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Evaluation of the antimicrobial efficacy of neutral electrolyzed water on pork products and the formation of viable but nonculturable (VBNC) pathogens

Dong Han [a], Yen-Con Hung [b], Luxin Wang [a, *]

[a] 210 Upchurch Hall, Department of Animal Sciences, Auburn University, Auburn, AL 36849, United States
[b] Department of Food Science & Technology, University of Georgia, Griffin, GA 30223, United States

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ABSTRACT

The goals of this study were to evaluate the antimicrobial efficacy of Neutral electrolyzed oxidizing (NEO) water on *E. coli* O157:H7, *Salmonella* Enteritidis and *Yersinia enterocolitica* in both pure culture and on inoculated pork chops and skin samples, and to investigate the formation of viable but nonculturable (VBNC) pathogens after treatments. Both the plate count method and flow cytometry were used to evaluate antimicrobial efficacy on pure cultures. Different concentrations of NEO water were prepared by diluting the original NEO water (100%) with sterilized deionized water. The antimicrobial efficacy increased as the concentrations of NEO water increased. The flow cytometry results showed that treating with diluted NEO water led to the formation of VBNC cells. No VBNC cells formed when treating pure cultures with 50% or 100% NEO water. *Yersinia* cultures were found to be more resistant to NEO treatments than *Salmonella* and *E. coli* O157:H7 cultures, with *Yersinia* cultures showing lower reductions and higher levels of VBNC cells after treatments. The antimicrobial efficacy of NEO water was significantly better on skin samples than on pork chops. The differences in protein content and structure between pork chops and skin samples serve as major factors impacting the NEO water's efficacy.

1. Introduction

Meat safety continues to be a major food safety concern in recent years. Highly publicized outbreaks of foodborne disease, especially outbreaks caused by pathogenic bacteria such as *Escherichia coli* O157:H7 and *Listeria monocytogenes*, have brought meat safety and associated issues to the forefront of societal awareness (Sofos, 2008). While traditional microbial pathogens remain the dominant concern, there are new, emerging or evolving pathogens, such as non-O157 shiga-toxin-producing *E. coli* serovars, that have attracted increasing attention. To control microbial contamination in final meat products, both pre-harvest and post-harvest intervention strategies have been developed and implemented. These intervention strategies have been verified as efficient for decontaminating cattle hide or the slaughtered and eviscerated carcasses (Koohmaraie et al., 2005; Huffman, 2002; Wheeler et al., 2014; Stopforth and Sofos, 2006; Wang et al., 2014).

Microbial decontamination is usually accomplished with chemical or physical approaches, including body cleaning (Bosilevac et al., 2004), chemical and physical dehairing (Belk, 2001), and rinsing with hot water and/or chemicals (Huffman, 2002). Unfortunately, traditional decontamination methods are often problematic because of their negative impact on the quality of the carcasses or meat. To address these concerns about negative quality impacts, novel carcass decontamination technologies, such as pulsed light, ultrasound, cold atmospheric plasma, ozone and electrolyzed oxidizing (EO) water, have been investigated for their potential application for microbial decontamination (Gomez-Lopez et al., 2007; Troy et al., 2016; Turantaş et al., 2015; Huang et al., 2008). Among these novel methods, electrolyzed water (EO) has attracted a significant amount of attention because of its advantages over traditional cleaning agents. EO water is an effective disinfectant that is easy to use, relatively inexpensive, and environmentally friendly (Huang et al., 2008). EO water is generated by electrolysis of a diluted NaCl solution. It can be acidic (AEW) with a pH as low as 2.3 and a high oxidation-reduction potential (ORP, >1000 mV), or it can be neutral (NEO), with a pH value between 6 and 8 and an ORP value of 700–900 mV (Al-Haq et al., 2005; Hsu,

* Corresponding author.
E-mail address: lzw0022@auburn.edu (L. Wang).

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2005). Because of the neutral pH, NEO water is more stable and does not contribute as aggressively as AEW to the corrosion of processing equipment and the irritation of hands (Ayebah and Hung, 2005; Len et al., 2002). NEO water has demonstrated efficacy in reducing foodborne pathogens on fresh produce (Afari et al., 2015; Deza et al., 2003; Abadias et al., 2008), shrimp (Ratana-Arporn and Jommark, 2014), broiler carcasses (Rasschaert et al., 2013), and plastic and wooden cutting boards (Deza et al., 2007).

To evaluate the efficacy of electrolyzed water, previous studies have used the traditional plate count method. The survival of pathogens after treatment was determined by plating treated bacteria populations on non-selective or selective agar plates. Unfortunately, this method may underestimate the real number of live bacteria, which includes both culturable and viable but nonculturable (VBNC) cells. Pathogens in the VBNC state may still retain their virulence and resuscitate under appropriate conditions, posing a risk to public health (Aurass et al., 2011). Therefore, a method that can quantify both the culturable and VBNC cells is needed in order to fully evaluate the efficacy of treatment with NEO water. In 2017, Li et al. used flow cytometry to evaluate the efficacy of slightly acidic electrolyzed water treatment on *Staphylococcus aureus*. The flow cytometry method successfully distinguished the different physiological states of the treated *S. aureus* (Li et al., 2017). Together with other previous studies (Falcioni et al., 2008; Morono et al., 2013), these results indicated that flow cytometry is a reliable method and can provide more insight into the stress-induced changes that occur during the course of sanitation. To summarize, the goals of this study were to 1) evaluate the antimicrobial efficacy of NEO water on *E. coli* O157:H7, *Salmonella* Enteritidis and *Yersinia enterocolitica* both in pure cultures and on inoculated pork chops and skin samples, and 2) investigate and calculate the VBNC pathogens formed under different concentrations of NEO water treatments.

2. Materials and methods

2.1. Bacterial cultures

E. coli O157:H7 505B, *Salmonella* Enteritidis PT 30 (ATCC BAA-1045) and *Yersinia enterocolitica* strain 729 (obtained from Dr. Stuart Price at Auburn University School of Veterinary Medicine) were used. Strains were maintained in trypticase soy broth (TSB) supplemented with 10% glycerol (BD Difco, Sparks, MD, USA) in a −80 °C freezer before use. Fresh *E. coli* O157:H7 and *Salmonella* Enteritidis cultures were revived by transferring 100 μl of each frozen culture into 10 ml of TSB and incubating at 37 °C for 18 h. To prepare fresh *Yersinia enterocolitica* culture, 100 μl frozen culture was transferred into 10 ml of TSB and incubated at 30 °C for 48 h. Fresh overnight cultures were then prepared by transferring the revived cultures into new TSB tubes and incubating at 37 or 30 °C for additional 24 h.

2.2. NEO water generation

NEO water was generated by electrolyzing 5% NaCl solution using a GenEon™ Instaflow generator (GenEon Technologies, San Antonio, TX, USA). The final pH and the oxidation-reduction potential (ORP) values were measured using a dual-channel FE20 FiveEasy with both the pH (LE409) and ORP (LE501) probes installed (Mettler Toledo, Columbus, OH, USA). The free chlorine concentrations were determined using a total chlorine test kit CN-21P (Hach, Chicago, IL, USA).

2.3. Antimicrobial efficacy on pure cultures

Overnight fresh bacterial cultures were washed by centrifugation at 3000 × *g* for 10 min at 20 °C (Model Eppendorf 5810R, Eppendorf, Hauppauge, NY, USA). The cell pellets obtained were washed with sterilized 0.85% NaCl solution by mixing and centrifuging twice. The washed bacterial pellets were then resuspended in 5 ml of sterilized deionized water (DW). The optical density (OD) value of each resuspended culture was measured at the wavelength of 600 nm using an Ultrospec® 10-cell density meter (Amersham Biosciences, Piscataway, NJ, USA) and was adjusted so that all three cultures had approximately the same concentrations (~8.5 log CFU/ml).

Different concentrations of NEO water (1%, 3%, 6%, 10%, 25% and 50%) were prepared by diluting the original NEO water (100%) with sterilized deionized water (DW). To treat the pure cultures, 2.5 mL of each bacterial suspension was mixed with 7.5 mL of each of the diluted NEO waters or the original undiluted NEO water. After 5 min of reaction, 0.5 mL of 0.5% sodium hyposulfite ($Na_2S_2O_3$) was added to the 10 mL of reaction mixture to terminate the redox-based reaction. Serial dilutions were prepared by transferring 1 mL of the reaction mixture to 9 mL of 0.1% buffered peptone water (BD Difco, Sparks, MD, USA). The surviving culturable bacterial population was determined by plating two 100 μL of each serial dilution on two trypticase soy agar plates (TSA, BD Difco, Sparks, MD, USA). Treated cells were also enriched by adding 40 mL of TSB to the treated culture and incubating the mixture at 37° or 30 °C for 48 h. The enriched broth was then streaked onto TSA plates to check the presence or absence of culturable bacterial cells.

2.4. Flow cytometry examination

Flow cytometry (FCM) was used to determine the total number of viable cells after NEO water treatments. To prepare the dead cell standard, a fresh overnight culture of each pathogen (1 mL) was first washed and pelleted by centrifuging liquid cultures at 10,000 × g for 2 min (Eppendorf Centrifuge 5810 R, Eppendorf AG, Hamburg, Germany). Cell pellets were then treated with 1 mL of 70% isopropyl alcohol (Fisher Scientific Company, Fair Lawn, NJ, USA) for 30 min. The generated dead cells were washed with 1 mL of 0.85% NaCl solution before flow cytometry analysis. The live cell standards were prepared by resuspending washed overnight cell cultures in 1 mL of 0.85% NaCl solution.

To stain the bacteria cells, every 1 mL of bacterial culture was mixed with 1.5 μL of 20 nmol/mL propidium iodide (PI) (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). The mixtures were stored in a light-proof environment at room temperature for 5 min. After that, each PI-stained bacterial suspension was washed with 0.85% NaCl solution by centrifugation at 10,000 × g for 2 min. Washed pellets were then fixed with 300 μL of 4% glutaraldehyde saline (made with 0.85% NaCl) by incubating the mixture at room temperature for 10 min. Fixed cells were washed twice with 0.85% NaCl solution and then resuspended in 1 ml of 0.85% NaCl. FCM was carried out on an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) at the wavelength of 488 nm. The PI fluorescence was collected at 635 nm wavelength. The thresholds of the forward scatter (FSC) and side scatter (SSC) values were set at 20 k.

The percentages of viable but nonculturable (VBNC) cells were analyzed following methods described by Khan et al. (2010) and Wang et al. (2010). The equation is listed below: