Application of Neutral Electrolyzed Oxidizing Water as a Post-Harvest Intervention Strategy to Control Foodborne Pathogens Associated with Meat Products

by

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Abstract

The meat industry has been viewed as an important component of human society and eat safety continues to be a major food safety concern in recent years. Nowadays, due to the increasing demand and preference of “fresh-like” food, novel non-thermal decontamination technologies that can further secure food safety without producing significant negative impact on food quality have attracted increasing research attentions. Among these nonthermal treatments, neutral oxidizing electrolyzed (NEO) water has been studied on different types of food and demonstrated its great antimicrobial efficacy and application potentials. In this project, antimicrobial efficacy and general influence of NEO water on *E. coli* O157:H7, *Salmonella* Enteritidis and *Yersinia enterocolitica* were analyzed in both pure culture and on inoculated pork chops and skin samples. Moreover, experiments were carried out to investigate viable but nonculturable formation, sublethally injured pathogens formation, bacterial transcriptional response after NEO water treatments. The results verified the potential of NEO water in inactivating foodborne pathogens both in pure cultures and on inoculated skin samples. High percentage of post-NEO treatment viable but nonculturable state and sublethal injury bacteria was verified by employed methods. In conclusion, NEO water has the potential to be applied as a post-harvest intervention to reduce the risk from pathogenic bacteria, especially when appropriately implemented in hurdle technologies.

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Chapter I: Literature Review

1. Introduction

The meat industry has been historically viewed as an important component of society in the United States as well as for the rest of the world. Preliminary and experimental data verifies that bacterial pathogens are major causes of foodborne diseases (Champiom et al., 2005; Linscott, 2011; Lynch et al., 2009; Williams et al., 2013). In 2014, the Foodborne Diseases Active Surveillance Network reported 19,542 infections, 4,445 hospitalizations and 71 deaths in the U.S. that were mainly caused by *Salmonella* (15.45%), *Campylobacter* (13.45%), *Shigella* (5.81%), *Cryptosporidium* (2.44%), and shiga toxin-producing *Escherichia coli* (2.35%) (Crim et al., 2015). Meat products are identified as one of the major sources of these pathogens due to multifaceted reasons (Turantaş et al., 2015). *Yersinia enterocolitica* is another type of pathogenic bacteria that can cause severe gastrointestinal diseases. *Yersinia enterocolitica* outbreaks are traditionally related to pork products since *Yersinia enterocolitica* is mainly carried by porcine animals. Meat safety issues will remain a concern for many years to come and major challenges from these issues come from microbial meat-borne illnesses (Sofos, 2008).

Post-harvest intervention has been verified as an effective implement to decontaminate slaughtered and eviscerated carcasses and secure the microbial safety of meat products (Koohmaraie et al., 2005, Huffman, 2002, Wheeler et al., 2014 and Stopforth and Sofos, 2006). Traditionally, microbial decontamination is usually associated with application of chemical and physical approaches, including carcass cleaning (Brown, 2011), chemical and

physical dehairing (Belk, 2001), and rinsing with hot water and/or chemicals (Huffman, 2002). However, traditional decontamination methods often face problems due to negative quality impacts on targeted animal carcasses and parts and lacks effectiveness. Negative impacts caused by these mothed could cause value loss in meat product. For example, retail beef devalued for nearly 15% due to surface discoloration and delayed color change can prevent significant economic loss (Mancini and Hunt, 2005). Novel technologies, including pulsed light, ultrasound, cold atmospheric plasma, ozone and electrolyzed oxidizing (EO) water have been thoroughly investigated for potential application in food decontamination (Gomez-Lopez et al., 2007, Troy et al., 2016, Turantaş et al., 2015 and Huang et al., 2008). To date, acidic electrolyzed water (AEW) and neutral electrolyzed water (NEW) have been studied and applied as antimicrobial tools in many aspects of the food industry (Huang et al., 2008). Both methods are generated by the electrolysis of diluted NaCl solution with an electrolyzed membrane (for AEW) (Hsu, 2005) or a nonelectrolyzed membrane (for NEW) (Gil et al., 2015). This article will summarize existing studies to estimate the potential of comprehensive applications of EO water to reduce pathogenic bacterial risks in fresh cut meat products. U.S. companies producing EO water and related equipment have only recently connected with U.S. Environmental Protection Agency (EPA) to seek registrations. Furthermore, quality and economic impacts of EO water application to the meat industry will also be discussed.

2. Electrolyzed water production

Electrolyzed water has been widely adopted and applied as a sanitizer in Japan since the 1980s and numerous types of commercial electrolyzed water generation devices have been invented since then (Al-haq et al., 2005, Huang et al., 2008, Hricova et al., 2008 and Rahman et al., 2016). Electrolyzed water is produced based on simple electrochemical principles. When a continuous sodium chloride solution flows through an electrolyzed cathode and anode,

electric current will generate a series of electrochemical reactions between Cl^-, H^+ and OH^- (Al-haq et al., 2005, Hricova et al., 2008, Huang et al., 2008 and Rahman et al., 2016). $2Cl^- - 2\ e^- \rightarrow Cl_2$ and $Cl_2 + H_2O \rightarrow HOCl + H^+ + Cl^-$ are the two major reactions used to produce Hypochlorous acid (HOCl). HOCl is considered a major antimicrobial substance in EO water (Park et al., 2002, Koide et al., 2009, Cao et al., 2009 and Guentzel et al., 2008). The electrolysis reactions to produce acidic electrolyzed oxidizing water (AcEOW) and alkaline electrolyzed reducing water (AlERW) are often carried out in a chamber that is partitioned by a membrane (Fig. 1).

EO water recovered from the anode side of the chamber has a low pH (2-3) and high Oxidation-reduction potential (ORP) (1000 to 1100 mV or higher) (Al-haq et al., 2005). Meanwhile, electrolyzed reducing (ER) water is generated from the cathode side with high pH (10-11.5) and low ORP (-800 to -900 mV) (Hsu, 2003 and Huang et al., 2008). Properties of EO water generated by a membrane partitioned generator can be affected by the change of NaCl solution concentration and flow rate, even though the electric potential and power consumption remain stable. Electrolysis efficiency decreases when the NaCl solution concentration and flow rate increase. ORP increases or decreases in the opposite direction with the change of NaCl solution flow rate. Also, free chlorine increases with the increasing NaCl concentration (Hsu, 2003 and 2005 and Huang et al., 2008).

In contrast to AcEOW and AlERW generated from a membrane partitioned generator, neutral electrolyzed oxidizing (NEO) water is usually generated from a single cell without a membrane or diaphragm. When NaCl solution flows through a single cell generator, the same electrochemical reactions occur compared to membrane partitioned generators but because there is no membrane to limit the movement of H^+ and OH^-, neutralization reaction turn the pH of the end product to near neutral (Fig. 2). NEO water often possesses a pH of 6-8 and ORP of

700-900 mV according to researches (Al-haq et al., 2005, Hsu, 2005 and Rahman et al., 2016). Also, NEO water can be produced by partially transferring product from the anode side into the cathode chamber to neutralize the pH. NEO water is not as corrosive as AEW in the corrosion of processing surfaces or the irritation of human skin and the chlorine concentration in NEO water is more stable in NEW due to its neutral pH (Abadias et al. 2008).

Much research was dedicated to the improvement of EO water production efficiency. Forghani et al. (2015) studied the impact of water hardness and electrolysis parameters on slightly acidic electrolyzed water properties. The authors concluded that using 5% HCl, 2M NaCl along with a 1.5 ml/ min flow through rate to electrolyze low hardness water is optimimum and pre-heating could also be useful. Also, EO water can maintain its disinfection activity and chlorine concentration when stored properly. Len et al. (2002) concluded that an alkaline (pH 9.0) environment, closed conditions and no agitation could decrease the chlorine loss of EO water for two-months of storage. Also, lower temperature (4°C) also decreased the chlorine loss compared to higher temperature (25°C) (Fabrizio and Cutter, 2003).

3. Electrolyzed oxidizing water: advantages and disadvantages

Since its invention and adoption, EO water has been generally recognized as a safe and effective antimicrobial sanitizer. EO water, especially NEO water with a neutral pH, is much less harmful and toxic to human skin and processing surfaces compared to sodium hypochlorite, hydrogen peroxide and formaldehyde. Once EO water is left open in the environment or contacts organic matter, the EO water loses its toxicity rapidly mainly because the chlorine in EO water exists in rather unstable forms (Park et al., 2002, Allende et al., 2006, Shimmura et al., 2000 and Rahman et al., 2016). Also, dilution between EO water and water (underground water and tap water) will cause EO water to lose its weak toxicity and corrosiveness which

makes EO water friendly to the environment (Rahman et al., 2011, Stopforth et al., 2008 and Huang, et al., 2008). EO water is also inexpensive compared to most sanitizers. Traditional sanitizers are usually prepared by diluting concentrated stock solutions with water. Many advantages of EO water come from its on-site generation that eliminates any expense and risk in transport, storage and dilution (Jeong et al., 2007 and and Rahman et al., 2016). For EO water, once the electrolysis device is purchased and installed, the only expenses are electricity, tap water and sodium chloride for electrolysis. Sakurai et al. (2002) estimated that EO water is about 226 times less expensive compared to glutaraldehyde.

EO water loses its antimicrobial effectiveness soon after production because of the unstable forms of chlorine in EO water. Organic matter contact and an open environment for storage are two of the most important issues that are associated with loss of effectiveness. These problems cause a need for continuous supplementation of EO water from electrolysis to ensure an effective decontamination process (Huang, et al., 2008 and Walker et al., 2003). Moreover, Acidic EO water could cause skin discomfort and eye injury mainly because of its acidity (Al-haq et al., 2005 and Rahman et al., 2016). Also, chlorine emission, surface corrosion and protein degradation are minor problems but all are considered controllable. It is known that EO water becomes normal water after using, so there is no need for special treatment post-disinfection and bacteria tend not to develop resistance to EO water because of its broad-spectrum antimicrobial properties (Hricova et al., 2008). As concluded by Huang et al. (2008) and Rahman et al. (2016), the reaction between NEO (or any form of electrolyzed oxidizing water) and organic matter is the major issue to compromise the antimicrobial efficacy in NEO water decontamination. The formation of combined chlorine (chloramines) caused by the NEO-organic matter reaction leads NEO water to lose its ability to reduce bacterial population in organic matter rich conditions (Huang et al., 2008). Existing studies and current applications

of electrolyzed chlorine sanitizers are heavily designated to decontaminate fruit (Koseki et al. 2004), vegetable (Koide et al., 2009) and egg (Park el al., 2005) products. Obviously, these products provide much less exposed organic matter compared to common meat products when treated with NEO water.

4. Electrolyzed oxidizing water: antimicrobial mechanism

EO water provides high antimicrobial effectiveness and is often employed to control foodborne pathogens in related facilities. However, the mechanism of EO water antimicrobial efficacy remains unclear. Many studies indicated that HOCl is the functional molecule in antimicrobial activities (FUKUZAKI, 2006, Park et al., 2002 and Rahman et al., 2016). Len et al. (2000) reported that EO water has the highest HOCl concentration at pH 4 and the highest *Bacillus cereus* population reduction was also observed around pH 4 in EO water treatment. Fenner et al. (2006) (Fig. 3.) concluded that Cl_2 becomes HOCl through hydrolysis in aqueous solutions and HOCl is considered one of the most effective antimicrobial oxidizing agents. Available HOCl is predominantly decided by the pH, which determines the electrolytic balance between hydrogen (H^+) and hypochlorite ion (OCl^-). At 20 °C and pH 9, 96% of the free chlorine in EO water is OCl^- that results in a very poor disinfectant effectiveness. At 20 °C and pH 8.3, undissociated HOCl in EO water is 16.1% that was observed to have a mediocre disinfectant effectiveness. At pH below 5 at 20 °C, the percentage of undissociated HOCl in EO water is approximate 99.7% (Fenner et al., 2006). However, Park et al. (2004) found that experimental results indicate that chlorine in EO water has a more significant effect on inhibition of bacteria compared to the effect of pH. Cui et al. (2009) described that NEW had relatively sustainable bactericidal activity compared to AEW. After 30 days' storage, all types of EO water demonstrated strong antibacterial activity except AEW stored in an open environment.

An electron microscopy method has been employed to demonstrate the EO water bactericidal mechanism. Nan et al. (2010) studied the inactivation effectiveness of EO water on *Escherichia coli* O157:H7 and *Staphylococcus aureus and t*reated bacteria were observed with a transmission electron microscopy. Although complete inactivation was detected for both types of bacteria, clear cell wall destruction was only observed in *Escherichia coli* O157:H7. *Staphylococcus aureus* cell structure remained intact visually. Osafune et al. (2006) observed *Staphylococcus saprophyticus*, *Bacillus sphaericus* and *Micrococcus luteus*. The scanning electron microscope captured images that showed that the outer layer of bacteria cell walls was obviously wrinkled and the inner layer was distantly detached from the cell wall. The authors drew the conclusion that destruction of cellular structure was one of the major reasons for the inactivation of bacteria. Keskinen et al. (2009) observed 20 or 200 ppm chlorine EO water washed Romaine lettuce with scanning electron microscope. Results indicated that biofilm formation of *Escherichia coli* O157:H7 and wounds in the lettuce have a strong influence on EO water antibacterial effectiveness.

5. Electrolyzed oxidizing water in pure bacterial culture

Zhang et al. (2010) concluded that HOCl in EO water may adhere to bacterial cell walls and increase the cell membrane permeability. This phenomenon causes a rapid leakage of DNA, K^+, and proteins in cytoplasm that eventually kill the bacteria. As a weak acid, HOCl concentration depends on the ionization equilibrium between HOCl, hypochlorite ions ($^-$OCI) and protons (H^+) that is decided by EO water pH. Yang et al. (2003) summed that bactericidal efficacy of EO water application has significantly better antimicrobial efficacy than treatments using chlorinated water and oxidizing agent concentrations at the same pHs. Also, the ORP value was decreasing in EO water with increasing pH. However, the authors found no evidence in the study that the ORP along with the ratio of ClO /HClO contributes to the higher

bactericidal efficacy of EO water than the chlorinated water at the same pH. Research (Graca et al., 2011) demonstrated that HOCl is the major functioning substance of EO water. Also, HOCl is considered as one form of free available chlorine that can inhibit a broad range of bacteria. Higher concentration of HOCl produces more hydroxyl radicals. The combination of high ORP and low pH may provide more effective antimicrobial activity of EO water treatments. Vorobjeva et al. (2004) suggested that EO water has bactericidal effects because of its high ORP, dissolved chlorine gas and HOCl and it can overcome drug resistance due to its broad spectrum bactericidal properties. Ding et al. (2016) examined the disinfection efficacy of slightly acidic electrolyzed water (pH 6.1) on *Staphylococcus aureus*. In comparison with HCl and NaClO, slightly acidic electrolyzed water reduced the *Staphylococcus aureus* population by 5.8 $\log_{10}$ CFU/mL compared to 3.26 $\log_{10}$ CFU/mL (NaClO) and 2.73 $\log_{10}$ CFU/mL (HCl). The authors also concluded that EO water disrupted the permeability of bacterial cellular membranes, while low pH was responsible for the cytoplasmic disruptions. Xuan el al. (2017) compared the effect of slightly acidic electrolyzed water (pH 6.3) and heat treatment on the recovery of *Listeria monocytogenes* using the Baranyi growth model. The result demonstrated that EO water treatments had a stronger influence on the treated bacteria compared to the heat treatment despite the similar injury level. Another study (Tango et al., 2015) determined the antimicrobial efficacy of EO water (pH 6.29) against *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli* O157:H7 and *Salmonella* Typhimurium. Complete inactivation of EO water on all four bacterial pathogens was achieved when chlorine concentration was above 20 ppm while gram-positive pathogens (*Staphylococcus aureus* and *Listeria monocytogenes*) tended to be more resistant to the EO water treatment compared to gram-negative pathogens (*Escherichia coli* O157:H7 and *Salmonella* Typhimurium).

Park et al. (2002) applied EO water (50 mg/L residual chlorine) to pure *Campylobacter jejuni* (7.5 CFU/ ml) cultures. After ten seconds of reaction, complete inactivation (verified by enrichment) of *C. jejuni* was observed. Also, authors concluded that a lower concentration of chlorinated water (25 mg/L residual chlorine) has less antibacterial effectiveness compared to EO water at the same concentration. In an in vivo experiment, the authors found that EO water and chlorinated water were both equally capable of decreasing *C. jejuni* population by approximately 3 $\log_{10}$ CFU/g on chicken carcass. Issa-Zacharia et al. (2010) analyzed the sanitization effectiveness of slightly acidic electrolyzed water (SAEW) on *S. aureus* and *E. coli* pure cultures and the potency was compared to strong acidic electrolyzed water or sodium hypochlorite solution. The result showed that slightly acidic electrolyzed water treatment caused a more than 5 $\log_{10}$ CFU/ml reduction of *S. aureus* and *E. coli*, although both strong acidic electrolyzed water and sodium hypochlorite solution had slightly stronger potencies. Although EO water demonstrated strong antimicrobial and bactericidal efficacy, the reactions between EO water and organic materials could be problematic because these reactions will dramatically compromise the antimicrobial efficacy of EO water (Gómez-López, 2017). Oomori et al. (2000) tested the remaining available chlorine of EO water after the reactions between EO water and nutrient broth, proteose, glycine, sucrose, glucose, and corn oil. In this study, all tested food-related substances significantly decreased the remaining available chlorine in EO water except sucrose and glucose. The authors concluded that the presence of organic matter, including amino acids, proteins, and possibly unsaturated fatty acid chains could be responsible for the loss of bactericidal activity of EO water. This means the presence of organic matter, especially protein-rich substances, should be taken into consideration when EO water is applied. A recent study (Jo et al., 2018) tested the effect of organic materials on chlorine loss in EO water during the washing process. Both free chlorine concentration and pathogenic bacterial inactivation efficacy were recorded to demonstrate the impact of organic

material. The results showed that protein-rich organic matter was more detrimental to the chlorine concentration and antimicrobial efficacy when compared to lipids and carbohydrates. Because of this, the authors drew the conclusion that the optimization of EO water sanitization efficacy should consider that the presence of organic matter, especially proteins and meats, were more likely to decrease the disinfection effectiveness of EO water compared to vegetable products.

Vorobjeva et al. (2004) reported that treatment of EO water (4.5 ml) on bacterial suspensions (0.5 ml, approximate 8 $\log_{10}$ CFU/ml) can completely inactivate a variety of gram-positive and gram-negative bacteria strains (gram-negative: *Pseudomonas aeruginosa, Escherichia coli, Citrobacter freundii, Flavobacter* sp., *Proteus vulgaris, Alcaligenes faecalis, Aeromonas liquefaciens;* gram-positive: *Enterococcus faecalis, Staphylococcus aureus, Bacillus cereus* (spores)) in a reaction lasting 30 seconds. However, results also indicated that gram-positive bacteria *Bacillus cereus* (6.72 ± 0.02 $\log_{10}$ CFU/ml) required a longer reaction time (5 min) to achieve complete inactivation. Abadias et al. (2008) studied the antimicrobial activity of NEW compared with a standard sodium hypochlorite treatment. The results showed that when NEW contains more than 48 ppm of free chlorine, all strains (*Salmonella, Listeria innocua, E. coli* O157:H7 and *Erwinia carotovora*) populations were reduced by more than 5 $\log_{10}$ CFU/ml. In addition, reaction time increased the reduction effect with lower free chlorine concentration (28 ppm) of NEW under ambient (20 ± 2°C) temperature. Also, when tested in laboratory conditions, bacterial culture preparation and general handling may have an impact on the experiment. Zhang et al. (2016) detected the antimicrobial activity of EO water on *Bacillus subtilis* and *E. coli* O157:H7 with different bacterial concentrations and different times of pre-centrifugation. The author pointed out that current studies of EO water concentrated on the chlorine concentration, pH, ORP, presence of organic material, treatment

time and temperature. The results indicated that both initial bacterial concentration and pre-centrifugation times are important to the final population reduction result and should be take into consideration in related antimicrobial efficacy studies. Lehto et al. (2017) evaluated the efficiency of decontamination of NEO water on *Escherichia coli, Yersinia pseudotuberculosis, Yersinia enterocolitica,* and *Candida lambica* with or without the interference 1% carrot juice. Result showed that more than 3 log CFU/ml population reductions were observed after NEO water treatment for all four types of microorganism tested with or without the carrot juice interference.

Fabrizio and Cutter (2003) detected free chlorine concentrations in EO water after 1 days "aging" at 4 and 25 °C after generation. Free chlorine increased at 4 °C but decreased at 25 °C after 24 h. The authors also applied EO water along with other types of chlorinated water to pure cultures of *Salmonella* Typhimurium and *Listeria monocytogenes* at both 4 and 25 °C. Results demonstrated that EO water could significantly reduce *Salmonella* Typhimurium and *Listeria monocytogenes* (more than 8 $\log_{10}$ CFU/ ml reduction) under different conditions. Ovissipour et al. (2015) compared resistance of *Escherichia coli* O104:H4, *Listeria monocytogenes, Campylobacter jejuni, Aeromonas hydrophila,* and *Vibrio parahaemolyticus* to EO water in pure culture. Results verified the strong inactivation ability of EO water to all of the bacteria strains tested. However, *E. coli* O104: H4 clearly has stronger resistance compared to alkaline electrolyzed water compared to acidic electrolyzed water. However, Jadeja et al. (2013) reported that O157:H7 has the highest resistance compared to other shiga toxin-producing *E. coli* (STEC) (O26, O45, O103, O111, O121, O145) strains and enteroaggregative STEC O104: H4. The authors also claimed that *E. coli* O104:H4 did not demonstrate any more resistance than many other STEC strains in this project and EO water treatment is fully capable of killing O157:H7 as well as other STECs.

The disruptive effect of EO water on biofilm was also studied by many researchers because biofilms are a potential source of contamination in food products and it can survive surface sanitization due to its unique properties. Ayebah et al. (2005) investigated the inactivation efficacy of the combination of alkaline and acidic EO water in *Listeria monocytogenes* biofilms (2 by 5 cm) on stainless steel surfaces. The results show that using either alkaline or acidic EO water alone has lower biofilm cleaning efficacy compared to using the combination of alkaline and acidic EO water and the time of exposure is important to *Listeria monocytogenes* population reduction. This finding indicated that the tested combination has the potential to achieve better biofilm inactivation efficacy. Another study (Li et al., 2017) tested the acidic EO water disinfection efficacy on *Bacillus cereus* biofilms located on stainless steel surfaces and comparing it to chemically modified chlorine water. In summary, EO water displayed much better inactivation efficacy against tested biofilms compared to chemically modified chlorine water at similar pH, chlorine concentration and ORP. However, the study also found that the presence of bovine serum albumin, which can be classified as a type of protein-rich organic material, negatively affected the inactivation and cleaning efficiency of EO water on *Bacillus cereus* biofilms after 15 min of reaction time on a stainless steel surface.

6. Meat industry safety and post-harvest intervention

Nowadays, consumers' demands for completely safe high-quality meat products have raised the production standard for the entire meat industry. As a high water-activity and diversely nutritional food, meat, especially fresh meat product, is considered to have an ideal environment for bacterial growth (Saucier, 2015). Microbial contaminations are well recognized as the most important threat to meat safety in the modern era compared with traditional safety challenges (chemical and physical contaminants) in meat production. Novel technologies, like ozone, high pressure treatment and irradiation etc., provide new approaches

to further secure product safety without high level quality compromise. Post-harvest microbial interventions have been studied to verify the effectiveness in many institutions. A report disclosed that 420 to 960 million cases of foodborne illnesses have been estimated by World Health Organization in 2015 (WHO, 2015). Although thermal processing is among the traditional methods and could be effective in microorganism disinfection, it inevitably causes quality changes in treated meat products. Recently, many novel thermal processing methods were employed for preserving and decontaminating meat products, like microwave, including radiofrequency, infrared heating, and ohmic heating. (Troy et al., 2016). Thus, thermal treatments still offer tremendous usefulness in meat production and consumption and should taken under serious consideration when applied alone or along with other processing techniques in hurdle technologies. Meat safety also relies on inspection and sampling analysis since the production chains are implemented with safety assurance systems. However, not all products will be thoroughly investigated to guarantee complete safety. Among all challenges, bacterial pathogens associated with meat products are the major sources of diseases that originate from contaminated meat consumption. *Salmonella* spp. is considered as a type of important pathogenic bacteria that is responsible for approximate 1 million illness cases in the U.S (Scallan et al., 2011). *E. coli* O157:H7 is a well-recognized type of foodborne pathogenic bacteria that results in diarrhea and hemorrhagic colitis. These *E. coli* O157:H7 caused diseases has been traditionally related to beef, pork and poultry products. *Yersinia enterocolitica* is a bacterial pathogen that is responsible for human diseases and has been historically affected post production (Bonardi et al., 2018). The emerging demand for higher meat quality and safety undeniably challenges meat processors to utilize innovative techniques to sustainably produce meat products while decreasing environmental impacts and has drawn a lot of attention. The modern meat industry should increase engagement and commitment in novel innovative meat

processing and storage methods to further improve quality and safety attributes in meat products (Troy et al., 2016).

As the most consumed meat, per capita consumption of pork remained stable over the past 20 years at about 60 pounds per year in the United States (Baer et al. 2013). Pork is also consumed by people in European Union and China more than other types of meat and the consumption is increasing in many of these regions (Scharff, 2010). According to the USDA (Custer 2015), in 2013, the United States exported over 453 million pounds of pork. Because pork is consumed in large quantities and is an important U.S. export, safety of the pork supply is one of many critical control points in the entire food industry. Foodborne pathogens are one of the major contributors to illnesses, hospitalizations and deaths. The Centers for Disease Control and Prevention (CDC 2012) published that 48 million illnesses and 3000 deaths are caused by foodborne pathogens in the year 2011. As for bacterial pathogens associated with pigs or pork products, *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter* spp. and *Staphylococcus aureus* are among the top well-documented ones that cause the most illness and deaths (Baer et al. 2013). Also, a study confirmed that pork can be contaminated by Pathogenic *Yersinia enterocolitica* in the slaughter process (De Boer and Nouws 1991). Also, beef is recognized as one of the most important meat products with lower fat and higher protein content compared to other major products. Beef is also a source of other valuable nutrients, like minerals, fatty acid and vitamins (Lyu et al., 2016). Color and appearance are two of the most influential factors that could impact consumers' purchase decisions and ability to judge shelf life of fresh beef. Minimizing negative impacts on color and appearance in beef products is always important to any post-harvest intervention.

High pressure processing has been studied as a post-harvest treatment to muscle foods. Roman et al. (2013) concluded that high pressure processing can introduce different structural

changes in muscle food products especially in proteins. These changes are mainly caused by and depend on the level of the treatment times, pressures and temperatures. High pressure processing has been verified as a treatment that can effectively sanitize the targeted products. However, the influences introduced by high pressure processing inevitably affect consumption qualities through dramatic textural and other property changes. Ionizing radiation processing is considered as an under-utilyzed technique in the meat industry (Ehlermann, 2016). Even though evidence shows that radiation has a lot of antimicrobial potential, public concerns and other issues (e.g. labeling legislation) hold it back in terms of large scale application. Ozone is a strong oxidation agent and is classified as a safe microbial population control method. Studies have revealed that ozone is fully capable of preventing or limiting microbial growth to ensure products' safety (Lyu et al., 2016). Jaksch et al. (2004) found that ozone effectively decreased the growth of bacteria on contaminated fresh pork cutlets.

Over the last few decades, people's supermarket shopping habits have been altered from daily trips to once-a-week shopping because of the changing lifestyles of consumers (Coles et al. 2003). Using synergistic efforts to control microorganisms, especially bacterial pathogens, has garnered a lot of attention. Securing the safety and quality of food during non-freezing storage has been a major issue due to this change, especially for meat products. New technologies, like modified atmosphere packaging (MAP) and ozone treatment, have been invented and studied intensively to fulfill the demand (Appendini and Hotchkiss 2002) and many of these novel methods have the potential to be applied along with EO water to further secure food safety and prolong shelf-life. MAP is designed to create a low oxygen and high carbon dioxide and/or high nitrogen storage atmosphere to limit the growth activity of microorganisms. Both ozone and electrolyzed water treatments aim to provide free radical to the food matrix to kill or limit microorganisms. Pork spoilage bacteria activity under different

ORP atmospheres has been well studied by researchers. Adams et al. (2015) found that Lactic acid bacteria dominated the spoilage microflora in different dissolved CO_2 and O_2 concentrations in the purge of vacuum packaged pork chops, followed by Enterobacteriaceae. *Pseudomonas* spp., *Enterobacteriaceae* and *Enterococcus* spp. dominated the spoilage of ozone treated commercial samples of pork meat and determined the shelf life for such products (Jaksch et al. 2004). Li et al., 2017 studied the synergistic antimicrobial effects of ultrasound and EO water on *Staphylococcus aureus*. The authors employed flow cytometry cell sorting and electron microscopy methods to evaluate treated bacterial cells and the result demonstrated the inactivation effectiveness of the tested decontamination combination. Also, as high as 45.75% of cells entered a viable but non-culturable state when ultrasound is applied alone, while 0.07% cells were in a viable but non-culturable state when the combination is used. This finding suggested that not only EO water can be applied individually as a disinfection reagent but also has the potential to be utilyzed as a major component in a decontamination combination. Fermented meat product safety is another important aspect in the industry. These products have unique tastes primarily due to the presence of low molecular weight flavor compounds, like peptides, amino acids, aldehydes, and organic acids. Degradation of protein and other high molecular weight substances generate these flavors and could potentially raise the risk of contamination because of the existance of varieties of microorganism (Ojha et al., 2015).

7. Electrolyzed water in meat-related researches

Ever since its invention, EO water has been studied to control bacteria particularly pathogenic bacteria on fresh products. It is known that EO is less affective in eliminating microbes on fresh meat products compared to vegetables and fruits due to abundant organic matter and absence of an epidermis. Some studies have been conducted to explore the potential for application of EO water in meat products. Ding et al. (2010) established a model of

Escherichia coli O157:H7 growth in fresh beef with EO water treatment at different storage temperatures. They detected that EO water decreased the population of *E. coli* O157:H7 on fresh beef for 1.64 to 1.72 $\log_{10}$ CFU/ g and that strong antibacterial effects were observed despite the pH of EO water. Kim et al. (2005) evaluated the EO water on bactericidal effectiveness on *Campylobacter jejuni*, a traditionally poultry related pathogenic bacteria. The meat ingredient was chicken carcasses after dehairing. The EO water significantly decreased the *Campylobacter jejuni* by 2.33 $\log_{10}$ CFU/g (from 4.92 $\log_{10}$ CFU/g). A similar study was carried out by Wang et al. (2012) looking at an EO water treatment and *Listeria monocytogenes* on lean pork. After dipping the pork in EO water for 10 minutes, the lean pork portions were air-packaged and stored at different temperatures. An initial *Listeria monocytogenes* population reduction of 1.72 to 1.74 $\log_{10}$ CFU/ g was observed and the population difference between EO treated lean pork samples and untreated pork samples remained stable throughout the entire storage period at all of the temperature settings. Veasey et al. (2016) applied EO water to pure cultures of *Listeria monocytogenes*, *E. coli* O157:H7, and *Salmonella* and inoculated fresh beef carcasses, frankfurters, and processing blades. Little to no bacterial population reduction efficacy was observed on inoculated meat products but populations on processing blades were significantly reduced after EO water treatment. A follow-up study of protein and EO water reaction demonstrated that the presence of protein organic matter compromised the antimicrobial efficacy of EO water by eliminating free available chlorine. Shimamura et al. (2015) conducted an experiment to evaluated antimicrobial efficacy of EO water and alkaline electrolyzed water on *Salmonella* Enteritidis, *Escherichia coli*, and *Staphylococcus aureus* inoculated chicken breast and beef liver. The treatment reduced the bacterial population by more than 1 $\log_{10}$ CFU/g and significantly suppressed the expression of a toxin (staphylococcal enterotoxin A) production gene in specific strains. Also, the

treatment had no impact on freshness, texture score, and odor of the meat samples according to the sensory evaluations.

As one of the major pathogenic bacteria source, swine slaughtering and processing are highly regarded as critical control points in the entire swine production system. The application attempts of EO water in swine industry have been mentioned and studied in a few occasions. Rahman et al. (2013) evaluated the bactericidal effectiveness and overall quality influence of EO water along with other popular antimicrobial substances (distilled water (control), aqueous ozone, lactic acid and calcium lactate in fresh pork. In this project, gram negative pathogen *E. coli* O157:H7 and gram-positive pathogen *Listeria monocytogenes* were inoculated into the tested fresh pork to implement population enumeration and sensory experiments. The results indicated that EO water alone and in combination with calcium lactate are highly effective against *E. coli* O157:H7 in fresh pork. The application also improved the shelf-life and resulted in acceptable sensory scores. These researches indicated that EO water has the potential to overcome exposed organic matter interference to be effective on controlling bacteria population. Fabrizio and Cutter (2004) investigated the bactericidal efficacy of EO water spray on fresh pork belly artificially inoculated with *Listeria monocytogenes, Salmonella typhimurium* or *Campylobacter coli*. The antimicrobial effectiveness of EO water was compared against distilled water (control), chlorinated water and lactic acid immediately after application and vacuum package storage or anaerobic storage for up to 7 days. The study verified the ability of EO water to reduce the population of *Campylobacter coli.* Also, the author concluded that longer and more intense reaction between EO water and fresh pork may be required to achieve better bactericidal results. Another study (Mansur et al., 2015) evaluated the antimicrobial efficacy of the treatment combination of EO water and fumaric acid against *Escherichia coli* O157: H7, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella* Typhimurium in fresh pork. The tested combination was carried out by dipping

the pork sample in a treatment broth at 40 °C for 3 min. Results showed that the combination (EO water + fumaric acid) not only achieved the highest bacterial population reduction, but also significantly prolonged the shelf-life of treated pork samples.

Applications of EO water washing on animal hides or skin also have been studied by some groups. Bosilevac el al. (2005) conducted an experiment to study the decontamination efficacy of EO water washing on randomly selected beef hides. The bactericidal effectiveness of EO water was compared with ozonated water. EO water washing resulted in a higher population reduction in both aerobic plate counts (2.1 $\log_{10}$ CFU/ 100 cm^2 and 3.5 $\log_{10}$ CFU/ 100 cm^2, respectively) and *Enterobacteriaceae* counts (3.4 $\log_{10}$ CFU/ 100 cm^2 and 4.3 $\log_{10}$ CFU/ 100 cm^2, respectively). Both washing methods displayed much higher decontamination effectiveness compared to the control (water washing) on both aerobic plate (0.5 - 1.0 $\log_{10}$ CFU/ 100 cm^2 and *Enterobacteriaceae* counts (0.9 $\log_{10}$ CFU/ 100 cm^2). The EO washing also decreased the *E. coli* O157:H7 prevalence (from 89 % to 31%) and no prevalence reduction was observed in the control. Park et al. (2002) evaluated the EO water bactericidal efficacy on killing *Campylobacter jejuni* on chicken skin with designed washing process using original or diluted EO water. The overall effectiveness was compared against chlorinated water and deionized water (control). Results showed that EO water washing led to a complete elimination of *Campylobacter jejuni* (verified with enrichment) on 3 $\log_{10}$ CFU/ g inoculated chicken and reduced the *Campylobacter jejuni* population to minimal but detectable on 4 $\log_{10}$ CFU/ g inoculated samples. Studies focused on EO water hide and skin washing revealed tremendous potential in related applications in the meat industry. These findings indicated that EO water with lower acidity has the potential to be utilized in related carcasses decontaminations. Al-Holy and Rasco (2015) applied acidic (pH =2.3) EO water on trout, chicken legs, and beef meat surfaces that were inoculated with *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* Typhimurium. The EO water wash (1.6-2.0 log reduction) achieved a much higher

bacterial population reduction compared to traditional tap water wash (0.2-1.0 log reduction). This research showed that EO water wash has the potential to be applied as a water substitute in washing and cleaning during processing in the meat industry. Wang et al. (2018) developed and installed a novel spray cabinet to apply EO water on chicken carcasses. The novel washing design successfully decreased the total microbial load on chicken carcasses 1.0 $\log_{10}$ CFU/cm^2 due to high spray pressures and all-around spray angles. Interestingly, slightly acidic EO water achieved similar bacterial population reduction efficacy compared to acidic EO water despite the free available chlorine concentration in slightly acidic EO water being significantly lower (30 mg/l vs 60 mg/l).

Raising and slaughtering facilities are the two most important locations in the meat production chain. Sanitation of the facilities and prevention of cross-contamination is vital to secure the safety of the end product. Zheng et al. (2013) employed EO water as the air-cleaning spray agent to decrease the population of airborne bacteria in egg laying houses. The overall outcome was compared with didecyl dimethyl ammonium bromide. The EO spray decreased the temperature by approximate 1 °C and increased the humidity by 3% in 30 minutes. More importantly, the airborne bacteria population decreased by 0.7 $\log_{10}$ CFU/ m^3 and is considerably better than didecyl dimethyl ammonium bromide spray (0.37 $\log_{10}$ CFU/ m^3). The results suggested that EO water can be utilized as an environment friendly sanitizer in raising facilities and manage to control the bacteria related safety risk. In the meat industry, especially slaughtering facilities, processing surfaces are crucial to sanitation and safety in the end products. Cleaning, decontamination and sustained hygiene of these surfaces are and always will be of the most importance for the whole industry. Li et al. (2016) analyzed the bactericidal efficacy of EO water on *E. coli*, *Salmonella* Typhimurim, *Staphylococcus aureus*, and bacterial cocktail inoculated stainless steel surfaces and total aerobic bacterial population in animal transportation vehicles. The result showed that EO water has the potential to be applied as an

environment friendly disinfection reagent in animal related activities. New technologies and strategies are continuously studied and introduced to related areas. Jiménez-Pichardo et al. (2016) were designated to optimize the disinfection effectiveness of EO water on stainless steel surfaces, one of the most important processing surfaces in meat industry. Bacteria cell removal was recorded to analyze effectiveness in different settings of temperature, concentration, reaction time and surface type. Results demonstrated EO water was a powerful sanitizer that can possibly be broadly applied onto steel-based surfaces, whereas electropolished steel surface were shown to require shorter reaction times of EO water and easier to be disinfected compared to non-electropolished steel surface.

8. Viable but nonculturable (VBNC) state bacteria and food safety

The terminology of viable but non-culturable (VBNC) state has attracted increasing attention in microbiology researches, especially in food microbiology, since its presentation (Xu et al., 1982; Zhao et al., 2017). To date, the enumeration of specific bacteria in food samples has been largely relied on conventional medium-based assays (Ferro et al., 2018) and cultivation and plate count method remains as one of the most important analytical technique in microbiology (Buck et al., 1979; Li et al., 2014). In these conventional enumeration method, researchers count the total bacterial population based on the bacterial viability that enable a single cell to develop into a colony (Talaro et al., 2018). In contrast to viable cells, bacteria in VBNC state are still biologically alive but unable to grow into colonies in respective mediums (Oliver, 2000). Resuscitation of bacterial cells in the VBNC state may occur so that these cells become culturable again (Pinto et al., 2015). Many studies discovered that VNBC bacteria have many characteristics that dead cells lack: intact cell membrane (Heidelberg et al., 1997; Wang, et al., 2010); wholesome gene and gene expression (Lleò et al., 2000); metabolic respiration (Mukamolova et al., 2003) and ATP activity (Lindbäck et al., 2010). These underlying

properties of VBNC bacteria present considerable challenges to the food industry because the targeted bacteria can be underestimated or undetected when conventional enumeration methods are employed (Ferro et al., 2018; Li et al., 2014).

Many studies addressed the association between VBNC state and foodborne pathogens. Pinto et al. (2015) summarized that a total of 68 bacterial species have been historically described to enter a VBNC state, many of which can cause food related illnesses and outbreaks (Nicolò and Guglielmino, 2012). Makino et al. (2000) found that *Escherichia coli* O157:H7 associated with salted salmon roe consumed in Japan has been consequently underestimated because *Escherichia coli* O157:H7 enters VBNC state in a high NaCl environment and this food item could be dangerous. Temperature is also important to the transformation of the VBNC state, Dinu and Bach (2011) discovered that *Escherichia coli* O157:H7 maintained strong culturability on green leaves at 16°C but shifted toward a VBNC state at 8 °C and as high as 70% of the total bacterial population entered a VNBC state in this study. Another study (Nicolò et al., 2011) revealed that *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Shigella flexneri* could enter a VBNC state when inoculated in grapefruit juice while *Listeria monocytogenes* lost its culturability and viability within 24 h of incubation in grapefruit juice. An acidic environment is considered one of the causes for the VBNC state of bacteria (Ferro et al., 2018; Zhao et al., 2016). Cunningham et al. (2009) found that potassium sorbate forced *Listeria monocytogenes* to enter a VBNC state when grown at pH 4.0. In the study of Zhao and Matthews (2000), the environment of low pH along with low temperature was detected to have the strongest impact on the VBNC state formation of *Escherichia coli* O157:H7. Pawlowski et al. (2011) illustrated that *Yersinia pestis* can enter a VBNC state and become undetectable at low temperature. The existance of a VBNC state of a pathogen can also present risks in meat production, in a study by Jang et al. (2005) *Campylobacter jejuni* cells not only changed into a coccoid shape but lost cell viability under

an undesired nutritional environment. The authors also found that *Campylobacter jejuni* entered a VBNC state both in a pure culture experiment and on inoculated chicken skin which could lead to risk in human consumption. Meat processing facilities have been viewed as a critical area of safety for meat products (Bustillo-Lecompte et al., 2015) and a related study demonstrated (Peneau et al., 2007) that link between bacteria in a VBNC state and disinfection of meat processing facilities. As a self-protection mechanism for bacteria, biofilms have been of interest in food hygiene and food safety (Shi and Zhu, 2009). Study (Gião et al., 2014) found that *L. monocytogenes* formed biofilms and entered a VBNC state in tap water after 48 hours which means the pathogen can be undetectable when using conventional culture methods. In another study (Wang et al., 2010), both electrochemical and chemical chlorine disinfection methods generated the VBNC population in *Escherichia coli* and *Legionella beliardensis*. This study showed that chlorine-based disinfection method has the potential to create VBNC population in targeted bacteria.

The detection and verification of VBNC state cells depend on two key characteristics: viability and non-culturability, and the quantification of VBNC cells is usually carried out by comparing the viable bacterial population and the culturable population (Li et al., 2014). Thus, an assay designed to calculate the VBNC population generally includes two major components: conventional medium-based experiment and a bacterial viability assessment experiment. Once the population is estimated by viable assessment assay and a medium-based assay, the VBNC population can be detected with the difference (Zhao et al., 2017). Bacterial viability can be tested by many methods, such as bacterial membrane integrity and metabolic activity tests using dye staining and many researchers using this methodology employed flow cytometry to quantitatively count targeted bacterial cells. Over the last few decades, flow cytometry has become an important analytical method in microbiology, biotechnological engineering, food analysis, and chemical disinfection assessment since it allows single-cell analysis in a short

time frame (Berney et al., 2007). When carrying out a flow cytometer experiment, cells (eukaryotic or prokaryotic) are passing through a laser light beam in a thin channel one by one. The interaction between laser and cells causes reflection and the reflection is measured by light receptors by light scatter and fluorescence level. Different fluorescent and scatter signals from the same cell will ideally demonstrated the cell characteristics as recorded by the flow cytometer (Laerum and Farsund, 1981). Wang et al. (2010) employed multiple staining methods along with flow cytometry to analyze the esterase activity, membrane integrity and polarization. They concluded that flow cytometry and an adequate staining method is a useful tool to estimate the percentage of VBNC bacteria in water. Also, LIVE/DEAD® BacLight™ staining kit with or without flow cytometry sorting has been widely used in bacterial viability detection, which discriminate dead bacterial cells from live ones based on cellular membrane intactness (Berney et al., 2007; Boulos el a., 1999; Karkashan et al., 2015). Other methods, like housekeeping genes mRNA detection, global gene expression profile and surface protein identification may also be used in bacterial viability analysis (Nocker and Camper, 2009; Trevors, 2011). Patrone et al. (2013) studied the membrane protein CadF by using reverse transcription polymerase chain reaction (RT-PCR) and the experiments demonstrated that *Campylobacter jejuni* can enter a VBNC state without losing its adhesion ability. The current studies and understanding on VBNC bacteria, like foodborne pathogens associated with EO water treatment, raise the demand to improve the knowledge and detection assay of VBNC bacteria to further secure the food safety.

9. Sublethally injured bacteria and hurdle technology in food safety

Much like the VBNC state, bacterial sublethal injury is another phenomenon that is viewed to be associated with cell viability and self-replicate ability (Wesche elt al., 2009). When bacteria face a harsh growth environment or are exposed to chemical or physical stresses,

sublethal injury can occur to bacterial cells and these injuries are generally considered as transient because they can be repaired in desirable conditions (Espina et al., 2016; Mackey, 2000). Also, sublethal injury is well recognized as an important phenomenon because the presence of injured bacterial cells could present a food safety risk just like fully viable bacteria and maybe not able to be recovered by selective mediums (Silva et al., 2015). Injured bacteria, especially sublethally injured ones, can remain viable and carried out reproduction in food matrices due to the ideal environment in foods: high water activity, neutral pH, moderate ORP, and high nutrition (Wesche et al., 2009). Many foodborne bacterial pathogens have been viewed that can be sublethally injured in stressful environment according to existing publications: *Escherichia coli* (Bi, et al., 2015; Espina et al., 2016; Gayán et al., 2016), *Salmonella* spp. (Espina et al., 2016; Wang et al., 2017; Saldaña et al., 2010), *Listeria monocytogenes* (Zhao et al., 2013; Noriega et al.,2013), *Campylobacter jejuni* (Lu et al., 2011; Liu et al., 2012), and *Yersinia enterocolitica* (Kalchayanand et al., 1992; García et al., 2005).

Post-harvest treatments have been verified to be effective in inactivating bacterial vegetative cells, they usually are incapable of providing the complete elimination of food-borne pathogens, especially in industrial applications. The post-treatment surviving bacterial population is, very likely, sublethally injured bacteria (Jasson et al., 2007; Manas and Pagan, 2005). In the sublethal injury state, bacterial cells become more sensitive to environmental stresses which healthy cells are resistance to. Total populations for bacteria in the sub-lethal injury state could be underestimated in selective media enumeration because injured bacterial cells' culturability can be affected by respected selective media in contrast to non-selective media (García 2005). Also, sublethal injury caused by a single treatment can be utilized in hurdle decontamination technology because higher decontamination efficacy could be achieved with less treatment intensities due to synergistic effects (Ukuku, 2010, Khan, 2017).

The common method to enumerate a sublethal population is based on the comparison between populations calculated on conventional media and modified media (Gelaw et al., 2014). However, new technologies and methodologies have emerged to improve the quantification and understanding of sublethal injury of bacteria in foods. Espina et al. (2016) employed traditional selective and non-selective plating methods alone with media modification and flow cytometry cell sorting to detect the sublethal injury in heat shocked *Escherichia coli*. Results and statistical analysis confirmed the adequacy of current and improved enumeration methods in sublethal injury quantification. A study (Li et al., 2017) was carried out to investigate the synergistic antimicrobial efficacy of ultrasound and mild thermal treatment against *Staphylococcus aureus*. The results demonstrated that sonication can cause immediate death of the bacterial cell while heating can sublethally injure *Staphylococcus aureus*. Also, the combination of sonication and heating can synergistically reduce the bacterial population due to severe cellular damages. Shi et al. (2015) studied lactic acid induced sublethal injury in *Escherichia coli*. After 60 min of exposure to lactic acid, *Escherichia coli* become widely persisted to lower pH values (3-5) and all tested *Escherichia coli* strains could be fully recovered after incubation.

Bi et al. (2014) applied high pressure carbon dioxide to *Escherichia coli* O157:H7 cultures and enumerated the bacterial population by both selective medium (tryptic soy agar + 3% NaCl) and non-selective medium (tryptic soy agar). Much more population reduction was observed on selective medium (5.18 $\log_{10}$ CFU cycles) compared to non-selective medium (1.21 $\log_{10}$ CFU cycles). This finding suggested that high pressure carbon dioxide can cause sublethal injury to *Escherichia coli* O157:H7 and the injury could be repaired in liquid enrichment mediums. García et al. (2005) evaluated a total of eight strains of bacteria including *Yersinia enterocolitica* using selective and non-selective mediums after pulsed electric field treatment. Results indicated that the tested treatment induced sublethal injury to *Yersinia enterocolitica*

at pH 4. A large quantity of sublethally injured bacteria existed after pulsed electric field treatments and the injury level was dependant on the types of bacterial strain and the pH. To quantify and compare these sub-lethal injuries may provide useful information to not only improve the pathogenic bacterial identification and enumeration but also help to establish better hurdles to reduce the risk involve in food processing and production.

10. Bacterial molecular response to chlorine-induced stresses

Chlorine and chlorine involved disinfection methods, including EO water treatment, most likely functions as oxidants to disrupt bacterial functions, such as electron transport systems, gene expression, metabolic activities, and cellular membrane structure (Wang et al., 2009). For example, hypochlorous acid (HOCl) has been historically viewed as a powerful oxidant that can interact with important cellular structures due to the chemistry of reactive chlorine species reactions (Gray et al., 2013). It is believed that HOCl works as the major antimicrobial substance in EO water (Al-haq et al., 2005, Huang et al., 2008 and Rahman et al., 2016). The reaction between HOCl and amines generate chloramines (-NHCl). And because chloramines are four to five times less reactive than HOCl, the antimicrobial efficacy could be significantly compromised due to this reaction (Gray et al., 2013; Deborde and Von Gunten, 2008). The formation of chloramines between HOCl and DNA and RNA nucleotide bases are detrimental to bacteria since high volumes of HOCl will eventually lead to DNA and RNA breakage (Gray et al., 2013).

Many researchers studied the molecular level bacterial response to chlorine-based oxidative stresses to provide better understanding in relation to bacterial defense mechanisms, cellular stress adaptation, and future applications. A series of studies (Wang et al. 2009; Wang et al., 2010) using microarray and related techniques detected that the global transcriptomic response of *Escherichia coli* O157:H7 and *Salmonella enterica* to sodium hypochlorite induced. The

results indicated that genes of *Escherichia coli* O157:H7 associated with antibiotic resistance, cysteine synthesis, and Fe-S cluster assembly and genes of *Salmonella enterica* associated with Fe-S cluster assembly, stress response, ribosome formation, cysteine synthesis, and biofilm formation were up- or down- regulated significantly under tested oxidative stresses. The authors concluded that these findings extended the basic understanding of the resistance mechanism of *Escherichia coli* O157:H7 and *Salmonella enterica* to chlorine introduced stressful environments. Karumathil et al. (2014) investigated the survival ability of *Acinetobacter baumannii* after the exposure to different free chlorine concentrations. Also, real-time quantitative PCR study was conducted to target a group of antibiotic resistance genes. Survival of *Acinetobacter baumannii* was observed in all tested strains and up-regulation of certain genes indicated the correlation between chlorine resistance and antibiotic resistance.

Winter et al. (2008) demonstrated HOCl induced stress can lead to the unfolding of Hsp33. This HOCl-mediated protein unfolding transfers the inactive Hsp33 into a highly active chaperone state to protects essential *Escherichia coli* proteins against the HOCl aggregation and increases *Escherichia coli* survivability. This finding suggested that the antimicrobial effect of HOCl largely depend on its ability to aggregate bacterial cellular proteins. Bodet et al. (2012) analyzed *Legionella pneumophila* transcriptional response to sodium hypochlorite induced stress. DNA arrays was employed to profile the transcriptome response and the results showed that chlorine caused 391 genes' significant up- or down-regulation. Genes of general stress response, bacterial virulence, metabolic activity, bacterial information pathways and nutrient transport were observed to have the most up- or down- regulations. The finding indicated that chlorine can lead to expression of proteins involved in cellular defense mechanisms and these gene expression regulations may be involved in adaptation and

resistance to chlorine-based treatments. Future studies are needed to further explore the many bacteria responses to chlorine-induced stresses, like EO water.

11. Conclusion and objectives

As a non-corrosive, safe, inexpensive and environment friendly novel disinfection strategy, EO water generation and applications have been well documented and broadly adopted in fruits, vegetables and egg industries. However, limited attempts have been focused on meat industries, such as pork, beef and poultry. Recent evidences indicate that appropriate approaches and systematic engineering can help to increase the current effectiveness of EO water in post-harvest meat processes applications. Also, the ability of EO water to form a VBNC state in bacteria and to cause sublethal bacterial injury largely remains unclear. New discoveries in molecular level bacterial response to EO water induced stress are likely to present more valuable information which could benefit our understanding in bacteria-chlorine interactions. To reduce the risk from pathogenic bacteria caused diseases and firmly secure meat product safety with EO water application, more studies related to EO specific applications (beef, pork, lamb and poultry), application method (washing, spray, rinse and dip) and in-plant performance (biofilm disintegration, processing surface disinfection efficacy and biological stress responses) are required to move forward.

This study is designed to evaluate the antimicrobial efficacy of NEO water on *E. coli* O157:H7, *Salmonella* Enteritidis and *Yersinia enterocolitica* in both pure culture and on artificially contaminated pork products. Also, VBNC and sublethally-injured bacteria population are investigated using documented and modified methods. Expression of selected pathogenic bacterial genes are also analyzed using quantification PCR method. In the end, combinations of diluted NEO water and other stresses are evaluated for their synergistic antimicrobial effects.

Table 1 EO water antimicrobial efficacy evaluated in pure culture studies

Microorganisms	Free chlorine concentration (ppm)	pH	Reaction time (second)	ORP (mV)	Population reduction (log CFU/mL)	Reference
Bacillus subtilis ATCC 19659	30	5.59-6.36	30	891-963	8.44	Zhang et al. (2016)
Candida lambica VTTC 00360	30	neutral	30	Unclear	3	Lehto et al. (2017)
Escherichia coli O157:H7 NCTC 12900	48	8.40	60	736	>5.4	Abadias et al. (2008)
Escherichia coli NBRC3301	50.3	2.6	60	1139.4	6.02	Issa-Zacharia et al. (2010)
Escherichia coli NBRC3301	23.7	5.6	60	940.0	5.07	Issa-Zacharia et al. (2010)
Escherichia coli 0157:H7 five strains	30	2.71-2.82	30	925-1128	8.48	Zhang et al. (2016)
Escherichia coli ATCC 11229	30	neutral	60	Unclear	5	Lehto et al. (2017)
Escherichia coli ATCC 25922	30	6.1	30	863.5	6.02	Liao et al. (2017)
Escherichia coli ATCC25922	60	6.40	300	900	5.91	Ye et al. (2017)
Erwinia carotovora ssp. carotovora CECT-225	48	8.40	60	736	>5.7	Abadias et al. (2008)

Listeria innocua CECT-910	48	8.40	60	736	>5.5	Abadias et al. (2008)
Listeria monocytogenes ATCC 19114-3	30	6.3	30	867.4	4.7	Xuan et al. (2017)
Salmonella choleraesuis ATCC BAA-709	48	8.40	60	736	>5.5	Abadias et al. (2008)
Salmonella enterica NBRC13245	50.6	2.6	60	1140.0	6.12	Issa-Zacharia et al. (2010)
Salmonella enterica NBRC13245	21.4	5.7	60	942	5.18	Issa-Zacharia et al. (2010)
Staphylococcus aureus NBRC12732	50.3	2.6	60	1139.4	5.93	Issa-Zacharia et al. (2010)
Staphylococcus aureus NBRC12732	23.7	5.6	60	940	4.83	Issa-Zacharia et al. (2010)
Staphylococcus aureus ATCC 25923-3	30	6.4	60	834.9	5.8	Ding et al. (2016)
Staphylococcus aureus ATCC 25923	30	6.1	30	863.5	5.83	Liao et al. (2017)
Yersinia enterocolitica EELA 56	30	neutral	150	Unclear	5	Lehto et al. (2017)
Yersinia pseudotuberculosis EELA 472	30	neutral	300	Unclear	5	Lehto et al. (2017)

Table 2 EO water applied on meat and poultry products

Microorganisms	Product type	Free chlorine concentration (ppm)	pH	Reaction time (min)	ORP (mV)	Population reduction (log CFU)	Reference
Campylobacter coli	Pork belly with skin	50	2,6	Spray for 15 s	1150	1.81/cm^2	Fabrizio and Cutter (2004)
Escherichia coli	Beef liver	14	2.5 (combined with alkaline EW)	3	960	3.72/g	Shimamura et al. (2016)
Escherichia coli	Chicken breast	14	2.5 (combined with alkaline EW)	3	960	3.63/g	Shimamura et al. (2016)
Escherichia coli 0157:H7	Boneless pork loin	100.1	6.8	5	700	1.7/g	Rahman et al. (2013)
Escherichia coli 0157:H7	Boneless pork loin	30	6.29	Up to 5	826	1.19-1.55/g	Mansur et al. (2015)
Escherichia coli 0157:H7	Chicken leg skin	38	2.30	10	Unclear	0.8/g	Al-Holy and Rasco (2016)
Escherichia coli 0157:H7	Beef	38	2.30	10	Unclear	1.4/g	Al-Holy and Rasco (2016)
Listeria monocytogenes	Boneless pork loin	100.1	6.8	5	700	1.7/g	Rahman et al. (2013)
Listeria monocytogenes	Boneless pork loin	30	6.29	Up to 5	826	1.19-1.55/g	Mansur et al. (2015)
Listeria monocytogenes	Chicken leg skin	38	2.30	10	Unclear	1.1/g	Al-Holy and Rasco (2016)

Listeria monocytogenes	Beef	38	2.30	10	Unclear	1.3/g	Al-Holy and Rasco (2016)
Salmonella Typhimurium	Boneless pork loin	30	6.29	Up to 5	826	1.19-1.55/g	Mansur et al. (2015)
Salmonella Typhimurium	Chicken leg skin	38	2.30	10	Unclear	1.5/g	Al-Holy and Rasco (2016)
Salmonella Typhimurium	Beef	38	2.30	10	Unclear	1.4/g	Al-Holy and Rasco (2016)
Salmonella Enteritidis	Chicken breast	14	2.5 (combined with alkaline EW)	3	960	3.25/g	Shimamura et al. (2016)
Salmonella Enteritidis	Beef liver	14	2.5 (combined with alkaline EW)	3	960	3.22/g	Shimamura et al. (2016)
Staphylococcus aureus	Chicken breast	14	2.5 (combined with alkaline EW)	3	960	3.92/g	Shimamura et al. (2016)
Staphylococcus aureus	Beef liver	14	2.5 (combined with alkaline EW)	3	960	3.12/g	Shimamura et al. (2016)
Staphylococcus aureus	Boneless pork loin	30	6.29	Up to 5	826	1.19-1.55/g	Mansur et al. (2015)
Total aerobic count	Boneless pork loin	100.1	6.8	5	700	1.2/g	Rahman et al. (2013)
Yeast and mold	Boneless pork loin	100.1	6.8	5	700	0.9/g	Rahman et al. (2013)

Table 3 Verified VBNC state of foodborne pathogens

Pathogens	Inducing conditions	Viability detection methods	Culturability (C) and viability (V) reduction	Reference
Campylobacter jejuni ATCC 33291	Microcosm conditions at 4 °C	CTC-DAPI double staining and antibody technique	Unclear	Shimamura et al. (2016)
Escherichia coli ATCC 25922	Atmospheric pressure plasma jet	LIVE/DEAD® BacLight™ staining kit and fluorescent microscopy	C=100%; V=45%	Dolezalova and Lukes (2015)
Escherichia coli ATCC 25922	Ultrasound treatment	Flow cytometry and Transmission electron microscopy	As high as 23.59% in VBNC state	Li et al. (2017)
Escherichia coli O157:H7 six strains	Low temperature	LIVE/DEAD® BacLight™ staining, and 6-CFDA staining	C=6-9 log; V=2 log	Dinu and Bach (2011)
Listeria monocytogenes 6 strains	Starvation in microcosm conditions	LIVE/DEAD® BacLight™ staining, ATP assay, RT-PCR	1% in VBNC state	Lindbäck et al. (2010)
Salmonella enterica ATCC 14028	Peracetic acid, lactic acid	LIVE/DEAD® BacLight™ staining kit and microscopy	0.5 log CFU difference	Purevdorj-Gage et al. (2018)
Salmonella Enteritidis environmental isolate	H_2O_2 induced stress	CTC, 2-NBDG, EdU analyzed by flow cytometry	As high as 85.8% in VBNC state	Morishige et al. (2015)
Staphylococcus aureus ATCC 25923	Ultrasound treatment	Flow cytometry and Transmission electron microscopy	As high as 21.87% in VBNC state	Li et al. (2017)
Yersinia pestis Harbin 35 strain	Microcosm conditions at 4 °C	LIVE/DEAD® BacLight™ staining, DNA expression activity, and electron microscopy	C=6 log; V= about 1 log	Pawlowski et al. (2011)

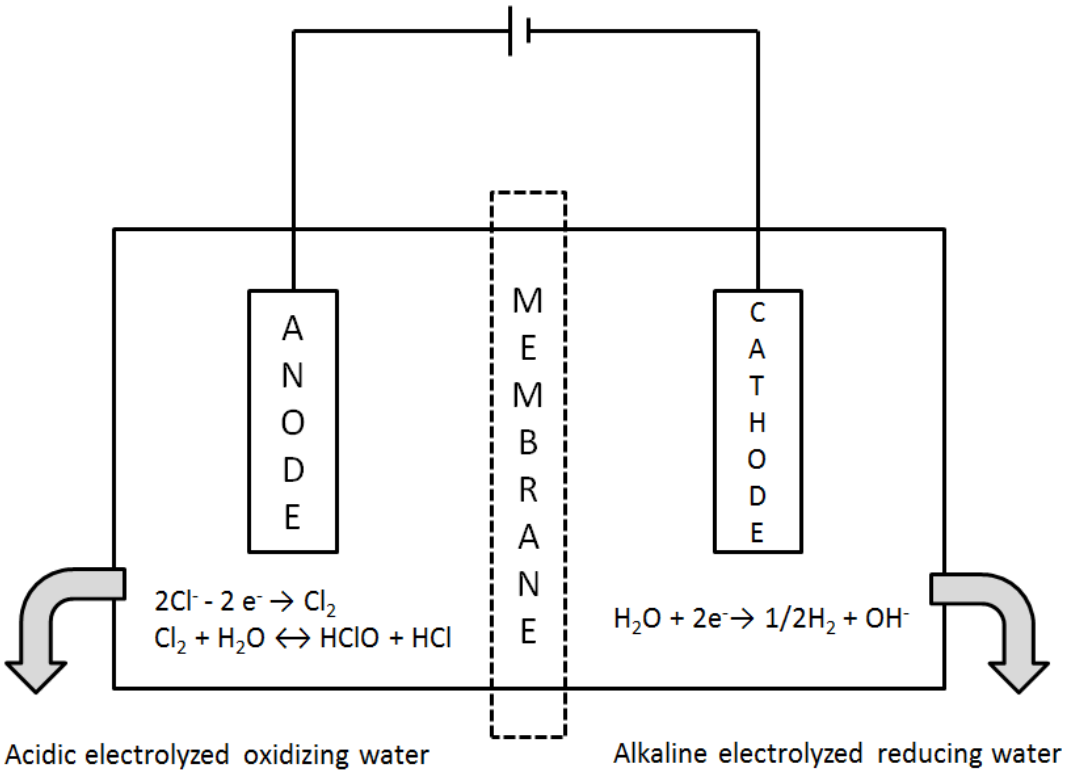

Fig. 1. Acidic electrolyzed oxidizing water (AcEOW) and alkaline electrolyzed reducing water (AlERW) generated from electrolysis

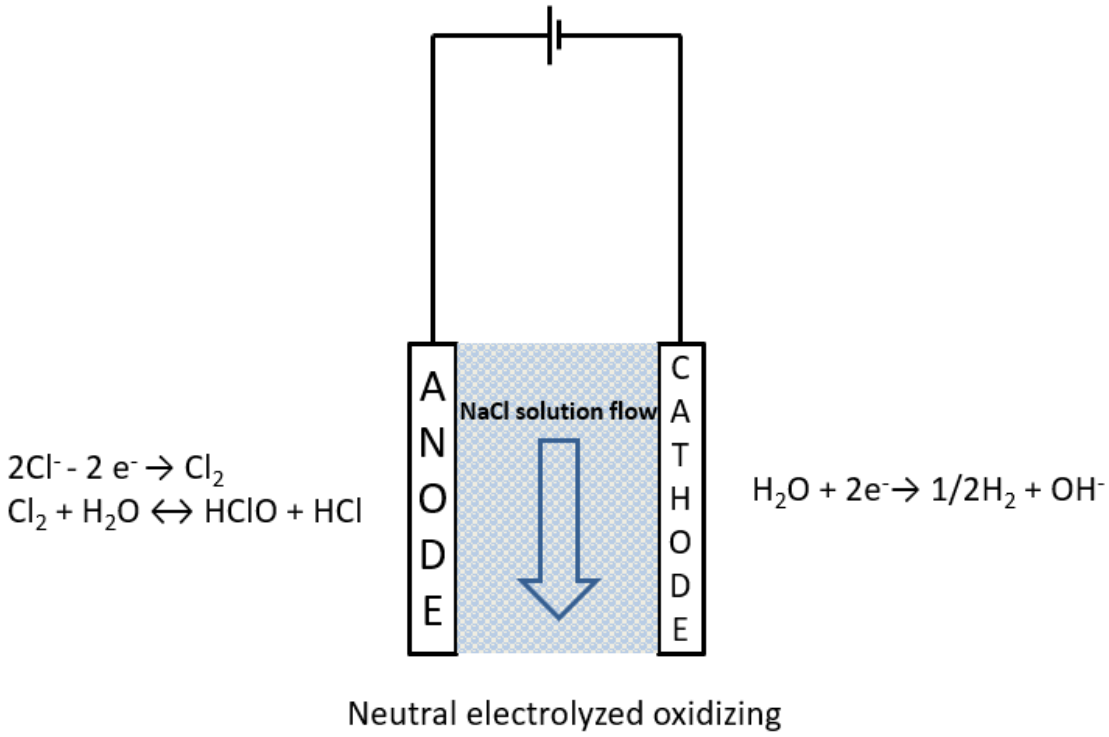

Fig. 2 Neutral electrolyzed oxidizing water generated by single cell generator

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Chapter II: Evaluation of the Antimicrobial Efficacy of Neutral Electrolyzed Water on Pork Products and the Formation of Viable but Nonculturable (VBNC) Pathogens

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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.

Keywords: Neutral electrolyzed water, pork, VBNC, pathogens

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 and 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 6-8 and an ORP value of 700-900 mV (Al-Haq et al., 2005; Hsu, 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 hours.

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 3,000 × *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 hours. 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 minutes. 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 20k.

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:

$$\text{Percentage of VBNC cells} = \frac{\text{Total viable cells identified by FCM} - \text{Culturable cells calculated by plating method}}{\text{Total cell population identified by FCM}}$$

2.5. Pork product inoculation

Fresh pork chops and dehaired pork belly skin were obtained from the Auburn University Lambert-Powell Meats Laboratory (Auburn, AL, USA). Pork chops and skin samples were cut into 5 cm × 5 cm square, 1-cm-thick portions. The extra subcutaneous fat was trimmed off from the back side of skin samples. All meat samples were checked for the potential presence of *E. coli* O157:H7, *Salmonella*, and *Yersinia enterocolitica* following the FDA Bacteriological Analytical Manual. Pork chops and skin samples were proven to be negative for *E. coli* O157:H7, *Salmonella* and *Yersinia enterocolitica* before use.

Pork sample inoculation was carried out by adding 100 µL of each pure culture inoculum onto the surface of each pork chop and skin sample. Spreaders were used to evenly distribute the inoculum on the sample surface (VWR Scientific, San Francisco, CA, USA). After that, the inoculated pork chops and skin samples were air-dried in a biosafety cabinet (Labconco, Kansas City, MO, USA) at ambient temperature for 30 min. The final bacterial densities of the inoculated meat samples were determined by plating the inoculated samples immediately after drying.

2.6. NEO water treatment efficiency on inoculated pork products

NEO water treatments on inoculated pork products were carried out by first adding 10 mL of the original NEO (100%) water into a disposable Petri dish (100 mm × 15 mm, VWR Scientific, San Francisco, CA, USA). The inoculated side of each meat sample was then immersed in the NEO water for 2 min or 10 min. A set of inoculated pork chops and skin samples was “treated” with sterilized deionized water for use as the control. During the treatment, Petri dishes with meat samples were shaken gently once every minute to ensure complete contact between the NEO water and the inoculated pork chops or skin samples.

After the 2-min or 10-min immersion, each treated meat sample together with the NEO water in the petri dish was transferred into a sterile stomacher bag (model 400, 6041/STR, Seward Limited, London, UK). To ensure that all of the pathogen cells were recovered, every petri dish was washed twice with 20 mL of 0.1% peptone water. The washing liquid was also transferred into the same stomacher bag. To enumerate the surviving culturable pathogens, 50 mL of 0.1% peptone water was added to each stomacher bag. Samples were homogenized using a smasher at 230 rpm for 2 min (Model 400, Seward^{TM}, Riverview, FL, USA). Serial dilutions were made for plating. The number of surviving *E. coli* O157: H7 cells were determined by plating samples on sorbitol MacConkey agar (SMAC) agar, the surviving *Salmonella* Enteritidis cells were determined by plating samples on xylose lysine deoxycholate (XLD) agar, and the surviving *Yersinia enterocolitica* cells were determined using the cefsulodin-Irgasan-novobiocin (CIN) agar. The SMAC and XLD plates were incubated at 37 °C for 24 hours while the CIN plates were incubated at 30 °C for 48 hours. The final colony counts were confirmed again after additional 24 hours incubation at the temperatures described above.

2.7. Color measurement of treated pork chops

Uninoculated pork chops were treated with NEO water as described above, and these treated pork chops were used for color measurement during storage. After treatment, each sample was individually packaged in a Genpak #1 Supermarket Tray (Genpak LLC., Glens Falls, NY, USA) and overwrapped with Performance Plastic Meat Film 70 Gauge (ID: WP-MWH18, US Packaging and Wrapping LLC., Austin, AR, USA). Treated meat samples were stored at 4° C for 7 days. Color measurement parameters L* (brightness to darkness), a* (redness to greenness), and b* (yellowness to blueness) were measured using a Hunter Miniscan XE Plus (Hunter Laboratories, Reston, VA, USA, D_{65} illuminant using 10^0 observance angle, 2.54 cm in aperture) on day 0, 2, 4 and 7. Untreated samples and samples

treated with DW were also prepared, packaged and stored under the same conditions. Their color evaluations were used for comparison with the samples treated with NEO water.

2.8. Statistical analysis

Two independent trials were conducted for all NEO water treatment experiment with two repeats in each trial. Color evaluation results were recorded from four independent repeats. Cell concentrations generated by the plate counting method were converted to logarithmic form before analysis. The analysis of variance was carried out by conducting single-factor ANOVA using a SPSS statistic software package (SPSS Statistics for Windows 19.0.0; SPSS Inc., Chicago, IL, USA). Significant differences were determined when *P* values were less than 0.05 based on the Duncan's multiple-range test.

3. Results

3.1. Antibacterial efficacy of NEO water on pure culture

The NEO water generated had a pH value of 7.64 ± 0.07 and an ORP value of 818 ± 5.57 mV and contained 74 ± 2 mg/L of free chlorine. The initial concentrations of washed *E. coli* O157: H7, *Salmonella* Enteritidis and *Yersinia enterocolitica* cultures were 8.46 ± 0.02 log CFU/ml, 8.41 ± 0.04 log CFU/ml and 8.49 ± 0.02 log CFU/ml, respectively. No significant difference was observed within the initial populations ($P > 0.05$). When three cultures were treated with 3% NEO water, an approximate 0.8 log CFU/ml reduction ($P < 0.05$) was observed in *Salmonella* samples. No significant reduction was observed in the treated *E. coli* O157:H7 (3% NEO, $P > 0.05$), and a 0.22 log CFU/ml reduction ($P < 0.05$) was detected in *Yersinia* cultures (Table 1).

After being treated with 15% NEO water, more than 4 log CFU/ml reductions were observed for *E. coli* O157: H7 and *Salmonella* Enteritidis, while a 2.59-log CFU/ml reduction was observed for the *Yersinia* samples. Statistical analysis showed that the numbers of surviving *E. coli* O157: H7 and *Salmonella* Enteritidis were significantly lower compared to *Yersinia enterocolitica* ($P < 0.05$). When treated with 25% NEO water, the culturable cells from all three treated bacterial strains fell below the limit of enumeration (1.62 log CFU/ml); however, results from the enrichment analysis indicated that live cells still existed in all three of the treated cultures. When the three pathogens were treated with 50% and 100% NEO water, no growth was observed after plating the 48-h enrichment broth on non-selective agar.

3.2 Flow cytometry

In this study, flow cytometry was employed to detect propidium-iodide-stained dead pathogenic bacteria. Propidium iodide (PI) can penetrate into bacterial cells that have lost their membrane integrity, and then bind with their double-stranded DNA (Berney et al., 2007). Isopropyl-alcohol-treated cells were used to establish the standard for each bacteria strain. In each small figure, live viable cells were located in PI-negative regions, while dead cells were presented in PI-positive regions.

As shown in Figures 1 (NEO-water-treated *E. coli* O157:H7), 2 (NEO-water-treated *Salmonella* Enteritidis) and 3 (NEO-water-treated *Yersinia enterocolitica*), the proportion of the dead cells shown in the PI-positive section increased as the concentration of NEO water increased. When treated with 1% NEO water for 5 min, 90.85 ± 0.05 % of the treated *E. coli* O157:H7 cultures, 88.05 ± 0.15 % of the treated *Salmonella* Enteritidis cultures and 98.75 ± 0.05 % of the treated *Yersinia enterocolitica* remained alive. After being treated with 25% and 50% NEO water, more than 90% of the cells located in the PI-positive areas. No flow cytrometry cell sorting was carried out for the 50% and 100% NEO-water-treated *Salmonella*

Enteritidis samples, since the NEO treatments resulted in unpelletable cultures that could not be used for fixation and flow cytometry. The original (100%) NEO water treatment figures for *E. coli* O157:H7 and *Yersinia* are not included in Figure 1 and 3 because the results were the same with the 50% NEO water treatment.

3.3 Calculation of the viable but nonculturable (VBNC) cells

The percentage of VBNC cells after treatment with NEO water was calculated by comparing the differences between the flow cytometry data and the plate count results. Higher percentages of viable *Yersinia* cells were observed when treated with 6%, 10% and 15% NEO water compared to the viable cell percentages of treated *E. coli* O157:H7 and *Salmonella* cultures. The calculated VBNC population percentages for all the NEO treatments are shown in Figure 4. As shown in Figure 4, approximately 68.31% of *Salmonella* Enteritidis cells become VBNC when treated with 3% NEO water. Approximately 56% of *E. coli* O157: H7 cells and 58% of *Yersinia enterocolitica* cells become VBNC when treated with 6% NEO water. When cultures were treated with 25% NEO water, less than 10% of the bacterial cells remained viable and were identified by the flow cytometry method. However, they were not detected by the plating method before enrichment. No VBNC cells were detected when treated with 50% or 100% NEO water.

3.4 Efficacy of NEO water on inoculated pork products

The inoculated pork chops and skin samples were treated with the original NEO water (100%) for either 2 min or 10 min. Samples were plated immediately after treatments. After drying, the initial *E. coli* O157: H7 inoculation level was 5.17 ± 0.02 log CFU/cm^2 on skin samples and was 5.16 ±0.04 log CFU/cm^2 for pork chops. The initial inoculation level of *Salmonella* Enteritidis was 3.97 ± 0.05 log CFU/cm^2 for skin samples and was 4.02 ± 0.07 log

CFU/cm^2 for pork chops. The initial inoculation level of *Yersinia enterocolitica* was 5.06 ± 0.04 log CFU/cm^2 for skin samples and was 4.94 ± 0.01 log CFU/cm^2 for pork chops.

As shown in Figure 5, a 2.12 log CFU/cm^2 reduction was observed in the *E. coli* O157:H7 inoculated skin samples after 2 min of treatment, and a 2.59 log CFU/cm^2 reduction was observed after 10 min. Similar results were observed for skin samples inoculated with *Salmonella* Enteritidis, with a 2.22 log CFU/cm^2 reduction observed after 2 min and a 2.37 log CFU/cm^2 reduction ($P < 0.05$) observed after 10 min treatments. For the *Yersinia*-inoculated skin samples, a 1.74 log CFU/cm^2 was observed after the 2 min treatment, and a 1.81 log CFU/cm^2 reduction was observed after the 10 min treatment. No significant difference was observed between the 2 min and the 10 min treatments ($P > 0.05$), regardless of the pathogens used.

Compared to the inoculated skin samples, although reductions were observed after treating the inoculated pork chops, the reduction was much smaller compared with the skin samples. For the *E. coli* O157: H7-inoculated pork chops, a 0.32-log CFU/cm^2 reduction was observed after 2 min and a 0.29 log CFU/cm^2 reduction was detected after 10 min of treatment. For *Yersinia-enterocolitica*-inoculated chops, an average of a 0.17-log CFU/cm^2 reduction was observed after 2 min of treatment, and a 0.15-log CFU/cm^2 after 10 min of treatment. No significant reduction was observed on *Salmonella*-inoculated pork chops. The treatment time did not generate significant impact on the antimicrobial efficacy either ($P > 0.05$).

3.5 Color measurement of treated pork chops during storage

Color measurement (L*, a*, and b*) was carried out for treated samples and controls at days 0 (measured immediately after treatment), 2, 4, and 7. As shown in Table 2, for L* (brightness), no differences among the untreated (control), DW-treated, and NEO- treated samples were observed on day 0, 2, and 4. After 7 days of storage, no difference ($P > 0.05$) was

observed between the DW-treated and NEO-treated samples, although L* decreased significantly ($P < 0.05$) for the NEO-treated sample compared to the untreated control. For a* (redness), treatment with both DW (11.20 ±1.06) and NEO (11.19 ±0.25) significantly ($P < 0.05$) decreased the redness values compared to the untreated control (12.81 ±0.92) on day 0; however, the change in redness could not be detected by human eyes. After 7 days of storage, a statistical difference was observed only between the DW-treated samples and the control samples, with no difference observed between DW-treated and NEO-treated samples or between NEO-treated samples and the control. For b* (yellowness to blueness), DW and NEO treatment significantly decreased the b* values on day 0. Differences between NEO-treated samples and control samples, as well as differences between DW treated samples and control samples, decreased as storage time increased. However, none of these color changes measured by equipment could be detected by the naked eye.

4. Discussion

Pork remains the most consumed meat in the world (Delgado et al., 2001; FAO, 2014). In 2007, the per capita consumption of pork in the U.S. was 50.5 pounds (American Meat Institute, 2009). *Salmonella spp.*, *Listeria monocytogenes*, *Campylobacter spp.*, and *E. coli* O157:H7 are among the top pathogens causing foodborne illness and death annually (Scallan, 2015). These pathogens have been well documented as being associated with pork products (Baer et al., 2013). In 2014, an outbreak of illnesses caused by *Escherichia coli* O157:H7, which sickened 119 people in Alberta, Canada, was found to be associated with pork and pork production environments. Contaminated pork products were identified at all key investigation points (Honish et al., 2017). *Yersinia enterocolitica* is another zoonotic pathogen that can cause foodborne intestinal illness in humans. The symptoms of yersiniosis include fever, abdominal pain, and diarrhea, and sometimes patients may experience nausea and vomiting (Smego et al.,

1999). Pigs and pork products are also major sources of yersiniosis (Laukkanen-Ninios et al., 2014). *Y. enterocolitica* has been identified along with *Salmonella spp.*, *Toxoplasma gondii*, and *Trichinella spp.* in a qualitative risk assessment as one of the most relevant biological hazards in meat inspection of pigs (EFSA BIOHAZ, 2011). Unfortunately, there has been very limited research to evaluate the efficacy of different intervention strategies on *Y. enterocolitica*.

NEO water is a type of electrolyzed water with neutral pH. Its main biocidal reagents are HOCl, ClO^-, HO_2, and $^{\bullet}O_2$. NEO water is more stable than acidic electrolyzed water because the chlorine loss is significantly reduced at pH 6-9 (Len et al., 2002). Most importantly, because of its neutral pH, NEO water is less corrosive compared to other sanitizers (Ayebah and Hung, 2005). In this study, the antimicrobial efficacy of different concentrations of NEO water was evaluated on both pure cultures and inoculated pork products. For the pure culture study, treatment efficacy was investigated via both traditional plating and flow cytometry. As shown in Table 1, based on the plating method, significant reductions were observed in *E. coli* O157:H7 cultures treated with NEO water that was at least 6%. *Salmonella* cultures showed a 3-log reduction when treated with 6% NEO water. For *Yersinia*, a minimum concentration of 15% NEO water was needed in order to reach a 2.5-log reduction within 5 min of treatment. Significantly higher numbers of culturable cells were recovered from the treated *Yersinia* cultures than from the treated *E. coli* O157:H7 and *Salmonella* cultures when treated with 6%, 10% or 15% NEO water (Table 1).

NEO water's antimicrobial efficacy was also evaluated using flow cytometry. Treatments with 1%, 3%, 6%, 10%, and 15% NEO water all resulted in the formation of VBNC cells, indicating that the traditional plating method overestimated the treatment's antimicrobial efficacy. This finding is in agreement with many previous studies. It has been shown that when healthy bacterial cells, such as *Listeria monocytogenes* (Cunningham, et al. 2009), *Campylobacter jejuni* and *Escherichia coli* O157:H7 (Cook and Bolster, 2007) and *Escherichia*

coli K-12 (Desnues et. al, 2003), are exposed to sub-lethal environmental stresses, some cells became unrecoverable when the traditional plating method was used. When comparing the percentages of viable cells and the percentages of VBNC cells, higher percentages of viable and VBNC cells were seen in the *Yersinia* cultures compared to the *E. coli* O157:H7 and *Salmonella* cultures after the treatments with 6%, 10% and 15% NEO-water. Our results indicated that *Yersinia* is more resistant to treatment with NEO water when compared to *E. coli* O157:H7 and *Salmonella*. *Yersinia* is a psychrotrophic, facultative anaerobic bacterium that can survive and grow at temperatures as low as -5° C. As reported by Bergann et al. (1995), when 90 different *Y. enterocolitica* strains were incubated for 3 weeks in nutrient broth, the initial plate counts doubled for about 85% of the strains at -1 or -2° C, 59% at -3° C, nearly 16% at -4° C, and about 13% at -5° C. All of these previous results, together with our findings, suggest that hurdle techniques are needed in order to control *Y. enterocolitica* contamination in pork products.

When artificially inoculated pork chops and skin samples were treated with NEO water, significant reductions for all three tested pathogens were observed from treated skin samples. However, the treatments showed very limited antibacterial efficacy when applied to inoculated pork chops. Clearly, the presence of organic matter on the pork chops negatively impacted the efficacy of NEO water treatment. As indicated by Huang et al. (2008) and Rahman et al. (2016), the reaction between NEO (or other forms of electrolyzed oxidizing water) and organic matter is a major factor that can compromise the antimicrobial efficacy of NEO water. The formation of combined chlorine (chloramines) generated from the NEO-organic matter reaction causes the NEO water to lose its ability to reduce the bacterial population in organic-matter-rich conditions (Huang et al., 2008).

In this study, inoculated skin samples and pork chops were immersed in NEO water for two different treatment times, the results showed no significant difference in efficacy between the

2 min and the 10 min treatments. The findings are in agreement with existing literature stating that a short reaction time (~ 2 min) is sufficient to reach the antimicrobial potential of NEO water (Rahman et. al, 2010 and 2016) and that the maximum bacterial reduction potential is mainly determined by the chlorine concentration instead of the reaction time (Quan et al., 2010).

Undesired color changes caused by any disinfecting procedures have been an important issue directly associated with consumers' acceptance of products (Kumar et al., 2014). Color measurement results (Table 2) illustrated that, when applied to pork chops, NEO water treatments did not cause significant color changes compared to DW treatments. This finding, along with previous evidence (Navarro-Rico et al., 2014; Gil et al., 2015; Mansur et al., 2015), verified that NEO water is a relatively mild sanitizer that has low impact on the quality of food products.

5. Conclusion

In conclusion, NEO water has shown its potential in inactivating foodborne pathogens both in pure cultures and on inoculated skin samples. Its antimicrobial efficiency is closely related to NEO water concentrations. Utilizing both the plate counting method and the flow cytometry method allowed the observation and calculation of VBNC cells. The formation of VBNC cells was confirmed from cultures treated with 1%, 3%, 6%, 10%, and 15% of NEO water. When treated with 25% of NEO water, less than 10% of the cells remained viable and were detected by flow cytometry method. The traditional plating method showed its limitation for identifying stressed and VBNC cells; therefore, flow cytometry serves as a more reliable way to evaluate antimicrobial efficacy. Among all three pathogens tested, *Yersinia enterocolitica* was more resistant to the NEO water treatments compared to *E. coli* O157:H7 and *Salmonella* cultures, indicating that hurdle technologies or synergistic treatment methods are needed in order to control *Yersinia* contamination. When applying the original NEO water to inoculated pork

chops and skin samples, NEO water treatment showed better antimicrobial efficacy on inoculated skin samples than on pork chops, indicating that the organic matter present on pork chops significantly interfered with the treatment's antimicrobial efficacy. The color evaluation on treated pork chops proved that the sensory or quality changes caused by NEO water treatments are minor, NEO water serves as an efficient sanitizer for decontaminating the skin part of pig carcasses and will also work well as a part of the synergistic treatment plan for the meat part of carcasses.

Table 1. Survival of pathogens after being treated with different concentrations of NEO water for 5 min. Limit of enumeration: 1.62 log CFU/ml.

NEO water concentration	Free chlorine concentration	Pathogenic bacterial strains (log CFU/mL)		
		E.coli	*Salmonella*	*Yersinia*
0	0 ppm	8.46 ± 0.02^{a}	8.41 ± 0.04^{a}	8.49 ± 0.02^{a}
1%	0.74 ppm	8.29 ± 0.03^{aB}	8.26 ± 0.00^{bB}	8.49 ± 0.02^{aA}
3%	2.22 ppm	8.20 ± 0.03^{aA}	7.61 ± 0.01^{cB}	8.27 ± 0.03^{bA}
6%	4.44 ppm	5.91 ± 0.17^{bB}	5.57 ± 0.09^{dB}	8.08 ± 0.06^{cA}
10%	7.40 ppm	5.29 ± 0.04^{cB}	4.57 ± 0.01^{eC}	7.79 ± 0.01^{dA}
15%	11.10 ppm	4.31 ± 0.08^{dB}	3.93 ± 0.01^{fC}	5.90 ± 0.02^{eA}
25%	18.50 ppm	+	+	+
50%	37.00 ppm	-	-	-
100%	74.00 ppm	-	-	-

Results were presented as mean ± standard deviation.

+: Positive after enrichment.

-: Negative after enrichment.

$^{a-f}$ represent significant difference within each column ($P < 0.05$).

$^{A-C}$ represent significant difference within each row ($P < 0.05$).

Table 2. Color measurement of the untreated, sterile deionized water (DW)-treated and NEO water-treat pork chops.

Color measurement	Day	Control	DW-treated	NEO-treated
L*	0	58.81±4.08	58.87±4.98	55.43±3.53
	2	57.40±0.40	57.49±5.23	57.73±3.79
	4	56.03±1.99	57.51±1.38	54.84±1.31
	7	58.95±4.32^{A}	56.54±0.76^{AB}	53.96±0.86^{B}
a*	0	12.81±0.92^{bA}	11.20±1.06^{bB}	11.19±0.25^{cB}
	2	15.66±0.46^{a}	14.46±1.17^{a}	14.37±0.44^{a}
	4	12.53±0.50^{b}	13.89±0.62^{a}	12.96±0.95^{b}
	7	11.62±1.39^{bB}	13.56±0.57^{aA}	13.28±0.71^{abAB}
b*	0	14.66±1.73^{bA}	11.99±1.03^{bB}	12.06±0.37^{bB}
	2	17.19±0.24^{a}	17.23±0.40^{a}	16.60±0.65^{a}
	4	15.69±0.19^{abC}	17.41±0.26^{aA}	16.39±0.43^{aB}
	7	15.98±0.24^{abC}	16.99±0.32^{aA}	16.49±0.18^{aB}

Results were presented as mean ± standard deviation.

$^{a-c}$ represent significant difference within each column ($P < 0.05$).

$^{A-C}$ represent significant difference within each row ($P < 0.05$).

Fig. 1 Flow cytometry results of *E. coli* O157: H7 cultures treated with 0, 1%, 3%, 6%, 10%, 25% and 50% NEO water. X-axis: red fluorescence collected at 635 nm; Y-axis: forward light scatter.

Fig.2 Flow cytometry results of *Salmonella* Enteritidis cultures treated with 0, 1%, 3%, 6%, 10% and 25% NEO water. X-axis: red fluorescence collected at 635 nm; Y-axis: forward light scatter.

Fig. 3 Flow cytometry results of *Yersinia enterocolitica* cultures treated with 0, 1%, 3%, 6%, 10%, 25% and 50% NEO water. X-axis: red fluorescence collected at 635 nm; Y-axis: forward light scatter.

Fig. 4 Percentages of viable but nonculturable bacteria populations after NEO water treatments, calculated based on the flow cytometry data.

Fig. 5 The changes of culturable pathogenic bacteria before and after 2 min and 10 min of NEO water treatments. E-skin: skin samples inoculated with *E. coli* O157:H7; E-meat: pork chops inoculated with *E. coli* O157:H7; S-skin: skin samples inoculated with *Salmonella*; S-skin: pork chops inoculated with *Salmonella*; Y-skin: skin samples inoculated with *Yersinia*; Y-meat: pork chops inoculated with *Yersinia*. Values with distinct letters (a, b) within each treatment represent significant difference ($P < 0.05$).

Fig. 1

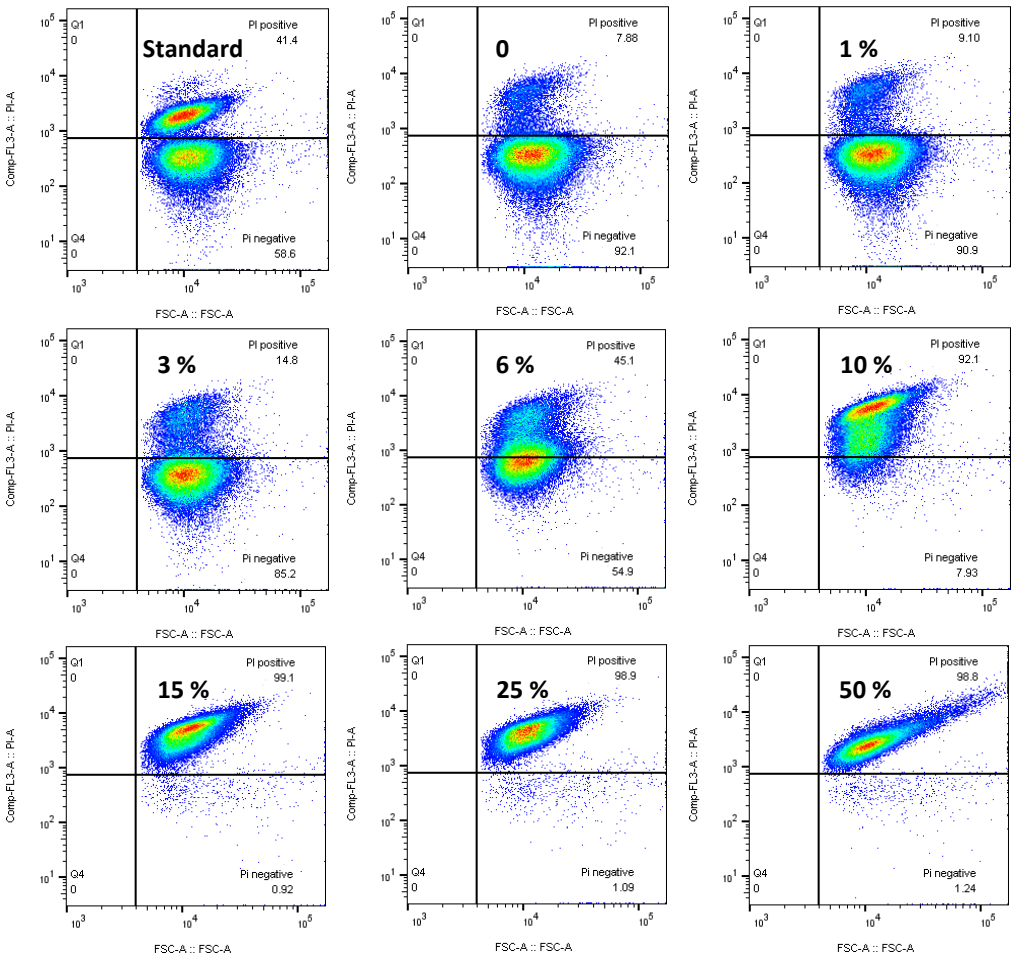

Standard
PI positive 41.4
Pi negative 58.6
0
PI positive 7.88
Pi negative 92.1
1 %
PI positive 9.10
Pi negative 90.9
3 %
PI positive 14.8
Pi negative 85.2
6 %
PI positive 45.1
Pi negative 54.9
10 %
PI positive 92.1
Pi negative 7.93
15 %
PI positive 99.1
Pi negative 0.92
25 %
PI positive 98.9
Pi negative 1.09
50 %
PI positive 98.8
Pi negative 1.24
Comp-FL3-A :: PI-A
FSC-A :: FSC-A

Fig. 2

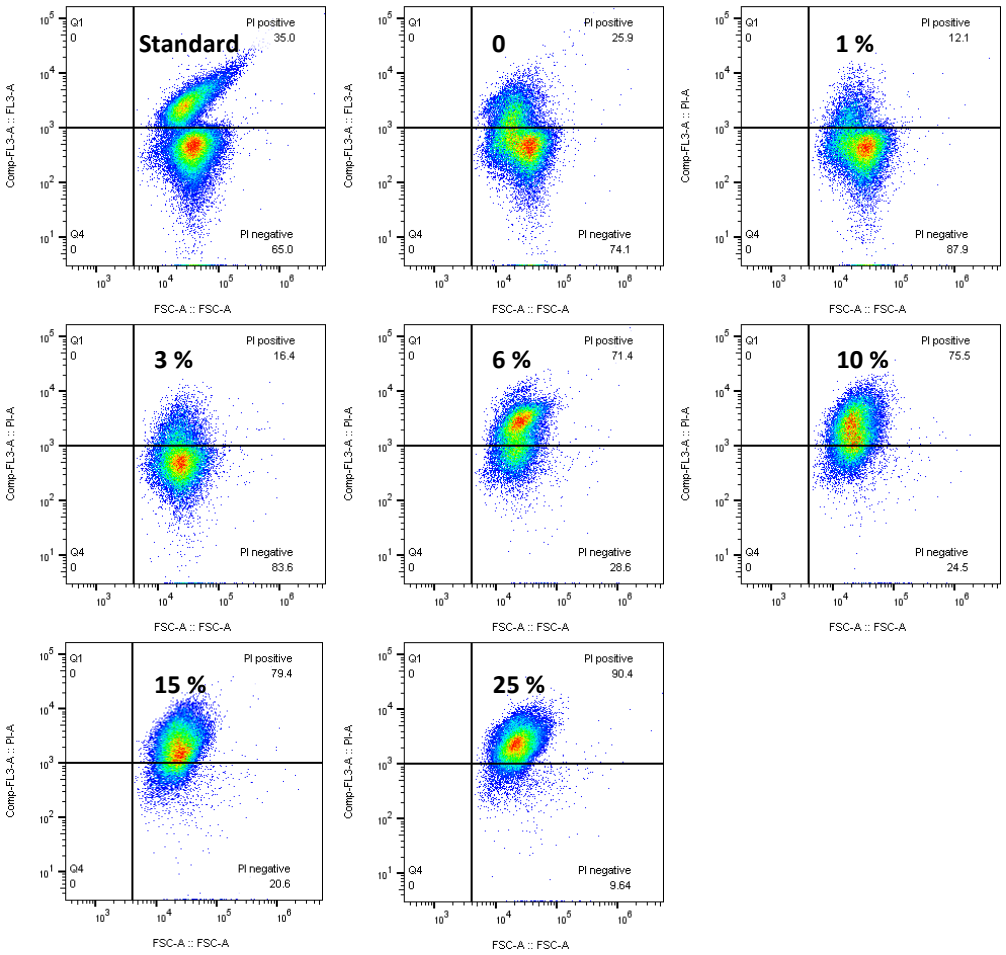

Standard
PI positive 35.0
PI negative 65.0
0
PI positive 25.9
PI negative 74.1
1 %
PI positive 12.1
PI negative 87.9
3 %
PI positive 16.4
PI negative 83.6
6 %
PI positive 71.4
PI negative 28.6
10 %
PI positive 75.5
PI negative 24.5
15 %
PI positive 79.4
PI negative 20.6
25 %
PI positive 90.4
PI negative 9.64
FSC-A :: FSC-A
Comp-FL3-A :: PI-A

Fig. 3

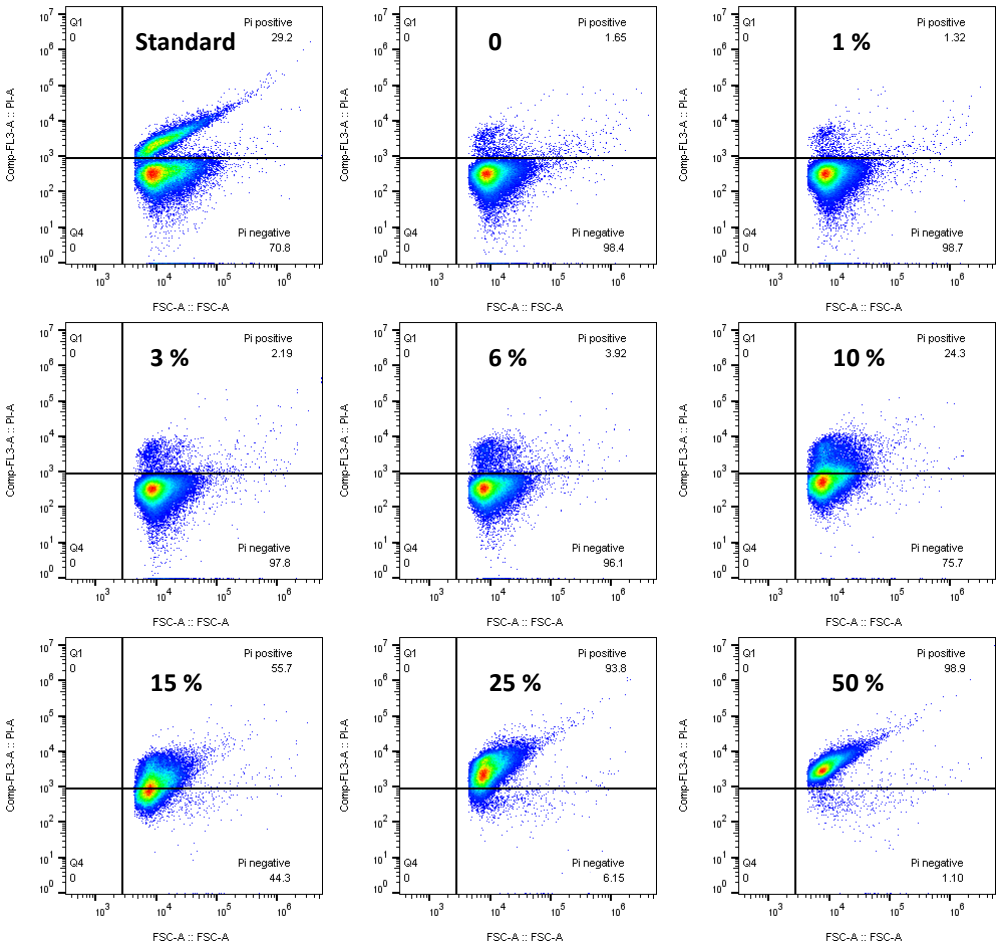
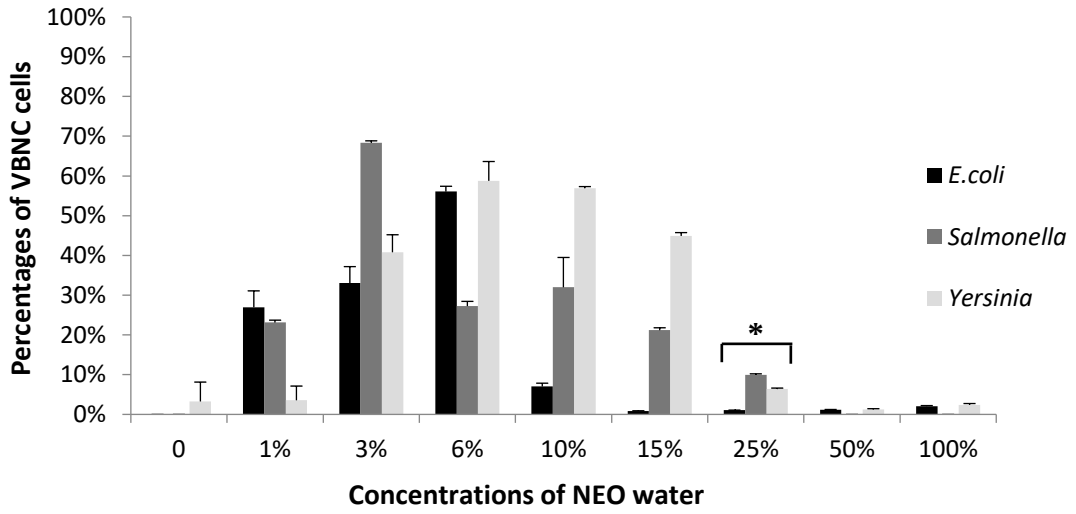
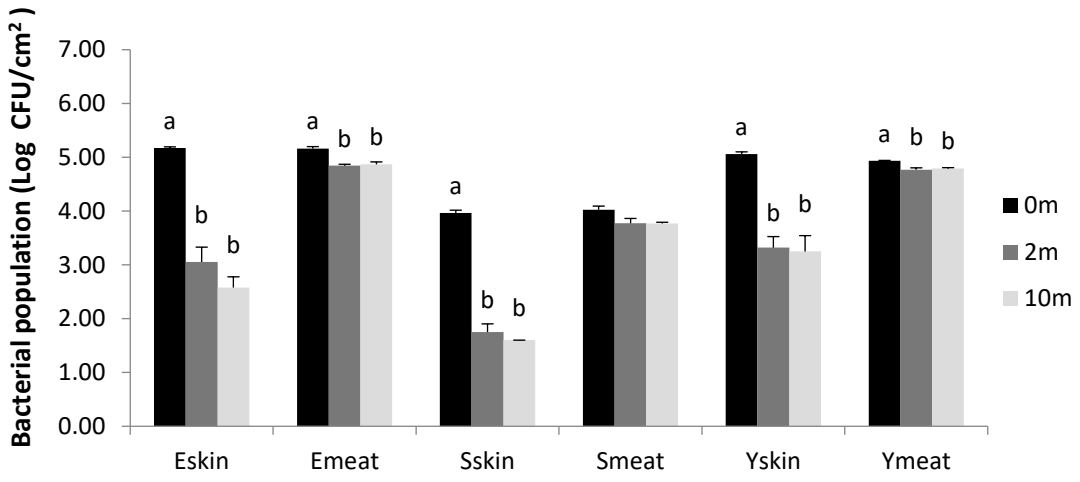

Standard
Pi positive 29.2
Pi negative 70.8
0
Pi positive 1.65
Pi negative 98.4
1 %
Pi positive 1.32
Pi negative 98.7
3 %
Pi positive 2.19
Pi negative 97.8
6 %
Pi positive 3.92
Pi negative 96.1
10 %
Pi positive 24.3
Pi negative 75.7
15 %
Pi positive 55.7
Pi negative 44.3
25 %
Pi positive 93.8
Pi negative 6.15
50 %
Pi positive 98.9
Pi negative 1.10
Comp-FL3-A :: PI-A
FSC-A :: FSC-A

Fig. 4



*When treated with 25% of NEO water, less than 10% of the treated cells remained viable and were enumerated only by the flow cytometry method.

Fig. 5


Bacterial population (Log CFU/cm²)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
a
b
b
a
b
b
a
b
b
a
b
b
a
b
b
Eskin
Emeat
Sskin
Smeat
Yskin
Ymeat
0m
2m
10m

Reference:

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Chapter III: Formation of Sublethally Injured *Yersinia enterocolitica*, *Escherichia coli* O157:H7, and *Salmonella* Enteritidis Cells after Neutral Electrolyzed Oxidizing Water Treatments

Note: This chapter has been accepted by the journal of "Applied and Environmental Microbiology". It is available at: http://aem.asm.org/content/early/2018/06/25/AEM.01066-18.short?rss=1 Accessed on July 5, 2018.

Abstract

The impact of neutral electrolyzed oxidizing (NEO) water treatments on the formation of sublethally-injured *Yersinia enterocolitica*, *E. coli* O157:H7 and *Salmonella* Enteritidis was evaluated. When treating pathogens with 6% NEO water, approximately 38% of the treated Yersinia population and 25% of the treated Salmonella population became sublethally-injured. The highest sublethally-injured population was found when Salmonella cultures were treated with 3% NEO water. Regardless of the NEO water concentrations, no sublethally-injured E. coli O157:H7 was found. To evaluate the sensitivity of NEO-water-treated cells, four additional stresses (heat treatment, pH, NaCl and bile salt) were tested. NEO water treatments did not generate any cross-protection on treated cells against other stresses. The diluted NEO water treatments in combination with the heat treatment at 51º C for 10 min led to the best synergistic antimicrobial effects with a combined reduction of 7 log. The gene expression results showed that NEO water treatments led to the up-regulation of ompR, ail and ycfR. These genes are known for their involvement in cells' environmental stress responses. In summary, this study investigated the sublethal injury in pathogenic cells caused by NEO water treatments. Although sublethal injury was discovered, when combined with other mild stresses, the synergistic antimicrobial effects were able to further reduce viable pathogenic cells. These results demonstrate the great application potential of NEO water as a non-thermal and less corrosive antimicrobial treatment.

Keywords: Neutral electrolyzed oxidizing water, sub-lethal injury, stress response, hurdle technology, pathogens

1. Introduction

As consumers' demand for and consumption of "fresh-like" food has increased in the last decade, intensive research attention has been paid to novel non-thermal decontamination methods that can enhance food safety without having a significant negative impact on food quality (Abadias et al, 2008; Manas and Pagan, 2005). Among these nonthermal treatments, neutral electrolyzed water (NEO) water has been tested on different types of food and has demonstrated its great antimicrobial efficacy and application potentials. As a neutral solution, the pH of NEO water stays around 8.5 ± 0.5, making it less corrosive to processing equipment and less of a skin contact irritant (Abadias et al., 2008). In addition, its neutral pH enables reduced chlorine loss and extended shelf-life (Abadias et al., 2008; Ayebah and Huang, 2005; Len et al., 2002). Food products on which the antimicrobial efficacy of NEO water has been tested include tomatoes (Deza et al., 2003; Vásquez-López et al., 2016), romaine and iceberg lettuce (Afari et al., 2015), shredded carrots and spinach (Guentzel et al., 2008), fresh-cut endive, corn salad, "four season" salad (Abadias et al., 2008), dates (Bessi et al., 2013), blueberries (Chiabrando et al, 2017), apples (Graca et al., 2011), pork chops and pork skin products (Han et al., 2018). NEO water treatments have also been applied to the surfaces of plastic and wooden cutting boards (Deza et al., 2007), bamboo board (Monnin et al., 2012), and plates, spoons, forks, knives, and drinking glasses (Handojo et al., 2009). All of these previous studies have demonstrated the great antimicrobial efficacy of NEO water on different types of food and food contact surfaces.

The disinfection mechanism of NEO water is based on its high oxidation-reduction potential (ORP), its hypochlorous acid (HClO) content and its available chlorine concentrations (Len et al., 2000; Liao et al., 2007; Park et al., 2004). The ORP value of NEO water is usually between 800 to 1,100 mV (Bügener et al., 2014). It is believed that the germicidal activity of

HOCl or –OCl is due to its inhibition of enzyme activities that are essential for microbial growth, the damage it causes to the membrane and DNA, and the deterioration it causes to the cells' membrane transport capacity (Rahman et al., 2016). In recent years, in addition to the continuous evaluation and optimization of NEO water treatments, several studies have been carried out in order to find out the potential "side effects" of NEO water treatments. Lin et al. (2017) showed that low levels of chlorination (0.5 mg/L) in drinking water could lead to the formation of viable but non-culturable (VBNC) *Escherichia coli*. While such low chlorination level caused the reduced metabolic activity of *E. coli*, it, on the other hand, enhanced the persistence of *E. coli* to nine antibiotics, including ampicillin, gentamicin, polymyxin, ciprofloxacin, terramycin, tetracycline, rifampicin, clarithromycin, and chloromycetin (Lin et al., 2017). Similarly, the formation of VBNC cells was also found in this research group's previous study (12). When *E. coli* O157:H7, *Salmonella* Enteritidis and *Yersinia enterocolitica* were treated with diluted NEO water (1%, 3%, 6%, 10%, 15%, and 25%), the formation of VBNC cells was confirmed using flow cytometry (Han et al., 2018). VBNC pathogenic bacteria are considered a threat to public health and food safety because they continue to retain their viability and ability to express their virulence (Ramamurthy et al, 2014). Results of these previous studies highlight the importance of better understanding the impact of NEO water treatments, especially the changes it might bring to cells that survive the treatment.

Bacterial cells are frequently impaired by sublethal injury as a result of being exposed to adverse conditions caused by physical or chemical treatments during food processing (Wesche et al, 2009). The metabolic injury that occurred within cells made the sublethally-injured populations unable to form colonies on the selective agar (Jay, 1986; Kang and Siragusa, 1999). The differential in counts between selective and nonselective media has been used to determine the degree to which bacterial cells are sublethally-injured and to calculate sublethally-injured populations (Andrews and Ray, 1989; Kang and Siragusa, 1999; McCleer and Rowe, 1995).

Cells in the sublethal injury state are more sensitive to agents or stresses to which they would show resistance in their healthy state (Jasson et al., 2007). Thus, understanding the sensitivities of sublethally-injured populations provides new opportunities for developing mild processing technologies that can further reduce pathogenic cells without changing food quality. Such mild processing technologies can be utilized in combination with NEO water treatments to form hurdle decontamination strategies that lead to synergistic antimicrobial effects (Khan, 2017; Ukuku, 2010).

Thus, the first objective of this study was to evaluate the formation of sublethally-injured cells under different NEO water treatments. The second objective was to evaluate the sensitivity of populations that survived after the NEO-water treatments and to discover which additional mild stresses (including temperature, pH, NaCl and bile salt stresses) could further reduce the number of sublethally-injured cells. To complete this investigation, the third objective of this study was to evaluate gene expression in three pathogens after being treated with NEO water. Such information will help us understand the survival mechanisms of VBNC and sublethally-injured cells. Real-time RT-PCR was utilized for monitoring gene expression. Based on the literature, four target genes (*gsr*A, *omp*R, *rpo*S, and *ail*) were selected for *Y. enterocolitica* (Brzostek et al., 2003; Dorrel et al., 1998; Iriarte et al., 1995; Kirjavainen et al, 2008; Pierson and Falkow, 1993; Miller et al., 1990; Yamanoto et al., 1996). Five genes, (*ybi*J, *cys*D, *cys*J, *ycf*R and *osm*B) were chosen for *E. coli* O157:H7 (Boulanger et al., 2005; Jung et al.,1990; Malo and Loughlin, 1990; Mchugh et al., 2003; Ostrowski et al., 1989; Wang et al, 2009; Zhang et al., 2007). Five genes (*cys*K, *yfh*P, *nif*S, *ycf*R and *nif*U) were monitored for *S.* Enteritidis (Boyd et al., 2008; Nilsson et al., 2002; Tai et al., 2008; Vergnes et al., 2017; Wang et al, 2010; Zhang et al., 2007).

2. Materials and Methods

2.1 Bacterial cultures

Bacterial strains used in this study included *E. coli* O157:H7 505B, *Salmonella* Enteritidis PT 30 (ATCC BAA-1045) and *Yersinia enterocolitica* strain 729 (provided by Dr. Stuart Price from the College of Veterinary Medicine at Auburn University). Prior to the experiment, all strains were kept in trypticase soy broth (TSB, Catalog# 211768, BD Difco, Sparks, MD) supplemented with 10% glycerol (Catalog# BDH1172-1LP, VWR, West Chester, PA) in a −80° C freezer. For culture revival, 100 μl of every completely thawed frozen culture was transferred into 10 mL of TSB-supplemented 0.6% yeast extract (Catalog# 210933, BD Difco, Sparks, MD) (TSBYE). Cultures were incubated at 37° C for 18 h to grow *E. coli* O157:H7 and *S.* Enteritidis. *Y. enterocolitica* cultures were incubated at 30° C for 48 h. After the revival step, fresh overnight cultures were prepared by transferring 100 μl of each revived culture into 10 mL TSBYE and incubated at 37 or 30° C for another 24 h.

2.2 NEO water preparation

The original undiluted NEO water was prepared by electrolyzing 5% NaCl solution with the GenEon™ Instaflow generator (GenEon Technologies, San Antonio, TX). FE20 FiveEasy with both the pH (LE409) and the ORP (LE501) probes was used to check the pH and the oxidation-reduction potential (ORP) of both the freshly made and the diluted NEO water (Mettler Toledo, Columbus, OH). The CN-21P kit (Hach, Chicago, IL) was used to monitor free chlorine concentrations of the NEO water. The original NEO water had a pH value of 7.35 ± 0.11, ORP value of 829.7 ± 4.5 mV, and free chlorine concentration of 59.1 ± 0.1 mg/L. To make different NEO water dilutions, the original NEO water (100%) was mixed with different volumes of autoclaved deionized water (DW) to generate 1%, 3%, 6%, 10%, 15%, 25% and 50% NEO water dilutions.

2.3 Determination of sublethally injured cells

Overnight fresh bacterial cultures were harvested and washed twice with 10 ml of sterilized 0.85% NaCl solution by centrifugation in 15 ml Falcon™ tube (Corning™, Tewksbury, MA, USA) at 3,000 × *g* for 10 min at 20° C (Model Eppendorf 5810R, Eppendorf, Hauppauge, NY, USA). The cell pellets were resuspended in 5 ml of 0.85% NaCl solution, and 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). The OD values were adjusted so that all three cultures had approximately the same concentrations. Their final concentrations (~ 8.5 log CFU/ ml for each pathogen) were enumerated by plating cultures on trypticase soy agar (Catalog# 211043, BD Difco, Sparks, MD) supplemented 0.6% yeast extract (TSAYE).

To treat the pure cultures, 2.5 mL of each bacterial suspension was mixed with 7.5 mL of each of diluted or the original NEO water. The bacterial concentrations in the mixtures were approximately 7.9 Log CFU/ml. After 5 min of reaction at the ambient temperature, 0.5 mL of 0.5% sodium hyposulfite ($Na_2S_2O_3$) was added to each 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 (BPW, Catalog# 218103, BD Difco, Sparks, MD). The surviving bacterial population was determined by plating two 100 µL of each serial dilution on two TSAYE and an additional two 100 µL of each serial dilution on selective agar. For this step, sorbitol MacConkey (SMAC, Catalog# 279100, BD Difco), xylose lysine deoxycholate (XLD, Catalog# 278850, BD Difco, Sparks, MD) and cefsulodin-Irgasan-novobiocin (CIN, Catalog# C5391, Hardy Diagnostics, Santa Maria, CA) agar were selective media used for enumerating *E. coli* O157:H7, *S.* Enteritidis and *Y. enterocolitica* respectively. Plates were incubated at 37º C for *E. coli* O157:H7 and *Salmonella* and 30º C for *Yersinia* for 48 hours. Colonies were counted after 24 hours and were confirmed after 48 hours.

2.4 Stress selection for NEO-water-treated pathogens

To determine the stress levels used for further reducing NEO-water-treated cells, different stresses and stress levels were studied, first using healthy overnight non-NEO-water-treated cells. The idea was to determine the mildest stress levels at which only the NEO-water-treated cells would be reduced. To determine the mildest stress levels, a total of 24 treatments (stress and stress level combinations) were prepared, including six different NaCl concentrations (1%, 2%, 3%, 4%, 5%, and 6%) (Amresco, Solon, OH), six bile salt concentrations (0.05%, 0.1%, 0.15%, 0.2%, 0.3%, and 0.4%) (Sigma-Aldrich, 48305–50G-F, New Zealand), six pH values (3, 3.6, 4, 4.6, 5.3, 5.8) (HCl, Fisher Scientific, Fairlawn, NJ), and six temperatures (45° C, 48° C, 51° C, 54° C, 57° C, and 60° C).

To create these stresses, modified TSAYE agar with different concentrations of NaCl, bile salt or of different pH values were prepared. To create different NaCl stress levels, 1%, 2%, 3%, 4%, 5%, or 6% of NaCl was added to the liquid TSAYE agar before pouring plates. Similar strategies were used for creating bile salt stresses; 0.05%, 0.1%, 0.15%, 0.2%, 0.3%, or 0.4% of bile salts was added to the liquid TSAYE agar before pouring the plates. To create pH challenges, different volumes of 37% HCl were gradually added to the liquid agar to adjust the pH value; the final pH values of the liquid agar were 3, 3.6, 4, 4.6, 5.3, or 5.8. To expose heathy bacterial cells to these three stresses, washed fresh overnight cultures and their serial dilutions (made by diluting the cultures in 9 ml of 0.1% peptone water) were plated onto TSAYE and modified TSAYE plates. Plates were then incubated for 48 hours at 37º C for *E. coli* and *Salmonella* and 30º C for *Yersinia*. Colonies were counted after 24 hours and then confirmed after an additional 24-hour incubation. The colony counts obtained from the TSAYE were compared with the counts obtained from the modified TSAYE.

Thermal treatments were carried out by incubating the washed overnight cultures and their dilutions for 10 min at different designated temperatures, including 45° C, 48° C, 51° C, 54°

C, 57° C, and 60° C. Cultures were kept in 15-ml Falcon™ tubes (Tewksbury, MA, USA) and incubated in the waterbath in the Eppendorf Thermomixer (model Thermomixer R, Brinkmann Instruments, NY) with agitation at 300 rpm. Cells were then plated on TSAYE and incubated for 48 hours at 37º C for *E. coli* and *Salmonella* and 30ºC for *Yersinia*. Tables 5, 6 and 7 in the supplemental materials show the results obtained using healthy bacteria cells. Stress levels at which no significant difference in population was observed between the treated and untreated cells (using healthy overnight cultures) were selected for following studies. Stresses and stress levels used for evaluating the sensitivity of NEO-water-treated cells are listed in Table 2.

2.5 Exposure of NEO water-treated cells to different stresses

To evaluate the sensitivity of the NEO-water-treated cells, these treated cells were first made into serial dilutions using 0.1% peptone water. After that, both the original treated cultures and their dilutions were exposed to the stresses listed in Table 2 following the procedures described previously.

2.6 Transcriptional level responses analysis

To evaluate the gene expression of NEO-water-treated cells, overnight fresh cultures were treated with 50% or 100% NEO water. Treated cultures were then collected by centrifugating 1 ml of each treated culture at 10,000 × g for 5 min at 4º C. The pellet was then washed and resuspended in 1 ml of RNAprotect Bacteria Reagent (Qiagen, Valencia, CA) to stabilize the RNA. Total RNA was extracted using the RNeasy Mini Kit following the manufacturer's manual (Qiagen, Valencia, CA), and the genomic DNA was removed using the gDNA Wipeout Buffer from QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). The concentration and purity of RNA samples were analyzed using a NanoVue Plus spectrophotometer (GE, Healthcare, Piscataway, NJ). The quality of the RNA samples were also checked by running regular PCR reactions in order to make sure that there was no DNA contamination in RNA

samples. The 16S primers listed in Table 4 and the PCR kit of AccuStart™ II PCR Supermix (2x) (Quanta BioSciences, Beverly, MA) were used. In each 0.1ml PCR tube, 5 µL of the RNA template, 0.25 µL of each forward and reverse primer, 12.5 µL of AccuStart™ II PCR Supermix (2x), and 6 µL of nuclease-free water were mixed. The PCR reaction was carried out in an Applied Biosystems® Veriti® 96-Well Fast Thermal Cycler (Life Technologies™, U.S.A.). The reaction process started with an initial 3 min 94°C denaturation period and then followed by 30 cycles of 30 sec. 94°C denaturation, 30 sec. 60°C annealing, and 1 min 72°C. PCR products were then held at 4°C. The presence of potential DNA contamination was checked by running PCR products in a 2% agarose gel for 40 min. No DNA contamination was found in RNA samples.

For the gene expression evaluation, a two-step RT-qPCR was conducted, with the cDNA being synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). SYBR Real-time PCR was carried out on the ABI 7500 (Applied Biosystem, Foster City, CA). In each reaction tube, there were 12.5 µl of 2 X reaction mix of PerfeCTa® SYBR® Green SuperMix (Quanta BioScience Inc., Gaithersburg, MD), 0.3 µl of each forward and reverse primers, 3 µl of cDNA templates, and Milli-Q water (total 25 μl). Real-time PCR was conducted following the program of an initial denaturing period at 90^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 s and 60^{o}C for 1 min. The reference gene used was 16S rRNA (Castelijn et al., 2012). The gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Data was presented as the fold change in gene expression normalized to the reference gene and compared to the control, $\Delta\Delta Ct = (Ct_{target} - Ct_{reference})_{test} - (Ct_{target} - Ct_{reference})_{control}$ (Livak and Schmittgen, 2001). The $\log_2$ fold change ≥ 2 was considered significant (Meng et al., 2015). Genes selected for each species were based on the previous reports (Boulanger et al., 2005; Brzostek et al., 2003; Iriarte et al., 1995; Jung et al.,1990; Kirjavainen et al, 2008; Malo and Loughlin, 1990; Mchugh et al., 2003; Meng et al., 2015;

Ostrowski et al., 1989; Pierson and Falkow, 1993; Wang et al, 2009; Wang et al., 2010; Yamanoto et al., 1996; Zhang et al., 2007). Primers used for the real-time PCR are listed in Table 4. The primer sets for *gsr*A, *omp*R, *ail*, *rpo*S, *ycf*R, *ybi*J, *cys*D, *cys*J, and *osm*B were designed using Primer3Plus (available at http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and verified using Basic Local Alignment Search Tool (BLAST). The primer efficiency was also calculated based on methods described by (Bustin , 2000; Chen and Jiang; Pfaffl, 2001). Three trials were conducted for this part of the study with three biological replicates in each trial for every NEO-water-treatment and pathogen combination. Three replicate reactions were done for every biological replicate in order to generate the one Ct value used for analysis.

2.7 Statistical analysis

Three independent trials were conducted for every experiment. For sublethally-injured cell enumeration study, there were three replicates in each trial. The bacterial populations detected by the plate counting method were converted to logarithmic form before statistical analysis. Since the limit of enumeration was 1.62 CFU/ml, the number of 1.62 was used for all "ND" samples when conducting statistical analyses for comparing "ND" samples with samples that had actual enumeration counts. The two-tailed Student t-test was employed when comparing cell counts obtained from TSAYE with the cell counts obtained from selective agar. A difference was considered significant when the *P* value was less than 0.05. The comparison between different NEO water treatments and among the different NEO water and stress combinations was conducted using single-factor analysis of variance (ANOVA), and means were compared with Duncan's multiple-range test. Statistical analysis was carried out using a SPSS statistic software package (SPSS Statistics for Windows 19.0.0; SPSS Inc., Chicago, IL).

3. RESULTS

3.1 Formation of sublethally injured cells

Different concentrations of NEO water were used to treat pure cultures of *Y. enterocolitica*, *E. coli* O157:H7 and *S.* Enteritidis. As shown in Table 1, as the concentrations of the NEO water increased, the surviving cell number decreased. For *Yersinia*, significant differences between colony counts obtained from TSAYE and colony counts obtained from CIN were observed when cultures were treated with NEO water of concentrations equal to or above 6%. More than a 1 log difference between TSAYE and CIN was observed when *Yersinia* was treated with 10% or 25% NEO water. When calculating the percentage of cells that became sublethally-injured, approximately 38% of the *Yersinia* population was sublethally-injured when treated with 6% NEO water. No difference in *E. coli* O157:H7 populations were observed between TSAYE and SMAC regardless of the NEO water concentrations. For *Salmonella*, a 0.4 log difference between TSAYE and XLD was observed when the culture was treated with 3% NEO water. Under this 3% NEO water treatment, 52% of the *Salmonella* population became sublethally-injured. When all three pathogens were treated with 50% or 100% NEO water, no colony was found on either the TSAYE or the selective agar, indicating that both the 50% and the 100% NEO water reduced all pathogen cells to the undetectable level after 5 min treatment.

3.2 Stress selection for treating NEO-water-treated pathogens

As shown in supplement Table 5, heat treatment of 51º C, a pH value of 4.6, NaCl concentration of 3% and bile salt concentration of 0.4% were stress levels that did not generate impacts on healthy non-NEO water treated *Yersinia* cultures. They were then chosen for the following studies in which their impacts on NEO-water-treated cells were investigated. For *E. coli* O157:H7 (Table 6), heat treatment of 51º C, a pH challenge of 3.6, an NaCl challenge of

2% and a bile salt concentration of 0.4% were stress levels that did not reduce the viable cell counts in healthy overnight *E. coli* O157:H7 cultures. Similar results were seen when exposing *S.* Enteritidis to the same stress conditions, except that the highest bile salt concentration that did not generate a negative impact on healthy *Salmonella* cells was 0.1%. Table 2 summarizes the stress and stress levels that were used for challenging NEO-water-treated cells. These stress levels did not impact healthy pathogen cells and were expected to impact or kill NEO-water-treated cells.

3.3 Survival of NEO water-treated pathogenic cells after being exposed to additional stresses

The numbers of surviving pathogenic cells that were first treated with different concentrations of NEO water and then challenged with additional stresses were investigated. Taking *Yersinia* as an example first, as shown in Table 3, when no NEO water was applied to the culture, the original cell concentration was 8.68 ± 0.05 Log CFU/ml. When these non-NEO water-treated cells were exposed to different stresses, no reduction in cell numbers was observed regardless of the stress applied. However, cells that were first treated with NEO water were more vulnerable to the additional stresses applied. Taking the 10% NEO-water-treated *Yersinia* cells as an example, the NEO water treatment step led to an approximately 1.3-log reduction when comparing the 7.39 ± 0.05 log CFU/ml with the original 8.68 ± 0.05 log CFU/ml. When these surviving *Yersinia* cells were further exposed to the 10 min 51º C heat treatment, an additional 2.53-log reduction was observed. Similarly, when exposing these 10% NEO-water-treated cells to other stresses by plating them on modified TSAYE agar (TSAYE + 3% NaCl or TSAYE + 0.4% bile salt), an additional 0.69 log and an additional 1.7-log reduction were observed respectively. For *Yersinia*, the pH change did not cause further reduction of the cells after being first treated with 10% NEO water, as there was no significant difference between cell counts obtained from the pH-adjusted TSAYE vs. regular TSAYE. For

Yersinia, the maximum synergistic effect was found when combining the 25% NEO-water-treatment with the 10 min 51º C heat treatment; the number of surviving *Yersinia* cells fell below the limit of enumeration (1.62 CFU/ml), indicating a 7-log reduction in total.

Similar observations in *E. coli* O157:H7 was made. The concentration of the original non-treated E. *coli* O157:H7 cells was 8.76 ± 0.01 log CFU/ml. When treated with 10% NEO water, a 1.64-log reduction was observed. After exposing the NEO-water-treated cells to the 10 min 51º C heat treatment, an additional reduction of approximately 5.5 log was observed, with the surviving cells falling below the limit of enumeration. Treatment combinations, including 10, 15, or 25% NEO plus heat treatment at 51º C for 10 min, and the 25% NEO plus either 3% NaCl, 0.4% bile salt or 3.6 pH, have all reduced pathogenic cells to levels that could not be enumerated.

For *Salmonella*, 25% NEO water alone was able to achieve a 6-log reduction. When combining the 15% NEO water treatment with the heat treatment, this additional 10 min 51º C treatment led to an additional 1.82-log reduction on top of the reduction obtained from the NEO-water-treatment alone. The 0.1% bile salt challenge also led to a reduction of approximately 1.82 log. All of these observations demonstrated the synergistic antimicrobial effects achieved by combining diluted NEO water treatments with other stresses.

3.4 Transcriptional level analysis

Four genes of *Yersinia*, five genes of *E. coli*, and five genes of *Salmonella* were selected for RT-qPCR. In *Yersinia*, up-regulation of *rpoS*, *ail* and *ompR* were observed when treating cultures with 50% and 100% NEO water. However, only the 100% NEO water-treated cells had their *ail* gene (2.23-$\log_2$ fold) and *omp*R gene (2.38-$\log_2$ fold) significantly up-regulated (> 2 Log fold change and $P < 0.05$). No significant ($P > 0.05$) up- or down-regulation was detected

in NEO water-treated *E. coli*. For *Salmonella*, after being treated with 100% NEO water, the *ycf*R (1.99-$\log_2$ fold) were significantly up-regulated ($P < 0.05$).

4. DISCUSSION

This study evaluated the formation of sublethally-injured pathogenic cells after being treated with different concentrations of NEO water. It has been reported that sublethally-injured bacteria become sensitive to agents to which they would otherwise be resistant to (Kurbanoglu and Algur, 2006; Osmanagaoglu, 2005). The presence of a variety of selective ingredients, such as novobiocin and bile salts, in the selective agar can be harmful to injured cells, leading to retarded growth of injured cells (Adams, 2005; Jasson et al., 2007; Restaino et al., 2001). The under-estimation of the sublethally-injured pathogenic cells poses serious food safety concerns, as these injured organisms may be capable of repairing themselves when the environmental stress is removed, as a result keeping or regaining their pathogenicity and posing hazards to human health (Bozoglu et al, 2004; Vermeiren et al., 2006). Much research has focused on enhancing the ability to enrich, detect, and enumerate sublethally-injured cells (Kang and Siragusa, 1999; Osborne and Bremer, 2002; Restaino et al., 2001; Uytendaele et al., 2001). However, limited information is available in the literature about the conditions under which sublethally-injured cells are formed and the characteristics of these sublethally-injured cells. In a study conducted by Izumi et al. (2016), the percentage of chlorine-injured *Enterobacter cloacae*, *E. coli* and *E. coli* O157:H7 cells in pure culture was shown to be between 69-77%. Fungicides, including Topsin-M, Sumilex, and Oxirane, also caused the formation of injured cells. The percentage of the injured population was 45-97% for Topsin-M, 80-87% for Sumilex, and 50-97% for Oxirane. These results demonstrate the importance of choosing not only the right sanitizer but also the right sanitizer concentrations so that the

treatment will kill target microorganisms completely rather than just injure the cells (Izumi et al., 2016).

NEO water has been reported as an efficient and less corrosive antimicrobial agent and has been tested on a variety of food products ranging from meat to fresh fruit and vegetables. In our previous study, we reported that low concentrations of NEO water can lead to the formation of viable but nonculturable cells (VBNC) (Han et al., 2018). In the VBNC state, cells cannot be detected by either selective or non-selective agar. We found that when treating *E. coli* O157:H7, *Salmonella*, and *Yersinia* cultures with 6% NEO water, approximately 58%, 30%, and 62% of treated cells entered into the VBNC state (Han et al., 2018). In this study, the same NEO water treatments were applied, and the percentages of the sublethally-injured populations formed as a result of treatment were calculated. As shown in Table 2, when treating *E. coli* O157:H7, *Salmonella* and *Yersinia* cultures with 6% NEO water, 0% of the *E. coli* culture, approximately 25% of the *Salmonella* culture, and approximately 38% of the *Yersinia* culture became sublethally-injured. Based on our previous and current studies, it can be concluded that diluted NEO water treatments can generate both VBNC and sublethally-injured cells.

It is worth mentioning here that the percentages of sublethally-injured cells can also be impacted by the selective agar used. In this study, CIN, SMAC and XLD were used for the three pathogens tested. Different selective media were used for other studies. For example, Jasson et al. (2007) used Sorbitol MacConkey Agar supplemented with Cefixime Tellurite (SMAC-CT) for enumerating *E. coli* O157:H7, and the sublethally-injured population was calculated based on the colony counts difference between SMAC-CT and non-selective agar TSAYE.

The second part of this study sought to characterize the sublethally-injured cells and determine a way to further reduce them. Four stresses (including temperature, pH, NaCl, and bile salt) and six stress levels for each stress were chosen based on the previous literature

(Arroyo et al., 2010; Ghate et al., 2015; Jasson et al., 2007; Stephens et al., 1998; Ukuku and Geveke, 2010; Weissinger et al., 2000;). We started by first evaluating a series of stress levels on healthy pathogenic cells and then chose the levels that did not impact the healthy cells. We then applied these stresses to cells that had been treated with NEO water. The idea is that by combining such mild stresses with NEO-water-treatments, we can create a series of hurdle treatments and achieve synergistic antimicrobial effects. As discussed already by Espina et al. (63), in hurdle techniques, pathogenic populations that are sublethally-injured by one treatment can be further inactivated by other hurdle treatments. As shown in Table 3, combining NEO water treatments with mild temperature treatment (51º C for 10 min), pH challenges (pH value of 3.6 or 4.6), and NaCl or bile salt challenges have all achieved synergistic antimicrobial effects. Among them, diluted NEO water treatments in combination with the mild 51º C heat treatment achieved the highest reduction regardless of the pathogens tested. Results from this study, together with other previous reports ((Espina et al., 2016; Shigapova, 2005; Wesche et al., 2009), indicated that NEO water treatments combined with moderate thermal treatment have strong synergistic effects and could potentially be used together to effectively secure post-harvest food safety.

"Stress-induced cross-protection" has been one of the concerns when designing and applying mild antimicrobial treatments (Wesche et al., 2009). Sykes attributed the survival of bacteria in adverse environments to either sublethal treatments that were insufficient to kill the cells or to the bacteria's innate protective mechanisms (Sykes, 1963). It was hypothesized that bacterial cells could adapt or acquire resistance to different conditions by modifying metabolic activities, adjusting nutrient utilization or using enzymes that were in a recessive role (23). For example, Chen and Jiang (2017) reported that the desiccation-adapted *S.* Typhimurium in broiler litter had cross-protection against high temperature treatments and the *rpo*S gene was involved in this process. Jenkins et al. (1988) reported that starvation or adaptive treatments

with heat, H_2O_2, or ethanol could protect *E. coli* against further oxidative stress (H_2O_2). Mazzotta found that the adaptation at pH 5.0 for 18-24 hours increased the heat resistance of *E. coli* O157:H7 and *Salmonella* at 56º, 58º, and 60º C (Mazzotta, 2001). In our study, cross-protection was not found. After NEO-water-treated cells from all three pathogens were exposed to additional salt, pH or heat challenges, further reductions were seen. These results illustrate the great potential for using diluted NEO water as a step in systematic hurdle techniques.

The third section of this study was to better understand the NEO-water-treated cells from the molecular level. To do this, real-time RT-PCR was conducted to monitor the expression of selected genes for each pathogen. The 50% and 100% NEO water were used and applied to pathogens, so that enough oxidative stresses would be generated on pathogens for gene expression evaluation (Wang et al., 2010). For *Yersinia*, genes *gsr*A, *omp*R, *rpo*S and *ail* were selected because of their involvement in responses to extracellular stresses such as heat, oxidative conditions, high salt concentrations and low pH (Brzostek et al., 2003; Dorrel et al., 1998; Iriarte et al., 1995; Kirjavainen et al, 2008; Pierson and Falkow, 1993; Miller et al., 1990; Yamanoto et al., 1996). Among these four genes, *ail* and *omp*R were significantly up-regulated when Y. *enterocolitica* was treated with 100% NEO water (Figure 1). *Omp*R encodes a transcriptional regulatory protein that is related to the bacteria's sensitivity to high osmolarity, stresses from heat and low pH, and macrophage phagocytosis (Brzostek et al., 2003; Dorrel et al., 1998). *Ail* encodes a 17 kDa outer-membrane-surface protein that has been proven to be involved in serum stress resistance, adhesion and invasion of eukaryotic cells (Kirjavainen et al, 2008; Miller et al., 1990; Pierson and Falkow, 1993). According to Pierson and Falkow (2008), *ail*-homologous sequences are present only in pathogenic species and strains of *Yersinia*. Resistance to serum stress is very critical for pathogens to be able to survive and cause infections in the host. *ail* is also known for its ability to promote resistance to complement killing (Bliska and Falkow, 1992; Kirjavainen et al, 2008; Pierson and Falkow,1993). The up-

regulations of these two genes highlighted the importance of establishing hurdle techniques to further reduce the number of cells that survive NEO water treatments.

The gene of *ycf*R encodes a putative outer membrane protein present in both *E. coli* and *Salmonella*. It plays an important role in biofilm formation and stress responses in *E. coli* O157:H7 (Zhang et al., 2007). For *Salmonella*, this gene is known for its involvement in surface attachment and chlorine resistance (Salazar et al., 2013). As shown in Figure 1, its expression was significantly up-regulated in *S.* Enteritidis only after the cells were treated with 100% NEO water. HOCl and ClO^- are the major functional antimicrobial components in NEO water (Huang et al., 2008; Rahman et al., 2016). Salazar et al. (2013) have found that the deletion of *ycf*R in *S.* Typhimurium significantly decreased the bacteria's chlorine resistance and its attachment ability.

Previous studies have suggested that the transcriptomic activities of genes responsible for cysteine and iron-sulfur-cluster biosynthesis could be highly associated with the chlorine-induced bacterial stress response (Wang et al., 2009; Wang et al., 2010). Thus, the expression of *ybi*J, *cys*D, *cys*J, and *osm*B in *E. coli* O157:H7 and the expression of the *cys*K, *yfh*P, *nif*S and *nif*U genes in *Salmonella* were monitored as well. However, their expression was not significantly up-regulated or down-regulated in this study, indicating that NEO water treatment did not cause any significant changes in cysteine or iron-sulfur-cluster biosynthesis. One limitation about this study that needs to be mentioned here is that only one strain was used for each pathogen. The insignificant expression of certain selected genes only applied to the particular strains tested. Future study might want to look into the differences in gene expression between different strains within each species to better interpret the different responses observed between different species after the NEO-water-treatments.

This study evaluated the impact of NEO water treatments on the formation of sublethally-injured pathogens. When *Y. enterocolitica* was treated with 6%, 10%, 15% and

25% NEO water, sublethally-injured cells were confirmed by plating the treated cultures on non-selective (TSAYE) and selective agar (CIN). Sublethally-injured *S.* Enteritidis was found when the pure culture was treated with 3%, 6% and 10% NEO water. No sublethally-injured *E. coli* O157:H7 was found in this study regardless of the concentrations of the NEO water tested. The 50% and 100% NEO water killed all culturable cells and did not generate sublethal injury in cells. Combining this observation with our previous results, it can be concluded that the formation of sublethally-injured cells and the formation of VBNC cells are genus-dependent. The up-regulation of the adhesion- and stress-response-related genes in *Y. enterocolitica* and *S.* Enteritidis highlighted the importance of developing hurdle techniques when using diluted NEO water as an antimicrobial treatment. No cross-protection was observed in this study. Combining the diluted NEO water treatments with heat, NaCl, bile salt or pH stresses led to additional reduction in pathogens. Among different combinations, diluted NEO water treatment in combined with the 51º C 10 min heat treatment was the most efficient hurdle technology, resulting in a 7-log reduction in pathogens.

TABLE 1. Formation of sublethally injured cells after being treated with different concentrations of NEO water.

*indicates significant difference existed ($P < 0.05$) between the counts obtained from TSAYE and the counts obtained from the corresponding selective agar by using a two-tailed student's t-test.

NEO water concentration	Free chlorine concentration	*Yersinia*			*E. coli* O157: H7			*Salmonella*		
		TSAYE	CIN	%[a]	TSAYE	SMAC	%	TSAYE	XLD	%
0	0	8.42±0.10	8.40±0.05	N/A	8.61±0.02	8.58±0.01	N/A	8.08±0.16	7.82±0.12	N/A
3%	1.8 ppm	8.33±0.08	8.27±0.08	N/A	8.55±0.03	8.54±0.05	N/A	8.01±0.09	7.60±0.07*	52.00
6%	3.5 ppm	8.08±0.10	7.30±0.13*	38.12	7.91±0.04	7.85±0.06	N/A	7.54±0.11	6.71±0.08*	24.57
10%	5.9 ppm	7.13±0.12	6.14±0.12*	4.566	6.71±0.03	6.69±0.04	N/A	5.78±0.09	5.48±0.07*	0.250
15%	8.9 ppm	5.91±0.04	5.41±0.05*	0.211	4.89±0.16	4.78±0.03	N/A	3.61±0.21	3.04±0.32	N/A
25%	14.8 ppm	3.90±0.12	2.70±0.35*	0.002	ND	ND	N/A	ND	ND	N/A

[a] Percentage of sublethally injured cells. Percentages were only calculated when significant differences in cell counts were observed between the selective agar and the non-selective agar.

TABLE 2. Highest mild stress conditions used to challenge the NEO water-treated pathogenic cells.

	Temp. (°C)	pH	NaCl	Bile salt
Yersinia	51	4.6	3%	0.40%[a]
E. coli	51	3.6	2%	0.40%
Salmonella	51	3.6	2%	0.10%

[a] The highest bile salt concentration tested was 0.40%. Although it did not significantly ($P >$ 0.05) impact the population of *Yersinia* and *E. coli*, it was still chosen for the following experiment.

TABLE 3. Survival of NEO water-treated *Y. enterocolitica*, *E. coli* O157:H7, and *S.* Enteritidis populations after being exposed to additional temperature (Temp.), NaCl, bile salt (BS), and pH stresses (limit of enumeration: 1.62 CFU/ml)*.

		Y. enterocolitica				
NEO water concentrations	**Free chlorine concentrations**	**NEO only**	**NEO + Temp.**	**NEO + NaCl**	**NEO + BS**	**NEO + pH**
0	0	8.68 ± 0.05^{Aa}	8.66 ± 0.02^{Aa}	8.68 ± 0.03^{Aa}	8.67 ± 0.02^{Aa}	8.67 ± 0.04^{Aa}
3%	1.8 ppm	8.67 ± 0.01^{Aa}	8.65 ± 0.05^{Aa}	8.69 ± 0.02^{Aa}	7.79 ± 0.02^{Bb}	8.63 ± 0.03^{Aa}
6%	3.5 ppm	8.57 ± 0.04^{Aa}	6.98 ± 0.17^{Cb}	8.14 ± 0.01^{Bb}	6.78 ± 0.06^{Cc}	8.47 ± 0.05^{Aa}
10%	5.9 ppm	7.39 ± 0.05^{Ab}	4.86 ± 0.23^{Dc}	6.70 ± 0.07^{Bc}	5.69 ± 0.19^{Cd}	7.13 ± 0.22^{ABb}
15%	8.9 ppm	6.07 ± 0.16^{Ac}	3.60 ± 0.31^{Cd}	5.66 ± 0.08^{Ad}	4.61 ± 0.10^{Be}	6.01 ± 0.04^{Ac}
25%	14.8 ppm	4.12 ± 0.09^{Ad}	ND^{Ce}	3.20 ± 0.49^{Be}	2.68 ± 0.26^{Bf}	3.45 ± 0.26^{ABd}

		***E. coli* O157:H7**				
NEO water concentration	**Free chlorine concentration**	**NEO only**	**NEO + Temp.**	**NEO + NaCl**	**NEO + BS**	**NEO + pH**
0	0	8.76 ± 0.01^{Aa}	8.75 ± 0.02^{Aa}	8.74 ± 0.02^{Aa}	8.73 ± 0.04^{Aa}	8.75 ± 0.02^{Aa}
3%	1.8 ppm	8.73 ± 0.03^{Aa}	8.56 ± 0.03^{Bb}	8.72 ± 0.02^{Aa}	8.65 ± 0.02^{ABa}	8.68 ± 0.03^{Aa}
6%	3.5 ppm	8.00 ± 0.05^{Ab}	5.01 ± 0.19^{Bc}	7.96 ± 0.11^{Ab}	7.71 ± 0.07^{Ab}	7.89 ± 0.05^{Ab}
10%	5.9 ppm	7.12 ± 0.07^{Ac}	ND^{Dd}	7.10 ± 0.03^{Ac}	6.76 ± 0.03^{Cc}	6.90 ± 0.02^{Bc}
15%	8.9 ppm	4.97 ± 0.13^{Ad}	ND^{Cd}	3.69 ± 0.27^{Bd}	4.00 ± 0.24^{Bd}	4.74 ± 0.10^{Ad}
25%	14.8 ppm	3.02 ± 0.00^{Ae}	ND^{Bd}	ND^{Be}	ND^{Be}	ND^{Be}

		***S.* Enteritidis**				
NEO water concentration	**Free chlorine concentration**	**NEO only**	**NEO + Temp.**	**NEO + NaCl**	**NEO + BS**	**NEO + pH**
0	0	8.53 ± 0.03^{Aa}	8.50 ± 0.04^{Aa}	8.52 ± 0.03^{Aa}	8.50 ± 0.04^{Aa}	8.52 ± 0.04^{Aa}
3%	1.8 ppm	8.49 ± 0.03^{Aa}	8.01 ± 0.04^{Bb}	8.46 ± 0.04^{Aa}	8.50 ± 0.03^{Aa}	8.02 ± 0.11^{Bb}
6%	3.5 ppm	7.67 ± 0.10^{Ab}	7.11 ± 0.04^{Bc}	6.98 ± 0.07^{Bb}	6.18 ± 0.07^{Db}	6.51 ± 0.04^{Cc}
10%	5.9 ppm	5.87 ± 0.15^{Ac}	3.39 ± 0.24^{Dd}	5.40 ± 0.11^{ABc}	4.56 ± 0.05^{Cc}	5.08 ± 0.21^{BCd}
15%	8.9 ppm	3.44 ± 0.37^{Ad}	ND^{Ce}	2.75 ± 0.19^{Bd}	ND^{Cd}	2.81 ± 0.16^{ABe}
25%	14.8 ppm	ND^{e}	ND^{e}	ND^{e}	ND^{d}	ND^{f}

*Different capitalized letters within each row represent significant differences when comparing numbers with each other in the same row, while the different lowercase letters represent significant difference within a column ($P < 0.05$). ND represents that the survived cell numbers were below the limit of enumeration.

TABLE 4. Primer sets used in the real-time RT-PCR.

Target	Gene	Functions and related stresses	Primer	Sequence 5' to 3'	Primer efficiency (%)	Amplicon size (bp)	Reference
All	16S	Reference gene	16S_F 16S_R	CGATCCCTAGCTGGTCTGAG GTGCAATATTCCCCACTGCT	*Yersinia*: 94 E. *coli*: 94 *Salmonella*: 93	93	Castelijn et al., 2012
Y. enterocolitica	*gsr*A	Serine endoprotease; environmental stresses	F_gsrA R_gsrA	GACGGTTCTCCGTTCCAAGG CACGGAAATCCTGCTTGCTG	91	85	Yamamoto et al., 1996, this study
	*omp*R	Transcriptional regulator; environmental stresses	F_ompR R_ompR	TGCTCGACCTGATGTTACCG CACCCTTTGCCGTCACCATA	93	102	Brzostek et al., 2003, Dorrell et al., 1998, this study
	ail	Adhesion and invasion; serum stress	F_ail R_ail	AGCCTTTATGGATTACTGGGGG CCCGTATGCCATTGACGTCTT	94	96	Pierson and Falkow, 1993, this study
	*rpo*S	Polymerase sigma factor; external stresses	F_rpoS R_rpoS	CAGAACGCGGTTTCCGTTTC CAGACGGATGGTACGGGTTT	95	95	Iriarte et al, 1995, this study
E. coli O157:H7	*ycf*R	Outer membrane protein; chlorine stress	EC_F _ycfR EC_R_ycfR	GTCATTTGCCAGCTTTGCGG AGATTTGTCCCCGCGTTAGC	93	93	Wang et al., 2009, Zhang et al., 2007, this study
	*ybi*J	Putative periplasmic protein; iron metabolism	EC_F _ ybiJ EC_R_ ybiJ	TTGCTGCTATGGCTCTTTCA GAAACCACGCCGATTTTATT	96	100	McHugh et al., 2003, Wang et al., 2009, this study
	*cys*D	Cysteine biosynthesis; oxidative stress	EC_F _ cysD EC_R_ cysD	GCCAGATATCCTGCTCGGTC TGGCACAACTATAACGGGCA	93	85	Wang et al., 2009, Malo et al., 1990, this study
	*cys*J	Sulfite reductase; oxidative stress	EC_F _ cysJ EC_R_ cysJ	GTGTTTCACTGCGGGTAAGC TGAACGAAGCGCTACAGTGG	91	90	Ostrowski et al., 1989, Wang et al., 2009, this study
	*osm*B	Osmotic stress inducible protein; multiple stresses	EC_F _ osmB EC_R _ osmB	TACCCAACGTACTGCCATCG TCTAAACGGGACCGCAACAC	92	85	Boulanger et al., 2005, Wang et al., 2009, Jung et al., 1990, this study

S. Enteritidis	*ycf*R	Outer membrane protein; chlorine stress	F _ycfR	ACGCCAGAAGGTCAACAGAA	94	134	Salazar et al., 2013, Wang et al.,2010,
			R_ycfR	GGGCCGGTAACAGAGGTAA			
	*cys*K	Cysteine synthase A; oxidative stress	F _ cysK	CGCTATTCAGAAAGCCGAAG	99	121	Tai et al., 2008, Wang et al.,2010,
			R_ cysK	CATCGGTGTCTTCCCAGATT			
	*yfh*P	Transcriptional regulator IScR; oxidative stress	F _ yfhP	TTACCTTAGGCGAGCTGGTG	91	104	Vergnes et al., 2017, Wang et al.,2010,
			R_ yfhP	GCGCGTAATTTAACGTCGAT			
	*nif*S	Cysteine desulfurase; oxidative stress	F _ nifS	ATCGCGAAAGAAGAGATGGA	95	123	Nilsson et al., 2002, Wang et al.,2010,
			R_ nifS	TCGCCGTTCAGGTAAACTTC			
	*nif*U	Fe-S cluster assembly protein; oxidative stress	F _ nifU	AACGACGATAACGTGGGAAG	92	136	Boyd et al., 2008, Wang et al.,2010,
			R _ nifU	GCAGCCGTAAGTCTTGAAGC			

TABLE 5. Culturable *Y. enterocolitica* after being treated with different stresses.

Temp. (°C)		**pH**		**NaCl**		**Bile salt**	
Control	8.87±0.01	Control	8.83±0.01	Control	8.83±0.01	Control	8.83±0.01
45	8.84±0.01	5.8	8.79±0.01	1%	8.83±0.00	0.05%	8.83±0.01
48	8.89±0.00	5.3	8.81±0.03	2%	8.83±0.01	0.10%	8.84±0.00
51	8.88±0.00	4.6	8.80±0.01	3%	8.80±0.03	0.15%	8.85±0.05
54	8.57±0.0*	4	ND*	4%	ND*	0.20%	8.82±0.05
57	ND*	3.6	ND*	5%	ND*	0.30%	8.79±0.01
60	ND*	3	ND*	6%	ND*	0.40%	8.78±0.00

*indicates significant reduction was detected by using one-tailed student's t-test ($P < 0.05$) when compared with the un-treated control in the same column.

TABLE 6. Culturable *E. coli* O157:H7 after being treated with different stresses.

Temp. (°C)		pH		NaCl		Bile salt	
Control	8.81±0.03	Control	8.75±0.05	Control	8.75±0.05	Control	8.75±0.05
45	8.78±0.02	5.8	8.71±0.01	1%	8.76±0.00	0.05%	8.71±0.01
48	8.76±0.01	5.3	8.68±0.12	2%	8.75±0.02	0.10%	8.73±0.11
51	8.76±0.01	4.6	8.70±0.03	3%	8.31±0.03*	0.15%	8.74±0.06
54	8.40±0.02*	4	8.75±0.01	4%	ND*	0.20%	8.77±0.00
57	8.26±0.09*	3.6	8.73±0.02	5%	ND*	0.30%	8.78±0.01
60	ND*	3	ND*	6%	ND*	0.40%	8.68±0.02

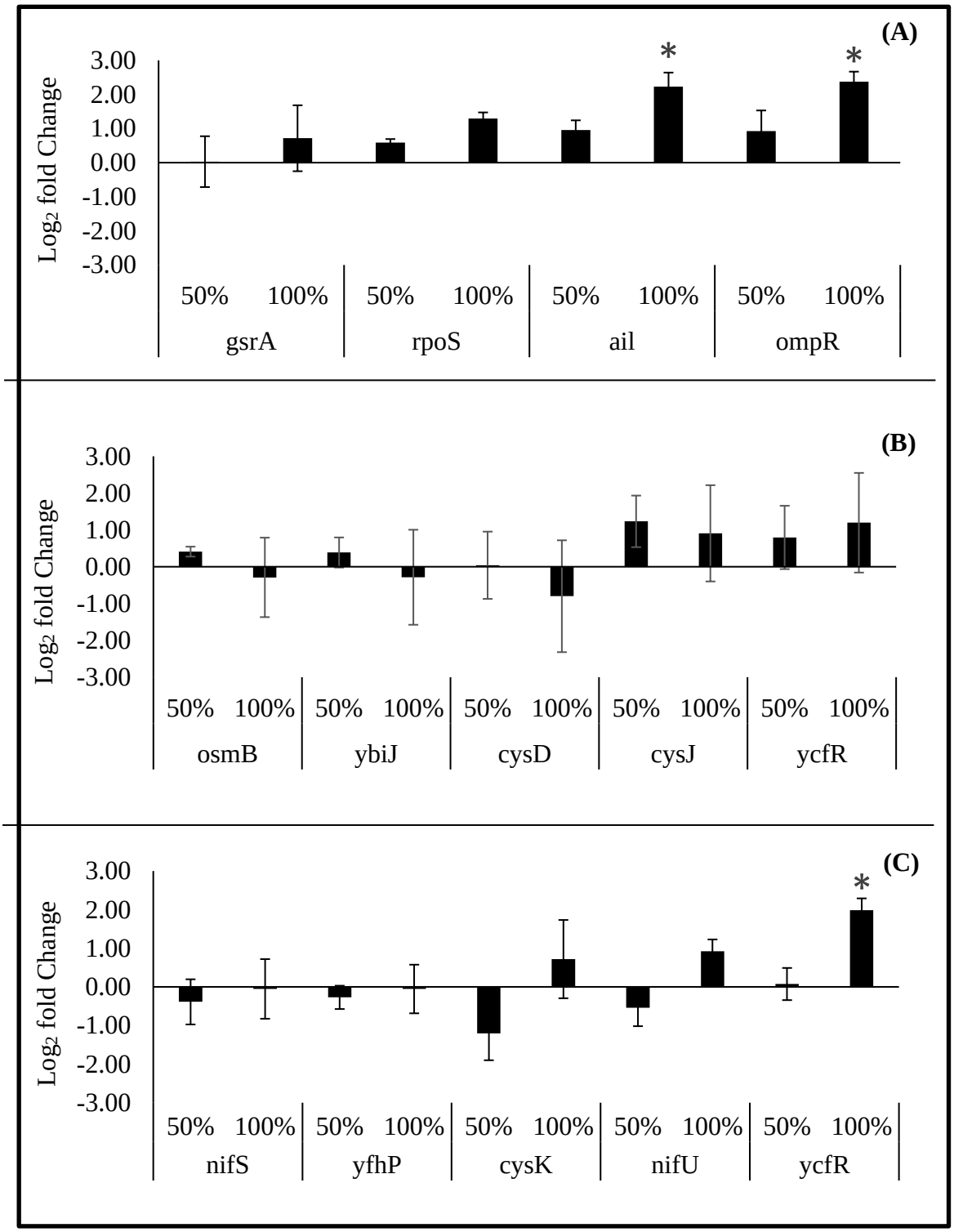
*indicates significant reduction was detected by using one-tailed student's t-test ($P < 0.05$) when compared with the un-treated control in the same column.

TABLE 7. Culturable *S.* Enteritidis after being treated with different stresses.

Temp. (°C)		**pH**		**NaCl**		**Bail salt**	
Control	8.72±0.05	Control	8.69±0.02	Control	8.69±0.02	Control	8.69±0.02
45	8.69±0.00	5.8	8.70±0.05	1%	8.75±0.04	0.05%	8.75±0.03
48	8.73±0.06	5.3	8.74±0.05	2%	8.70±0.03	0.10%	8.71±0.03
51	8.71±0.03	4.6	8.71±0.01	3%	8.56±0.02*	0.15%	8.18±0.05*
54	7.87±0.02*	4	8.68±0.03	4%	ND*	0.20%	ND*
57	ND*	3.6	8.71±0.05	5%	ND*	0.30%	ND*
60	ND*	3	ND*	6%	ND*	0.40%	ND*

*indicates significant reduction was detected by using one-tailed student's t-test ($P < 0.05$) when compared with the un-treated control in the same column.

FIG. 1 Evaluation of the gene expression in Y. enterocolitica (A), E. coli O157:H7 (B) and S. Enteritidis (C) after being treated with 50% or 100% NEO water. * represents significant up-regulation ($P < 0.05$).



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Conclusion, limitation and future studies

In conclusion, my research finds out that NEO water has strong capability in inactivating foodborne pathogens and has great potential to be applied as a disinfection reagent. The antimicrobial efficiency of NEO water is dependent upon the NEO water concentrations but not on treatment time. *Yersinia enterocolitica* showed resistance to the NEO water treatments, indicating that the application of hurdle technologies or other treatment methods with synergistic effects are needed to control *Yersinia enterocolitica*. Utilizing traditional plate counting method and flow cytometry cell sorting method allows the calculation of cells in VBNC state. The identification of sublethally-injured bacterial cells needs the use of both selective and non-selective agar. *E. coli* O157:H7 does not generate sublethally-injured cells when treated with low concentrations of NEO water. High percentages of VBNC and sublethally-injured bacteria are formed when cells are treated with sublethal dosages of NEO water. NEO water treatment works better on the contaminated pork skin samples. In general, the formation of VBNC or sublethally-injured cells is genus-dependent. The up-regulation of the stress response, adhesion, and virulence related genes in *Y. enterocolitica* and *S.* Enteritidis highlights the importance of developing adequate hurdle techniques when using NEO water as an antimicrobial treatment. Combining the diluted NEO water treatments with traditional stress hurdles, like heat, NaCl, bile salt, and low pH, led to additional reduction in targeted bacterial pathogens. Among different combinations, diluted NEO water treatment in combination with mild heat treatment was the most efficient hurdle technology combination.

The first part of this study only investigated the efficacy of applying NEO water treatments on artificially inoculated meat products. The second part of this study only investigated the

formation of sublethally-injured cells by using pure cultures. Future work needs to better consider the real-world conditions. In addition, experiments such like the impact of NEO water on biofilm removal and inactivation of “persister” cells need to be done and will provide additional useful information to the existing knowledge about NEO water. Global gene expression evaluations of bacterial pathogens, like *Yersinia enterocolitica*, under NEO-water-induced stresses, will also offer more insights on the mechanism of NEO water treatment.