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FOOD MICROBIOLOGY

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

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ABSTRACT The impact of neutral electrolyzed oxidizing (NEO) water treatments on the formation of sublethally injured *Yersinia enterocolitica*, *Escherichia coli* O157:H7, and *Salmonella enterica* serovar Enteritidis cells was evaluated. When pathogens were treated 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 concentration used, no sublethally injured *E. coli* O157:H7 cells were 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 of treated cells against the other stresses. The diluted NEO water treatments in combination with heat treatment at 51°C for 10 min led to the best synergistic antimicrobial effects with a combined reduction of 7 logs. The gene expression results showed that NEO water treatments led to the upregulation 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 the numbers of viable pathogenic cells. These results demonstrate the great application potential of NEO water as a nonthermal and less corrosive antimicrobial treatment.

IMPORTANCE Neutral electrolyzed oxidizing (NEO) water is a nonthermal and less corrosive antimicrobial treatment that has been demonstrated to have efficacy in reducing microbial contamination in food, including meat, fresh fruit, and vegetables. However, NEO water treatments can cause sublethal injury to pathogenic cells, resulting in cells that retain their viability. Consequently, these sublethally injured pathogenic cells become a serious food safety concern. This study evaluated the formation of sublethally injured *Yersinia enterocolitica*, *Escherichia coli* O157:H7, and *Salmonella enterica* serovar Enteritidis cells by NEO water treatments and the potential cross protection against heat, pH, NaCl, or bile salt stresses that it may generate. No cross protection was observed. By combining NEO water treatments with sublethal levels of additional stresses, significant synergistic antimicrobial outcomes were achieved. These results indicate that mild processing treatments, when combined, can effectively reduce pathogen populations while minimizing the negative impacts on food quality.

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KEYWORDS neutral electrolyzed oxidizing water, sublethal injury, stress response, hurdle technology, pathogens

As consumers' demand for and consumption of fresh-like food have increased in the last decade, intensive research attention has been paid to novel nonthermal decontamination methods that can enhance food safety without having a significant negative impact on food quality (1, 2). Among these nonthermal treatments, neutral electrolyzed oxidizing (NEO) water treatment has been tested on different types of food and has been demonstrated to have great antimicrobial efficacy and application potential. As a neutral solution, the pH of NEO water stays at about 8.5 $\pm$ 0.5, making it less corrosive to processing equipment and less of an irritant when it comes into contact with skin (2). In addition, its neutral pH enables reduced chlorine loss and an extended shelf-life (2–4). Food products on which the antimicrobial efficacy of NEO water has been tested include tomatoes (5, 6), romaine and iceberg lettuce (7), shredded carrots and spinach (8), fresh-cut endive, corn salad, "Four Seasons" salad (2), dates (9), blueberries (10), apples (11), and pork chops and pork skin products (12). NEO water treatments have also been applied to the surfaces of plastic and wooden cutting boards (13), bamboo board (14), and plates, spoons, forks, knives, and drinking glasses (15). 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 (16–18). The ORP value of NEO water is usually between 800 and 1,100 mV (19). 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 that it causes to the membrane and DNA, and the deterioration that it causes to the cells' membrane transport capacity (20). 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 the potential side effects of NEO water treatments. Lin et al. (21) showed that low levels of chlorination (0.5 mg/liter) in drinking water could lead to the formation of viable but nonculturable (VBNC) *Escherichia coli* cells. While such a 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, rifampin, clarithromycin, and chloromycetin (21). Similarly, the formation of VBNC cells was also found in this research group's previous study (12). When *E. coli* O157:H7, *Salmonella enterica* serovar 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 (12). 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 (22). The results of these previous studies highlight the importance of better understanding the impact of NEO water treatments, especially the changes that they 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 (23). The metabolic injury that occurs within cells makes the sublethally injured populations unable to form colonies on selective agar (24, 25). 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 (25–27). Cells in the sublethal injury state are more sensitive to agents or stresses to which they would show resistance in their healthy state (28). Thus, understanding the sensitivities of sublethally injured populations provides new opportunities for developing mild processing technologies that can further reduce the numbers of pathogenic cells without changing food quality. Such mild processing

TABLE 1 Formation of sublethally injured cells after treatment with different concentrations of NEO water

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

[a]Different letters within each column represent significant differences between treatments with different NEO water concentrations ($P < 0.05$). *, a 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. ND, the surviving cell numbers were below the limit of enumeration.

[b]Percentage of sublethally injured cells. Percentages were calculated only when significant differences in cell counts between the selective agar and the nonselective agar were observed. NA, not applicable.

technologies can be utilized in combination with NEO water treatments to form hurdle decontamination strategies that lead to synergistic antimicrobial effects (29, 30).

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 reverse transcription (RT)-PCR was utilized for monitoring gene expression. On the basis of findings presented in the literature, four target genes (*gsrA*, *ompR*, *rpoS*, and *ail*) were selected for *Y. enterocolitica* (31–37), five genes (*ybiJ*, *cysD*, *cysJ*, *ycfR*, and *osmB*) were chosen for *E. coli* O157:H7 (38–44), and five genes (*cysK*, *yfhP*, *nifS*, *ycfR*, and *nifU*) were monitored for *S.* Enteritidis (39, 45–49).

RESULTS

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 concentration of the NEO water increased, the surviving cell number decreased. For *Yersinia*, significant differences between the colony counts obtained from Trypticase soy agar supplemented with 0.6% yeast extract (TSAYE) cultures and the colony counts obtained from cefsulodin-irgasan-novobiocin (CIN) cultures were observed when cultures were treated with NEO water at concentrations equal to or above 6%. More than a 1-log difference between TSAYE and CIN cultures 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 it was treated with 6% NEO water. No difference in *E. coli* O157:H7 populations was observed between TSAYE and sorbitol MacConkey (SMAC) agar regardless of the NEO water concentration used. For *Salmonella*, a 0.4-log difference between the counts on TSAYE and xylose lysine deoxycholate (XLD) cultures was observed when the cultures were 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 TSAYE or the selective agar, indicating that both 50% and 100% NEO water reduced the numbers of cells of all pathogen to an undetectable level after 5 min of treatment.

Stress selection for treating NEO water-treated pathogens. As shown in Table S1 in the supplemental material, heat treatment at 51°C, a pH value of 4.6, an NaCl concentration of 3%, and a bile salt concentration of 0.4% were stress levels that did not generate impacts on healthy non-NEO water-treated *Yersinia* cultures. These stresses

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

Organism	Temp (°C)	pH	NaCl concn (%)	Bile salt concn (%)
Y. enterocolitica	51	4.6	3	0.40[a]
E. coli O157:H7	51	3.6	2	0.40
S. Enteritidis	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 *Y. enterocolitica* and *E. coli* O157:H7, it was still chosen for use in the experiment.

were then chosen for use in the following studies, in which their impacts on NEO water-treated cells were investigated. For *E. coli* O157:H7 (Table S2), heat treatment at 51°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 conditions and stress levels that were used to challenge NEO water-treated cells. These stress levels did not impact healthy pathogen cells and were expected to impact or kill NEO water-treated cells.

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

TABLE 3 Survival of NEO water-treated *Y. enterocolitica*, *E. coli* O157:H7, and *S.* Enteritidis populations after being exposed to additional temperature, NaCl, bile salt, and pH stresses

Organism and NEO water concn	Free chlorine concn (ppm)	Count (log CFU/ml)[a]				
		NEO only	NEO + temp	NEO + NaCl	NEO + bile salts	NEO + pH
Y. enterocolitica						
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	8.67 ± 0.01 Aa	8.65 ± 0.05 Aa	8.69 ± 0.02 Aa	7.79 ± 0.02 Bb	8.63 ± 0.03Aa
6	3.5	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	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	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	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						
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	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	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	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	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	3.02 ± 0.00 Ae	ND Bd	ND Be	ND Be	ND Be
S. Enteritidis						
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	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	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	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	3.44 ± 0.37 Ad	ND Ce	2.75 ± 0.19 Bd	ND Cd	2.81 ± 0.16 ABe
25	14.8	ND e	ND e	ND e	ND d	ND f

[a]The limit of enumeration was 1.62 CFU/ml. Different capitalized letters within each row represent significant differences when comparing the numbers with each other in the same row, while the different lowercase letters represent significant differences within a column ($P < 0.05$). ND, the surviving cell numbers were below the limit of enumeration.

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 51°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 (TSAYE plus 3% NaCl or TSAYE plus 0.4% bile salt), an additional 0.69-log reduction and an additional 1.7-log reduction were observed, respectively. For *Yersinia*, the pH change did not cause a further reduction of the cells after they were first treated with 10% NEO water, as there was no significant difference between cell counts obtained from the pH-adjusted TSAYE and those obtained from regular TSAYE. For *Yersinia*, the maximum synergistic effect was found when combining the 25% NEO water treatment with the 10-min 51°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 were made for *E. coli* O157:H7. The concentration of the original nontreated *E. coli* O157:H7 cells was 8.76 ± 0.01 log CFU/ml. When the cells were treated with 10% NEO water, a 1.64-log reduction was observed. After exposing the NEO water-treated cells to the 10-min 51°C heat treatment, an additional reduction of approximately 5.5 logs was observed, with the amount of surviving cells falling below the limit of enumeration. Treatment combinations including 10, 15, or 25% NEO plus heat treatment at 51°C for 10 min and 25% NEO plus either 3% NaCl, 0.4% bile salt, or pH 3.6 all reduced the amount of pathogenic cells to levels that could not be enumerated.

For *Salmonella*, 25% NEO water alone was able to achieve a 6-log reduction. When the 15% NEO water treatment was combined with the heat treatment, this additional 10-min 51°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 logs. All of these observations demonstrate the synergistic antimicrobial effects achieved by combining diluted NEO water treatments with other stresses.

Transcription-level analysis. Four genes of *Yersinia*, five genes of *E. coli*, and five genes of *Salmonella* were selected for RT-quantitative PCR (qPCR). In *Yersinia*, the upregulation of *rpoS*, *ail*, and *ompR* was observed when the cultures were treated with 50% and 100% NEO water. However, only the 100% NEO water-treated cells had their *ail* gene (2.23-$\log_2$-fold) and *ompR* gene (2.38-$\log_2$-fold) significantly upregulated (>2-log-fold change and $P < 0.05$). No significant up- or downregulation was detected in NEO water-treated *E. coli* O157:H7 cells. For *Salmonella*, after they were treated with 100% NEO water, *ycfR* (1.99-$\log_2$-fold) was significantly upregulated.

DISCUSSION

This study evaluated the formation of sublethally injured pathogenic cells after the cells were 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 (50, 51). 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 the retarded growth of injured cells (28, 52, 53). The underestimation 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 (54, 55). Much research has focused on enhancing the ability to enrich, detect, and enumerate sublethally injured cells (25, 53, 56, 57). 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. (58), the proportion of chlorine-injured *Enterobacter cloacae*, *E. coli*, and *E. coli* O157:H7 cells in pure culture was shown to be between 69 and 77%. Fungicides, including thiophanate-methyl (Topsin-M), procymidone (Sumilex), and ethylene oxide (Oxirane), also caused the formation of injured cells. The percentage of the injured population was 45 to 97% for thiophanate-methyl-treated

cells, 80 to 87% for procymidone-treated cells, and 50 to 97% for ethylene oxide-treated cells. These results demonstrate the importance of choosing not only the right sanitizer but also the right sanitizer concentrations so that the treatment kills target microorganisms completely rather than just injures the cells (58).

NEO water has been reported to be 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) (12). In the VBNC state, cells cannot be detected by either selective or nonselective 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, respectively, entered into the VBNC state (12). In this study, the same NEO water treatments were applied, and the percentages of the sublethally injured populations that 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* cells in the culture, approximately 25% of the *Salmonella* cells in the culture, and approximately 38% of the *Yersinia* cells in the culture became sublethally injured. On the basis of the findings of 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. (28) used sorbitol MacConkey agar supplemented with cefixime tellurite (SMAC-CT) for enumerating *E. coli* O157:H7, and the sublethally injured population was calculated on the basis of the colony count difference between SMAC-CT and the nonselective 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 on the basis of the previous literature (28, 29, 59–62). We started by first evaluating the effects of 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 was that by combining such mild stresses with NEO water treatments, we could create a series of hurdle treatments and achieve synergistic antimicrobial effects. As already discussed 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 (51°C for 10 min), pH challenges (a pH value of 3.6 or 4.6), and NaCl or bile salt challenges all achieved synergistic antimicrobial effects. Among them, diluted NEO water treatments in combination with the mild 51°C heat treatment achieved the highest reduction regardless of the pathogens tested. Results from this study, together with other previous reports (23, 63, 64), indicated that NEO water treatments combined with moderate thermal treatment have strong synergistic effects and could potentially be used together to effectively secure postharvest food safety.

Stress-induced cross protection has been one of the concerns when designing and applying mild antimicrobial treatments (23). Sykes attributed the survival of bacteria in adverse environments either to sublethal treatments that were insufficient to kill the cells or to the bacteria's innate protective mechanisms (65). 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 (66) reported that the desiccation-adapted *S*. Typhimurium in broiler litter had cross protection against high-temperature treatments and that the *rpoS* gene was involved in this process. Jenkins et al. (67) 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 adapta-

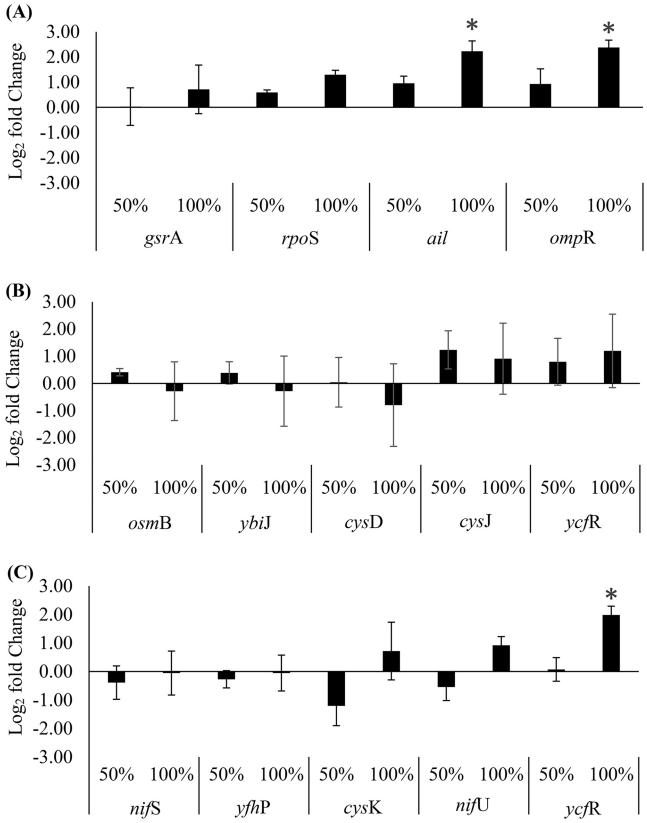

FIG 1 Evaluation of gene expression in *Y. enterocolitica* (A), *E. coli* O157:H7 (B), and *S.* Enteritidis (C) after they were treated with 50% or 100% NEO water. *, significant upregulation ($P < 0.05$).

tion at pH 5.0 for 18 to 24 h increased the heat resistance of *E. coli* O157:H7 and *Salmonella* at 56°C, 58°C, and 60°C (68). 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 by each pathogen. The 50% NEO water and 100% NEO water were used and applied to the pathogens so that enough oxidative stresses would be generated on the pathogens for gene expression evaluation (45). For *Yersinia*, the *gsrA*, *ompR*, *rpoS*, and *ail* genes were selected because of their involvement in the organism's responses to extracellular stresses, such as heat, oxidative conditions, high salt concentrations, and low pH (31–37). Among these four genes, *ail* and *ompR* were significantly upregulated when *Y. enterocolitica* was treated with 100% NEO water (Fig. 1). *ompR* encodes a transcriptional regulatory protein that is related to the bacterium's sensitivity to high osmolarity, stresses from heat and low pH, and macrophage phagocytosis (32, 33). *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 (35–37). According to Pierson and Falkow (35), sequences homologous to *ail* 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 (35, 37, 69). The upregulation of these two genes highlighted the importance of establishing hurdle techniques to further reduce the number of cells that survive NEO water treatments.

The *ycfR* gene 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 (39). For *Salmonella*, this gene is known for its involvement in surface attachment and chlorine resistance (70). As shown in Fig. 1, its expression was significantly upregulated 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 (20, 71). Salazar et al. (70) found that the deletion of *ycfR* in *S.* Typhimurium significantly decreased the bacterium'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 (38, 45). Thus, the expression of *ybiJ*, *cysD*, *cysJ*, and *osmB* in *E. coli* O157:H7 and the expression of the *cysK*, *yfhP*, *nifS*, and *nifU* genes in *Salmonella* were monitored as well. However, their expression was not significantly upregulated or downregulated 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 applied only to the particular strains tested. A 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, the presence of sublethally injured cells was confirmed by plating the treated cultures on nonselective (TSAYE) and selective (CIN) agar. 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 upregulation 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 reductions in pathogen levels. Among the different combinations, diluted NEO water treatment in combination with the 10-min 51°C heat treatment was the most efficient hurdle technology, resulting in a 7-log reduction in pathogens.

MATERIALS AND METHODS

Bacterial cultures. The 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 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 number 211768; BD Difco, Sparks, MD) supplemented with 10% glycerol (catalog number 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 number 210933; BD Difco, Sparks, MD) (TSBYE). The 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 incubating at 37 or 30°C for another 24 h.

NEO water preparation. The original undiluted NEO water was prepared by electrolyzing a 5% NaCl solution with a GenEon Instaflow generator (GenEon Technologies, San Antonio, TX). An FE20 FiveEasy instrument 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). A CN-21P kit (Hach, Chicago, IL) was used to monitor the free chlorine concentrations of the NEO water. The original NEO water had a pH value of 7.35 $\pm$ 0.11, an ORP value of 829.7 $\pm$ 4.5 mV, and a free chlorine concentration of 59.1 $\pm$ 0.1 mg/liter. 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.

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 a 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 a 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 number 211043; BD Difco, Sparks, MD) supplemented with 0.6% yeast extract (TSAYE).

To treat the pure cultures, 2.5 ml of each bacterial suspension was mixed with 7.5 ml each of the 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 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 number 218103; BD Difco, Sparks, MD). The surviving bacterial population was determined by plating two 100-μl aliquots of each serial dilution onto two TSAYE plates and an additional two 100-μl aliquots of each serial dilution onto selective agar. For this step, sorbitol MacConkey (SMAC; catalog number 279100; BD Difco), xylose lysine deoxycholate (XLD; catalog number 278850; BD Difco, Sparks, MD), and cefsulodin-irgasan-novobiocin (CIN; catalog number C5391, Hardy Diagnostics, Santa Maria, CA) agars were the selective media used for enumerating *E. coli* O157:H7, *S.* Enteritidis, and *Y. enterocolitica*, respectively. The plates were incubated at 37°C for *E. coli* O157:H7 and *Salmonella* and 30°C for *Yersinia* for 48 h. Colonies were counted after 24 h and were confirmed after 48 h.

Stress selection for NEO water-treated pathogens. To determine the stress levels used for further reducing the number of 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 cells treated only with NEO water 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%) (catalog number 48305-50G-F; Sigma-Aldrich, New Zealand), six pH values (3, 3.6, 4, 4.6, 5.3, and 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 with different concentrations of NaCl or bile salts or different pH values was prepared. To create different NaCl stress levels, 1%, 2%, 3%, 4%, 5%, or 6% NaCl was added to the liquid TSAYE before the plates were poured. Similar strategies were used for creating bile salt stresses; 0.05%, 0.1%, 0.15%, 0.2%, 0.3%, or 0.4% bile salts was added to the liquid TSAYE before the plates were poured. To create pH challenges, different volumes of 37% HCl were gradually added to the liquid agar to adjust the pH value; the final pH value of the liquid agar was 3, 3.6, 4, 4.6, 5.3, or 5.8. To expose healthy 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. The plates were then incubated for 48 h at 37°C for *E. coli* and *Salmonella* and 30°C for *Yersinia*. Colonies were counted after 24 h, and then the counts were confirmed after an additional 24-hour incubation. The colony counts obtained from 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 a water bath in an Eppendorf Thermomixer (model Thermomixer R; Brinkmann Instruments, NY) with agitation at 300 rpm. Cells were then plated on TSAYE and incubated for 48 h at 37°C for *E. coli* and *Salmonella* and 30°C for *Yersinia*. Tables S1, S2, and S3 in the supplemental material show the results obtained using healthy bacterial cells. The stress levels at which no significant difference in the populations between the treated and untreated cells was observed (using healthy overnight cultures) were selected for the following studies. The stresses and stress levels used for evaluating the sensitivity of NEO water-treated cells are listed in Table 2.

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 above.

Transcription-level response 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 centrifuging 1 ml of each treated culture at 10,000 × *g* for 5 min at 4°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 an RNeasy minikit following the instructions in the manufacturer's manual (Qiagen, Valencia, CA), and the genomic DNA (gDNA) was removed using the gDNA Wipeout buffer from the 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 was also checked by running regular PCRs in order to make sure that there was no DNA contamination in the RNA samples. The 16S rRNA-specific primers listed in Table 4 and the AccuStart II PCR Supermix (2×) from the PCR kit (Quanta BioSciences, Beverly, MA) were used. In each 0.1-ml 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 (2×), and 6 μl of nuclease-free water were mixed. The PCR was carried out in an Applied Biosystems Veriti 96-well Fast thermal cycler (Life Technologies, USA). The reaction process

TABLE 4 Genes and primer sets used in the real-time RT-PCR

Target	Gene	Function, related stress	Primer	Sequence (5′ to 3′)	Primer efficiency (%)	Amplicon size (bp)	Reference(s) or source
All	16S rRNA	Reference gene	16S_F 16S_R	CGATCCCTAGCTGGTCTGAG GTGCAATATTCCCCACTGCT	*Yersinia*, 94; *E. coli*, 94; *Salmonella*, 93	93	72
Y. enterocolitica	*gsrA*	Serine endoprotease, environmental stresses	F_gsrA R_gsrA	GACGGTTCTCCGTTCCAAGG CACGGAAATCCTGCTTGCTG	91	85	31, this study
	ompR	Transcriptional regulator, environmental stresses	F_ompR R_ompR	TGCTCGACCTGATGTTACCG CACCCTTTGCCGTCACCATA	93	102	32, 33, this study
	ail	Adhesion and invasion, serum stress	F_ail R_ail	AGCCTTTATGGATTACTGGGGG CCCGTATGCCATTGACGTCTT	94	96	35, this study
	rpoS	Polymerase sigma factor, external stresses	F_rpoS R_rpoS	CAGAACGCGGTTTCCGTTTC CAGACGGATGGTACGGGTTT	95	95	34, this study
E. coli O157:H7	*ycfR*	Outer membrane protein, chlorine stress	EC_F_ycfR EC_R_ycfR	GTCATTTGCCAGCTTTGCGG AGATTTGTCCCCGCGTTAGC	93	93	38, 39, this study
	ybiJ	Putative periplasmic protein, iron metabolism	EC_F_ybiJ EC_R_ybiJ	TTGCTGCTATGGCTCTTTCA GAAACCACGCCGATTTTATT	96	100	38, 40, this study
	cysD	Cysteine biosynthesis, oxidative stress	EC_F_cysD EC_R_cysD	GCCAGATATCCTGCTCGGTC TGGCACAACTATAACGGGCA	93	85	38, 41, this study
	cysJ	Sulfite reductase, oxidative stress	EC_F_cysJ EC_R_cysJ	GTGTTTCACTGCGGGTAAGC TGAACGAAGCGCTACAGTGG	91	90	38, 42, this study
	osmB	Osmotic stress-inducible protein, multiple stresses	EC_F_osmB EC_R_osmB	TACCCAACGTACTGCCATCG TCTAAACGGGACCGCAACAC	92	85	38, 43, 44, this study
S. Enteritidis	*ycfR*	Outer membrane protein, chlorine stress	F_ycfR R_ycfR	ACGCCAGAAGGTCAACAGAA GGGCCGGTAACAGAGGTAA	94	134	45, 70
	cysK	Cysteine synthase A, oxidative stress	F_cysK R_cysK	CGCTATTCAGAAAGCCGAAG CATCGGTGTCTTCCCAGATT	99	121	45, 46
	yfhP	Transcriptional regulator IScR, oxidative stress	F_yfhP R_yfhP	TTACCTTAGGCGAGCTGGTG GCGCGTAATTTAACGTCGAT	91	104	45, 47
	nifS	Cysteine desulfurase, oxidative stress	F_nifS R_nifS	ATCGCGAAAGAAGAGATGGA TCGCCGTTCAGGTAAACTTC	95	123	45, 48
	nifU	Fe-S cluster assembly protein, oxidative stress	F_nifU R_nifU	AACGACGATAACGTGGGAAG GCAGCCGTAAGTCTTGAAGC	92	136	45, 49

started with an initial denaturation for 3 min at 94°C, and then this was followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and 1 min at 72°C. The PCR products were then held at 4°C. The presence of potential DNA contamination was checked by running the PCR products in a 2% agarose gel for 40 min. No DNA contamination was found in the RNA samples.

For the gene expression evaluation, a two-step RT-qPCR was conducted, with the cDNA being synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). SYBR real-time PCR was carried out on an ABI 7500 system (Applied Biosystems, Foster City, CA). In each reaction tube, there were 12.5 μl of a 2× reaction mix of PerfeCTa SYBR green SuperMix (Quanta BioScience Inc., Gaithersburg, MD), 0.3 μl each of the forward and reverse primers, 3 μl of cDNA templates, and Milli-Q water (total volume, 25 μl). Real-time PCR was conducted following the program of an initial denaturing period at 90°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The reference gene used was 16S rRNA (72). The gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ threshold cycle (C_T) method. Data were presented as the fold change in gene expression normalized to the expression of the reference gene and compared to the expression of the control, $\Delta\Delta C_T = (C_{T\text{target}} - C_{T\text{reference}})_{\text{test}} - (C_{T\text{target}} - C_{T\text{reference}})_{\text{control}}$, where $C_{T\text{target}}$ is the C_T value for the target gene and $C_{T\text{reference}}$ is the C_T value for the reference gene (73). A $\log_2$ fold change of ≥2 was considered significant (74). The genes selected for each species were based on previous reports (31, 33–35, 38–45, 74). The primers used for the real-time PCR are listed in Table 4. The primer sets specific for *gsrA*, *ompR*, *ail*, *rpoS*, *ycfR*, *ybiJ*, *cysD*, *cysJ*, and *osmB* were designed using the Primer3Plus program (available at http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and verified using the Basic Local Alignment Search Tool (BLAST). The primer efficiency was also calculated on the basis of methods described previously (66, 75, 76). Three trials were conducted for this part of the study, with three biological replicates being used 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 C_T value used for analysis.

Statistical analysis. Three independent trials were conducted for every experiment. For the 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, a value of 1.62 CFU/ml was used for all samples in which bacteria were not detected (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 treatment 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 an SPSS Statistics software package (SPSS Statistics for Windows, version 19.0.0; SPSS Inc., Chicago, IL).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01066-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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