The Effect of pH on Inactivation of Pathogenic Bacteria on Fresh-cut Lettuce by Dipping Treatment with Electrolyzed Water

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ABSTRACT: Fresh-cut lettuce samples inoculated with *S.* Typhimurium, *E. coli* O157:H7 or *L. monocytogenes* were dipped into 300 ppm electrolyzed water (EW) at pH 4 to 9 and 30 °C for 5 min. The effects of treatment pH on bacterial reduction and visual quality of the lettuce were determined. The treatments at pH 4 and 8 resulted in the most effective inactivation of *E. coli* O157:H7, but the effect of pH was not significant ($P > 0.05$) for *S.* Typhimurium and *L. monocytogenes*. The treatment at pH 7 retained the best visual quality of lettuce, and achieved a reduction of approximately 2 log CFU/g for above 3 bacteria.

Keywords: fresh-cut vegetables, electrolyzed water, dipping treatment, pH, bacterial reduction

Introduction

OUTBREAKS OF FOODBORNE ILLNESS ASSOCIATED WITH CONsuming raw vegetables occur more frequently in recent years in the United States (Park and Beuchat 1999; Mead and others 1999). Fresh-cut vegetables are highly susceptible to microbial contamination because of microbial cross-contamination through the shredders and slicers (Garg and others 1990) and the exposure of inner tissues to microbial attachment and growth after cutting (Brackett 1987). The pathogenic bacteria of the most concern in fresh vegetables include *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* Typhimurium (CDC 1997; Beuchat 1996; Farber and others 1990).

Chemical agents such as sodium hypochlorite (Zhang and Farber 1996), chlorine dioxide (Zhang and Farber 1996), sodium bisulfite (Krahn 1977), sulfur dioxide (Bolin and others 1977), organic acids (Adams and others 1989; Zhang and Farber 1996), calcium chloride (Izumi and Watada 1995), trisodium phosphate (Zhang and Farber 1996), ozone (Nagashima and Kamoi 1997), and cetylpyridinium chloride (Wang and others 2001) have been studied as potential disinfectants of fresh-cut vegetables. Sodium hypochlorite with a maximum concentration of 2000 ppm is the only chemical agent currently allowed by federal regulations for washing fruit and vegetables (FDA 1996). Chlorinated water of 50 to 200 ppm is widely used for reducing bacterial contamination on whole fruits, vegetables, and fresh-cut produces on the commercial processing (Beuchat and others 1998). A problem with chlorine treatment is that a buildup of organic materials in wash water inactivates chlorine rapidly (Gelinas and Goulet 1983).

Electrolyzed Water (EW) contains a mixture of inorganic oxidants such as HClO, OCl^-, Cl_2, OH, and O_3, which are effective for inactivating a variety of microorganisms (Wang and Doyle 1998; Yang and others 1999; Vinkitanarayanan and others 1999; Len and others 2000). The ability of EW to provide better microbial reductions also attributes to the fact that EW is freshly produced and used immediately. As a disinfectant for fresh-cut vegetables, EW of < 50 ppm was first evaluated by Izumi (1999), and the results of total plate count on squash were reported with a reduction of 0.6 to 2.6 log CFU/g after the treatment. Later, EW was compared with acidified chlorinated water (Park and others 2001), and chlorinated and ozonated water (Koseki and others 2001) for the application in the treatment of lettuce. No significant difference ($P > 0.05$) in bacterial reduction and the color of lettuce was found between the treatments using EW and chlorinated water at oxidant concentration of 45 ppm and treatment pH of 2.5 (Park and others 2001). EW with oxidant concentration of 30 ppm and pH of 2.6 showed a similar bactericidal effect as 150 ppm chlorinated water of pH 9.3, and provided a better bactericidal effect than 5 ppm ozonated water of pH 6.6 (Koseki and others 2001). In our previous study (Swem and others 2002), bacterial reductions on fresh-cut lettuce, broccoli, and sprouts were tested for EW treatment with different oxidant concentrations (100 and 300 ppm), treatment temperatures (25, 30, and 35 °C) and treatment times (3 and 5 min) at a neutral pH of 7. *Listeria monocytogenes*, *E. coli* O157:H7, and *S.* Typhimurium on fresh-cut lettuce were reduced by 2.5 to 4.0 log CFU/g after 5-min dipping treatment with 300 ppm EW at 30 °C.

The research by Len and others (2000) indicated that pH value determined the equilibrium of hypochlorous acid (HClO) and hypochlorite ion (OCl^-) in buffered EW solution, and in turn, affected the reduction of bacteria in the solution. However, no literature has been found for the effects of pH in EW treatment of vegetable on the bacterial reduction, and the color of vegetables. In our previous study (Swem and others 2002) and in the research by Park and others (2001), vegetables were treated with EW in approximately 1 h after inoculation. In nature, vegetables may be contaminated for a longer time before or during processing and bacterial biofilm may be developed on the vegetables during that period of time. In a study on EW treatment of lettuce, Koseki and others (2001) did observe bacterial biofilm on lettuce under scanning electron microscope (SEM). Bacterial biofilm may present more difficulties for bacterial inactivation with chemical agents (Koseki and others 2001). No literature has been found for the EW treatment of lettuce to reduce *L. monocytogenes*, *E. coli* O157:H7, and *S.* Typhimurium with biofilm. The objectives of this research are to determine an optimal pH for EW treatment of fresh-cut lettuce considering both bacterial reduction and visual quality of the vegetable, as well, to determine bactericidal effect of EW on *L. monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium with biofilm at the optimal treatment pH.

Materials and Methods

EW and chlorinated water

An electrochemical system, STEL-80 (Electrochemical Technologies, Ltd., Las Vegas, Nev., U.S.A.), was used to generated EW in a broad range of pH. The system consisted of an electrochemical reactor incorporated of 8 electrochemical cells, known as Flow-through Electrolytic Modules (FEM elements). Every FEM element consisted of an internal rod electrode as the anode, an external tubal electrode as the cathode, and a ceramic diaphragm placed between them. The electrodes were made of titanium. The anode was coated with iridium ruthenium oxide on the top of titanium to catalyze the conversion of chloride ion (Cl^-) into oxidizing species (Cl_2, ClO^-, HClO, O_3, OH+). Tap water was mineralized with a 7.5% sodium chloride solution online to a salinity level of 0.01 to 0.05% and flowed through 8 FEM elements connected in parallel at a flow rate of 80 L/h. A 40 V of direct current was applied to operate the system. Inside the electrolytic cell electrochemical synthesis of neutral anolyte took place around the anode, generating inorganic chemical compounds such as HClO, ClO^-, OH^+, and O_3. Anolyte was collected as EW for the treatment. The oxidant concentration of 300 ppm in the EW was obtained by controlling the amperage at 6 A, and the pH of EW was adjusted by the amount of alkaline catholyte (containing OH^-) allowed into the runoff of the anolyte.

For comparison with EW, chlorinated water with 300 ppm of available chlorine was prepared using sodium hypochlorite (8.4%, Ecolab, St. Paul, Minn., U.S.A.) and tap water. An 8.5% phosphoric acid solution was used to adjust the pH of chlorinated water.

The total oxidant concentration, in terms of available chlorine, was measured with an ion specific meter (Model H193711, Hanna Instruments, Woonsocket, R.I., U.S.A.). The pH value and oxidation-reduction potential (ORP) were measured with a pH/ion/conductivity meter (Accument® model 50, Fisher Scientific, Pittsburgh, Pa., U.S.A.) with pH and ORP electrodes, respectively. The absorbance of EW and chlorinated water at 235 and 292 nm was measured using a UV-visible spectroscopy system (Hewlett Packard, Waldbronn, Germany) to determine the concentrations of HClO and OCl^-, respectively, in the solutions, based on Beer's law equation (Len and others 2000) and the molar absorptivities reported by Morris (1966).

Inoculation of lettuce

The stock cultures of nalidixic acid (N) resistant mutant *Salmonella* Typhimurium (ATCC 14029), *Escherichia coli* O157:H7 (ATCC 43888), and *Listeria monocytogenes* (FDA 10143) were maintained at 4 °C. To determine the effect of treatment pH on each of 3 bacteria, a pure culture for 1 of above organisms was grown in brain-heart infusion at 37 °C for 18 to 20 h, and suspended into 0.85% physiological saline solution (PSS) for an inoculum of about 10^7 CFU/ml. To compare the competitive attachment, the pure cultures of 3 bacteria were mixed and suspended into PSS to obtain an inoculum containing about 10^7 CFU/ml cells for each of the bacteria.

Fresh-cut romaine lettuce was obtained from a local market on the day of the experiment. The lettuce was preweighed to 25-gram samples, and each sample was submerged into 150 ml of inoculum in a 250 ml beaker for 1 min. After removal from the inoculum, the lettuce samples were stored at 7 °C for 24 h for the formation of bacterial biofilm. Then, samples were rinsed with double deionized (DD) water for 30 s to remove loosely attached bacteria. Lettuce samples prepared above were used in EW dipping treatment, as well, in SEM test for the observation of biofilm. As the controls without biofilm, lettuce samples were used immediately after inoculation without storage.

Dipping treatment

Six trials were conducted, each with single test pH within the range of 4 to 9. In each trial, 24 lettuce samples were divided into 4 groups. Each groups consisted of 6 samples, 3 as controls and 3 for EW treatment. Group 1 was used without inoculation to determine the color change of lettuce after the treatment. Groups 2, 3, and 4 were inoculated with *L. monocytogenes*, *E. coli* O157:H7, and *S.* Typhimurium, respectively, to determine the reduction of individual bacterial specie due to the treatment. For the treatment, lettuce sample was dipped into a 2 L beaker containing 800 ml of 300 ppm EW for 5 min at 30 °C. Treatment temperature was achieved by running EW through tubing suspended in a water bath (Fisher Scientific Isotemp Model 101M, Fisher, Pittsburg, Pa., U.S.A.). Samples were actively agitated using glass stirring-rods during the treatment, and rinsed for 30 s using tap water to wash off the possible residual of EW after the treatment.

Microbial enumeration

Each control and treatment sample was suspended in 225 ml of 0.1% buffered peptone water (Remel, Lenexa, Kans., U.S.A.) in a stomaching bag (Nasco Whirlpak®, 71/2" by 12"), and stomached for 1 min in a stomacher (model 400, Seward Medical Ltd, London, U.K.). Stomaching water of the control samples was collected, diluted to 10^{-2}, and then plated on the 150 mm (in dia) plates, while the stomaching water of the treatment samples was directly plated without the dilution. Plating was performed by a Wasp Spiral Plater (DW Scientific, W. Yorkshire, U.K.). MacConkeys Agar (Difco, Detroit, Mich., U.S.A.), Oxford Agar (Oxoid, Basingstoke, Hampshire, U.K.), and Trypticase Soy Agar (TSA, EM Science, Gibbs Town, N.J., U.S.A.) with 200 ppm nalidixic acid were used for enumeration of *E. coli* O157:H7, *L. monocytogenes*, and (N) *S.* Typhimurium, respectively. The plates were incubated at 37 °C, and colonies were counted with a ProtoCol plate counter (Symbiosis, Cambridge, U.K.) after 24 h for *E. coli* O157:H7 and (N) *S.* Typhimurium, and after 48 h for *L. monocytogenes*. For the better recovery of injured bacteria, colonies were counted again after incubation for another 24 h.

Scanning electron microscopy (SEM)

Aseptically cut lettuce leaves (0.5 × 0.5 cm^2) were immersed in 2% Karnovsky's fixative for 2 h and washed twice with 0.05 *M* cacodylate buffer (pH 7.2) (Polysciences, Warrington, Pa., U.S.A.) for 20 min each. Samples were post-fixed with 1% osmium tetroxide (Electron Mcroscopy Sciences, Fort Washington, Pa., U.S.A.) in 0.05 *M* cacodylate buffer for 2 h and washed with distilled water for 1 to 2 min. The fixed samples were dehydrated with ethanol (once in 30, 50, 70, 80, and 95%, and 3 times in 100%) (Fisher Scientific, Fair Lawn, N.J., U.S.A.) for 15 min each, washed 3 times with hexamethyldisilazane (Electron Microscopy Sciences, Fort Washington, Pa., U.S.A.) for 5 min each, and then dried. The samples were mounted on aluminum stubs, sputter coated with gold, and viewed by ISI-60 scanning electron microscope (International Scientific Instrument, Japan) at 15 kV with magnification factor of 3000 times.

Evaluation of the color of lettuce

The color of the lettuce at outer edges of the leaves (without stem) was measured using a Chroma Meter colorimeter (Model Cr-300, Minolta Co., Ramsey, N.J., U.S.A.), and expressed as Hunter-Lab (*L**) for whiteness and darkness, Chroma for richness and dullness, and Hue for the actual perceived color such as green or yellow.

Statistical analysis

The reduction of bacteria on fresh-cut lettuce after EW treatment with the tested treatment pH were calculated by subtracting the

mean populations of bacteria (Log CFU/g) in the treatment samples (triplicates) from the mean population in the control samples (triplicates). The means of color values were calculated for control and treatment samples, based on the triplicates for each pH. The significant difference between the treatments in terms of bacterial reduction and the change in the color of the lettuce were analyzed by Student *t*-test at a significance level of $\alpha = 0.05$ using JMP software (SAS institute, Cary, N.C., U.S.A.).

Results and Discussion

Bacterial competitive attachment

The numbers of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* recovered from lettuce samples were close when these 3 bacteria were inoculated separately (Figure 1), which demonstrated that 3 bacteria had similar attachment to lettuce when they were alone. However, when these 3 bacteria were inoculated together, the average numbers of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* attached to lettuce were 6.1, 6.4, and 3.8 log CFU/g, respectively (Figure 1). *Salmonella* Typhimurium and *E. coli* O157:H7 retained similar attachment as they were inoculated alone, whereas, *L. monocytogenes* showed an attachment of 2 log CFU/g less. Takeuchi and others (2000) reported that *E. coli* O157:H7 and *L. monocytogenes* attached preferentially to cut edge of lettuce, and *S.* Typhimurium had similar preferences on the intact surface and cut edge. Obviously, *L. monocytogenes* failed to compete with *S.* Typhimurium and *E. coli* O157:H7 in their attachment to the lettuce.

Bacterial reduction

The treatment with 300 ppm EW at pH 7 and 30 °C reduced *S.* Typhimurium, *E. coli* O157:H7, and *L. monocytogenes* by 2.0, 2.0, and 2.1 log CFU/g, respectively, in 5 min (Table 1). Lower bacterial reductions were obtained comparing to the results of a previous study (Swem and others 2002), in which the reductions of above 3 bacteria were 2.5, 3.0, and 4.0 log CFU/g, respectively, under the same treatment condition. The difference is that in previous study, lettuce samples were treated at 30 min after inoculation, but in this study inoculated lettuce samples were stored at 7 °C for 24 h before the treatment. Similar numbers of bacteria (approximately 6 log CFU/g) were recovered from inoculated lettuce before and after storage (Figure 2). This result indicated that bacteria neither multiplied nor died during the storage. With scanning microscopy, bacterial biofilm, a sticky and threadlike substance surrounding the bacterial cells, was observed on the inoculated lettuce samples after 24-h storage (Figure 3a-2, 3b-2, and 3c-2). No biofilm was observed on the control samples without the 24-h storage (Figure 3a-1, 3b-1, and 3c-1). Biofilm consists of an adhesive extra-cellular polymer called glycocalyx, which produced by bacteria within a liquid phase contiguous to a surface (Costerton 1987). Biofilm envelops bacterial community, benefits it by concentrating nutrients from liquid phase (Costerton 1987), and protects it from many bactericidal agents (Fatemi and Frank 1999). During 24-h storage, bacteria within the water phase contiguous to the surface of lettuce developed the biofilm, and it might become more resistant to EW treatment.

The effects of pH on the reductions of *S.* Typhimurium and *L. monocytogenes* were not statistically significant within the tested range ($P > 0.05$). The log reductions of *S.* Typhimurium and *L. monocytogenes* ranged from 1.5 to 2.0 and 1.7 to 2.1, respectively, with the treatment pH ranging from 4 to 9 (Table 1). The variation of bacterial reduction associated with the change in treatment pH was ± 0.5 log CFU/g. However, the pH of EW significantly ($P < 0.05$) affected the survival of *E. coli* O157:H7. The reduction of 2.0 to 2.2 log CFU/g was achieved for *E. coli* O157:H7 at either pH 4 or pH 7, while a reduction of 1.3 log CFU/g, was obtained at pH 5 to 6 (Table 1).

Color change of lettuce

No obvious color change of lettuce was visually observed after EW treatment. The mean hue values of treated and untreated lettuce were 117° and 118 to 121°, respectively (Table 2). Above hue values described the yellow-green color of lettuce. The lower L^* and Chroma values for treated samples (Table 2) indicated that the color of lettuce became darker and duller after treatment. The lettuce samples treated at pH 7 had color values most close to the values of control samples (Table 2).

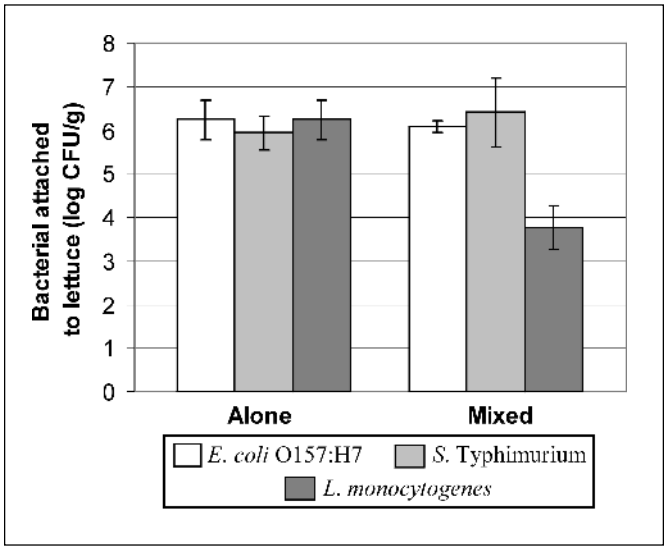

Figure 1—Bacterial attachment to lettuce when 3 bacteria inoculated alone and together. Each mean value and standard deviation was calculated based on 6 samples in 2 trials.

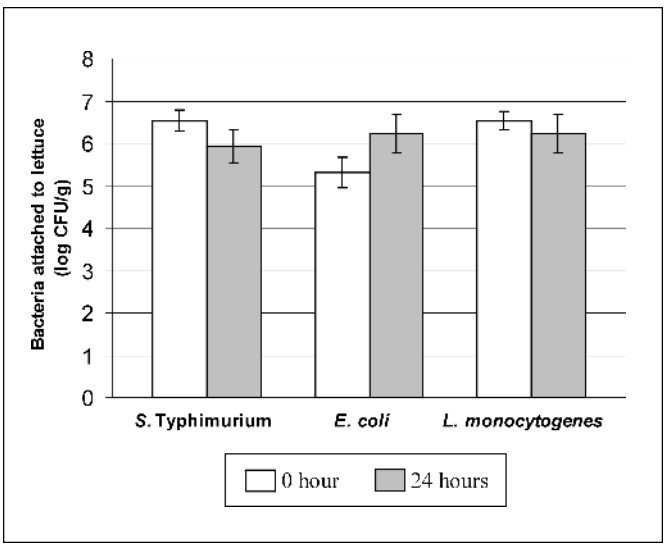

Figure 2—Bacterial attachment to lettuce immediately after inoculation (0-h) and after inoculation followed by 24-h storage (24-h). Each mean value and standard deviation was calculated based on 6 samples in 2 trials.

Properties of EW and chlorinated tap water

The results of our previous study (Swem and others 2002) showed that bactericidal efficacy of EW treatment was significantly ($P < 0.05$) higher than the treatment with chlorinated water at the same pH (pH 7) and oxidant concentration (300 ppm). Table 3 shows that EW and chlorinated water with an oxidant concentration of 300 ppm had similar ORP values at the same pH. The ORP was decreasing with the increasing pH. Although the total chlorine in chlorinated water was approximately 30 ppm lower than that in the EW due to the errors introduced during EW generation and chlorinated water preparation, the equilibrium of ClO^-/HClO in EW and chlorinated water showed the same trend verse the pH (Table 3).

Table 1—The reduction of bacteria on fresh-cut lettuce after electrolyzed water treatment with different pH

	Bacterial reduction, log cycle		
pH	*S.* Typhimurium	*E. coli*	*L. monocytogenes*
4	$1.7^a \pm 0.4$	$2.2^a \pm 0.3$	$2.0^a \pm 0.1$
5	$1.8^a \pm 0.1$	$1.3^b \pm 0.2$	$1.9^a \pm 0.4$
6	$1.8^a \pm 0.3$	$1.3^b \pm 0.3$	$1.9^a \pm 0.2$
7	$2.0^a \pm 0.3$	$2.0^a \pm 0.1$	$2.1^a \pm 0.3$
8	$1.5^a \pm 0.4$	$2.2^a \pm 0.1$	$1.7^a \pm 0.1$
9	$1.7^a \pm 0.3$	$2.1^a \pm 0.1$	$1.6^a \pm 0.3$

*Values of reduction within the same column with the same letter are not significantly different ($P > 0.05$).

Table 2—The color of lettuce samples before (control) and after treatment (treatment) with different pH.

Sample		L	Chroma	Hue
Control		61.94 ± 3.78	39.39 ± 2.79	117.41 ± 1.08
Treatment	pH 4	44.27 ± 1.07	31.27 ± 1.65	118.74 ± 0.12
	pH 5	43.07 ± 4.14	28.69 ± 0.29	120.52 ± 0.02
	pH 6	43.94 ± 6.41	30.21 ± 3.19	121.96 ± 2.44
	pH 7	65.81 ± 0.45	40.53 ± 4.07	116.62 ± 0.75
	pH 8	48.94 ± 4.85	31.19 ± 1.87	121.21 ± 2.02
	pH 9	44.51 ± 3.56	36.23 ± 3.29	120.52 ± 1.02

Table 3—The properties of 300 ppm chlorinated water and electrolyzed water at different pH

	Chlorinated water				Electrolyzed water			
pH	ORP* (mv)	HClO (ppm)	ClO^- (ppm)	HCLO + ClO^- (ppm)	ORP (mv)	HClO (ppm)	ClO^- (ppm)	HCLO + ClO^- (ppm)
4	1122	225	18	243	1150	269	22	291
5	1048	216	16	232	1000	258	21	279
6	987	223	20	243	964	252	35	287
7	924	196	54	250	880	231	75	306
8	870	91	155	246	780	120	174	294
9	769	43	206	249	750	68	198	266

*Oxidation-reduction potential

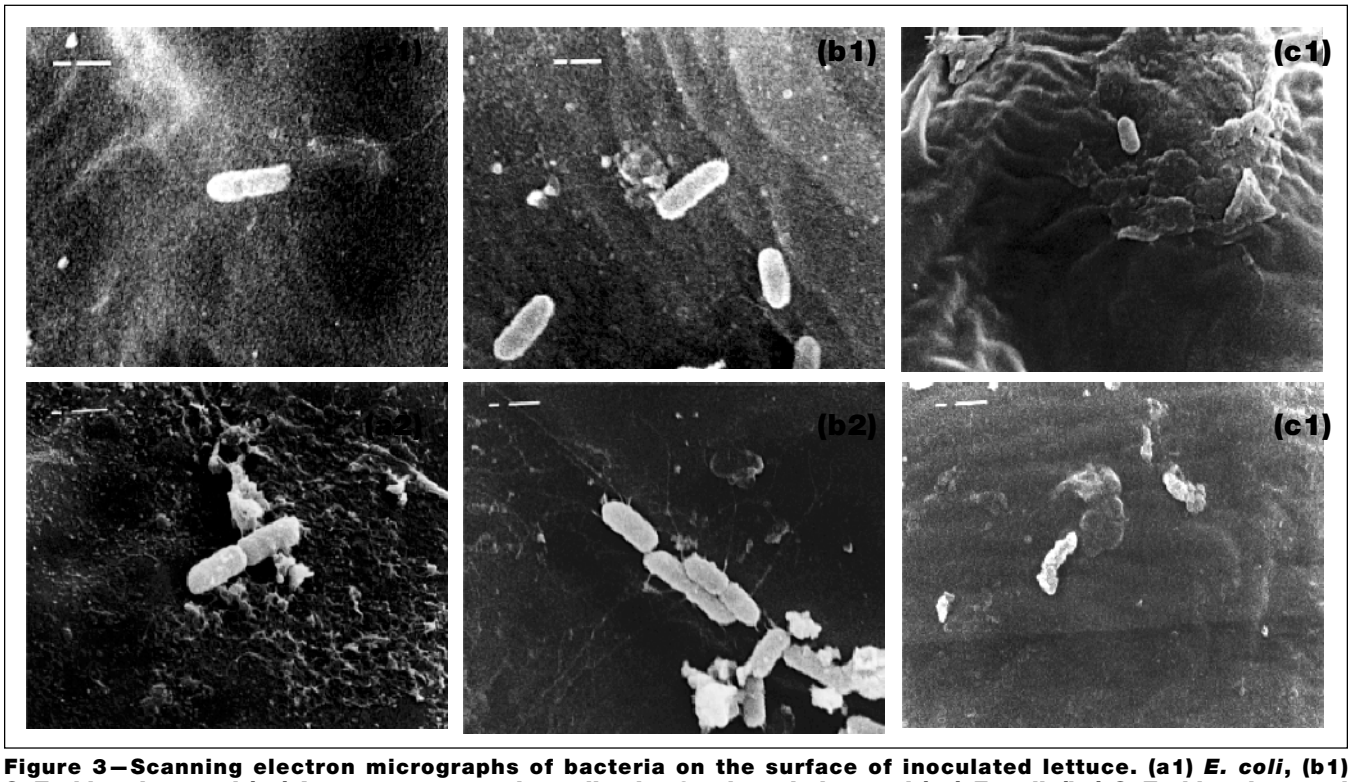

Figure 3—Scanning electron micrographs of bacteria on the surface of inoculated lettuce. (a1) *E. coli*, (b1) *S.* Typhimurium, and (c1) *L. monocytogenes* immediately after inoculation, and (a2) *E. coli*, (b2) *S.* Typhimurium, and (c2) *L. monocytogenes* after the inoculation followed 24-h storage at 7 °C.

When pH value was lower than 7, the majority of chlorine presented in the form of HClO, and the concentration of ClO^- was lower than 100 ppm with a total chlorine of 300 ppm. The concentration of ClO^- and HClO approached to equal between pH 7 and 8. When pH increased to a value higher than 8, the majority of the chlorine presented in the form of ClO^-. ORP and relative concentrations of chlorine species have been reported as important factors in EW treatment (Kim and others 2000; Len and others 2000). However, no evidence was found in this study that the ORP and the ratio of ClO^-/HClO were the factors attributed to the higher bactericidal efficacy of EW comparing to the chlorinated water with the same pH and oxidant concentration.

Conclusions

IN SUMMARY, A PH OF 7 WAS SUGGESTED FOR THE ELECTROLYZED WAter dipping treatment of lettuce to achieve the greatest reduction on *E. coli* O157:H7 and retain the best visual quality of the lettuce. Under this condition, an approximately 2-log reduction was achieved for *S.* Typhimurium, *E. coli* O157:H7, and *L. monocytogenes* with biofilm after 5 min treatment.

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