Effects of Bacterial Concentrations and Centrifugations on Susceptibility of *Bacillus subtilis* Vegetative Cells and *Escherichia coli* O157: H7 to Various Electrolyzed Oxidizing Water Treatments

© 2015. This manuscript version is made available under the Elsevier user license
<http://www.elsevier.com/open-access/userlicense/1.0/>

Chunling Zhang^a, Baoming Li^{a*}, Ravirajsinh Jadeja^b, Jianlong Fang^c, Yen-Con Hung^{b*}

^a Key Laboratory of Bio-environmental Engineering, Ministry of Agriculture, China Agricultural University, P.O. Box 67, Beijing 100083, P.R. China

^b Department of Food Science and Technology, University of Georgia, 1109 Experiment Street, Griffin, GA 30223, USA

^c Institute for Environmental Health and Related Product Safety, Chinese Center for Disease Control and Prevention, No.7 Panjiayuananli, Chaoyang District, Beijing, 100021, P.R. China

* Corresponding authors.

Yen-Con Hung, Professor, Department of Food Science and Technology, University of Georgia,
1109 Experiment Street, Griffin, GA 30223-1797, USA.

Phone: 770-412-4739, Fax: 770-412-4748, E-mail: yhung@uga.edu

Baoming Li, Professor, 17 Qinghua East Road, Haidian District, Beijing 100083, P.R. China.

Phone: 86-010-62736904, E-mail: libm@cau.edu.cn

Abstract

This study discussed the effects of different bacterial concentrations and centrifugations on the antimicrobial efficacy of electrolyzed oxidizing water (EO) on *Bacillus subtilis* and *Escherichia coli* O157: H7. Overnight grown bacterial cultures were centrifuged 1 to 3 times and bacterial concentrations were adjusted to approximately 9 (high), 7 (medium), or 5 (low) log₁₀ CFU/mL. Antimicrobial efficacy of acidic (AEW) and neutral pH (NEW) EO water containing 0.25 to 30 mg/L available chlorine was determined. In order to ascertain the effects of AEW and NEW on targeted pathogens cellular properties at bio-molecular levels were also studied. The results showed that the susceptibility of both pathogens decreased significantly with increasing bacterial concentrations. AEW with 10, 0.25 and 0.25 mg/L and NEW with 30, 0.5 and 0.25 mg/L available chlorine were needed for high, medium and low bacterial concentrations, respectively to non-detectable levels by direct plating for *E. coli* O157:H7. *B. subtilis* was found more resistant to both EO water treatments and only 4.1 and 3.8 log reductions were achieved for AEW and NEW containing 30 mg/L available chlorine. On the other hand, it was observed that as centrifugation time increased, both bacteria became significantly more sensitive to EO water treatments. When centrifugation period increased from 1 to 3 times, additional 2.67 and 3.38 log *E. coli* O157:H7 reductions were observed for AEW and NEW treatments, respectively. A similar trend was observed for *B. subtilis*. DNA and protein leakage increased when pathogens were treated by AEW and NEW with increasing available chlorine concentration, but decreased DNA and protein leakage were observed with increased centrifugation times. These results indicate that initial bacterial concentration and the centrifugation time are two important factors and should be carefully considered in chlorine-based antimicrobial efficacy testing.

Keywords: EO water, *E. coli* O157:H7, *B. subtilis*, initial bacterial concentration, centrifugation time

1. Introduction

Escherichia coli O157:H7 is a Gram-negative Shiga toxin-producing strain and has been recognized as a major foodborne pathogen (Kaper & Karmali, 2008; Scallan, et al., 2011). In the United States it leads to approximately 73,000 illnesses each year (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005). Cattle have been identified as primary reservoir of this pathogen. *E. coli* O157:H7 infections are transmitted through food and water contaminated by cattle feces and cause disease ranging from abdominal cramps, bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (Griffin & Tauxe, 1991; Lathem, Bergsbaken, Witowski, Perna, & Welch, 2003).

Bacillus subtilis is a Gram-positive, non-pathogenic endospore-forming soil bacterium (Driedger, et al., 2001). *B. subtilis* is difficult to control as their spores are resistant to many antimicrobial treatments and may subsequently germinate and grow when environment is suitable. This spore-forming capability of *B. subtilis* makes them a well-known food spoilage organism in variety of processed food products (Jagannath, Tsuchido, & Membre, 2005; Minh, Dantigny, Cornet, & Gervais, 2010). Therefore, it is crucial to develop improved methods for reduction of *E. coli* O157 and *B. subtilis* in food products.

Electrolyzed oxidizing (EO) water has been recognized as an environmentally friendly antimicrobial agent with a broad spectrum antimicrobial activity. EO water is generated by electrolysis of diluted sodium chloride solution in a chamber with anode and cathode electrodes separated by a membrane. EO water collected from anode is known as acidic electrolyzed water (AEW) with a low pH value (<2.7), high oxidation reduction potential (ORP) (>1000 mV) and high available chlorine concentration (ACC), whereas alkaline EO water is collected from cathode (Jadeja, Hung, & Bosilevac, 2013). Near neutral EO water (NEW) with pH of 5.0 to 6.5 is produced by electrolysis of a mixture of anode solution and cathode solution, and it contains high concentration of hypochlorous acid. EO water has been considered as an effective alternative to antimicrobial based treatment used in the food industry (Hsu, 2003). It has been widely used to control pathogens on fresh fruits and vegetables (Forghani & Oh,

2013; Guentzel, Lam, Callan, Emmons, & Dunham, 2010; Hung, Tilly, & Kim, 2010; Pangloli, Hung, Beuchat, King, & Zha, 2009; Park, Alexander, Taylor, Costa, & Kang, 2008, 2009; Stopforth, Mai, Kottapalli, & Samadpour, 2008; Udompijitkul, Daeschel, & Zhao, 2007; Zhang, et al., 2011), meat (Fabrizio & Cutter, 2004, 2005; Rahman, Park, Song, Al-Harbi, & Oh, 2012), eggs (Bialka, Demirci, Knabel, Patterson, & Puri, 2004; Cao, Zhu, Shi, Wang, & Li, 2009; Park, Hung, Lin, & Brackett, 2005; Russell, 2003) and seafood (Lin, et al., 2013; Ren & Su, 2006). EO water has been demonstrated to have strong disinfectant activity against wide range of pathogens. When *Salmonella enteritidis* bacterial suspension was treated by EO water with ACC more than 4 mg/L for 2 min, a reduction of 8.2 log₁₀ CFU/mL was observed (Cao, Zhu, Shi, Wang, & Li, 2009). In a different study when, EO water with pH ranging from 2.6 to 7.0 and ACC with more than 2 mg/L, completely eliminated *E. coli* and *L. monocytogenes* from pure culture (Park, Hung, & Chung, 2004). Many EO water studies (Guentzel, Liang Lam, Callan, Emmons, & Dunham, 2008; Hao, et al., 2013; Iwasawa & Nakamura, 1996; Kiura, et al., 2002; Tamaki, Bui, Ngo, Ogawa, & Imai, 2014; Zeng, et al., 2011; Zhang, et al., 2011) only focused on the effect of organic matter, available chlorine concentration, pH, ORP, treatment time, temperature on EO water antimicrobial efficacy but, very limited information is available on factors such as bacterial concentration and inoculum preparation.

The primary focus of this study was to evaluate the effects of different initial bacterial (*E. coli* O157:H7 and *B. subtilis* vegetative cell) concentrations and centrifugation times on bacterial inactivation by AEW and NEW with different ACC.

2. Materials and methods

2.1. Bacterial culture

In this study a five strain of *E. coli* O157 was used, which consisted of E009 (beef isolate), 932 (human isolate), 1 (beef isolate), 4 (human isolate) and 5 (human isolate). *B. subtilis* ATCC 19659 was obtained from American Type Culture Collection. *E. coli* O157:H7 strains were subcultured in 10 mL of sterile tryptic soy broth (TSB, Difco, Becton Dickinson, MD, USA), whereas *B. subtilis* was cultured in

50 mL plastic tubes containing 35 mL of TSB. These bacterial were incubated at 37°C for 24 h in incubator shaker (New Brunswick Scientific, Edison, NJ, USA) at 150 rpm. Twenty-four-hour cultures of *E.coli* O157:H7 and *B. subtilis* were then sedimented twice by centrifugation (2,600×g, 15min). Cell pellets were suspended in phosphate buffered saline (PBS, pH 7) and final cell concentrations were adjusted to approximately 10⁹ CFU/mL, 10⁷ CFU/mL and 10⁵ CFU/mL. Counts of viable bacteria were obtained by plating 0.1 mL of 10-fold serial dilution of cultures onto sterile tryptic soy agar (TSA, Difco, USA). The plates were incubated at 37°C overnight before enumeration.

2.2. Preparation of EO water

AEW and NEW were produced by electrolyzing 0.03% NaCl solution using an EO water generator (Model #P30HST44T, EAU, GA, USA). The pH, ORP, and ACC of EO water was measured immediately before each bactericidal experiment. The pH and ORP of EO water samples were measured using an ACCUMET pH meter (AR50, Fisher Scientific, Pittsburgh, PA, USA) with a pH electrode and ORP electrode. The initial ACC of EO water was determined by a DPD-FEAS method (Hach Co., Loveland, Co., USA). AEW and NEW with ACC of 0.25, 0.5, 1, 2, 4, 6, 8, 10, 20, 30 mg/L were obtained from EO water dilution with deionized water in different proportions. The pH was adjusted to around 2.7 using 0.1mol/L HCl after AEW diluted.

2.3 EO water treatment

One mL of bacterial suspension was mixed with 9 mL of freshly prepared EO water in 15 mL sterile plastic tube and vortexed for 5 seconds to mix bacteria and EO water completely. At the end of 30s treatment, 1 mL of solution was taken out from treatment tube and mixed with neutralizer buffer containing 5 g/L Na₂S₂O₃ and 0.03 mol/L PBS to terminate any residual chlorine reaction. The mixture was taken and used for further analysis. Surviving cells were enumerated as previously described. All treatments of this experiment were repeated three times.

2.4 Treatment of centrifugation time

Ten mL of *E. coli* O157:H7 and *B. subtilis* cultures grown for 24 h, was centrifuged 1, 2 and 3 times (2,600×g, 15min). The supernatant was decanted and pellet was suspended in 10 mL PBS. Then 1 mL of bacterial suspension was added to a sterilized plastic tubes containing 9 mL of AEW and NEW and mixed for 30s, separately. The reaction was stopped and viable cells were enumerated as previously described. The mixture was also taken as the treated sample for further analysis.

2.5 Measurement of intracellular DNA leakage

Six mL of the analytical/treated sample was centrifuged at 14,000× g for 3 min and the supernatant was taken out to measure the absorbance at 260 nm in a 1 cm quartz cuvette using a DU 520 UV/Vis spectrophotometer (Beckman Coulter Inc., Brea, CA,USA) (Chen & Xue, 2002).

2.6 Measurement of intracellular protein leakage

The analytical sample was centrifuged at 14,000 × g for 3 min. One mL of the supernatant was taken out and stained with 5 mL of Bradford reagent (Bio-world, Dublin, OH, USA) for 5 min. Protein leakage was measured by UV/Vis spectrophotometry, 595 nm by the Bradford method (Zhou, 2003).

2.7 Measurement of conductivity

Ten mL of analytical sample was centrifuged at 14,000 × g for 3 min. The supernatant was collected into sterile tube and the conductivity was tested using an ACCUMET pH meter with conductivity electrode (Tang, et al., 2011).

2.8 Statistical analysis

All results presented were obtained from three independent repeated trials. Statistical analysis was performed using SAS software 9.2 (Institute, Inc., Cary, NC, USA). Least significance difference of means tests was used for multiple comparisons at the 0.05 probability level.

3. Results and discussion

*3.1 Reduction of *E. coli* O157:H7 at different concentration levels by AEW and NEW*

The effect of initial bacterial concentration on *E. coli* O157:H7 resistance to EO water treatment was evaluated by diluted bacteria from high concentration to low and the results were presented in Table 1. The results from Table 1 indicated that bacterial susceptibility to EO water is different at different bacterial concentrations. AEW and NEW containing only 0.25 mg/L available chlorine were able to completely inactivate all bacteria at low levels of initial concentration. At medium levels of initial bacterial concentration, reductions greater than 6.44 and 6.38 log were achieved by treatment with AEW and SAEW with ACC of 0.25 and 0.5 mg/L, respectively. Complete inactivation was achieved for medium concentration when AEW and NEW with more than 0.5 mg/L available chlorine were used. On the other hand, to achieve bacterial reductions to non-detectable levels by direct plating required AEW with ACC of 10 mg/L and NEW with ACC of 30 mg/L for the high inoculation levels. The reductions were significantly increased when the ACC of AEW and NEW increased from 2 to 30 mg/L ($p < 0.05$). On the other hand, the bactericidal activity of AEW was slightly higher than NEW with the same ACC. The bactericidal activity of AEW and NEW for *E. coli* O157:H7 decreased with the initial bacterial concentration increased. At the initial bacterial level of low, medium and high, 4.46, 6.44 and 1.04 log₁₀ CFU/mL and 4.41, 6.38 and 0.77 log₁₀ CFU/mL reductions were achieved by AEW and NEW treatment of with ACC of 2 mg/L, respectively. This could be because of elevated number of bacterial cells resulted in higher organic content in the solution and therefore, available chlorine gets neutralized. Moreover, more organic matter could protect cells from getting injured by EO water treatment. It was reported that organic matter in water increase the resistance of bacteria to chlorine by stabilizing the bacterial membranes and not allowing chlorine to enter into cell (Virto, Manas, Alvarez, Condon, & Raso, 2005). Available chlorine can react with organic matter in solution leading to production of chlorine derivatives, which are not as strong oxidant as free chlorine (Rule, Ebbett, & Vikesland, 2005).

It was observed that AEW was slightly more effective in inactivating *E. coli* O157:H7 than NEW at same available chlorine levels, which can possibly be explained by AEW's lower pH and/or higher ORP value than NEW. Initial bacterial concentration plays an important role in determining antimicrobial

efficacy of EO water (Table 1).

3.2 Reduction of *B. subtilis* at different concentration levels by AEW and NEW

As seen in Table 2, at high initial bacterial concentration, only 4.12 and 3.75 log reductions were achieved by AEW and NEW treatment even with ACC of 30 mg/L, while as for *E. coli* O157:H7 at the same cell concentration, AEW with only 10 mg/L (ACC) and NEW with 30 mg/L (ACC) was required to achieve complete elimination by direct plating method. The reason might be that *B. subtilis* as an endospore-forming, Gram-positive bacterium, which confer additional level of resistance towards EO water treatment. It was reported similar findings in which bacterial cells were treated for 10 s with EO water, *B. cereus* (spore-forming bacteria) was found to be more resistant than *E. coli* O157:H7 (Kim, Hung, & Brackett, 2000). These results were also in agreement with the earlier study conducted by Cloete et al. (2009) who also observed *B. subtilis* vegetative cells were more resistant to anolyte than *E. coli*. The resistance of *B. subtilis* increased to the treatments with increased bacterial concentrations. No *B. subtilis* colonies were detected on plates after treatment by AEW and NEW with low ACC, even with ACC of 0.25 mg/L at low initial *B. subtilis* concentration, whereas AEW and NEW with ACC of 0.5 mg/L was required to achieve no bacteria detection at medium initial bacterial concentration. In this study, medium and low bacterial concentrations were reached by diluting bacterial populations from high concentration. Possibly lowering the amount of organic matter in suspension facilitated better interaction of bacterial cells with available chlorine ions leading to higher efficacy. It was also reported that available chlorine could be consumed by organic matter, leading to reduction in the efficacy of EO water (Tosa & Yamasaki, 2000). Log reduction was increased with increasing ACC of EO water and a significant difference was observed from the data when treated by EO water with ACC more than 10 mg/L ($p < 0.05$). There was no significant difference between antimicrobial efficacy of AEW and NEW at the same ACC ($p > 0.05$). Initial bacterial concentration also plays an important role in disinfecting *B. subtilis* vegetative cell by EO water.

3.3 Effect of centrifugation time on inactivation of *E. coli* O157:H7 by EO water

Table 3 shows the reduction of *E. coli* O157:H7 treated by AEW and NEW with 6 mg/L available chlorine, after 1, 2 and 3 times centrifugation. The results indicated that resistance of *E. coli* O157:H7 to both EO water changed significantly after cells were centrifuged 1, 2 and 3 times. The initial bacterial concentrations were almost the same (9.63, 9.66 and 9.62 log₁₀ CFU/mL); after centrifuged for 1, 2 and 3 times, the log reduction of *E. coli* O157:H7 however, was increased with gradual increase in the centrifugation time. *E. coli* O157:H7 treated by AEW with ACC of 6 mg/L reduced the bacterial count population by 1.66, 3.47 and 4.33 log₁₀ CFU/mL after centrifugation of bacterial suspension for 1, 2 and 3 times, respectively. Reductions of 1.10, 3.11 and 4.48 log₁₀ CFU/mL were achieved by the treatment of NEW at centrifugation time 1, 2 and 3. No significant difference was observed between bacterial cells treated with AEW and NEW at the same centrifugation time ($p>0.05$). Peterson et al. (2012) reported that high pressure and shear forces acted onto the cells surface during compaction when centrifuged, making the cells more susceptible to EO water treatment. Although, there was no significant difference between two or three centrifugation time ($p>0.05$), additional 0.86 and 1.37 log reductions were achieved by AEW and NEW treatment, respectively. AEW and NEW were observed to be playing the same role in reducing *E. coli* O157:H7 since no significant difference was observed between these two types of EO water after different centrifugation times ($p>0.05$).

3.4 Effect of centrifugation time on inactivation of *B. subtilis* by EO water

B. subtilis cells after being centrifuged for 1, 2 and 3 times were treated by AEW and NEW with ACC of 10 mg/L, and the results are shown in Table 4. Initial bacterial concentrations for all three trails with three different centrifugation times were at the same level (8.73, 8.65 and 8.73 log₁₀ CFU/mL). Log reduction increased with increasing centrifugation times and there was significant difference among different centrifugation times ($p<0.05$) for both AEW and NEW treatment. After 1, 2 and 3 times centrifugation, reductions of 0.46, 1.42 and 2.64 log₁₀ CFU/mL were achieved for AEW treatment, and 0.17, 1.13 and 2.09 log reductions were obtained for NEW with the same ACC. There was still additional reduction of 0.96 log₁₀ CFU/mL obtained by AEW and NEW treatment, in comparing the first

centrifugation with the second one, although no significant difference between AEW and NEW for the first two centrifugations was observed. As can be seen in Tables 3 and 4, less log reductions of *B. subtilis* were achieved at each centrifugation time, even though *B. subtilis* was treated by EO water with higher ACC (10 mg/L). This could be because *B. subtilis* being a Gram-positive and spore forming bacteria possess a thick cell wall, conferring higher level resistant to EO water than *E. coli*, which is in agreement with previous publication (Cloete, Thantsha, Maluleke, & Kirkpatrick, 2009). Long-time centrifugation caused improved susceptibility of *B. subtilis* cells which was treated by EO water. Therefore, EO water could more easily enter the cells damaged by high pressure and shear forces generated during the long centrifugation process, results in enhanced bactericidal properties of EO water.

3.5 Bio-molecular test of *B. subtilis*

Changes in DNA, protein and conductivity of *B. subtilis* treated with AEW and NEW with ACC of 10, 20 and 30 mg/L were shown in Fig. 1 and Fig. 2. Concentration of DNA, protein and conductivity were measured immediately after EO water treatment. As seen in Figs. 1A and 1B, DNA and protein concentrations increased in response to the treatment of AEW with increased ACC at each centrifugation time. Significant difference were found between ACC of 10 mg/L and 30 mg/L in both Figs. 1A and 1B at centrifugation time 1, 2 and 3, respectively ($p < 0.05$). EO water could damage the membrane of bacteria due to its strong oxidizing property; therefore, cells treated with higher ACC might receive more injuries and damages leading to release of more DNA and protein outside of cells. Zeng et al, (2010, 2011) used transmission electron microscopy to study bactericidal mechanism of EO water. Results of the study indicated increased bacterial cell membrane permeability, leading to leakage of intracellular cell solutes after EO water treatment. However, DNA and protein leakage were reduced with increasing centrifugation time. The reason for this reduction could be that partial cell inclusion was released in the supernatant, which was decanted after centrifugation (data not shown). Variation in conductivity after *B. subtilis* was treated by AEW with ACC of 10, 20 and 30 mg/L was shown in Fig. 1C. Not much significant difference was found among different ACC and at different centrifugation times.

Variation of DNA, protein and conductivity after *B. subtilis* treated by NEW with ACC of 10, 20 and 30 mg/L was shown in Figs. 2A, 2B and 2C. As can be seen in Fig. 2, a similar trend for DNA, protein and conductivity of *B. subtilis* treated with NEW was observed as compared to Fig. 1 which was treated with AEW. Both AEW and NEW were effective disinfectants, causing inactivation of bacteria. Similar effects of AEW and NEW on bacterial cell injury was recorded in this study.

Similar studies evaluating the effect of EO water treatment at cellular level (DNA, protein leakage and conductivity) was previously studied on *E. coli*, *Staphylococcus aureus* and *Candida albicans* to evaluate the changes of DNA, protein and conductivity of *Candida albicans*, *Staphylococcus aureus* and *E. coli* treated by EO water have been performed (Zeng, et al., 2010; Zeng, et al., 2011). Tang et al., (2011) studied the effect of EO water on *B. subtilis* spores. All these previous studies found that DNA, protein and conductivity increased with increasing treatment time in the first minute. In our study, we found DNA, protein leakage and conductivity increased by increasing ACC from 10 to 30 mg/L. The results obtained in this study, further substantiate previous report that EO water treatment can inflict cell injury leading to increased cell permeability, resulting in higher leakage of DNA, protein and ions, caused by increasing ACC and treatment time.

3.6 Bio-molecular test of *E. coli* O157:H7

Changes in DNA and conductivity of *E. coli* O157:H7 treated by AEW and NEW with ACC of 2, 6 and 10 mg/L were shown in Figs. 3 and 4. DNA concentration significantly increased after *E. coli* O157:H7 was treated with AEW and NEW as ACC increased from 2 to 10 mg/L at the same centrifugation time ($p<0.05$). With increase in centrifugation time, a slight increase in DNA concentration in solution in response to the treatment with AEW was observed at the same ACC (Fig. 3A). In Fig. 3B, conductivity increased slightly in response to the treatment of AEW with ACC increasing from 2 to 10 mg/L at different centrifugation times, but no significant difference was observed. Treatment of AEW with the same ACC resulted in no significant difference with increasing centrifugation

from 1 to 3 times ($p>0.05$). Similar tendency for DNA and conductivity of *E. coli* O157:H7 treated by NEW with 2, 6 and 10 mg/L available chlorine was obtained.

DNA leakage and conductivity of supernatant were also measured after second and third centrifugation cycle (data not shown). It was observed that DNA and ion leakage increased with each additional centrifugation cycle. High shear force could be created by high rotating speed and pressure when cells were centrifuged. More impact force might be placed on cells with more centrifugation time (Peterson, Sharma, van der Mei & Busscher, 2012) and therefore, resistance of cells to EO water could be reduced.

4. Conclusions

Initial bacterial population and centrifugation time are two important factors that affect the antimicrobial efficiency of EO water, especially for laboratory assay. This study showed that initial bacterial concentration and centrifugation period are two important factors and should be carefully considered in chlorine-based antimicrobial efficacy testing.

Acknowledgements

This research was supported by State and Hatch funds allocated to the University of Georgia Agricultural Experiment Station, Griffin Campus and an Agriculture and Food Research Initiative Grant no. 2011-68003-30012 from the USDA National Institute of Food and Agriculture, Food Safety: Food processing technologies to destroy food-borne pathogens program (A 4131). We thank Dr. Prashant Singh for specific comments on the preparation of this manuscript.

References

- Bialka, K. L., Demirci, A., Knabel, S. J., Patterson, P. H., & Puri, V. M. (2004). Efficacy of electrolyzed oxidizing water for the microbial safety and quality of eggs. *Poultry Science*, 83(12), 2071-2078.
- Cao, W., Zhu, Z. W., Shi, Z. X., Wang, C. Y., & Li, B. M. (2009). Efficiency of slightly acidic electrolyzed water for inactivation of *Salmonella enteritidis* and its contaminated shell eggs. *International Journal of Food Microbiology*, 130(2), 88-93.
- Chen, R., & Xue, G. (2002). An experimental study on disinfecting mechanism of dimethylhydantoin. *Nanfang Journal of Nursing*, 5, 037.
- Cloete, T. E., Thantsha, M. S., Maluleke, M. R., & Kirkpatrick, R. (2009). The antimicrobial mechanism of electrochemically activated water against *Pseudomonas aeruginosa* and *Escherichia coli* as determined by SDS-PAGE analysis. *Journal of Applied Microbiology*, 107(2), 379-384.
- Driedger, A., Staub, E., Pinkernell, U., Marinas, B., Koster, W., & Von Gunten, U. (2001). Inactivation of *Bacillus subtilis* spores and formation of bromate during ozonation. *Water Research*, 35(12), 2950-2960.
- Fabrizio, K. A., & Cutter, C. N. (2004). Comparison of electrolyzed oxidizing water with other antimicrobial interventions to reduce pathogens on fresh pork. *Meat Science*, 68(3), 463-468.
- Fabrizio, K. A., & Cutter, C. N. (2005). Application of electrolyzed oxidizing water to reduce *Listeria monocytogenes* on ready-to-eat meats. *Meat Science*, 71(2), 327-333.
- Forghani, F., & Oh, D. H. (2013). Hurdle enhancement of slightly acidic electrolyzed water antimicrobial efficacy on Chinese cabbage, lettuce, sesame leaf and spinach using ultrasonication and water wash. *Food Microbiology*, 36(1), 40-45.
- Griffin, P. M., & Tauxe, R. V. (1991). The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiologic Reviews*, 13, 60-98.
- Guentzel, J. L., Lam, K. L., Callan, M. A., Emmons, S. A., & Dunham, V. L. (2010). Postharvest management of gray mold and brown rot on surfaces of peaches and grapes using electrolyzed oxidizing water. *International Journal of Food Microbiology*, 143(1-2), 54-60.
- Guentzel, J. L., Liang Lam, K., Callan, M. A., Emmons, S. A., & Dunham, V. L. (2008). Reduction of bacteria on spinach, lettuce, and surfaces in food service areas using neutral electrolyzed oxidizing water. *Food Microbiology*, 25(1), 36-41.
- Hao, X., Shen, Z., Wang, J., Zhang, Q., Li, B., Wang, C., & Cao, W. (2013). In vitro inactivation of porcine reproductive and respiratory syndrome virus and pseudorabies virus by slightly acidic electrolyzed water. *Veterinary Journal*, 197(2), 297-301.
- Hsu, S. Y. (2003). Effects of water flow rate, salt concentration and water temperature on efficiency of an electrolyzed oxidizing water generator. *Journal of Food Engineering*, 60(4), 469-473.

- Hung, Y. C., Tilly, P., & Kim, C. (2010). Efficacy of electrolyzed oxidizing (EO) water and chlorinated water for inactivation of *Escherichia coli* O157:H7 on strawberries and broccoli. *Journal of Food Quality*, 33(5), 559-577.
- Iwasawa, A., & Nakamura, Y. (1996). Bactericidal effect of acidic electrolyzed water--comparison of chemical acidic sodium hydrochloride (NaOCl) solution. *The Journal of the Japanese Association for Infectious Diseases*, 70(9), 915-922.
- Jadeja, R., Hung, Y. C., & Bosilevac, J. M. (2013). Resistance of various shiga toxin-producing *Escherichia coli* to electrolyzed oxidizing water. *Food Control*, 30(2), 580-584.
- Jagannath, A., Tsuchido, T., & Membre, J. M. (2005). Comparison of the thermal inactivation of *Bacillus subtilis* spores in foods using the modified Weibull and Bigelow equations. *Food Microbiology*, 22(2-3), 233-239.
- Kaper, J. B., & Karmali, M. A. (2008). The continuing evolution of a bacterial pathogen. *Proceedings of National Academy of Science of United States of America*, 105(12), 4535-4536.
- Kim, C., Hung, Y. C., & Brackett, R. E. (2000). Efficacy of electrolyzed oxidizing (EO) and chemically modified water on different types of foodborne pathogens. *International Journal of Food Microbiology*, 61(2-3), 199-207.
- Kiura, H., Sano, K., Morimatsu, S., Nakano, T., Morita, C., Yamaguchi, M., Maeda, T., & Katsuoka, Y. (2002). Bactericidal activity of electrolyzed acid water from solution containing sodium chloride at low concentration, in comparison with that at high concentration. *Journal of Microbiological Methods*, 49(3), 285-293.
- Lathem, W. W., Bergsbaken, T., Witowski, S. E., Perna, N. T., & Welch, R. A. (2003). Acquisition of stcE, a C1 esterase inhibitor-specific metalloprotease, during the evolution of *Escherichia coli* O157:H7. *Journal of Infectious Diseases*, 187(12), 1907-1914.
- Lin, T., Wang, J. J., Li, J. B., Liao, C., Pan, Y. J., & Zhao, Y. (2013). Use of acidic electrolyzed water ice for preserving the quality of shrimp. *Journal of Agricultural and Food Chemistry*, 61(36), 8695-8702.
- Minh, H. N. T., Dantigny, P., Cornet, J. M. P., & Gervais, P. (2010). Germination and inactivation of *Bacillus subtilis* spores induced by moderate hydrostatic pressure. *Biotechnology and Bioengineering*, 107(5), 876-883.
- Pangloli, P., Hung, Y. C., Beuchat, L. R., King, C. H., & Zha, Z. H. (2009). Reduction of *Escherichia coli* O157:H7 on produce by use of electrolyzed water under simulated food service operation conditions. *Journal of Food Protection*, 72(9), 1854-1861.
- Park, C. M., Hung, Y. C., Lin, C. S., & Brackett, R. E. (2005). Efficacy of electrolyzed water in inactivating *Salmonella enteritidis* and *Listeria monocytogenes* on shell eggs. *Journal of Food Protection*, 68(5), 986-990.
- Park, E. J., Alexander, E., Taylor, G. A., Costa, R., & Kang, D. H. (2008). Effect of electrolyzed water for reduction of foodborne pathogens on lettuce and spinach. *Journal of Food Science*, 73(6), M268-272.

- 385 Park, E. J., Alexander, E., Taylor, G. A., Costa, R., & Kang, D. H. (2009). The decontaminative effects of
386 acidic electrolyzed water for *Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Listeria*
387 *monocytogenes* on green onions and tomatoes with differing organic demands. *Food*
388 *Microbiology*, 26(4), 386-390.
- 389
390 Park, H., Hung, Y. C., & Chung, D. (2004). Effects of chlorine and pH on efficacy of electrolyzed water
391 for inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *International Journal of*
392 *Food Microbiology*, 91(1), 13-18.
- 393
394 Peterson, B. W., Sharma, P. K., van der Mei, H. C., & Busscher, H. J. (2012). Bacterial Cell Surface
395 Damage Due to Centrifugal Compaction. *Applied and Environmental Microbiology*, 78(1), 120-
396 125.
- 397
398 Rahman, S. M., Park, J., Song, K. B., Al-Harbi, N. A., & Oh, D. H. (2012). Effects of slightly acidic low
399 concentration electrolyzed water on microbiological, physicochemical, and sensory quality of
400 fresh chicken breast meat. *Journal of Food Science*, 77(1), M35-41.
- 401
402 Rangel, J. M., Sparling, P. H., Crowe, C., Griffin, P. M., & Swerdlow, D. L. (2005). Epidemiology of
403 *Escherichia coli* O157 : H7 outbreaks, United States, 1982-2002. *Emerging Infectious Diseases*,
404 11(4), 603-609.
- 405
406 Ren, T., & Su, Y. C. (2006). Effects of electrolyzed oxidizing water treatment on reducing *Vibrio*
407 *parahaemolyticus* and *Vibrio vulnificus* in raw oysters. *Journal of Food Protection*, 69(8), 1829-
408 1834.
- 409
410 Rule, K. L., Ebbett, V. R., & Vikesland, P. J. (2005). Formation of chloroform and chlorinated organics
411 by free-chlorine-mediated oxidation of triclosan. *Environmental Science & Technology*, 39(9),
412 3176-3185.
- 413
414 Russell, S. M. (2003). The effect of electrolyzed oxidative water applied using electrostatic spraying on
415 pathogenic and indicator bacteria on the surface of eggs. *Poultry Science*, 82(1), 158-162.
- 416
417 Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L., &
418 Griffin, P. M. (2011). Foodborne illness acquired in the United States-major pathogens. *Emerging*
419 *Infectious Diseases*, 17(1), 7-15.
- 420
421 Stopforth, J. D., Mai, T., Kottapalli, B., & Samadpour, M. (2008). Effect of acidified sodium chlorite,
422 chlorine, and acidic electrolyzed water on *Escherichia coli* O157:H7, *Salmonella*, and *Listeria*
423 *monocytogenes* inoculated onto leafy greens. *Journal of Food Protection*, 71(3), 625-628.
- 424
425 Tamaki, S., Bui, V. N., Ngo, L. H., Ogawa, H., & Imai, K. (2014). Virucidal effect of acidic electrolyzed
426 water and neutral electrolyzed water on avian influenza viruses. *Archives of Virology*, 159(3),
427 405-412.
- 428
429 Tang, W. W., Zeng, X. P., Zhao, Y. S., Ye, G. Q., Gui, W. C., & Ni, Y. M. (2011). Disinfection effect
430 and its mechanism of electrolyzed oxidizing water on spores of *Bacillus subtilis* var. *niger*. *Food*
431 *Science and Biotechnology*, 20(4), 889-895.
- 432
433 Tosa, N., & Yamasaki, Y. (2000). Effect of organic substances on the residual chlorine contained in the
434 strong acidic electrolyzed water. *Journal of the Japanese Society for Food Science and*
435 *Technology-Nippon Shokuhin Kagaku Kogaku Kaishi*, 47(4), 287-295.

- Udompijitkul, P., Daeschel, M. A., & Zhao, Y. (2007). Antimicrobial effect of electrolyzed oxidizing water against *Escherichia coli* O157:H7 and *Listeria monocytogenes* on fresh strawberries (*Fragaria x ananassa*). *Journal of Food Science*, 72(9), M397-406.
- Virto, R., Manas, P., Alvarez, I., Condon, S., & Raso, J. (2005). Membrane damage and microbial inactivation by chlorine in the absence and presence of a chlorine-demanding substrate. *Applied and Environmental Microbiology*, 71(9), 5022-5028.
- Zeng, X., Tang, W., Ye, G., Ouyang, T., Tian, L., Ni, Y., & Li, P. (2010). Studies on disinfection mechanism of electrolyzed oxidizing water on *E. coli* and *Staphylococcus aureus*. *Journal of Food Science*, 75(5), M253-260.
- Zeng, X., Ye, G., Tang, W., Ouyang, T., Tian, L., Ni, Y., & Li, P. (2011). Fungicidal efficiency of electrolyzed oxidizing water on *Candida albicans* and its biochemical mechanism. *Journal of Bioscience and Bioengineering*, 112(1), 86-91.
- Zhang, C. L., Lu, Z. H., Li, Y. Y., Shang, Y. C., Zhang, G., & Cao, W. (2011). Reduction of *Escherichia coli* O157:H7 and *Salmonella enteritidis* on mung bean seeds and sprouts by slightly acidic electrolyzed water. *Food Control*, 22(5), 792-796.
- Zhou, J. (2003). Basic Skills and Tactics of Molecular Biology. (1st ed.). *Science Press*, Beijing, China, (pp 51-200).

Table 1. Inactivation of *E. coli* O157: H7 at different concentration levels by AEW and NEW with different available chlorine concentrations.

Initial bacterial concentration level	Available chlorine concentration (mg/L)	Log reduction (log ₁₀ CFU/mL)	
		Acidic electrolyzed water	Near neutral electrolyzed water
Low	0.25		
	0.5		
	1	>4.46	>4.41
	2		
	4		
Medium	0.25		5.34±0.07
	0.5		
	1	>6.44	>6.38
	2		
	4		
High	2	A1.04±0.07a	B0.77±0.06a
	4	A3.88±0.05b	B3.17±0.16b
	6	A5.08±0.12c	A4.85±0.15c
	8	A6.94±0.70d	B5.90±0.13d
	10	A8.48±0.06e	B6.16±0.29d
	20	A8.48±0.06e	B6.67±0.40e
	30	A8.48±0.06e	A8.44±0.07f

Low (5.46 and 5.41 log₁₀ CFU/mL for AEW and NEW treatment), Medium (7.44 and 7.38 log₁₀ CFU/mL for AEW and NEW treatment), High (9.48 and 9.44 log₁₀ CFU/mL for AEW and NEW treatment). **AEW**: pH 2.71-2.82; ORP 925-1128 mV. **NEW**: pH 5.59-6.36; ORP 891-963 mV. Means not labeled with the same lowercase letter to the right of the number in each column are significantly different ($p<0.05$). Means not labeled with the same uppercase letter to the left of the number in each row are significantly different ($p<0.05$). Detection limit is 1.0 log₁₀ CFU/mL.

Table 2. Inactivation of *B. subtilis* at different concentration levels by AEW and NEW with different available chlorine concentrations.

Initial bacterial concentration level	Available chlorine concentration (mg/L)	Log reduction (log ₁₀ CFU/mL)	
		Acidic electrolyzed water	Near neutral electrolyzed water
Low	0.25		
	0.5		
	1	>4.20	>4.20
	2		
	4		
Medium	0.25	5.00±0.13	4.59±0.13
	0.5		
	1	>6.20	>6.20
	2		
	4		
High	2	A0.36±0.01f	A0.13±0.03e
	4	A0.64±0.18ef	A0.30±0.02de
	6	A0.94±0.23de	A0.51±0.06de
	8	A1.18±0.18cd	A0.83±0.01cd
	10	A1.55±0.36c	A1.17±0.03c
	20	A3.62±0.39b	A3.01±0.58b
	30	A4.12±0.15a	A3.75±0.34a

Low (5.2 log₁₀ CFU/mL), Medium (7.2 log₁₀ CFU/mL), High (9.2 log₁₀ CFU/mL). **AEW**: pH 2.69-2.82; ORP 1041-1152 mV. **NEW**: pH 5.63-6.39; ORP 905-949 mV. Means not labeled with the same lowercase letter to the right of the number in each column are significantly different ($p<0.05$). Means not labeled with the same uppercase letter to the left of the number in each row are significantly different ($p<0.05$).

Table 3. Effect of different centrifugation times on inactivation of *E. coli* O157:H7 by AEW and NEW

Centrifugation time	Bacterial concentration after centrifuged (Log ₁₀ CFU/mL)	Log reduction (log ₁₀ CFU/mL)	
		Acidic electrolyzed water	Near neutral electrolyzed water
1	9.63±0.14	A1.66±0.79b	A1.10±0.92b
2	9.66±0.11	A3.47±0.60a	A3.11±0.89a
3	9.62±0.13	A4.33±0.19a	A4.48±0.14a

AEW: ACC 6 mg/L; pH 2.84; ORP 1117 mV. NEW: ACC 6 mg/L; pH 6.01; ORP 905 mV. Means not labeled with the same lowercase letter to the right of the number in each column are significantly different ($p<0.05$). Means not labeled with the same uppercase letter to the left of the number in each row are significantly different ($p<0.05$).

Table 4. Effect of different centrifugation times on inactivation of *B. subtilis* by AEW and NEW

Centrifugation time	Bacterial concentration after centrifuged (Log ₁₀ CFU/mL)	Log reduction (log ₁₀ CFU/mL)	
		Acidic electrolyzed water	Near neutral electrolyzed water
1	8.73±0.12	A0.46±0.21c	A0.17±0.09c
2	8.65±0.08	A1.42±0.59b	A1.13±0.56b
3	8.73±0.17	A2.64±0.66a	B2.09±0.66a

AEW: ACC 10 mg/L; pH 2.82; ORP 1122 mV. NEW: ACC 10 mg/L; pH 5.87; ORP 930 mV. Means not labeled with the same lowercase letter to the right of the number in each column are significantly different ($p<0.05$). Means not labeled with the same uppercase letter to the left of the number in each row are significantly different ($p<0.05$).

Figure 1

Figure 2

Figure 3

Figure 4

Fig. 1 Bio-molecular test of *B. subtilis* after treated with AEW for 30s at different conditions.

Acidic electrolyzed water: pH 2.82-2.85; ORP 1147-1165 mV. Means not labeled with the same uppercase letter for the same centrifugation time are significantly different for different ACC concentration; Means not labeled with the same lowercase letter for the same ACC are significantly different for different centrifugation time ($p<0.05$).

Fig. 2 Bio-molecular test of *B. subtilis* after treated with NEW for 30s at different conditions.

Near neutral electrolyzed water: pH 6.0-6.14; ORP 911-923 mV. Means not labeled with the same uppercase letter for the same centrifugation time are significantly different for different ACC concentration; Means not labeled with the same lowercase letter for the same ACC are significantly different for different centrifugation time ($p<0.05$).

Fig. 3 Bio-molecular test of *E. coli* O157:H7 after treated with AEW at different conditions.

Acidic electrolyzed water: pH 2.80-2.81; ORP 1130-1151mV. Means not labeled with the same uppercase letter for the same centrifugation time are significantly different for different ACC concentration; Means not labeled with the same lowercase letter for the same ACC are significantly different for different centrifugation time ($p<0.05$).

Fig. 4 Bio-molecular test of *E. coli* O157:H7 after treated with NEW at different conditions.

Near neutral electrolyzed water: pH 5.56-5.91; ORP 916-926 mV. Means not labeled with the same uppercase letter for the same centrifugation time are significantly different for different ACC concentration; Means not labeled with the same lowercase letter for the same ACC are significantly different for different centrifugation time ($p<0.05$).