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Inactivation of *Listeria monocytogenes* in Brine and Saline by Alternating High-Voltage Pulsed Current

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The inactivating efficiency of alternating high-voltage pulsed (AHVP) current was investigated in brine (20 w/v% NaCl) and saline (0.9 w/v% NaCl) inoculated with 1× 10^7 cells/ml of *Listeria monocytogenes*. AHVP current at 12 V with 1 pulse completely inactivated *L. monocytogenes* in brine within 3 ms, while the bacteria in saline were fully inactivated by 10-pulsed electric treatment at 12 V within the same time. Electron microscopic observation demonstrated substantial structural damage of electrically treated *L. monocytogenes* in brine. These results suggest that AHVP treatment would be effective for the rapid and complete inactivation of *L. monocytogenes* in brine or saline solution.

Keywords: *Listeria monocytogenes*, alternating high-voltage pulsed current, brine, saline, inactivation

Listeria monocytogenes is a Gram-positive, nonspore-forming, facultatively anaerobic, psychrotrophic microorganism [1, 3, 7]. These bacteria may be able to survive longer under adverse environmental conditions, such as low pH [4, 16], high concentrations of NaCl [2, 10], refrigeration temperatures [3], or highly alkaline environment [17], than other nonspore-forming bacteria, and hence are a concern for food safety. These tolerance abilities of *L. monocytogenes* make the regulation of microorganisms particularly difficult.

Brine is frequently used in thermally processed foods in order to rapidly remove heat. Since heat and nutrients from products are permeated by the brine, however, the cooling solution may allow the growth or survival of harmful bacteria [5]. Brines are commonly recycled, which could lead to a contamination of products and increase of waste waters [10]. Saline is also used in the thaw process of freezing meat at a large scale. Recycled saline during a thaw process could lead to a contamination of products. In the present study, it was examined whether alternating high-voltage pulsed (AHVP) electric treatment could inactivate *L. monocytogenes* inoculated in either brine or saline for various times and at different voltages.

Both solutions of brine and saline were prepared at final concentrations of 20% (w/v) and 0.9% (w/v) sodium chloride (NaCl) in distilled water, respectively, and then sterilized by autoclaving at 121°C for 30 min. The strain of *L. monocytogenes* ATCC 15313 was maintained in brain heart infusion agar (BHI; Difco Laboratories, Detroit, MI, U.S.A.). Subcultures in its broth were incubated at 37°C for 24 h or lower until the end of the exponential growth phase [15] and inoculated in 2.5 ml of solution of either brine or saline to a density of 1×10^7 cells/ml prior to transferring into an electrolysis vessel.

As previously described [13], the electrolysis vessel was made as a small-batch treatment prototype and had two platinum electrodes (5 mm wide, 50 mm long), which were 3.5 mm apart from each other, with enough volume to contain 2.5 ml of solution. It was connected to a self-made converter of direct current into alternating current (AC), a power supply with rechargeable sealed lead-acid batteries, and a time controller. AHVP electric treatments were applied 1 or 10 times at intervals of 5 s in the range of 6 to 65 V for 0.02 to 5 ms, by which it was confirmed that AHVP current did not generate Cl_2 gas.

The number of viable bacteria in the AHVP current-treated solutions was determined as colony-forming units (CFU)/ml. After the electric treatment, each solution was 10-fold serially diluted in normal saline and 100 ml spread on the BHI agar, followed by incubation at 37°C for 48 h. The results were expressed as a log scale with the mean± standard deviation. In addition, the treated strains were sputter-coated with gold/platinum using an ion coater (E1010; Hitachi, Tokyo, Japan) and observed under an electron microscope (S-800; Hitachi).

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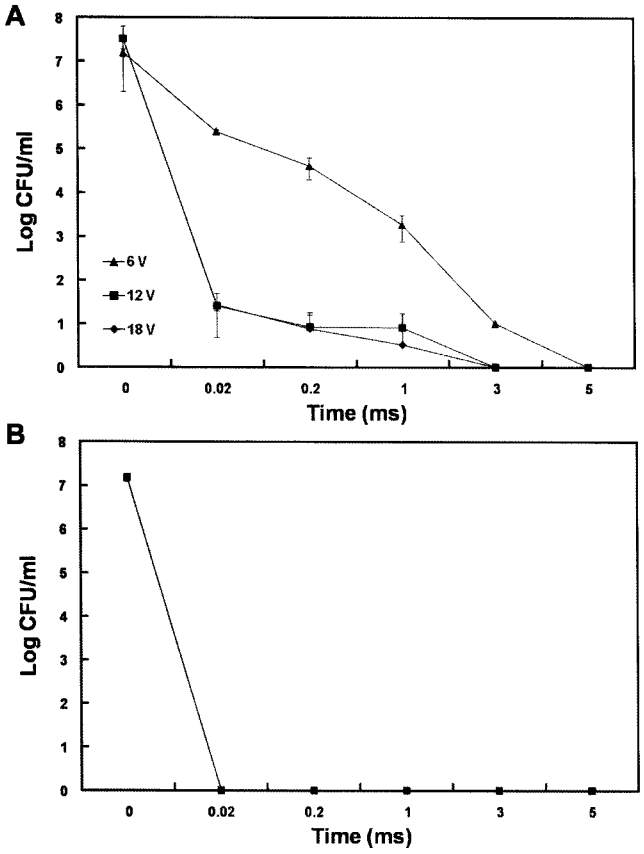

Fig. 1. Inactivation effects of alternating high-voltage pulsed current on *Listeria monocytogenes* in brine (20% NaCl) solution according to voltage intensity and duration time (**A**, 1-pulse; **B**, 10-pulse).
The detection limit was 10 CFU/ml. All variables were tested in three independent bacterial solutions of brine for each electric experiment, which was repeated twice (n=6).

L. monocytogenes in brine was decreased proportionally to the voltage intensity and duration. AHVP electric treatment at 6 V resulted in complete inactivation of the bacteria within 5 ms (Fig. 1A). At higher voltage settings of 12 V and 18 V, the bacterial population was steeply decreased after 0.02 ms and completely inactivated within 3 ms. Despite only 1-pulsed electric treatment, *L. monocytogenes* was entirely inactivated at the levels of ms and 6 V or above. Furthermore, 10-pulsed electric treatment fully inactivated *L. monocytogenes* within 0.02 ms, even at 6 V (Fig. 1B). The population levels of *L. monocytogenes* in brine without AHVP current treatment were not changed after incubation for 1 h at room temperature despite the high salinity.

This inactivation efficiency of AHVP current against *L. monocytogenes* in brine was then confirmed by observing the morphological alterations of *L. monocytogenes* with scanning electron microscopy (SEM). SEM micrographs of electrically treated *L. monocytogenes* revealed substantial structural damage at the cellular level (Fig. 2). One-pulsed AHV electric treatment with 6 V for 0.2 ms resulted in some pores and breaks on the membrane of *L. monocytogenes* (Fig. 2B). Treatment with 18 V for 5 ms was lethal enough to allow irreversible rupture of bacterial membrane at a number of locations with the apparent leakage of intracellular contents (Fig. 2C). Although these results have significant implications, the underlying mechanisms of microbial inactivation by AHVP electric treatment remain to be fully elucidated.

On the contrary, inactivation of *L. monocytogenes* in saline required a longer duration time and more pulse repetitions as well as higher intensity of voltage than that needed in brine. At higher voltage settings of 24 V,

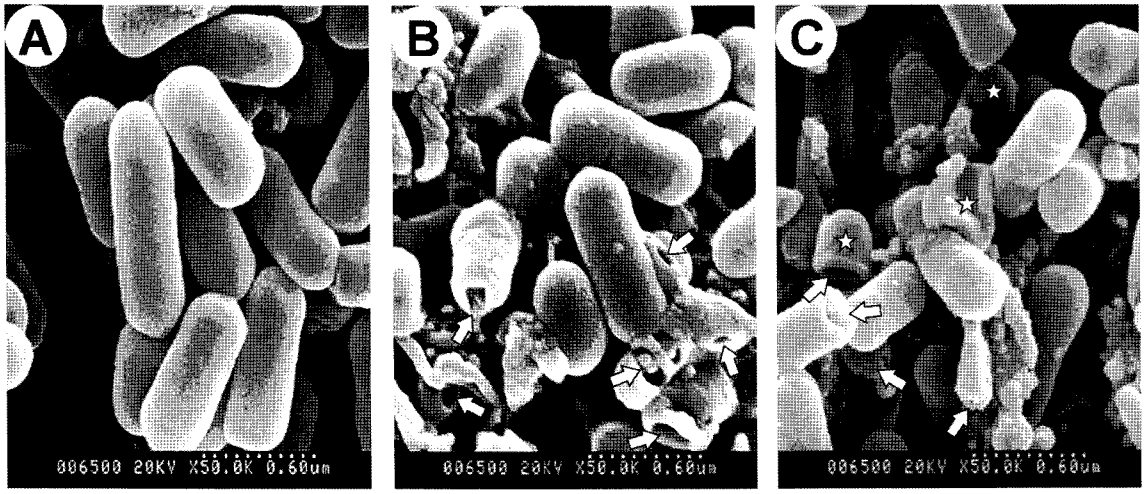

Fig. 2. SEM micrographs of *Listeria monocytogenes* in brine (20% NaCl) solution (**A**, non-treated; **B**, treated with 1-pulsed alternating high-voltage current at 6 V for 0.2 ms; **C**, treated with 1-pulsed alternating high-voltage current at 18 V for 5 ms).
Arrows indicate some pores and breaks on the membranes (in **B** and **C**). Stars indicate irreversible rupture of bacterial membrane with the apparent leakage of intracellular contents (in **C**). The images are representative of 6 independent experiments, showing similar results (magnification, ×50,000).

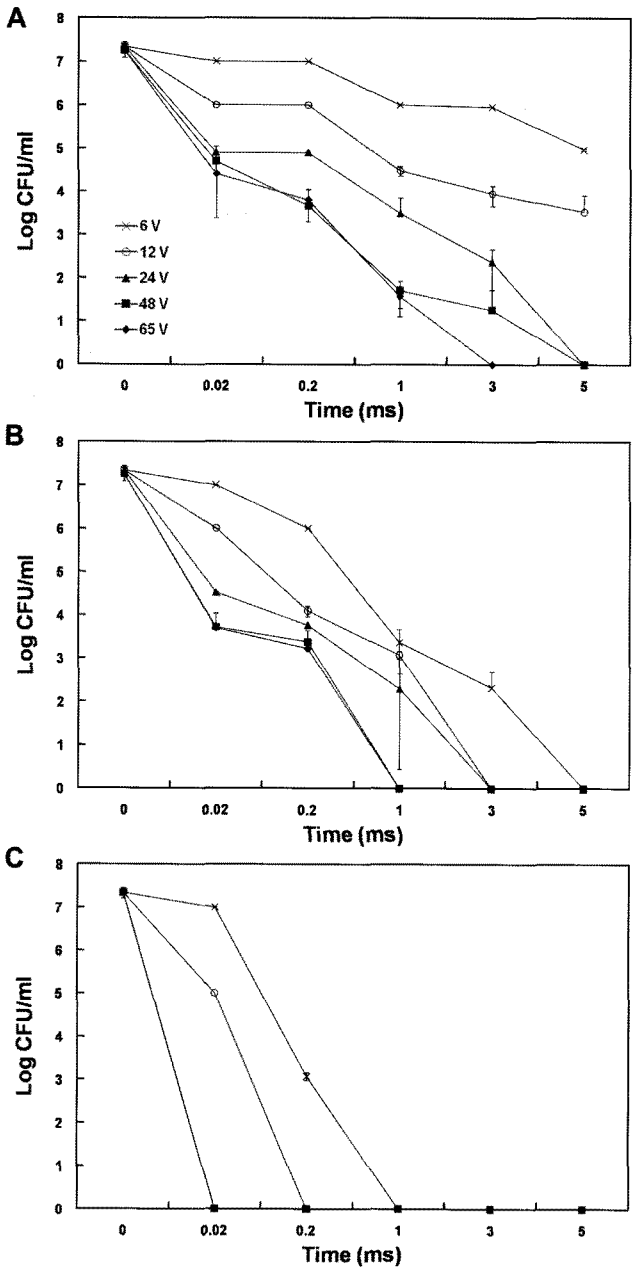

Fig. 3. Inactivation effects of alternating high-voltage pulsed current on *Listeria monocytogenes* in saline (0.9% NaCl) solution according to voltage intensity and duration time (**A**, 1-pulse; **B**, 10-pulse; **C**, 50-pulse).
The detection limit was 10 CFU/ml. All variables were tested in three independent bacterial solutions of saline for each electric experiment, which was repeated twice (n=6).

48 V, and 65 V with 1 pulse, it took at least 5 ms for *L. monocytogenes* to be fully inactivated (Fig. 3A). However, 10-pulsed AHV electric treatment resulted in a complete inactivation even at 6 V within 5 ms, as well as at 12 V or 24 V within 3 ms and at 48 V or 65 V within 1 ms (Fig. 3B). Moreover, *L. monocytogenes* was entirely inactivated within 1 ms when treated with 50 pulses irrespective of voltage settings (Fig. 3C). It seems that AHVP current would be as effective even at a short treatment time and low voltage settings as the increase in pulse repetition.

A recent report has demonstrated that electrolyzed oxidizing (EO) water could completely inactivate enterohemorrhagic foodborne pathogenic bacteria, indicating that the concentration of free chlorine present in EO water might be sufficient to bring about the reductions in bacterial counts achieved by EO water [12, 18]. Furthermore, it was revealed that the $^{\bullet}OH$ was the major lethal species responsible for the *E. coli* inactivation in the chloride-free electrochemical disinfection process [6]. In those electrolysis systems of water containing free chlorine, it took at least 5 min or longer to fully inactivate microorganisms. On the contrary, our previous and present studies revealed that AC electric current treatment at the levels of ms was enough to completely inactivate Gram-negative bacteria, including *Vibrio parahaemolyticus* [13, 14] and *E. coli* (our unpublished data) in seawater or saline, and Gram-positive *L. monocytogenes* in brine or saline.

Although AC with or without pulse has bactericidal effects, relatively little is known about the precise mechanisms of the inactivating effects of AHVP electric treatment against bacteria. The mechanism of electric current activity may include disruption of bacterial membrane integrity or electrolysis of molecules on the cell surface [8]. The most widely accepted model is that of severe electroporation, where local instabilities in the membranes of treated microorganisms are formed by electromechanical compression and electrical field-induced tension due to the applied voltage [19]. The permeabilization by pore formation of membrane can be reversible or irreversible, depending on the electrical field strength, treatment time, cell size, membrane surface charge, cytoplasm, and suspending liquid medium [9]. In this study, the lethal effects of AHVP current are attributed to specific conditions such as brine containing high concentrations of NaCl as electrolytes. It means that the greater the concentration of dissolved salts in the extracellular medium is, the greater will be the yield of electropermeated cells [11].

In conclusion, it is suggested that this AHVP electric treatment can prevent the microbial contamination in meat products through application to recycled brine and saline. It may also be utilized in practical industrial and clinical applications to resolve problems of resistance arising from the use of antibiotics and the increase of waste waters.

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