations of PAA and H_2O_2 were determined after treatments with and without the addition of *E. coli* (Table S1). Measurements to estimate H_2O_2 in laboratory-made wash water were more challenging to determine given the change in color during titration and the greenish hue of the water. Results showed that post-treatment, H_2O_2 and PAA were present in the water that had been used to wash lettuce leaf punches. A lower concentration of H_2O_2 and PAA is expected since the solution was not dosed into the water throughout the experiments.

During industrial-scale experiments, the PAA, H_2O_2, COD, and TOC concentrations of the PWW were measured for treatment 1 (*E. coli* excluded from the PWW) and treatment 2 (*E. coli* included in the PWW). During treatment 1, the PAA, H_2O_2, COD, and TOC concentrations appeared stable (Fig. S1.A, Fig. S1.B). The water temperature was controlled, ranging from 2.9–3.4 °C (data not shown). During treatment 2, the PAA, H_2O_2, COD, and TOC concentrations also appeared stable (Fig. S1.C, Fig. S1.D). The water temperature was controlled, ranging from 3.0–3.9 °C (data not shown). Similar to treatment 1, the pH of the water increased after 90 min due to the stopped supply of the PAA solution at 90 min. Ammonium-N and phosphate-P appeared stable, with a slight increase during treatment 2, due to the addition of *E. coli* at 90 min (Fig. S2).

3.2. Microbial reduction in the wash water

In a preliminary study, laboratory experiments evaluated the efficacy of PAA at 20 and 40 mg/L on *E. coli* added to potable water and

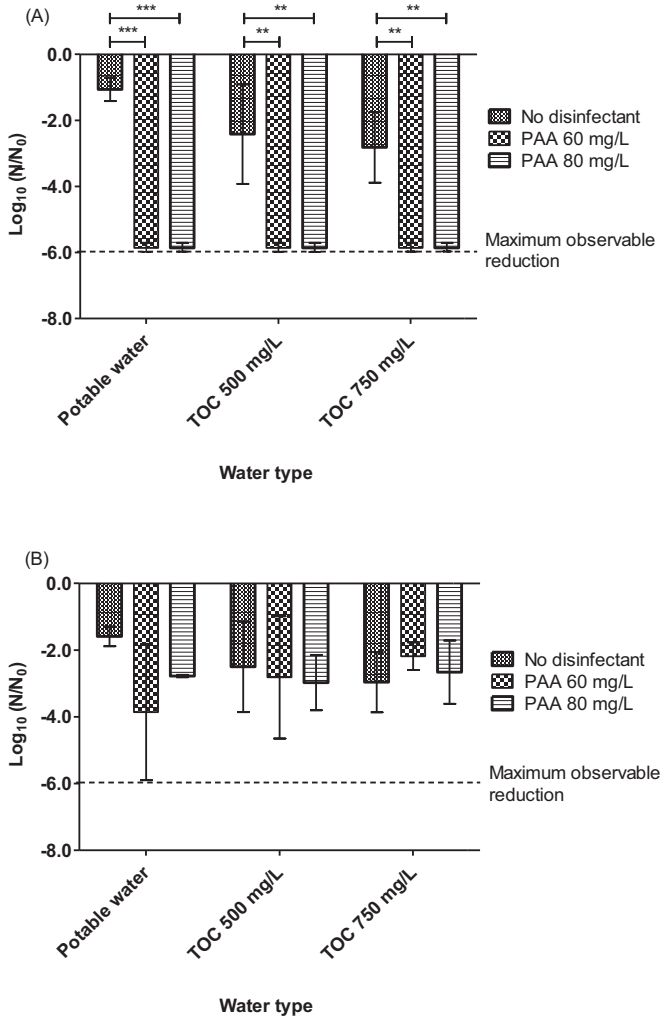

Fig. 2. Average *E. coli* reduction after a two-minute treatment at 4–5 °C with no disinfectant, a peracetic acid (PAA) solution of 60 mg/L, and 80 mg/L. Laboratory experiments were with tap water and laboratory-made wash water with total organic carbon (TOC) concentrations of 500 mg/L and 750 mg/L. (A) Reduction in the wash water of inoculated lettuce leaf punches. (B) Reduction on the inoculated lettuce leaf punches. The $\log_{10}$ (N/N_o) of 0 indicates that all bacterial cells remained in the water (A) or attached to the leaf punches (B). Data represent the average of four experiments and error bars represent standard deviation. Significant differences are denoted by asterisks, for $p < 0.01$ (**) and $p < 0.001$ (***). —, Maximum observable reduction.

laboratory-made wash water with TOCs of 500 and 750 mg/L. Results exhibited at least a 5-log reduction after 1 min. The control treatment (with no disinfectant) indicated no log reduction after 0 and 12 min (data not shown).

Results of the laboratory experiments varied for the reduction of *E. coli* in the wash water of the inoculated lettuce leaf punches (Fig. 2A). For treatments with no disinfectant, *E. coli* cells were recovered in all the wash water types, with a decreasing trend in cell recovery observed for wash waters with increasing TOCs. Average *E. coli* reduction was 1.1, 2.4, and 2.8 log CFU/mL, respectively, for treatments with no disinfectant in tap water, in laboratory-made wash water with a TOC of 500 mg/L and with a TOC of 750 mg/L. These results differ from the treatments with 60 and 80 mg/L of PAA, which both demonstrated that no *E. coli* cells were recovered given each of the three water types tested, indicating that almost a 6-log reduction occurred. Although results may be influenced by the non-use of neutralizers on PAA, serial dilutions in PPS were made before plating. Overall, there is a significant difference between the use of no disinfectant and the use of PAA for each of the concentrations tested.

Results of industrial-scale experiments where *E. coli* were excluded (treatment 1) and included (treatment 2) in the PWW at 90 min showed that no *E. coli* were detected in the PWW when analyzed on BESCA 1 min after the PAA solution supply stopped, *i.e.,* after 91 min of processing (data not shown). Moreover, the samples measured afterward (*i.e.,* at 93, 96, and 102 min of processing) for both treatments showed that no *E. coli* cells were detected. Similar to the lab experiments, neutralizers were not used, but serial dilutions in PPS were made before plating. Overall, treatment 2 experiments resulted in about a 5-log reduction of *E. coli* (data not shown). Also, swab samples for the four tested locations of the equipment were negative (*i.e.,* 0 CFUs of *E. coli* per 9 cm^2 were detected).

3.3. Microbial quality of the lettuce

Results for the laboratory experiments of the washed lettuce leaf punches showed that *E. coli* cells remained attached to the lettuce after treatments (Fig. 2B). For treatments with no disinfectant, *E. coli* decline averaged 1.6, 2.5, and 3.0 log CFU/punch, respectively, after washing in tap water, laboratory-made wash water with a TOC of 500 mg/L and with a TOC of 750 mg/L. *E. coli* were also recovered on the lettuce leaf punches following treatments with 60 and 80 mg/L PAA in all three water types. Treatments with 60 mg/L PAA indicated that *E. coli* decline on the lettuce averaged 3.9, 2.8, and 2.2 log CFU/punch, respectively, following washing with no disinfectants in tap water, laboratory-made wash water with a TOC of 500 mg/L, and with a TOC of 750 mg/L. Treatments with 80 mg/L PAA indicated that *E. coli* decline on the lettuce averaged 2.8, 3.0, and 2.7 log CFU/punch, respectively, following washing with no disinfectants in tap water, laboratory-made wash water with a TOC of 500 mg/L, and with a TOC of 750 mg/L. Although results may be influenced by the non-use of neutralizers on PAA, lettuce was washed with water before analyses. Overall, no significant differences were observed (Fig. 2B).

Results of industrial-scale experiments where *E. coli* were excluded (treatment 1) and included (treatment 2) to the PWW at 90 min on the recovery of *E. coli*, FP, and THB were measured from the lettuce during the experiments (Fig. 3). *E. coli* cells were not detected on the lettuce during experiments where *E. coli* had been excluded (treatment 1) (Fig. 3A). Similarly, for treatment 2, *E. coli* cells were not detected on the lettuce from the crate (data not shown) or on the lettuce sampled at 80 min (below the limit of detection). *E. coli* cells were detected on the lettuce samples taken after that, *i.e.,* at 91, 93, and 95 min (Fig. 3A), with significant differences observed between the treatments from samples at 93 min and samples at 95 min. Even though PAA appeared to influence *E. coli* counts on lettuce, the disinfection of the water with PAA did not result in the complete elimination of *E. coli* from the washed produce. Similar to the laboratory experiments, neutralizers were not used, but the lettuce was washed with water before analyses. With PAA application, a rinsing step after washing and before packaging reflects industrial practice. Moreover, considering the presence of microbial communities on the lettuce, FP (Fig. 3B) and THB (Fig. 3C) were detected before (80 min) and after (91, 93, and 95 min) the lettuce had been processed in the PAA disinfected washing water, with no significant differences observed between the treatments.

Also, *E. coli*, FP, and THB were measured on the lettuce after storage for 5 d at 4 °C (Fig. 4). *E. coli* cells were not detected on the lettuce during experiments where *E. coli* had been excluded (treatment 1) (Fig. 4A), which is expected. During experiments where *E. coli* had been included (treatment 2), *E. coli* cells were detected on the lettuce samples taken from the product chute. There was a significant difference in *E. coli* cells ($p < 0.001$) between treatments from samples taken from product chute at 92– 93 min (*i.e.*, after washing with PAA). This result is to be expected as *E. coli* was added to the washing tank at 90 min during treatment 2. PAA disinfection of the water in these experiments did not prevent the survival of *E. coli* in packaged lettuce samples. FP

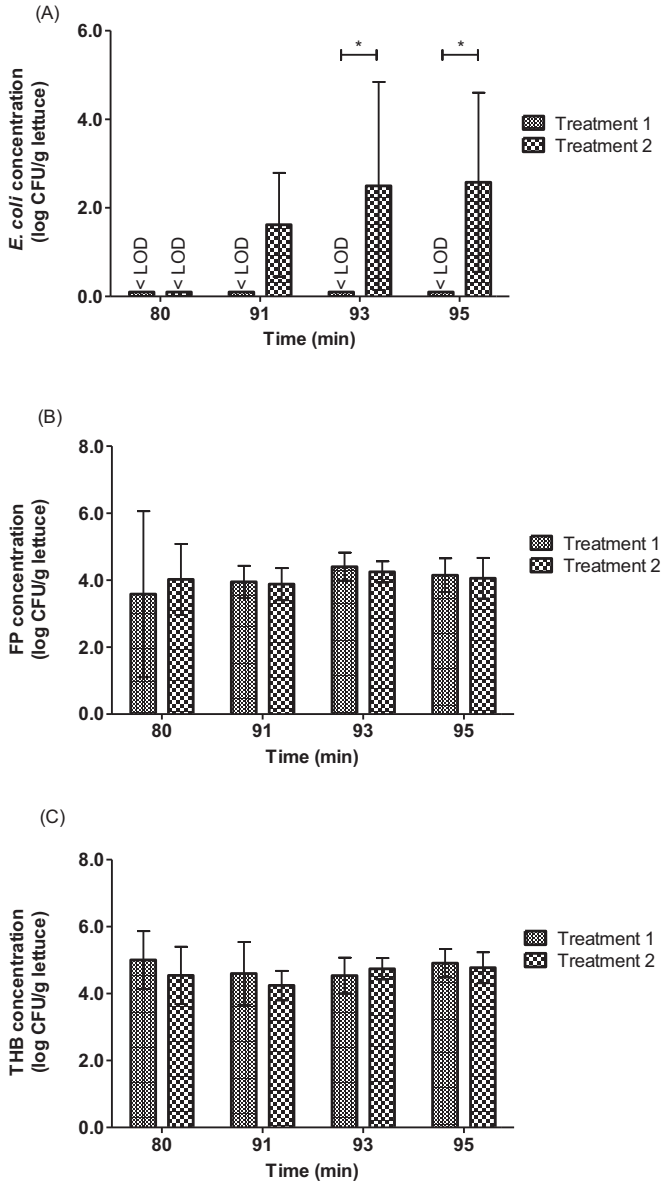

Fig. 3. Recovery of (A) *E. coli*, (B) fluorescent pseudomonads (FP), and (C) total heterotrophic bacteria (THB) from lettuce during industrial-scale washing in water treated with a peracetic acid solution. Treatment 1 excluded and treatment 2 included *E. coli* addition to the water in the wash tank at 90 min. Lettuce samples were collected during processing at the first, second, third, and fourth sampling points of 80, 91, 93, and 95 min ($n = 4$). Error bars represent standard deviation. Significant differences ($p < 0.05$) are denoted by asterisks (*). Values at zero are below the limit of detection (LOD) of c.a. 1 log CFU/g.

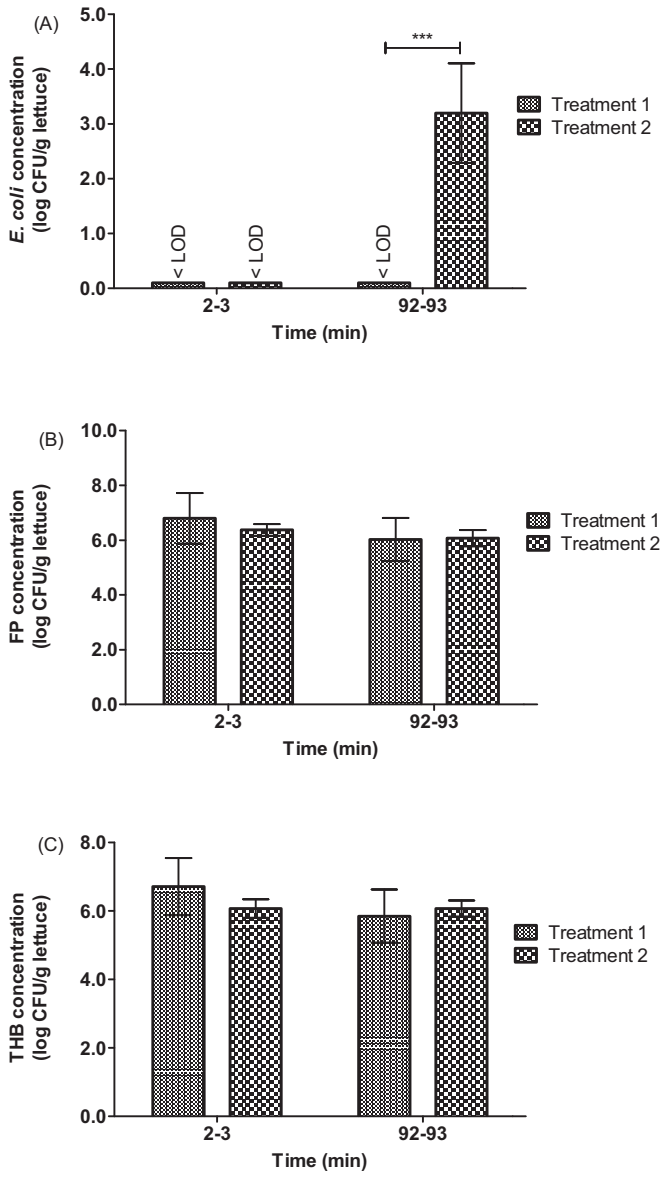

Fig. 4. Recovery of (A) *E. coli*, (B) fluorescent pseudomonads (FP), and (C) total heterotrophic bacteria (THB) from industrial-scale processed lettuce after storage for 5 d at 4 °C. Treatment 1 excluded and treatment 2 included *E. coli* addition to the water in the wash tank at 90 min. Lettuce samples were collected before and after washing with a peracetic acid solution, respectively, from the conveyor belt at 2–3 min ($n = 4$) and the product chute at 92–93 min ($n = 12$). Error bars represent standard deviation. Significant differences ($p < 0.001$) are denoted by asterisks (***). Values at zero are below the limit of detection (LOD) of c.a. 1 log CFU/g.

(Fig. 4B) and THB (Fig. 4C) were detected on stored lettuce samples before and after the lettuce had been processed in the PAA disinfected washing water, with no significant differences observed between the treatments.

4. Discussion

Our study examines the possibility of using a PAA (~75 mg/L) solution to disinfectant the water to prevent potential microbial cross-contamination during industrial-scale fresh-cut lettuce processing. An important implication of these findings is that PAA is shown to be an effective wash water disinfectant to aid in preventing cross-contamination. Other research has suggested only slight differences in the prevention of cross-contamination between the use of tap water and PAA solutions. For instance, a pilot-scale study observed minimal differences between that of tap water and water containing PAA (30 ppm) for *E. coli* O157:H7 in the PWW of (iceberg) lettuce. Results reflected a "best case" scenario during the early stages of processing in which the organic load was extremely low (0.0006% blended iceberg lettuce (wt/vol)) and where the incoming water temperature was 12–15 °C (Davidson et al., 2013). In our study, we opted for a "worst-case" scenario where the organic load of the wash water was built up over time, and contamination occurred near the end of processing. The higher concentration and continuous dose of PAA, albeit at lower water temperatures, most likely attributed to the higher log reductions of *E. coli* observed. Nevertheless, the differences between the studies concerning the lettuce type, *E. coli* analyzed, the composition of the PAA solution

(*e.g.*, Tsunami 100 with 11.2% H_2O_2 and 15.2% PAA (Ecolab, Inc., St Paul, MN) or 17.1% H_2O_2 and 15.2% PAA (Ecolab B.V., Nieuwegein, the Netherlands)), as well as the organic load of the water, should not be disregarded.

The survival of pathogenic bacteria during PWW disinfection has been shown to be dependent on variables like the organic load of the water, water temperature, and attachment and release to the produce (Banach et al., 2015; Banach et al., 2017). The *E. coli* strain used in our study is a model for pathogenic *E. coli*, as the use of a pathogenic strain inside an operating facility is a sanitary and safety concern. Our strain used may attach differently to the lettuce or have different resistance to chemicals than pathogenic strains. Challenge studies to validate this *E. coli* as a surrogate warrant further attention. Despite this limitation, previous research has motivated the use of environmental isolates as surrogates for foodborne pathogens (Cook et al., 2017). Our strain is an environmental isolate coming from surface water and could provide insight considering contamination routes *via* water. Another study also found that the conditions in which bacterial strains were grown before challenge studies with PAA had a larger effect on bacterial reduction than strain diversity (Harrand et al., 2019). Therefore, the factors that affect the strain, like the growth conditions, may even be more relevant than the strain itself.

Our results demonstrated that the tested PAA solutions were not adversely affected by the organic load of the water, as supported by the microbial reductions observed. Zhang et al. (2009) reported that during laboratory experiments, PAA (30 ppm; Tsunami 100, Ecolab, Inc., St Paul, MN) more effectively reduced *E. coli* O157:H7 cells in lettuce washing water *versus* that of tap or sanitizer-free sterile deionized water. This effect was also observed in our study, given the tested PAA concentrations for laboratory and industrial-scale experiments. However, Zhang et al. (2009) observed that PAA at 10 and 20 ppm in organically loaded water negatively affected the effectiveness of PAA on *E. coli* cells in the water and on the lettuce (Zhang et al., 2009). This result was not observed in our study. The difference is most likely due to a combination of higher concentrations of PAA and different organic loads of the wash water. Also, for industrial-scale experiments, the continuous dose of PAA could have contributed to this difference. Furthermore, a preliminary study evaluating 79 mg/L PAA (Tsunami 100) with tap water and *Lolla rossa* PWW (TOC = 50 mg/L) from the industrial-scale line that was tested in our study demonstrated > 5 log reduction of the same strain of *E. coli* when analyzed on BECSA (data not shown). This additional information shows that the effectivity of tested PAA solutions was not adversely affected by the organic load of the wash water. At the industrial scale, our results concur with previous research, which found that the organic load rarely affected the efficacy of PAA (50 ppm; Tsunami 100, Ecolab, St. Paul, MN) with reductions up to 5-log CFU/mL in the wash water observed on a small-scale production of lettuce (Davidson et al., 2017). Overall, the efficacy of the tested PAA solution was shown to be less affected by the organic load in the PWW. Factors like the concentration and dose, nonetheless, may influence its stability in organically loaded waters.

The dose of the PAA solution showed to be a relevant factor for the microbial reduction in the water during our experiments. When testing higher concentrations of PAA and COD of the wash water, López-Gálvez et al. (2009) reported in their laboratory study a 4 log reduction of *E. coli* in the water with PAA use (500 mg/L; Tsunami 100 containing PAA at 15% as the active compound, Ecolab, Barcelona, Spain) in processing waters with CODs of 700–1000 mg/L. This observation concurs with our results, albeit we tested lower concentrations of PAA and organic loads (in terms of COD and/or TOC) of the wash water. The results from our study showed a stable COD of between 444 and 538 mg/L during the processing of fresh(-cut) lettuce with PAA (~75 mg/L) at the industrial scale. When considering the same industrial-scale line and processing of the same type of lettuce, the COD of the wash water ranged from 350 to 800 mg/L when no disinfectant was used and between 250 and 280 mg/L when 3 mg/L ClO_2 was applied (Banach et al., 2018). The COD concentrations measured during PAA experiments at the industrial scale were higher than the lowest concentrations measurement when no disinfectant was used (350 mg/L) or when, *e.g.*, ClO_2 was tested in our previous research (250 mg/L) (Banach et al., 2018).

Compared to experiments testing 3 mg/L of ClO_2 (Banach et al., 2018), a higher COD concentration with the use of PAA was observed in our study. An increase in the organic load of the processing water with the use of PAA, and no effect with ClO_2 was also observed (Petri et al., 2015). Moreover, López-Gálvez et al. (2009) observed an increase in the organic load of PAA treated wash water. This increase has been explained by the presence of acetic acid, which is present in both PAA and its decomposition product (Kitis, 2004), yet also a result of the peracid itself (Beber de Souza et al., 2015; Luukkonen and Pehkonen, 2017). According to Luukkonen and Pehkonen (2017), an increase in TOC and COD is because of PAA dosing. Reported increases of COD are between 1.9 and 4.0 mg/L per 1 mg/L of PAA dosed; however, authors also noted that decreased COD could occur due to the oxidation of organic matter (Luukkonen and Pehkonen, 2017). Consequently, monitoring the physicochemical properties of the wash water is crucial to consider along with the dose of the PAA to be used. The use of PAA as a wash water disinfectant should not adversely affect its ability to prevent cross-contamination during processing. Hence, the PAA dose should also be monitored and kept stable for both microbiological reasons and to avoid unnecessary overdosing, which can contribute to higher operational costs.

Our study showed the effectiveness of a PAA solution in reducing cross-contamination during industrial-scale fresh-cut lettuce processing. Fewer *E. coli* were observed to attach to the lettuce during processing. This phenomenon is important to realize as an estimated 90% of *E. coli* O157:H7 have been reported to transfer *via* wash water during fresh-cut leafy green processing (Buchholz et al., 2012). In our study, we illustrated the recovery of *E. coli* lettuce after storage for 5 d at 4 °C. On average, the recovery of *E. coli* after washing (n = 12) was 3.2 log CFU/g. Given a similar experimental design, the control treatments (n = 2) without disinfectant, yet with the addition of *E. coli*, resulted in an average 4.5 log CFU of *E. coli*/g lettuce after storage for 5 d at 4 °C (Banach et al., 2018). The use of a PAA solution during washing resulted in > 1 log CFU/g fewer *E. coli* observed on stored lettuce. The data shows that washing with a PAA solution resulted in fewer *E. coli* recovered *versus* that of washing with no disinfection. This result supports PAA disinfection of the water during fresh-cut lettuce washing. Although the molecular mechanisms of PAA use during washing were not the focus of our study, and a restriction of our experimental design, other research has motivated PAA treatment during (fresh-cut) lettuce washing, showing the inactivation on lettuce of oxidative stress-related genes and proteins at early stages of storage (Daddiego et al., 2018). Similar to our study, the ability for *E. coli* to persist on the lettuce after PAA use has been reported (Al-Nabulsi et al., 2014; Davidson et al., 2013; Davidson et al., 2017; López-Gálvez et al., 2009; Rodgers et al., 2004; Vandekinderen et al., 2009; Zhang et al., 2009). The microbial load can be reduced about 1–2 logs with washing and disinfection (Doona et al., 2015; Fatica and Schneider, 2009); however, the notion that disinfectants such as PAA can be used to decontaminate lettuce to ensure end-product safety is misleading. Instead, the need to disinfect the wash water with, *e.g.*, a PAA solution, is to prevent potential cross-contamination during washing.

Previous research has indicated that phyllosphere bacterial communities of plants such as lettuce can be affected by season, irrigation, and other biological factors like the presence of *E. coli* O157:H7 (Williams et al., 2013). In our study, the effect that PAA (and *E. coli*) may have on the presence of the microbial communities in the lettuce was investigated. In our study, no difference was observed for culturable microbial communities on the lettuce directly measured before and after PAA washing, even when *E. coli* was added. Our result differs from Allende et al. (2008), which found a significant difference ($p \leq 0.001$) in the mesophilic reduction in Tsunami 100 at 80 μL/L (Ecolab,

Barcelona, Spain) applied in a submersion washing system *versus* that of the "water" wash. This difference between our studies can be attributed to, among other factors, the concentration of PAA used or the application system. Moreover, in our study, FP and THB were shown to be able to survive and grow during storage (5 d, 4 °C). This result concurs with the work of Allende et al. (2008), who reported no significant differences after eight days of storage (3 d at 5 °C and 5 d at 8 °C) given a dose of 40 and 80 μL/L in a submersion washing system.

Overall, our results showed that during the processing of fresh-cut lettuce, where the accumulation of soil, debris, and other plant exudates can negatively affect the washing system, the use of a PAA (~75 mg/L) solution was observed to be an effective wash water disinfectant. Moreover, the quality of the water used during washing (fresh-cut) produce is a crucial aspect that should be monitored. Similarly, the dose of the wash water disinfectants, such as PAA, to be used during fresh(-cut) lettuce processing needs to be well-controlled.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.108537.

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