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RESEARCH ARTICLE

Antibacterial Activity of Neutral Electrolyzed Water Against *Listeria monocytogenes*

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ABSTRACT

Inadequate cleaning and disinfection processes in the food industry are major causes of foodborne infections. Therefore, an effective cleaning process is essential for food production areas. Traditional disinfectants in the food industry have side effects like corrosion, irritation, toxicity, and residue problems. Therefore, electrolyzed water has become increasingly popular as an effective disinfectant in the food industry. Neutral electrolyzed water (NEW) contains hypochlorous acid and hypochlorite to inactivate pathogens effectively and has been proven to be stable. *Listeria monocytogenes*, a common foodborne pathogen, can be found throughout the food production chain at different points and conditions. This study examined the antimicrobial effect of NEW on *L. monocytogenes* at different concentrations, exposure times, and in vitro conditions. To assess the effectiveness of NEW, 0.1 %, 5 %, 10 %, 50 %, and 90 % concentrations were prepared, and the inactivation treatment was carried out for 30, 60, 90, and 120 min at room temperature. The results showed that at concentrations of 10 %, 50 %, and 90 %, the disinfectant significantly reduced the number of *L. monocytogenes* to undetectable levels within 30 minutes. However, about 1 log10 reduction was achieved at 0.1 % concentration in all applied times. This study reveals that NEW is highly effective for inactivating *L. monocytogenes* and should be used more in the food industry. In conclusion that NEW is a safe, environmentally friendly, and not harmful to food quality alternative to traditional disinfectants in the food industry, and recommended its increased use.

Keywords: Antimicrobial activity, *Listeria monocytogenes,* neutral electrolyzed water

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Nötral Elektrolize Suyun *Listeria monocytogenes*'e Karşı Antibakteriyel Etkinliği

ÖZ

Gıda kaynaklı enfeksiyonların başlıca nedenleri arasında gıda endüstrisindeki yetersiz temizlik ve dezenfeksiyon işlemleri yer almaktadır. Bu nedenle, gıda üreten işyerlerinde yerinde etkin temizlik çok önemlidir. Gıda endüstrisinde kullanılan geleneksel dezenfektanların tahriş meydana getirme, korozyon, toksisite ve kalıntı oluşturma gibi yan etkileri bulunmaktadır. Bu nedenle elektrolize su, gıda endüstrisinde etkin bir dezenfektan olarak popülerlik kazanmıştır. Nötral elektrolize su (NES), patojenleri etkili bir şekilde inaktive etmek için hipokloröz asit ile hipoklorit içermekte olup, ayrıca NES'in kararlı olduğu da kanıtlanmıştır. Gıda kaynaklı yaygın bir patojen olan *Listeria monocytogenes*, gıda üretim zinciri boyunca farklı noktalarda ve koşullarda bulunmaktadır. Bu çalışmada, NES'in farklı konsantrasyonlarda, farklı sürelerde ve in vitro koşullarda *L. monocytogenes* üzerindeki antimikrobiyal etkisi incelenmiştir. NES'in etkinliğini değerlendirmek için %0.1, %5, %10, %50 ve %90'lık konsantrasyonlar hazırlanarak inaktivasyon işlemi oda sıcaklığında 30, 60, 90, 120 dakikalık sürelerde gerçekleştirilmiştir. Elde edilen sonuçlar, dezenfektanın %10, %50 ve %90 konsantrasyonlarının *L. monocytogenes* sayısını 30 dakika içinde tespit edilemeyen seviyelere düşürdüğünü göstermiştir. Buna karşın, %0.1 konsantrasyonda ise tüm uygulanan sürelerde yalnızca 1 log10 azalma sağlanmıştır. Bu çalışma, NES'in *L. monocytogenes*'i inaktive etmek için oldukça etkili olduğunu ve gıda endüstrisinde daha fazla kullanılması gerektiğini ortaya koymaktadır. Sonuıç olarak, NES' in gıda endüstrisindeki geleneksel dezenfektanlara karşı güvenli, çevre dostu ve gıda kalitesine zarar vermeyen bir alternatif olduğu saptanmış olup, kullanımının artırılmasını tavsiye edilmektedir.

Anahtar Kelimeler: Antimikrobiyal aktivite, *Listeria monocytogenes*, nötral elektrolize su

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INTRODUCTION

Foodborne illnesses are a major concern in the food industry. One of the main causes of foodborne infections is inadequate cleaning and disinfection in the food industry, thus proper cleaning is essential. Disinfection procedures aim to eliminate pathogens and spoilage microorganisms (Mısırlı and Aydin 2011; Pedreira et al. 2021; Yuan et al. 2021).

Disinfectants used in the food industry (chlorine compounds, organic acids, quaternary ammonium compounds, hydrogen peroxide, peroxides, amphoteric compounds, etc.) can have high costs and side effects such as corrosive defects, irritation, toxicity, and residue problems (Mısırlı and Aydin 2011; Deshmukh et al. 2019; Carrascosa et al. 2021; Yuan et al. 2021).

In recent years, electrolyzed water (EW) has been increasingly used as an effective disinfectant in the food industry (Zhao et al. 2021). EW is produced by the electrolysis of diluted salt solution (tap water and NaCl solution) in an electrolytic cell. The electrolytic cell has two sides (anode and cathode) separated by a diaphragm (Chen and Wang, 2022). Acid-electrolyzed water (AEW) produced at the anode side has low pH and high oxidation-reduction potential (Xuan and Ling 2019; Iram et al. 2021). AEW has a strong bactericidal effect but can be corrosive to processing equipment and irritating to hands (Iram et al. 2021; Jee and Ha 2021). Alkaline electrolyzed water (AIEW), or basic electrolyzed water (BEW) generated on the cathode side, has a high pH value and a low redox potential (Xuan and Ling 2019). It is commonly used as a pre-treating and cleaning agent (Xie et al. 2012).

Neutral electrolyzed water (NEW) can be produced by using several methods. One of them is (1) mixing acidic and basic electrolyzed water and others, (2) using the electrolytic system with no separation membrane, and (3) redirecting the AEW to the cathode side (Ding and Liao 2019; Xuan and Ling 2019; Iram et al. 2021).

The pH of NEW is neutral and contains two important components to inactivate pathogens: hypochlorous acid (HOCl), the most effective chlorine compound, and hypochlorite (ClO). (Cui et al. 2009; Shiroodi et al. 2021). NEW is more stable than other electrolyzed water solutions because of reduced chlorine loss (Deza et al. 2005). Compared with other traditional agents, it has some advantages: strong bactericidal and antimicrobial effects, harmless to humans and the environment, low cost, easy to use, non-corrosive, and non-irritating structure (Chen and Wang 2022).

Many studies have shown the effectiveness of NEW against various foodborne pathogens and other microbiological contaminants on food contact surfaces (Al-Qadiri et al. 2019; Possas et al. 2021; Jee and Ha 2023) and foods (Medina-Gudiño et al. 2020; Sheng et al. 2020; Torres-Rosales at al. 2020; Hamidi et al. 2021).

Listeria monocytogenes can be found at different contact surfaces and conditions in the food production chain (Coban et al. 2019; Kara and Aslan 2021). It leads to major foodborne outbreaks and listeriosis with high mortality rates (20-30 %) (Martínez-Suárez et al. 2016; Şahin and Ayyıldız 2020). Listeriosis leads to severe sepsis, meningitis, meningoencephalitis, abortions, stillbirth, and death (Pogreba-Brown et al., 2022).

Since *L. monocytogenes* is considered one of the most important foodborne pathogens in the food industry, this current study aimed to investigate the in vitro effect of different concentrations of NEW at different periods on the viability of *L. monocytogenes*.

MATERIALS and METHODS

Bacterial strains and preparation of inoculum

NEW was obtained from commercial EW systems (Danish Clean Water, T-20 Series, Denmark). pH values and free active chlorine values of NEW were 8.5 and 499.42 mg.l-1, respectively. Concentrations of 0.1 %, 5 %, 10 %, 50 %, and 90 % of NEW were prepared using sterile deionized water immediately prior to challenge testing to demonstrate the minimum effective dose for *L. monocytogenes*. Sterile tap water was used as a control.

L. monocytogenes ATCC 19115 reference strain was used in this study's inactivation test (assay). *L. monocytogenes* was cultured on Tryptic Soy Agar (TSA; Oxoid, CM0131, Basingstoke, UK) plates at 37 °C for 24 h. After incubation, the bacterial cultures were transferred to a sterile tube containing 9 ml of sterile saline (0.9 % NaCI), and the suspensions were adjusted to a 0.5 McFarland (McF) of (approx. 1.5 x 10^8 CFU/ml). 1 ml suspensions were cultured on TSA plates at 37 °C for 24 h to verify the McF value.

In vitro microbial challenge (assay)

One ml of each bacterial suspension was added to the test solution containing a 9 ml mixture of NEW and deionized water. In addition, as a control group, 1 ml of each bacterial suspension was added to 9 ml of sterile tap water. All tubes were vortexed and left at room temperature (21-22 °C) for 10, 30, 60, and 90 min inactivation treatment. At the end of the treatment period, 1 ml of each sample was transferred to 9 ml of neutralizing solution (Phosphate buffered saline, Oxoid, BR0014G) and left at room temperature for 5 min to stop the activity of NEW. 0.1 % peptone water (Oxoid, LP0049) was used for serial dilutions. Each serial dilution representing the test group was plated onto TSA plates and incubated at 37 °C for 24 h. Colonies on TSA plates were counted. All the experiments were performed in duplicate (TSE EN 1276, 2019).

1 ml of each bacterial suspension was added to the test solution containing a 9 ml mixture of NEW and deionized water. At the end of the treatment, 1 ml of each mixture sample was transferred to a tube containing 9 ml of neutralizing solution, then vortexed and incubated for 5 min at room temperature to stop the activity of NEW.

Statistical analyses

In calculating the study's sample size, Power was determined by taking at least 80 % and a Type-1 error of 5 % for each variable. Shapiro-Wilk (n<50) and Skewness-Kurtosis tests were used to check whether the continuous measurements in the study were normally distributed, and Parametric tests were applied because the measurements were normally distributed. "ANOVA" was used in "periods (minutes)" and "between groups" comparisons, with "two-factor, one-factor repeated" measurements. The "Bonferroni posthoc test" was used to determine the periods and groups that account for the difference. The statistical significance level was assumed to be p<0.05 in the calculations, and the SPSS (IBM SPSS for Windows, ver.26) statistical package program was used for the analyses.

RESULTS

Our study examined the antimicrobial effect of NEW on *L. monocytogenes*, an important foodborne pathogen. The effectiveness of the disinfectant was measured at different dosages and exposure times.

Disinfectant concentrations of 0.1 % (4.99 mg.l-1), 5 % (24.97 mg.l-1), 10 % (49.94 mg.l-1), 50 % (249.71 mg.l-1), and 90 % (449.48 mg.l-1) were used to assess the efficacy of NEW on *L. monocytogenes*. The effectiveness of the disinfectant is affected by the level of free active chlorine. The level of free active chlorine in NEW used in our study is 499.42 mg.l-1. The free active chlorine level at each dilution level was calculated separately, and the disinfection efficacy was determined. The effect of NEW at different concentrations and times on the surviving population of *L. monocytogenes* is shown in Table 1. Table 1 shows two-way comparison results of the concentration measurements "according to Group and Period (minutes)".

According to the results of the study, a concentration of 0.1 % was not effective; a decrease of around 1 log10 CFU/ml was achieved in all minutes. The disinfectant was observed to have a strong effect at the 90th and 120th min at 5 % concentration. No statistically significant difference between the minute measurement periods was observed in the 0.1% concentration group (p=0.102). As a result; There was no significant change in the time-dependent measurements in this group. Similar; No statistically significant difference was observed between the minute measurement periods in the 5% concentration and control groups (p=0.113 and p=0.128) There was no significant change in the time-dependent measures in these groups.

At concentrations of 10 %, 50 %, and 90 %, the number of *L. monocytogenes* decreased to undetectable limits – as determined by plating procedures – at any time, including 30 min of exposure to NEW. In the control samples, no significant reduction in bacterial counts was achieved after 30, 60, 90, and 120 min.

In the 30th, 60th, and 90th minute of the concentration measurement; A statistically significant difference between the groups was observed (p=0.001).The groups that make up the differences are indicated with lowercase letters. In this period, the control group was found to be different from all other groups with the highest value. Despite that, In the 120th minute period, No statistically significant difference was observed "according to the groups" (p=0.056).

In the current study, the efficacy of disinfectant containing 24.97 mg.l-1 was determined at 90 min, but when the chlorine level was 49.94 mg.l-1, it decreased to 30 min. With increasing disinfectant concentration, it was observed that NEW strongly affected *L. monocytogenes* even in the short period of contact time. According to our results, NEW was effective for the concentrations and periods.

Table 1. Surviving population (log10 CFU/ml) of *L. monocytogenes*

Time (min)		30 *Med±SD	60 Med±SD	90 Med±SD	120 Med±SD	**p.
Concentrations %	0.1 %	6.98±.01 b	6.67±.04 b	7.15±.02 b	7.22±.00	.102
	5 %	3.16±.01 c	1.95±.00 c	*ND c	ND	.113
	10 %	ND d	ND d	ND c	ND	.
	50 %	ND d	ND d	ND c	ND	.
	90 %	ND d	ND d	ND c	ND	.
	Control	7.23±.02 a	7.38±.01 a	7.41±.01 a	7.30±.00	.128
	***p.**	**.001**	**.001**	**.001**	.056	

*ND: Not detected of viable *L. monocytogenes* on TSA plates

Med, median; SD, standard deviation (The significance level of the difference between the concentration groups in the same period (a,b,c: shows different groups according to Bonferroni Post Hoc test)

** The significance level of the difference between periods in the same concentration group

DISCUSSION

Listeria spp., which can cause meningitis, miscarriage, and death, is an important microorganism for the food industry. *L. monocytogenes* can be found in food and the environment, especially in the food industry (Buchanan et al. 2004; Kara and Aslan 2021; Sepin and Pamuk 2021). A good cleaning and disinfection process should be done to protect from *Listeria* spp. in terms of food and public health (Pedreira et al. 2021). NEW is at the forefront of new-generation disinfectants due to their strong bactericidal effect as well as not harming the environment, people, and animals (Al-Qadiri et al. 2019; Chen and Wang 2022). NEW is an efficient disinfectant in the food industry (Huang et al. 2008; Cui et al. 2009).

NEW has been widely used in studies on the inactivation of *L. monocytogenes* in various foods and surfaces. Ovissipour et al. (2018) reported that AEW and NEW inactivation of *L. monocytogenes* in salmon fillets were more effective, even at different temperatures. NEW had less effect on salmon protein quality than AEW. The number of surviving *L. monocytogenes* in tomatoes washed with NEW containing 89 mg.l-1 chlorine was reduced to <1 log CFU after 5 min (Deza et al. 2003). Rivera-Garcia et al. (2019) analyzed the efficacy of NEW for *L. monocytogenes* on the eggshell surfaces and reported that a reduction of 2.18 log10 CFU/egg was achieved, and no color change was observed in the eggshells.

The number of surviving *L. monocytogenes* on disinfected surfaces (glass and stainless steel surfaces) using NEW with an active chlorine concentration of 63 mg.l-1was determined to be 0.18-0.19 log CFU ml^{-1} in the study by Deza et al. (2005). Using NEW (64.92 mg.l-1), a reduction in *L. monocytogenes* biofilms on glass and stainless steel surfaces was determined, and *L. monocytogenes* decreased to undetectable levels in 10 min (Arevalos-Sánchez et al. 2012). Possas et al. (2021) found a reduction from 1.55 to 5.22 log CFU/cm^2 was achieved on stainless steel coupons. These results were consistent with present studies reporting that NEWs could be used as an effective disinfectant to inactivate *L. monocytogenes* in the food industry. Although a large number of studies have been conducted with NEW, many of these studies aimed to determine the efficacy of NEW *in vivo*. However, our results are consistent with those studies, despite the fact that the studies were conducted in vivo makes it difficult to compare their efficacy. Although our study conducted in vitro offers an advantage in terms of the direct contact of the disinfectant with bacteria but also represents a disadvantage since any bacteria present must be destroyed. In washing studies, effectiveness is expected to be lower as the number of bacteria to be controlled spreads to the surface.

In a study with acidic (pH 2.3) and basic (pH 11.5) electrolyzed water, AEW was found to be effective in inactivating *L. monocytogenes*, while BEW was not found to be ineffective. AEW applications detected 4 log bacteria for 15 min at 4 °C, although no bacteria were detected for 10 min at 25 °C (Fabrizio and Cutter 2003). Ovissipour et al. (2015) analyzed the effectiveness of acidic and alkaline electrolyzed water in cell suspensions for 2, 4, and 6 min at room temperature. According to this study, acidic EW is more effective than alkaline EW in inactivating pure cell suspensions of *L. monocytogenes*. In another study, EW with an available chlorine concentration of 5-10 mg.l-1and a pH of 6 achieved a reduction of 5-6 log CFU/ml after 30, 60, and 90 sec (Rahman et al. 2012). Although the AEW effects are high, the use of NEW in the food industry is recommended due to the disadvantages (corrosion on surfaces, irritation on mucous membranes, etc.) of acidic electrolyzed water.

In the current study, NEW was effective at all disinfectant concentrations and time periods by completely inactivating *L. monocytogenes*, while *L. monocytogenes* numbers survived without reduction in tap water control groups. In the present study, a concentration of 0.1 % NEW was ineffective in reducing *L. monocytogenes*, while concentrations of 10 %, 50 %, and 90 % reduced it to undetectable limits. The results of this study are consistent with previous studies confirming that NEW has potent antimicrobial activity against *L. monocytogenes* in vitro (as a pure culture). Deza et al. (2005) found that in pure cultures of *L. monocytogenes*, about 7 logs were reduced after 5 min exposure to NEW. In another study, *L. monocytogenes* was exposed to NEW (pH 6.8 and Cl 46 mg.l-1) for 30 seconds and under in vitro conditions. After treatment, a 6.1 log10 CFU/ml reduction was determined (Rivera-Garcia et al. 2019). Hamidi et al. (2021) reported that *L. monocytogenes* were completely inactivated by NEW in pure culture at concentrations of 50, 100, and 200 µg/ml and in 2 min contact time. Another in vitro assay reported that *L. monocytogenes* levels decreased to less than 3 log CFU/ml (Torres-Rosales et al. 2020). In vitro studies demonstrated that NEW could reduce the bacterial load and exerts a bactericidal effect on *L. monocytogenes*.

The effectiveness of the NEW disinfectant varies depending on contact time and concentration. In the current study, the effectiveness of a 25 µg/kg disinfectant was determined after 90 min, but it decreased to 30 min with a chlorine level of 50 µg/kg. These results are consistent with previous studies that reported that disinfectant efficacy increased with increasing application time and concentration (Sheng et al., 2020; Hamidi et al., 2021).

CONCLUSIONS

In conclusion, this study found that neutral electrolyzed water is highly effective in the

conditional inactivation of *L. monocytogenes* in vitro. In addition, the antimicrobial activity of NEW against *L. monocytogenes* increases as the concentration and application time increase. *L. monocytogenes*, an important food industry, and public health pathogen, can be easily inactivated by NEW disinfectant applied at appropriate concentrations and contact times. The use of NEW, which is easy to use and, does not cause harm to the environment, people, and food quality, unlike traditional disinfectants in the food industry, should be increased. The efficacy of NEW against other microorganisms should be evaluated in new studies, particularly in the in vitro setting. In this way, the effectiveness of the disinfectant can be proven by further studies.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: GÇ, AA and GMB contributed to the project idea, design of the study. GMB and ÇSN performed the experiments, data analysis and wrote the final manuscript. GÇ and GMB analyzed the experimental data and discussed the research results. GMB performed literature search, drafted and wrote the first draft of the manuscript. GÇ and AA supervised the work and reviewed/edited the manuscript critically. All the authors have read and approved the finalized manuscript.

Ethics Committee Information: "This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules."

Explanation: We have presented as a poster at the 10th Balkan Congress of Microbiology/Microbiologia Balkanica (2017).

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