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EFFECTIVENESS OF ELECTROLYZED OXIDIZING WATER FOR INACTIVATING *Listeria monocytogenes* IN LETTUCE

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ABSTRACT

The effectiveness of electrolyzed oxidizing (EO) water for the inactivation of *L. monocytogenes* in suspension and when inoculated on lettuce leaves was evaluated. An electrolytic cell for the production of EO water was built and a solution of 5% NaCl was used. The EO water produced had a residual chlorine concentration of 29 parts per million (ppm) and pH 2.83. Ten strains of *L. monocytogenes* isolated from processed chicken (10^9 CFU/ml) were inoculated into 9 ml of EO water or 9 ml of deionized water (control) and incubated at 15°C for 5, 10, 15 and 20 min. The surviving population of each strain was determined on Columbia agar. An exposure time of 5 min reduced the populations by approximately 6.6 log CFU/ml. The most resistant strains to sodium hypochlorite (NaOCl) were selected and used in a strain mixture (9.56 log CFU/ml, 10^9UFC/ml approximately) for the inoculation of 35 lettuce samples, by the dip inoculation method using distilled water as control. The population mean of *L. monocytogenes* after treatment with EO water and distilled water was reduced by 3.92 and 2.46 log CFU/ml respectively (p= 0.00001). EO water and 6% acetic acid (vinegar) were combined to improve the EO water effect on *L. monocytogenes* inoculated in lettuce; the effectiveness of this combination was examined. The results showed that there was a synergistic effect of both antimicrobial agents (population reduction by 5.49 log CFU/ml approximately) on the viability of *L. monocytogenes* cells.

Keywords: Disinfectant, EO water, Lettuce, *L. monocytogenes*.

RESUMEN

En este estudio se evaluó la efectividad del agua electrolizada oxidadora (EO) en la inactivación de *Listeria monocytogenes* en suspensión e inoculada en lechuga, para lo cual se construyó una celda electrolítica que permitiera la producción de agua EO a partir de una solución de NaCl al 5%, con una concentración de cloro residual de 29 partes por millón (ppm) y pH 2.83. Inicialmente se tomaron 10 cepas de *L. monocytogenes* aisladas de pollo procesado, las cuales fueron inoculadas en 9 ml de agua EO o 9 ml de agua desionizada estéril (control) e incubadas a 15°C durante 5, 10, 15 y 20 minutos. La población sobreviviente de cada cepa se determinó por recuento en placa en agar Columbia, obteniéndose una reducción de 6.6 UL en promedio a los 5 minutos de exposición. A partir de estas cepas se seleccionaron las cinco más resistentes a la acción del hipoclorito de sodio, las cuales fueron utilizadas como suspensión mixta (9.56 UL, 10^9UFC/ml aproximadamente), para inocular 35 lechugas por el método de inmersión. Después de la inoculación se sumergieron 6.25 g de cada lechuga en 375 ml de agua EO o agua destilada (control) a 15 °C durante 5 minutos. La población promedio de *L. monocytogenes* después del tratamiento con agua EO y con agua destilada, se redujo en 3.92 y 2. 46 UL respectivamente. Se

demostró que el agua EO tiene un efecto bactericida estadísticamente significativo (p=0.00001) Para mejorar el efecto del agua EO sobre *L. monocytogenes* inoculada en lechuga, se evaluó su efectividad en combinación con ácido acético al 6% (vinagre). Los resultados obtenidos (reducción de la población en 5.49 UL aproximadamente) muestran que hay un efecto sinergista de ambos agentes antimicrobianos sobre la viabilidad de las células de *L. monocytogenes*.

Palabras clave: Agua EO, Desinfectante, Lechuga, *L. monocytogenes*.

INTRODUCTION

Nowadays the consumption of vegetables in Colombia has become a critical factor in the development of a wide variety of enteric, parasitic and viral diseases, of different levels of seriousness like typhoid fever, amoeba infections and listeriosis (this last is statistically ascribed in 12% of the cases to the consumption of lettuce in the Cundiboyacense highlands) among others. *Listeria monocytogenes* is a food-borne pathogenic microorganism that has a high rate of mortality (23%); it is resistant to stress conditions, which makes it difficult to destroy, and it is easy to swallow through the consumption of vegetables; this microorganism is the causal agent of a generalized disease called Listeriosis (ICMSF, 1996). To solve this problem, washing processes and disinfection of food including the use of chemical substances like chlorine, organic acids and ozone have been implemented; in some cases these substances have reduced effects on pathogenic microorganisms, because those that have not been removed multiply; due to this problem, there is a need for an effective method to inactivate food-borne pathogens in food.

Electrolyzed Oxidizing Water (EO water) is the product of a new concept developed in Japan and it could be an effective alternative for disinfectant treatment of fresh products like vegetables (Venkitaranayanan *et al*., 1999a and Koseki *et al*., 2003). Electrolysis of deionized water containing a low concentration of sodium chloride in an electrolysis chamber where anode and cathode electrodes are separated by a diaphragm imparted strong bactericidal and virucidal properties to the water collected from the anode (EO water). Water from the anode normally has a pH of 2.7 or lower, an oxidation-reduction potential (ORP) greater than 1,100 mV, and a free chlorine concentration of 10 to 80 ppm in the form of hypochlorous acid. This use of electrolyzed oxidizing water in the disinfection of vegetables substantially eliminates food-borne pathogens, avoiding transmission of diseases (Cháves *et al*., 2004).

Previously, the effectiveness of EO water has been evaluated for inactivating different pathogens like *Escherichia coli, Salmonella enteritidis, and Listeria monocytogenes*, obtaining a considerable reduction in logarithmic units of CFU in comparison with the initial population in tomatoes, lettuce, kitchen cutting boards and *in vitro* experiments, at different temperatures and storage conditions. Cháves *et al* in 2004 designed an electrolytic chlorinator to produce EO water that was used efficiently for the elimination of microorganisms present in lettuce. The objective of this study was to evaluate the effectiveness of EO water produced in an electrolytic cell to inactivate *L. monocytogenes* in suspension and in lettuce leaves, and in this way, to standardize the action time required to inactivate the *L. monocytogenes* suspension.

2. MATERIALS AND METHODS

2.1. Electrolytic cell and EO water characterization

Following the parameters of Cháves *et al*., 2004, with the modification that two lateral holes were added for the exit of the two types of water, a 3.3 L glass cell was built with a PVC membrane (Darnel®) in order to separate the acid and alkaline electrolytes, allowing the ions to move to the electrodes. The material used as electrodes was graphite, the applied voltage was 14 volts and the current was 0.4 amperes. A 5% solution of sodium chloride was prepared to feed the electrolytic cell. During the electrolysis of water, the pH in both acid and alkaline water was determined at 3, 5, 7 and 10 minutes; a pH-meter previously calibrated made by the Center of Interfaculty Equipment (CEIF) of the Universidad Nacional de Colombia was used for these evaluations. The free available chlorine present in acidic electrolyzing water was also determined at the same times using, a photometric DPD kit (ref. 1.00598.0001 Merck, Darmstadt, Germany).

2.2. Selection and growth curve of *L. monocytogenes*

Ten strains of *L. monocytogenes* supplied by the Laboratorio de Microbiología de Alimentos de la Pontificia Universidad Javeriana, which were previously isolated by Correa and Fonseca in 2004 from processed chicken, were used. To build the growth curve, 3 ml of suspension were taken every 2 hours during the fermentation process of *L. monocytogenes* in Columbia broth supplemented with 50 mg/ml of nalidixic acid in order to determine the biomass by optical density at 540 nm, to establish the beginning of the log phase.

2.3. Effect of EO water for inactivating *L. monocytogenes* in culture

One ml aliquots from *L. monocytogenes* culture in the log phase were taken and serial dilutions in 9 ml of EO water from 10^{-1} to 10^{-6} were performed. The bactericidal activity of the EO water was evaluated at 5, 10, 15 and 20 min of exposure time at room temperature. After incubation time, the surviving cells of *L. monocytogenes* in each treatment were determined; 1 ml of each dilution was taken in order to do a recount on Columbia agar plates. *L. monocytogenes* colonies were counted after 48 h of incubation at 37°C. The recount was done in duplicate (Venkitanarayanan *et al*., 1999).

2.4. Effect of EO water for inactivating *L. monocytogenes* inoculated on lettuce leaves

The five strains most resistant to sodium hypochlorite were chosen from among the initial ten strains by a diffusion test using Waltman No. 3 paper disks saturated with hypochlorite concentrations (5250, 52.5 and 0.525 ppm), and these were selected and used in a strain mixture for the inoculation of 35 lettuce samples by the dip inoculation method as described by Park *et al*., 2001, Bari *et al*., 2003 and Koseki *et al*., 2003. Sterile distilled water was used as a control; each strain of *L. monocytogenes* was cultured in 10 ml of Columbia broth supplemented with 50 µg/ml of nalidixic acid at 37 °C during 5 hours at 120 rpm. Then, the suspension was transferred to 100 ml of Columbia broth in a 250 ml Erlenmeyer flask, and it was incubated under the same conditions during 12 hours. The cells were collected by centrifugation (3.500 rpm, 15 min at room temperature) and the resulting pellet was resuspended in 14 ml of sterile peptone water (0.1%, pH 7.5) distributed in seven tubes of 15 ml each. From each suspension,

equal volumes were taken and then combined to form an inoculum mix from the five strains with a final volume of 125 ml. The inoculum was maintained at room temperature, and it was applied the lettuce within one hour of preparation. The population of the *L. monocytogenes* inoculum was determined from a 10^{-8} dilution of the dip by recounting on Columbia agar plates in duplicate.

Thirty five units of lettuce were purchased from a local supermarket and were stored at 4 °C before being inoculated, within a maximum time of two days (Österblad *et al*., 1999). Following the inoculation method described by Koseki *et al* in 2003, treatment of the inoculated lettuce leaves was performed by immersing 6.25 g of lettuce in 375 ml of EO water or distilled water (control) in plastic bags during 5 minutes.

The previously treated lettuce samples were washed with sterile peptone water and macerated for one minute. 10^{-2} and 10^{-3} dilutions were made from the resultant solution from the EO water treatment and 10^{-4} and 10^{-6} dilutions were made from the distilled water treatment. 0.1% sterile peptone water was used for the dilutions. One ml of each dilution was taken for a recounting of the surviving population of *L. monocytogenes*. A dip recount on Columbia agar plates was carried out in duplicate, and the plates were incubated at 37 °C for 48 hours.

2.5. EO water and 0.6% acetic acid combined effect on *L. monocytogenes* inoculated on lettuce

Three treated lettuce samples were then exposed to a vinegar solution after EO water treatment or distilled water treatment. The microbiologic analysis was done as described earlier.

2.6. Minimal Inhibitory Concentration (MIC) Test

Ten 1:2 serial dilutions were made from a 5.6 % sodium hypochlorite solution using sterile distilled water; dilutions were inoculated with 10 µl containing *L. monocytogenes* suspension in lag phase; after a 5 minute exposure time, 10 µl from each dilution was transferred to tubes containing 2 ml of Columbia broth supplemented with 50 µg/ml of nalidixic acid. These tubes were incubated at 37 °C for 48 hours. After this, MIC was determined by turbidity at each sodium hypochlorite dilution (Lúnden, 2004). The MIC test was done in triplicate. This procedure was also done on the most sensitive strain in order to compare the results with the five strains used in this study.

2.7. Statistical Analysis

The results obtained were analyzed through a t student hypothesis test.

3. RESULTS AND DISCUSSION

3.1. Electrolytic cell and EO water

The electrolyzed water was obtained in an electrolytic cell that was built according to section 2.1. EO water was produced in a relatively short time with low power consumption, around 6 watts, (14 volts and 0.4 amperes). The required EO water should have had the same characteristics as that used in previous studies to inactivate *L. monocytogenes*, where EO water was used with a pH of 2.5 and 72 ppm residual chlorine, and with a pH of 2.63 and 43 ppm or 48.5 ppm residual chlorine (Venkitanarayanan *et al*.,1999, Venkitanarayanan *et al*., 1999a), with a pH of 2.5 and 45 ppm residual chlorine (Park *et al*., 2001) and with a pH of 2.6 and 30 ppm residual chlorine (Bari *et al*., 2003). Additionally, a disinfectant solution that would not change the lettuce flavor was sought.

First, 2.5%, 3.0%, 3.5%, 4.0%, 4.5%, 5.0% and 5.5% NaCl concentrations were evaluated. After 5 minutes of electrolysis, the pH was lower than 3.0 (see figure 1); therefore, at this time the first water sample was taken to determine the residual chlorine concentration present, and the second was taken after 10 minutes of electrolysis. The highest residual chlorine concentration (47.68 ppm) was obtained in a 5% NaCl solution, after 10 minutes of electrolysis, and the lowest (0.38 ppm) was obtained in a 2.5% NaCl solution after 5 minutes of electrolysis (see figure 2). The highest residual chlorine concentration obtained between 2.5% and 3.5% NaCl solutions was 5.83 ppm, obtained in a 3.5% NaCl solution; these solutions were discarded. A 5% NaCl solution was used in this study, because it had the highest chlorine concentration.

Once electrolysis time and NaCl concentration were determined, pH and residual chlorine stability were evaluated between 24 and 96 hours. A new electrolysis was done using a 5% NaCl solution, and a residual chlorine concentration of 47.33 ppm and a pH of 2.31 were obtained. The pH value remained on the same scale with a mean of 2.3. In contrast, the chlorine measurements were unstable, because the levels decreased drastically after 24 hours of storage, and additionally, values presented variability; therefore, measurements were stopped at 48 hours of storage because at this time the residual chlorine concentration was 5.48 ppm. These results were consistent with those described by Kirk *et al.* in 1962 who stated that in acid or alkaline solutions, the hypochlorous acid dissociates, forming hydrochloric and chloric acids, and in strongly acid solutions, chlorine, or chlorine and oxygen are released. Additionally, Rojas and Guevara, 1998, concluded that hypochlorous acid ionization has an equilibrium constant that depends on the pH solutions, which confirms the results obtained by Len *et al.* in 2002, who found that upon adjusting the pH of water from 2.5 to 4.0 using acetic acid solution, the stability of chlorine was greater by a nine-fold factor.

3.2. Effect of EO water for inactivating *L. monocytogenes* in culture

The bacterial population was reduced to undetectable levels in a 10^{-2} dilution (as determined by dip recount) after 5 minutes of EO water treatment; whereas in the control treatment, the logarithmic units of CFU/ml (log CFU/ml) were constant (see figure 3). The highest reduction was obtained with strain 227, which decreased to undetectable levels in a 10^{-1} dilution (as determined by dip recount), and the lowest reduction was obtained with strain 244, where after 5 minutes of exposure time, the population decreased by 5.0 log CFU/ml, and only after 10 minutes of exposure time, growth in 10^{-2} dilutions was not detected. An exposure time of 5 min reduced the populations by approximately 6.6 log CFU/ml. According to these results, it was possible to conclude that the most resistant strain to EO water effect was strain 244 and the most sensitive one was strain 227. These results were later confirmed by the Minimal Inhibitory Concentration test, using disks.

Between 5 and 20 minutes of exposure, there were no changes in *L. monocytogenes* populations. The microorganism recount in a 10^{-1} dilution was inconsistent, so only the 10^{-2} dilution values were taken.

An exposure time of 5 min reduced the populations by approximately 6.6 log CFU/ml, a high bactericidal effect, according to Delgado *et al* in 2003, who suggested that a disinfectant solution has to reduce the population by 5.0 log CFU. In this study, EO water with 28. 72 ppm of residual chlorine and pH 2.83 was used. Our

results are supported by Venkitanarayanan *et al* in 1999; in their study, the effect of EO water to inactivate *L. monocytogenes* was evaluated, and they determined that after 5 minutes of exposure time to EO water (48.5 ppm residual chlorine and pH of 2.63), the population decreased by 6.64 log CFU/ml.

From the results obtained in this phase, it was possible to determine that the treatment time for the evaluation of the EO water effect on *L. moncytogenes* inoculated on lettuce should be 5 minutes. Moreover, the most resistant strains to chlorine were selected by the MIC test using disks; with these strains, a mixed inoculum to inoculate lettuce samples was made. The most resistant strains were 155, 244, 91, 131 and 163.

3.3. Effect of EO water for inactivating *L. monocytogenes* inoculated on lettuce leaves

The five strain mixtures had a population mean of 9.56 log CFU/ml (10^9 CFU). The reduction mean of *L. monocytogenes* inoculated on lettuce was 3.92 log CFU/g (see figure 5). In sample number 31, the highest reduction was obtained at 5.82 log CFU/g, and in sample number 2 the lowest reduction was obtained at 2.48 log CFU/g. These results show that the EO water's effect on the *L. monocytogenes* culture is higher than the effect on *L. monocytogenes* inoculated on lettuce because the hypochlorous acid reacts with organic matter present in the lettuce, losing its disinfectant power (Snoeyink and Jenkins, 2002). However, the population reduction was statistically significant (p=0.00001).

Results of the present work are consistent with Oomori *et al*. in 2000, who demonstrated that available chlorine was transformed to N- Chlorates compounds by aminoacids and proteins present. A removal of available chlorine was observed by oxidation-reduction reactions with some vitamins, lipids and minerals. They also determined that the combined chlorine bactericidal effect on *E. coli* was lower than the free chlorine bactericidal effect. These authors suggest that for practical use of EO water in the food industry, the residual chlorine concentration must be increased to avoid the organic matter effect. Furthermore, the results obtained in lettuce treatments suggest that the action time of EO water must be increased to obtain a greater microbial reduction. The results of this study show the need of increasing the available chlorine concentration in EO water produced in an electrolytic cell for inactivation of *L. monocytogenes* to reduction levels lower than those observed by Park *et al.* in 2001, where after 3 minutes of exposure to EO water (45 ppm residual chlorine and pH 2.5) the *L. monocytogenes* population inoculated on lettuce decreased by 5.5 log CFU/g; in the present study the residual chlorine concentration was higher by 16 ppm. In the Bari *et al.*, 2003, study, chlorinated water (200 ppm) and EO water (30.3 ppm residual chlorine and pH of 2.6) effects were evaluated on *L. monocytogenes* inoculated on tomatoes. They obtained a reduction in population by 4.76 log CFU/g and 7.54 log CFU/g per tomato respectively. However, Beuchat and Brakett in 1990 proved the low effectiveness of chlorine solutions prepared with from 200 to 250m ppm of free chlorine for inactivation of *L. monocytogenes* inoculated on lettuce, reducing the population only by 1.36 log CFU/g. These results were lower than the ones obtained with EO water, due to its higher effect on microorganisms than a conventional chlorine solution, because of its properties like low pH and high ORP (Kim *et al.,* 2000 and Koseki *et al.,* 2002).

A population mean reduction of 2.46 log CFU/ml was observed in the control

treatment, obtained through the washing process of lettuce, in which many microorganisms were removed by water. Another reason could be osmotic stress due to hypotonic characteristics of distilled water; namely, a low solute concentration that produces cellular lysis (Madigan 2001). Additionally, the pH of distilled water was between 5 and 6, which contributed to the population decrease.

3.4. EO water and 0.6% acetic acid combined effect on *L. monocytogenes* inoculated on lettuce

Based on the previous results, the combined effect of EO water and 0.6% acetic acid on *L. monocytogenes* inoculated on lettuce was evaluated, and it was concluded that a high reduction in log CFU/g was obtained when lettuce was treated with acetic acid after EO water treatment. The *L. monocytogenes* population decreased by 5.74, 5.14 and 5.6 log CFU/g for the samples 36, 37 and 38 respectively; the mean value reduction was 5.49 log CFU/g. These results show that EO water can be employed as a disinfectant, but a combined treatment with another disinfectant like 0.6% acetic acid for improved bactericidal effect is suggested; other antimicrobial agent combinations like nisin and essential oils (carvacrol or tymol) have shown a synergistic effect for the inactivation of *L. monocytogenes* (Pol and Smid, 1999 cited by Delgado *et al.*, 2003)

3.5. CMI Test

Turbidity was observed from an 875 ppm NaClO dilution in strain 163, from 439.5 ppm in strain 121, from 218.75 ppm in strains 91 and 244, and finally from 109.37 ppm in strain 155. Strain 227 did not grow in any NaClO dilution, which confirms its sensitivity.

Selected strains for inoculation can present an acquired resistance to this disinfectant because they were isolated from processed chicken, and these were exposed to a high chlorine concentration during the prechiller and chiller processes, indicating that probably the cells had been exposed to a sublethal disinfectant concentration. Resistance could be due to a flux bomb or modifications in cell walls (Mc Donell and Russell, 1999).

3.6. Statistical Analysis

The reduction in *L monocytogenes* population was statistically significant after 5 minutes of immersing lettuce leaves in EO water (p= 0.00001 and CI=95%).

4. CONCLUSIONS

A 5 % NaCl solution had the highest residual chlorine concentration (mean of 29 ppm) and a pH value of 2.83 during 10 minutes of electrolysis.

The L. monocytogenes population had a mean decrease of 6.6 log CFU/ml after 5 minutes of exposure to EO water; however this reduction can probably be obtained in less time.

When lettuce was treated with 0.6% acetic acid after EO water treatment, there was a higher reduction in cell population (5.49 log CFU/g), which indicates that the combined treatment with another microbicidal agent improves the EO water effect.

According to MIC test results, trains used in this study show an acquired resistance to chlorine due to their place of origin.

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Aceptado: 2-02-2005

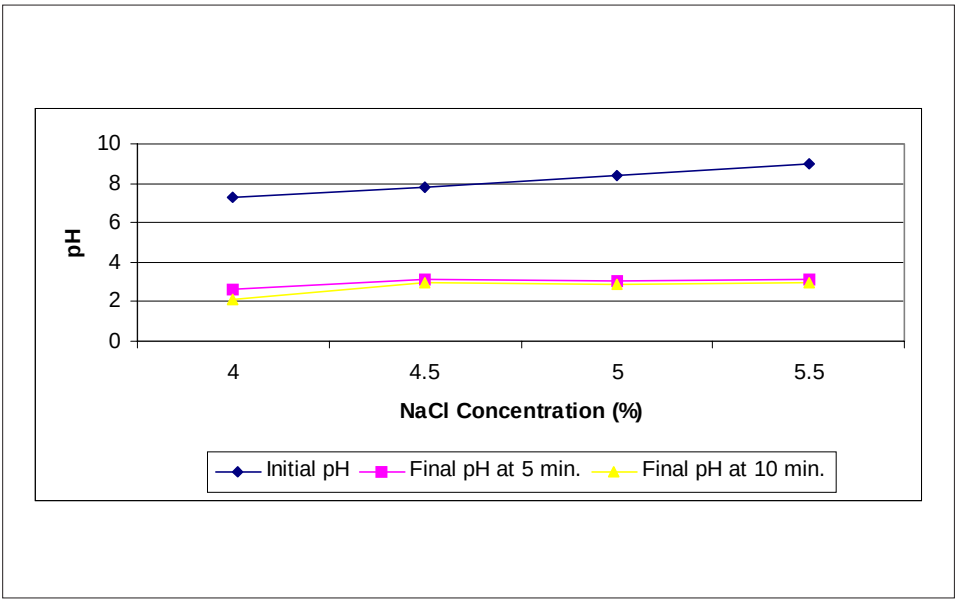

FIGURE 1. pH change of EO water during electrolysis process at different NaCl concentrations

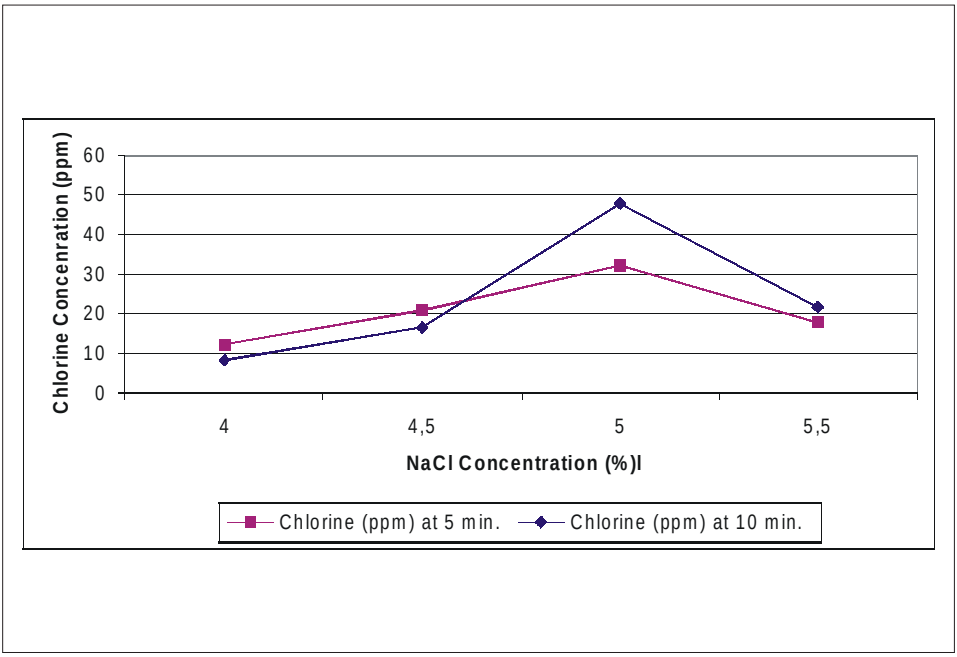

FIGURE 2. Residual chlorine concentration after electrolysis at different NaCl concentrations

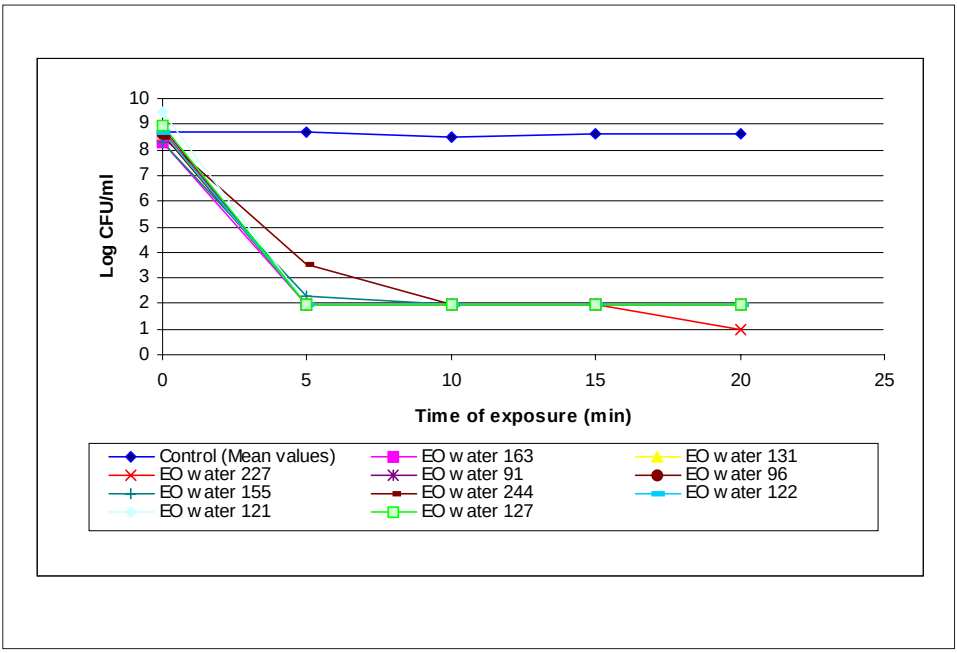

FIGURE 3. EO water effect upon the inactivation of *L. monocytogenes* suspension. (log CFU/ml) Strains 163, 131, 227, 91, 155, 244, 121, 127 and 132.

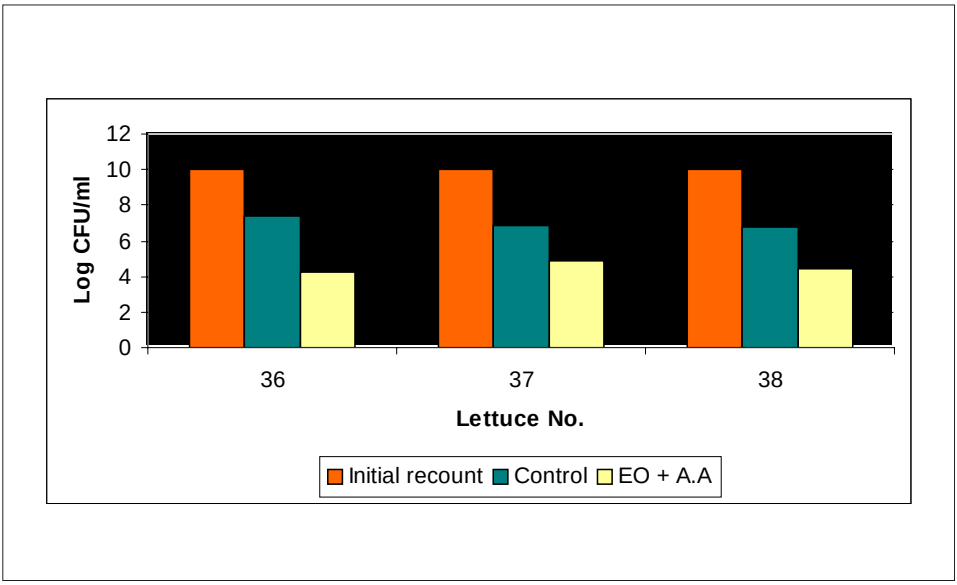

Figure 4. Combined effect of EO water and 0.6% acetic acid upon the inactivation of *L. monocytogenes* inoculated on lettuce.

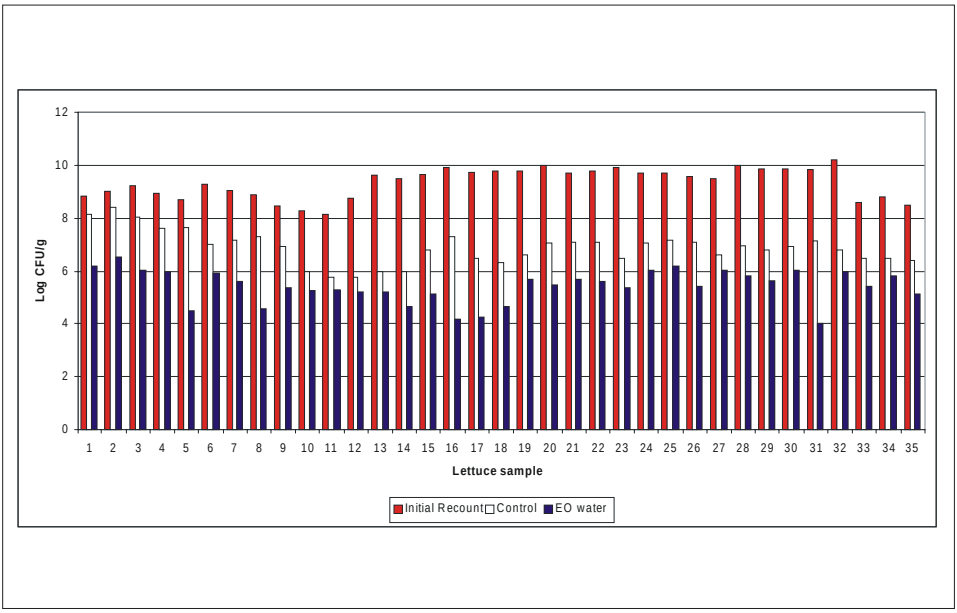

FIGURE 5. EO water effect on *L. monocytogenes* population inoculated on lettuce.