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Efficacy of neutral electrolyzed water (NEW) for reducing microbial contamination on minimally-processed vegetables

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Abstract

Consumption of minimally-processed, or fresh-cut, fruit and vegetables has rapidly increased in recent years, but there have also been several reported outbreaks associated with the consumption of these products. Sodium hypochlorite is currently the most widespread disinfectant used by fresh-cut industries. Neutral electrolyzed water (NEW) is a novel disinfection system that could represent an alternative to sodium hypochlorite. The aim of the study was to determine whether NEW could replace sodium hypochlorite in the fresh-cut produce industry. The effects of NEW, applied in different concentrations, at different treatment temperatures and for different times, in the reduction of the foodborne pathogens *Salmonella*, *Listeria monocytogenes* and *Escherichia coli* O157:H7 and against the spoilage bacterium *Erwinia carotovora* were tested in lettuce. Lettuce was artificially inoculated by dipping it in a suspension of the studied pathogens at 10^8, 10^7 or 10^5 cfu ml^{-1}, depending on the assay. The NEW treatment was always compared with washing with deionized water and with a standard hypochlorite treatment. The effect of inoculum size was also studied. Finally, the effect of NEW on the indigenous microbiota of different packaged fresh-cut products was also determined. The bactericidal activity of diluted NEW (containing approximately 50 ppm of free chlorine, pH 8.60) against *E. coli* O157:H7, *Salmonella*, *L. innocua* and *E. carotovora* on lettuce was similar to that of chlorinated water (120 ppm of free chlorine) with reductions of 1–2 log units. There were generally no significant differences when treating lettuce with NEW for 1 and 3 min. Neither inoculation dose (10^7 or 10^5 cfu ml^{-1}) influenced the bacterial reduction achieved. Treating fresh-cut lettuce, carrot, endive, corn salad and 'Four seasons' salad with NEW 1:5 (containing about 50 ppm of free chlorine) was equally effective as applying chlorinated water at 120 ppm. Microbial reduction depended on the vegetable tested: NEW and sodium hypochlorite treatments were more effective on carrot and endive than on iceberg lettuce, 'Four seasons' salad and corn salad. The reductions of indigenous microbiota were smaller than those obtained with the artificially inoculated bacteria tested (0.5–1.2 log reduction). NEW seems to be a promising disinfection method as it would allow to reduce the amount of free chlorine used for the disinfection of fresh-cut produce by the food industry, as the same microbial reduction as sodium hypochlorite is obtained. This would constitute a safer, 'in situ', and easier to handle way of ensuring food safety.

Keywords: Disinfection; Fresh-cut vegetables; *Escherichia coli*; *Listeria*; *Salmonella*; *Erwinia carotovora*

1. Introduction

Fresh fruit and vegetables are an essential part of the diet of people around the world. Nutritionists emphasize the importance of fruit and vegetables in healthy diets, and researchers and governmental publicity campaigns around the world tend to recommend consumption of at least five servings of fruit and vegetables per day. During recent decades, pre-prepared minimally-processed or fresh-cut fruit and vegetables have become increasingly popular amongst European consumers (EU Scientific Committee on Food, 2002). These products include pre-washed and pre-cut salads, which are generally eaten without processing. Concomitant with this trend and changes in people's eating habits, an increased number of microbial infections associated with the consumption of fresh-cut fruit and vegetables have been documented in recent years, for example in Japan (Nat'l. Inst. Inf. Dis., 1997; Gutierrez, 1997), USA (Cummings et al., 2001; De Roever, 1998; FDA, 2006), England and Wales (PHLS, 2000) and Europe (Pezzoli et al., 2007).

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To minimize the risk of infection or intoxication associated with the consumption of raw fruits and vegetables, potential sources of contamination from the environment to the table should be identified and specific measures and interventions to prevent and/or minimize the risk of contamination should be considered and correctly implemented (Beuchat, 1998). Surveys of minimally-processed fruit and vegetables and sprouts have demonstrated that fresh-cut produce could harbour high counts of bacteria and also foodborne pathogens such as *Salmonella*, *L. monocytogenes*, *Aeromonas hydrophila* and *E. coli* O157:H7 (Abadias et al., 2008; Beuchat, 1996; Francis et al., 1999; FEHD, 2002; Johannessen et al., 2002; Sagoo et al., 2003). Thus, sanitation of produce will play an important role in guaranteeing its quality and safety for human consumption.

A variety of disinfectants (including chlorine, hydrogen peroxide, organic acids and ozone) have been used to reduce bacterial populations on fruit and vegetables (Beuchat, 1998; EU Scientific Committee on Food, 2002). However, besides their potential toxicity, they have proved incapable of completely removing or inactivating microorganisms on fresh produce (Koseki and Itoh, 2001; Park et al., 2001). In the fresh-cut industry, chlorine is commonly used to disinfect produce at a concentration of 50–200 ppm, with a contact time of 1–2 min (Beuchat, 1998). Inhibitory or lethal activity depends on the amount of free available chlorine (in the form of hypochlorous acid, HOCl) present in the water that comes into contact with the microbial cells. The dissociation of HOCl depends on pH and chlorine is consumed on contact with organic matter. Moreover, it loses its activity with exposure to air, light and metals. Furthermore, a common concern among people who use chlorinated water as a disinfectant is that prolonged exposure to chlorine vapour may cause irritation to the skin and the respiratory tract.

In recent years, acidic electrolyzed water (AEW) and neutral electrolyzed water (NEW) have been studied as sanitizers. These solutions are generated by the electrolysis of a diluted NaCl solution passing through the anode of a membrane electrolyzer. AEW has a strong bactericidal effect on most known pathogenic bacteria due to its low pH (2–4) and high oxidation–reduction potential (ORP > 1000 mV) and because it also contains active oxidizers like hypochlorous acid (Kim et al., 2000b; Len et al., 2000). NEW is generated like AEW, but part of the product formed at the anode is then redirected into the cathode chamber. This produces a neutral solution (pH 8.0 ± 0.5) in which the main biocidal reagents are HOCl, ClO^-, HO_2 and $^{\bullet}O_2$. Because of its neutral pH, NEW does not contribute as aggressively as AEW to the corrosion of processing equipment or irritation of hands, and is also more stable as chlorine loss is significantly reduced at pH 6–9 (Ayebah and Hung, 2005; Len et al., 2002).

Several studies have revealed the efficacy of AEW, or electrolyzed oxidizing water (EOW), against different foodborne pathogens on several vegetable products, including lettuce (Park et al., 2001; Koseki et al., 2003, 2004a; Yang et al., 2003); cucumbers and strawberries (Koseki et al., 2004b) and tomatoes (Bari et al., 2003). It has also proved to be effective for the disinfection of several different food surfaces (Park et al., 2002; Venkitanarayanan et al., 1999) and the inactivation of biofilms (Ayebah and Hung, 2005; Kim et al., 2000a). Similarly, NEW has demonstrated its effectiveness as a method for reducing *E. coli* O157:H7, *Salmonella enteritidis* and *Listeria monocytogenes* on the surface of tomato (Deza et al., 2003) and also on plastic and wooden cutting boards (Deza et al., 2007). However, the efficacy of NEW has not been extensively studied with respect to fresh-cut vegetables.

The aim of this work was to determine whether NEW could replace sodium hypochlorite as a disinfectant for fresh-cut produce. Thus, we evaluated the efficacy of NEW in reducing populations of *Escherichia coli* O157:H7, *Salmonella*, *Listeria innocua* and *Erwinia carotovora* on pure cultures and on lettuce. We also tested its efficacy for reducing indigenous microbiota on lettuce, grated carrot, endive, corn salad and 'Four seasons' salad. This effectiveness was always compared with deionized water and with chlorinated water at a standard chlorine concentration (100 ppm).

2. Materials and methods

2.1. Microorganisms and preparation of inocula

Listeria innocua CECT-910 and *E. carotovora* ssp. *carotovora* CECT-225 from The Spanish Type Culture Collection (CECT), *Salmonella choleraesuis* subsp. *choleraesuis* (Smith) Weldin serotype Michigan, ATCC number BAA-709 and a non-toxicogenic strain of *E. coli* O157:H7 NCTC 12900 were used in this study.

E. coli and *Salmonella* were maintained at 5 ± 1 °C on Tryptone Soy Agar (TSA, Oxoid CM0131), *L. innocua* on TSA amended with 2.5 g l^{-1} of glucose, 6.0 g l^{-1} of yeast extract and 2.5 g l^{-1} of K_2HPO_4 (TSYEA, Tryptone Soy Yeast Extract Agar) and *E. carotovora* on Nutrient Agar (NA, Biokar BK185HA) until they were all used. When required, *E. coli* and *Salmonella* were subcultured for 24 ± 2 h at 37 ± 1 °C on TSA, inoculated in 10 ml of Tryptone Soy Broth (TSB, Oxoid CM0129) and then incubated for 18–20 h at 37 ± 1 °C. *L. innocua* was grown on TYSEA for 24 ± 2 h at 37 ± 1 °C and inoculated in 50 ml of TYSEB (TSB amended with 2.5 g l^{-1} of glucose, 6.0 g l^{-1} of yeast extract and 2.5 g l^{-1} of K_2HPO_4) and then incubated at 150 rpm for 24 ± 2 h at 37 ± 1 °C. *E. carotovora* was grown on NA for 48 ± 2 h at 30 ± 1 °C and then inoculated in 50 ml of TSB and incubated at 150 rpm for 24 ± 2 h at 30 ± 1 °C. Microorganisms were centrifuged at 8000 rpm for 10 min and the resulting pellet was resuspended in saline peptone (SP, 8.5 g l^{-1} NaCl and 1 g l^{-1} peptone). The concentration of microorganisms was estimated by measuring the suspension transmittance at 420 nm in a spectrophotometer and comparing this value with previously determined standard curves. Depending on the assay, suspensions of about 10^8 or 10^7 cfu ml^{-1} were prepared and the concentration applied was confirmed by plating 0.1 ml of appropriately diluted culture on TSA, TYSEA or NA.

2.2. Preparation of treatment solutions

Fresh NEW was generated using the Eurostel® EZ-90 Unit (Ecanet, Palamòs, Girona, Catalonia, Spain). Following manufacturer's instructions, a saturated sodium chloride solution and

tap water were simultaneously pumped into the generator at 90 l min^{-1}. The amperage was fixed at 30±5 A. Under these conditions, the water obtained contained approximately 280 ppm of free chlorine.

Depending on the assay, NEW was diluted in cold (5–8 °C) and/or ambient (20–22 °C) deionized water to obtain different chlorine concentrations. NEW treatments were compared with deionized water (DW) and a standard hypochlorite treatment of approximately 100 ppm of free chlorine. This standard sodium hypochlorite treatment (SH) was prepared by combining sodium hypochlorite (Conejo, commercial bleach, Henkel Iberica SA, Barcelona, Catalonia, Spain with 42.5 g of active chlorine per liter) and deionized water.

Free chlorine, pH and ORP were determined for all solutions. Free chlorine was measured using a free and total photometer (HI 93734, Hanna Instruments, Eibar, Spain). ORP and pH were determined using a pH/ion/conductivity meter (Model GLP-22, Crison), with a pH electrode (Crison, 52-01) or an ORP electrode (Crison, platinum Ag/AgCl electrode 52-61).

2.3. Effect of NEW on pure cultures

For each bacterial strain, suspensions of 10^8 cfu ml^{-1} were prepared as described above. One milliliter of each bacterial suspension was separately added to 9 ml of tested solution in triplicate: deionized water (DW, no residual chlorine, control), NEW 1:3 (volume NEW:total volume), NEW 1:6, NEW 1:10 and sodium hypochlorite (SH, 100 ppm). Each treatment was carried out at 20±2 °C and 5±2 °C. Samples were taken for each treatment after 0, 1, 3 and 5 min of exposure to NEW, DW and SH and the microorganism concentration was determined as follows. One milliliter of the suspension was transferred to 9-ml of neutralizing solution (sodium thiosulfate, ST, 0.5%, Deza et al., 2003). After 5 min of neutralization, a series of 10-fold dilutions were made in SP and 0.1 ml were spread-plated on TSA for *E. coli*, *Salmonella* and *E. carotovora* or on TSYEA for *L. innocua*. The plates were incubated at 37±1 °C for 24±2 h (*E. coli* and *Salmonella*) and 48±2 h (*L. innocua*) or at 30±1 °C for 48±2 h (*E. carotovora*). The effect of the neutralizing solution (ST) was also tested on bacterial cultures to ensure that the observed reduction was solely attributable to NEW and not due to sodium thiosulfate. In the assays at low temperature, the tubes were kept in a water bath at 5±2 °C to keep the temperature constant throughout the process. The experiment was repeated twice.

2.4. Effect of NEW on bacteria artificially inoculated on lettuce

Iceberg lettuces (*Lactuca sativa* L.) were purchased from a supermarket the day before the experiment and stored at 5±1 °C. Three or four of the wrapper leaves were removed from each lettuce head and discarded. Samples of lettuce subjected to inoculation were aseptically cut in a biosafety cabinet. Cut lettuce leaves were inoculated by dipping them in a 10^8 cfu ml^{-1} of each bacterial strain for 2 min at 150 rpm. They were then drained and left in a laminar flow biosafety cabinet on a wire screen until dry (for 1 h approximately). The inoculated cut lettuce leaves were then divided into 40-g samples, transferred to 1–l beakers and kept in an ice bath before treatment with the different sanitizers.

NEW solutions (at 1:3 and 1:6, volume NEW:total volume) were prepared by diluting NEW in cold deionized water (5–8 °C). Samples of inoculated lettuce were separately immersed in DW, NEW 1:3, 1:6 and SH (100 ppm) in a beaker, which was kept in an ice bath to maintain cold conditions and shook at 150 rpm for 1 or 3 min. The treatment temperature was monitored with a temperature probe. After treatment, the lettuce samples were drained off and rinsed with cold deionized water for 1 min at 150 rpm. After rinsing, the treated lettuce samples were left to dry in a flow cabinet (for approximately 1 h).

The concentration of each bacterium on lettuce was determined both before and after the treatment. Three 10-g samples of inoculated lettuce before treatment and from each inoculated lettuce after treatment were mixed with 90 ml of SP in a Stomacher bag and then pummeled in a Stomacher 400 (Seward, London, UK) for 2 min at 230 rpm. Serially diluted samples were surface plated and incubated in the media and at the temperatures recommended for each bacterium, as indicated above. Values were reported as cfu g^{-1} of lettuce.

Uninoculated controls were included to verify raw lettuces for the absence of the pathogens. For this, microbial counts of cut, uninoculated lettuce were also determined in triplicate. The bacterial concentration of DW, SH and NEW treatment solutions was also determined. This experiment was repeated twice.

2.5. Effect of NEW on bacterial mixtures artificially inoculated on lettuce

E. coli, *Salmonella* and *L. innocua* suspensions were prepared as previously described and their respective concentrations were estimated by measuring transmittance at 420 nm. They were then added to, and mixed into, 2-l of deionized water to achieve a final concentration of 10^7 or 10^5 cfu ml^{-1} each (high or low dose). The concentration of each pathogen in the bath was confirmed by plating 0.1 ml of appropriately diluted culture on Hektoen Agar (HK, Biokar BK067) for *Salmonella*, on Sorbitol MacConkey Agar (SMaC, CM0813, Oxoid) for *E. coli* O157:H7, and on Palcam Agar (Palcam Agar Base, Biokar BK145 with Palcam selective supplement BS00408) for *L. innocua*, followed by incubation at 37±1 °C. Samples of iceberg lettuce were prepared as previously described and dipped in the bath containing the mixture of the three pathogens for 2 min at 150 rpm. They were then drained and dried in a biosafety cabinet. Treatments with NEW at 1:3 and 1:5, SH (100 ppm) and DW under cold conditions (8±2 °C) for 3 min, followed by rinsing with cold deionized water for 1 min were carried out as previously described.

Concentrations of each pathogen, before and after treatment, were determined by mixing, in triplicate, 10 g of lettuce with 90 ml of SP in a Stomacher bag and pummeled for 2 min at 230 rpm. Serially diluted samples were surface plated on the selective media: HK for *Salmonella*, SMaC for *E. coli* and Palcam for *L. innocua*. The plates were incubated at 37±1 °C for 24 h (*E. coli* and *Salmonella*) and 24–48 h (*L. innocua*). The experiment was conducted two times.

Table 1
Physicochemical properties of tested solutions[a]

Treatment	Free chlorine (ppm)	ORP (mV)[b]	pH
NEW[c]	281±32	721±12	8.74±0.18
NEW 1:3	89±12	733±13	8.55±0.17
NEW 1:5	52±6	722±15	8.60±0.21
NEW 1:6	48±4	736±18	8.40±0.22
SH[d]	118±17	597±33	9.85±0.29

[a] Mean of all experiments±standard deviation.
[b] Oxidation reduction potential.
[c] Neutral electrolyzed water.
[d] Sodium hypochlorite solution.

2.6. Effect of NEW on the microbiota of different fresh-cut vegetables

Packaged fresh-cut iceberg lettuce, shredded carrot (*Daucus carota*), fresh-cut endive (*Chicorium endivia*), corn salad (*Valerianella locusta*) and 'Four seasons' salad (containing iceberg lettuce, carrot and cabbage) were purchased from a supermarket the day before the experiment and stored at 5±1 °C. Samples of fresh-cut vegetables were then divided into three portions, each of approximately 40 g, and kept in an ice bath until treated. SH (100 ppm) and NEW at 1:5 were prepared as previously described and stored at 5±1 °C until used. The treatments consisted of dipping the fresh-cut vegetable in DW, NEW at 1:5 or SH for 3 min at 150 rpm. After treatment, the samples were drained and rinsed with cold deionized water for 1 min at 150 rpm. Treatment temperature was monitored using a temperature probe. After rinsing, fresh-cut vegetable samples were left to dry in a flow cabinet.

The total aerobic mesophilic count of microorganisms for each product both before and after treatment was determined as follows: triplicate 10-g samples were mixed with 90 ml of SP and pummeled in a Stomacher for 2 min at 230 rpm. Serial dilutions were plated on PCA (Plate Count Agar) and incubated at 30±1 °C for 3 days. The experiment was repeated.

2.7. Statistical analysis

The reductions in bacteria on fresh-cut lettuce after NEW, SH and DW treatment were calculated by subtracting the initial mean bacteria population (log cfu g^{-1}) from the bacteria population (log cfu g^{-1}) after each treatment. Values represented the means of two different experiments with 3 replicates of the treatment per experiment. The General Linear Models (GLM) procedure of the Statistical Analysis System (SAS) was applied (v.8; SAS Institute, Cary, NC, USA). Significant differences between treatments with respect to bacterial reduction were analyzed by Duncan's Multiple Range test at a significance level of 0.05.

3. Results

3.1. Physicochemical properties of tested solutions

Pure NEW water obtained with the Eurostel EZ Unit contained 281 ppm of free chlorine, had a pH of 8.74 and 721 mV of ORP (Table 1). Pure NEW water was diluted at 1:3, 1:5 or 1:6 obtaining respective concentrations of free chlorine of 89, 52 and 48 ppm, without any significant changes in ORP or pH values. The hypochlorite solution tested (containing 118 ppm free chlorine) was more alkaline than the NEW solutions: it had a mean value of 9.85. It also had a lower ORP value, 597 mV, which indicated that it contained fewer active oxidizers than NEW.

3.2. Effect of NEW on pure cultures

In order to determine the best conditions (NEW concentration, temperature and time), the effect of NEW – diluted at 1:10, 1:6 and 1:3 for 1, 3 and 5 min, at ambient (20±2 °C) and low (5±2 °C) temperature – was studied in pure cultures of *Salmonella*, *L. innocua*, *E. coli* and *E. carotovora* and compared with SH and DW. The results obtained showed that no reduction in bacterial count was achieved in the DW suspension (Table 2). The sodium thiosulfate (ST) solution used to neutralize chlorine did not affect

Table 2
Effect of neutral electrolyzed water (NEW) concentration, temperature and time on the reduction (log cfu ml^{-1}) of pure cultures of *Salmonella*, *L. innocua*, *E. coli* O157:H7 and *E. carotovora* compared with sodium hypochlorite treatment containing 100 ppm of free chlorine (SH)

		Treatment (free chlorine)														
		DW/ST (0 ppm)			NEW 1:10[a] (28 ppm)			NEW 1:6 (48 ppm)			NEW 1:3 (89 ppm)			SH (118 ppm)		
		Time (min)			Time (min)			Time (min)			Time (min)			Time (min)		
Microorganism		1	3	5	1	3	5	1	3	5	1	3	5	1	3	5
Salmnella	T_a	0.0	0.0	0.0	2.1	3.2	>3.5	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5
	T_l	0.0	0.0	0.0	3.5	4.0	4.0	>5.3	>5.3	>5.3	>5.3	>5.3	>5.3	>5.3	>5.3	>5.3
L. inocua	T_a	0.0	0.0	0.0	2.5	2.5	2.3	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5
	T_l	0.0	0.0	0.0	3.7	3.7	3.7	−4.2	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5
E. coli	T_a	0.0	0.0	0.0	1.8	2.2	4.0	>5.4	>5.4	>5.4	>5.4	>5.4	>5.4	>5.4	>5.4	>5.4
	T_l	0.0	0.0	0.0	4.0	4.6	4.4	>5.3	>5.3	>5.3	>5.3	>5.3	>5.3	>5.3	>5.3	>5.3
E. carotovora	T_a	0.0	0.0	0.0	2.7	3.6	4.1	>5.7	>5.7	>5.7	>5.7	>5.7	>5.7	>5.7	>5.7	>5.7
	T_l	0.0	0.0	0.0	4.0	4.0	4.3	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5	>5.5

T_a: ambient temperature (20±2 °C).
T_l: low temperature (5±2 °C).
Deionized water (DW) and sodium thiosulfate (ST) were used as controls.
[a] Dilution, Volume NEW:Total volume.

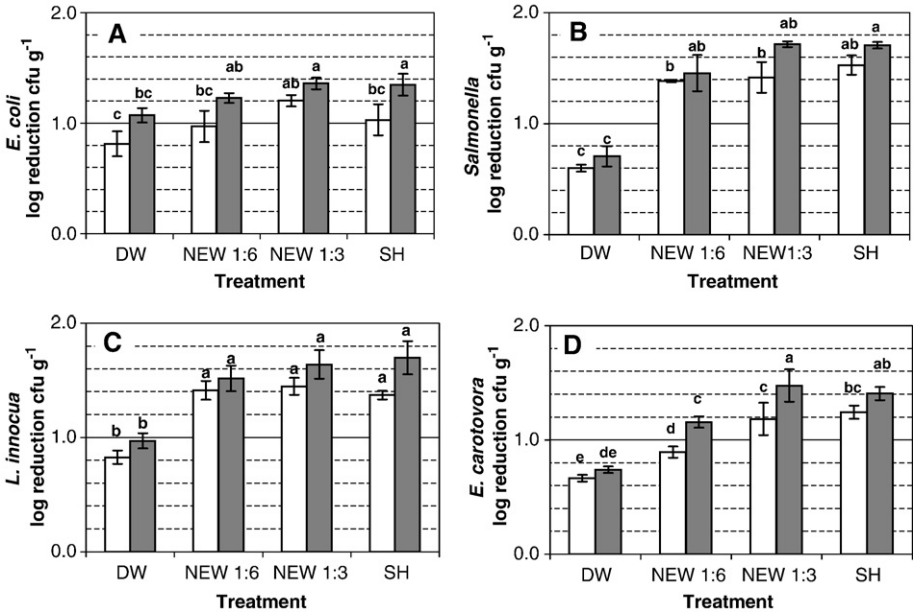

Fig. 1. Reduction of *E. coli* O157:H7 (A), *Salmonella* (B), *L. innocua* (C), and *E. carotovora* (D) in lettuce after treatment with cold (8±2 °C) neutral electrolyzed water (NEW), sodium hypochlorite 100 ppm (SH) and deionized water (DW) for 1 min (□) and 3 min (▩). Cut lettuce was inoculated by dipping it in a 10^8 cfu ml^{-1} suspension of each microorganism. For each microorganism, different letters indicate significant differences between treatments. Bars indicate standard error of means.

bacteria survival. All strains were reduced by >5 log cfu ml^{-1} when the cultures were mixed with SH, NEW at 1:3 and 1:6. Smaller reductions were observed when the NEW was diluted at 1:10 (approximately 30 ppm of free chlorine). A significant temperature effect was only observed with the treatment containing the lowest concentration of NEW tested (1:10). For this dosage, greater reductions were obtained at low temperature. In this treatment, an increase in treatment time from 1 to 5 min did not result in a major reduction of bacteria at low temperature. However, at ambient temperature, there was a greater reduction in *Salmonella*, *E. coli* and *E. carotovora* due to the increase in treatment time.

3.3. Effect of NEW on bacteria artificially inoculated on lettuce

The initial populations on fresh-cut lettuce, after inoculating and drying in a biosafety cabinet for about 1 h, were 7.2, 6.8, 6.7 and 7.3 log cfu g^{-1} for *E. coli*, *E. carotovora*, *L. innocua* and *Salmonella*, respectively (data not shown). Washing with DW for 1 min resulted in a reduction in populations of all strains by 0.6 to 0.8 log cfu g^{-1}, and by 0.7 to 1.1 log cfu g^{-1} when the treatment was extended to 3 min (Fig. 1). In all studied treatments, bacterial reductions were smaller than 2 log cfu g^{-1}.

Except with *E. coli*, application of the NEW treatment at 1:6 (48 ppm of free chlorine), significantly increased the reduction in bacteria with respect to the DW treatment. When NEW was used at a higher concentration (1:3, 89 ppm), the bacterial population inoculated on lettuce was reduced by ca. 1.2 to 1.5 log cfu g^{-1} and ca. 1.4 to 1.7 log cfu g^{-1} for 1 and 3 min treatments, respectively. In general, there was no significant increase in the reduction of bacteria with a longer treatment time; only slight differences were observed.

There were no significant differences in efficacy between applying NEW at 1:3 and SH for killing all of the tested strains on lettuce ($P \geq 0.05$). The treatment with NEW at 1:6 was only less effective than the SH treatment for reducing the *E. carotovora* population.

For each strain analyzed, no bacterium was detected in the washing solutions of NEW and SH after treatment (detection limit 10 cfu g^{-1}), whereas for deionized water an average count of 10^5 cfu ml^{-1} was found in the wash solution (data not shown).

Three samples from each lot of uninoculated lettuce used in all the studies were analyzed for the presence of native bacteria before inoculation. Contaminant bacteria were not detected in any of the samples analyzed (data not shown).

Table 3
Reduction (log10 initial concentration–log10 concentration after treatment) of foodborne pathogen population when inoculation was carried out by dipping fresh-cut lettuce in a mixture of the three strains at both a high (10^7 cfu ml^{-1}) and a low (10^5 cfu ml^{-1}) dose

	Salmonella		*E. coli*		*L. innocua*	
	High	Low	High	Low	High	Low
Initial[a]	5.7±0.2	3.7±0.3	5.3±0.0	3.4±0.2	5.1±0.2	3.7±0.4
DW	0.5[b]	0.6[b]	0.5[b,*]	1.0[b,*]	0.3[b]	0.9[b]
NEW	1.5	1.5[a]	1.6[a]	1.0[a]	1.3[a]	1.0[a]
NEW	1.3	1.7[a]	1.8[a]	1.1[a,*]	1.4[a,*]	1.1[a]
SH	1.6[a]	1.6[a]	0.8[a]	1.2[a]	1.1[a]	1.2[a]

Treatments: Deionized water (DW), neutral electrolyzed water (NEW) diluted at 1:5 and 1:3 (NEW volume:Total volume) and sodium hypochlorite treatment containing 100 ppm of free chlorine (SH). In the same column, different letters indicate significant differences between treatments. For each microorganism and treatment, an * indicates differences with respect to the inoculated dose.

[a] Initial concentration of the pathogen on lettuce, log cfu g^{-1}.

Finally, no changes in lettuce appearance were noted after applying any of the treatments.

3.4. Effect of NEW on bacterial mixtures artificially inoculated on lettuce

The initial concentrations of foodborne pathogens on lettuce when they were coinoculated at high dose (10^7 cfu ml^{-1}) were 5.7, 5.3 and 5.1 log cfu g^{-1} for *Salmonella*, *E. coli* and *L. innocua*, respectively, and 3.7, 3.4 and 3.7 log cfu g^{-1} when they were applied at low dose (10^5 cfu ml^{-1}, Table 3).

Again, there were no significant differences between NEW at 1:5, NEW at 1:3 and SH for any of the microorganisms or for either of the two inoculation doses. The reductions in bacteria obtained were slightly greater for *Salmonella* than for *E. coli* and *L. innocua*, and were similar to those obtained when the pathogens were individually inoculated at 10^8 cfu ml^{-1}.

Regardless of the inoculation dose (high or low), the reductions achieved were not significantly different, except for *E. coli*. When lettuce was inoculated with this bacterium and treated with DW or NEW at 1:3, treatment was more effective (produced a significantly greater reduction, $P<0.05$) when the initial *E. coli* population on lettuce was lower.

3.5. Effect of NEW on the native microbiota of different fresh-cut vegetables

The efficacy of NEW at 1:5 (52 ppm) for reducing the native microflora present in different packaged fruit and vegetable produce items was evaluated and compared with that of DW and SH (118 ppm). The total aerobic mesophilic count (Fig. 2) was higher for carrot (8.8 log cfu g^{-1}, data not shown) than for iceberg lettuce, endive, corn salad and 'Four seasons' salad (5.7, 5.5, 6.1 and 5.7 log cfu g^{-1}, data not shown). The reductions of indigenous microbiota on fresh-cut Iceberg lettuce were 0.4, 0.8 and 0.9 log cfu g^{-1} of total aerobic mesophilic counts for DW, NEW at 1:5 and SH, respectively. Similar results were obtained for 'Four seasons' salad (whose main ingredient was Iceberg lettuce). Microbial reductions for grated carrot and endive were slightly greater than those obtained for other produce. Microbial reductions in corn salad were very small for all treatments.

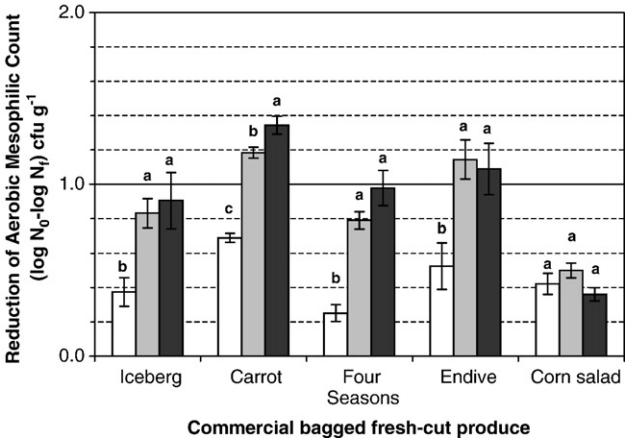

Fig. 2. Reduction of total aerobic mesophilic count (log cfu g^{-1}) in different packaged commercial fresh-cut produces after treatment with cold (8±2 °C) deionized water (DW, □), NEW 1:5 (▩) and sodium hypochlorite 100 ppm (SH, ■) for 3 min. For each fresh-cut product, different letters indicate significant differences between treatments. Bars indicate standard error of means.

With the exception of carrot, no significant differences were found between treatments with NEW at 1:5 and SH for any of the fresh-cut vegetables tested. Moreover, NEW at 1:5 and SH were more effective at reducing microbial populations than DW.

No changes in vegetable appearance were noted with any of the treatments.

4. Discussion

The results obtained in this study demonstrated that the bactericidal activity of diluted NEW (50 ppm of free chlorine approximately, pH 8.60) against *E. coli* O157:H7, *Salmonella*, *L. innocua* and *E. carotovora* on lettuce was similar to that of chlorinated water (120 ppm free chlorine). Moreover, our results revealed that immersing fresh-cut lettuce, carrot, endive, corn salad and 'Four seasons' salad in NEW at 1:5 (about 50 ppm) was equally effective in reducing indigenous microbiota as washing with chlorinated water at approximately 120 ppm. Using NEW would therefore make it possible to reduce the amount of free chlorine used by the fresh-cut industry for the disinfection of fruit and vegetables, while guaranteeing the same microbial reduction.

The NEW dilutions employed for the lettuce treatments were based on the studies carried out in pure cultures. We selected treatments that caused a reduction in all studied microorganisms of more than 5 log cfu ml^{-1}. The treatment at low temperature was selected because when NEW was used at a low concentration (1:10), bactericidal activity was higher than at ambient temperature. Moreover, this is particularly relevant for practical applications as low temperatures are currently used on processing lines to maintain the quality of fresh-cut produce. Large reductions (>5 log cfu ml^{-1}) in pure cultures of the four strains tested were observed when using NEW at 1:6 and 1:3 and SH (120 ppm).

The main aim of this study was to evaluate the efficacy of NEW as a disinfectant for fresh-cut vegetables. It has been demonstrated that the reductions obtained were smaller when the microorganisms were inoculated onto the vegetable target (1 to 2 log cfu g^{-1}) than when they were tested as pure cultures. In general, when microorganisms were individually inoculated onto lettuce, no significant differences were observed between treatments with NEW at 1:6, NEW at 1:3 and SH. Moreover, treatment time (1 or 3 min) did not significantly affect the antimicrobial activity of NEW and SH. Finally, similar reductions were observed for the four strains studied, indicating that NEW has a broad spectrum of action against bacteria.

Several studies have investigated the efficacy of using acidified electrolyzed water (AEW), but fewer have focused on the use of NEW. Moreover, the test conditions for these studies were not the same as for ours. The reductions in *S. typhimurium*, *E. coli* and *L. monocytogenes* populations on lettuce after treatment with EW, at a higher free chlorine concentration (300 ppm, pH 9, 750 mV) were 1.7, 2.1 and 1.6 log cycles, respectively

(Yang et al., 2003). Park et al. (2001) found greater reductions in *E. coli* O157:H7 and *L. monocytogenes* on lettuce (2.41 and 2.65 log cfu/lettuce) but they used AEW (pH 2.5, 1130 mV and 45 ppm residual chlorine). In that study, the lettuce was inoculated by dropping rather than dipping. Koseki et al. (2003) reported that the effectiveness of sanitizers, including AEW, was affected by the method of inoculation, with treatments being more effective when inoculation was done by dropping than by dipping. As bacterial cells would penetrate into the cut edges during dip inoculation, sanitizers could not easily access the bacteria (Koseki et al., 2003). In that study, Koseki et al. (2003) found similar results when testing the efficacy of a chlorine solution (200 ppm), acidic electrolyzed water (pH 2.6, 40 ppm) and deionized water against *E. coli* and *Salmonella* on lettuce at 4 and 20 °C. Koseki et al. (2004a) later reported greater pathogen reductions when combining a pre-wash treatment with alkaline EW followed by an acidic EW treatment at mild temperatures.

It was also notable that no cells of any of the microorganisms studied were detected in the NEW solution after treatment (detection limit 10 cfu ml^{-1}). This is very interesting and suggests that NEW could prevent cross-contamination of fresh produce in the fresh-cut industry. In contrast, bacteria counts of about 10^5 cfu ml^{-1} were found in the deionized water wash after treatment. Deza et al. (2003) and Park et al. (2001) reported similar results using NEW (86–92 ppm active chlorine, pH 7.99–8.19, ORP 745–760 mV) and AEW (pH 2.5, 1130 mV and 45 ppm residual chlorine), respectively.

In the study of the effect of NEW on lettuce artificially inoculated simultaneously with three foodborne pathogens at different doses, it was demonstrated that population size did not affect the efficacy of sanitizer on lettuce. Similar results were observed by Koseki et al. (2003) using AEW (pH 2.6, ORP 1130 mV, 40 ppm free available chlorine).

The effect of NEW on the established natural microbiota (total aerobic mesophilic count) of different fresh-cut vegetables was smaller than that obtained with artificially inoculated bacteria. This was probably because native microbiota in packaged fresh-cut produce could have produced biofilms and could therefore have been more attached to, or become trapped in, the vegetable tissue. Biofilm envelops the bacterial community and benefits it by concentrating nutrients from liquid phase (Costernon et al, 1987). It also protects it from many bactericidal agents (Fatemi and Frank, 1999). Effective washing and decontamination would therefore be more difficult in the presence of biofilm because biocides are not readily accessible to microorganisms forming biofilm. The total aerobic mesophilic count included not only bacteria but also yeasts and molds, which could be more resistant to electrolyzed water. Brackett and Splittstoesser (2001) pointed out that it is natural for sanitizing agents to be considerably more effective against molds and yeasts than against aerobic mesophilic microbiota, since mesophilic aerobic populations of vegetables, and particularly of those grown close to the soil surface, include several gram-positive spore-forming bacteria of the *Bacillus* genus, which are known to be more resistant to chemical sanitizing agents. More studies should be carried out to evaluate whether there are significant differences between the sensitivity to NEW of bacteria, yeast and molds. Izumi (1999) tested the effect of electrolyzed water (pH 6.8, 15 to 50 ppm available chlorine) on carrots, bell peppers, spinach, Japanese radish and potatoes and found reductions in total microbial counts ranged from 0.6 to 2.6 log cfu g^{-1}; the effectiveness was greater with spinach leaves than with chopped bell peppers and diced potatoes. Deza et al. (2003) also found that NEW was an effective method for controlling the presence of *E. coli* O157:H7, *L. monocytogenes* and *S. enteritidis* on the surface of tomatoes. Surface area, anatomy and tissue microstructure, which differ among vegetables as well as types of cut, could also affect the degree of contact between electrolyzed water and microorganisms (Izumi, 1999).

Although it was not an objective of this work, several authors have studied the effect of electrolyzed water on the quality of whole and fresh-cut vegetables and reported that this treatment did not affect tissue pH, surface color, general appearance or customer acceptance (Izumi, 1999, Bari et al., 2003; Deza et al., 2003). No differences were observed in the visual appearance of lettuce, carrot, endive and corn salad treated in our assays.

Our studies demonstrated that NEW exhibits a similar level of bactericidal efficacy to chlorinated water at lower free chlorine concentration, which could allow the fresh-cut industry to reduce the amount of chlorine used. This would help to improve the safety of both products and workers. Moreover NEW has the advantage of being non-corrosive (unlike acidified EW), and in comparison with sodium hypochlorite, it is safer, can be produced 'in situ' and is easier to handle. NEW could be used instead of sodium hypochlorite as an effective disinfectant for killing some foodborne pathogens such *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* that could be present on fresh-cut vegetables. However, neither the chlorinated water nor NEW treatments have proved to completely eliminate foodborne pathogens and natural microflora in these tests. It should be noted that in our experiment foodborne pathogens were artificially inoculated at a very high dose. Such high levels of bacteria are not normal under practical conditions when the fresh-cut industry implements GMP and HACCP programs. Moreover, a limited control of non-pathogenic indigenous microflora may also be desirable. Bennik et al. (1996) and Babic et al. (1997) reported that the presence of native microorganisms on fresh-cut vegetables had inhibitory effects on growth of *L. monocytogenes*. Further studies should be carried out to simulate typical commercial conditions.

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