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EFFICACY OF HOMEMADE ELECTROLYZED WATER DISINFECTANT FOR INACTIVATION OF FOODBORNE PATHOGENS

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ABSTRACT

Electrolyzed water, or hypochlorous acid, is a non-corrosive chlorinated disinfectant increasingly embraced by public and household consumers. This could be attributed to its effectiveness, economical, and ease of manufacturing. However, the antimicrobial efficacy of homemade EW (HEW) prepared using economical and easy-to-use EW generators has not been experimentally evaluated. The present study evaluated HEW solutions prepared and applied using different household generators and modes of application, respectively. HEW solutions were evaluated by spotting bacteria-inoculated agar, spraying bacteria-inoculated agar, and dipping/suspending bacteria into HEW solutions before colony-enumeration plating. Blood lysis/coagulation and total available chlorine concentration were investigated for bleach-equivalent toxicity using animal erythrocytes and free-chlorine-testing strips, respectively. The statistical significance of HEW antibacterial efficacy was determined using Student's t-test ($P<0.05$). Qualitative antibacterial analysis of fresh HEW spots exhibited growth inhibition zones with reducing inhibition visibility as dilution increased. Viable-culture-count comparative analyses revealed that the dipping method posted more antibacterial efficacy than spraying ($\geq$~5-log vs.$\leq$~3-log cfu/mL, $P<0.05$), suggesting that the dipping method be used for subsequent investigations. Dosage and shelf-life analyses demonstrated that HEW efficacy could increase and reduce significantly with dilutions ($\geq$1/8 v/v) and time, respectively, and was completely abolished at dilutions$\geq$1/16 (v/v). There was a significant HEW efficacy difference ($P<0.05$) among different generators. Comparative toxicity assays revealed that HEW ($\leq$1-month-old), unlike bleach, possessed much lesser total chlorine (>1000X) and did not cause blood complications. The

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findings suggest the effectiveness, friendliness, and optimization conditions of HEW and that its production needs a standardized evaluation.

Keywords: Homemade electrolyzed water (HEW), Food, Hypochlorous acid (HOCl), Electrolyzed water generators, Disinfectant, Antimicrobial, Antibacterial, Pathogens, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* species, *Staphylococcus aureus*

1. INTRODUCTION

Disinfectants: Disinfectants are globally recognized cleaning agents with multiple purposes, including cleaning (1), antimicrobial sanitizing (1,2), air freshening (ready-to-use spray) (3), and deodorizing (ready-to-use spray) (3). Their applications to deter bacterial (2), fungal, and viral (2,4) transmission and contamination of public areas (5), including healthcare facilities (2,6) and food processing plants (2,7), are particularly immense amid disease-prevalent seasons, such as COVID-19 pandemic (5,8), caused by a deadly severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (9, 10).

Ingredients: Diverse disinfectants are classified by the bioactive reagent compositions, concentrations, and application targets (i.e., bacteria, fungi, and/or viruses) (11,12). Most disinfectants consist of one or multiple active/potent ingredients, such as acids (13), ethylenediaminetetraacetic acid (EDTA) (14), alcohol (15,16), essential oil (17), chlorinated compounds (18,19-21), reactive oxygen species (ROS), and/or quaternary ammonium (11,12) that could render viability-detrimental membrane distortion, protein deformation, DNA denaturation, or multiple damages (22). The chlorinated compound hypochlorite ion, commonly called bleach (4), is a widely used disinfectant (22), attributed to its availability (4), cost-accessibility (4,23), and >1-century effectiveness history (6). However, they, including bleach, are corrosive to the environment and humans, causing irritations to the eye, respiratory tract, and skin (24). Specifically, long-term exposure to hydrogen peroxide, bleach, ethanol, and quaternary ammonium is deemed detrimental to human respiratory systems, causing health complications, including asthma (5). Chang et al. (25) reported that disinfectant poisoning had increased during the beginning of the COVID-19 outbreak in 2020.

Advantages and disadvantages of disinfectants and the alternative hypochlorous acid: Besides hypochlorite ions, active chlorines of chlorinated disinfectants include mixtures of different levels of hydrochloric acid, hypochlorous acid, and chlorine gas, which are biologically potent antimicrobials (18-21). Of all disinfectant potent active ingredients, hydrogen peroxide (26), hypochlorite ion (26), and hypochlorous acid (27,28) are naturally generated in micromolar in humans (26) while undergoing regular oxidation-reduction reactions. The semicromolar bioactives are essential to biological inflammation (26,29,30) and amine and protein synthesis

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(27). Hypochlorous acid, generally called electrolyzed water, is deemed a non-corrosive, effective chlorinated disinfectant against microorganisms (2,24) of public health concerns, including foodborne *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* species, *Campylobacter coli*, hepatitis B virus (2,18,19,21,31), bio-contaminants in drinking water (32), hospital-acquired infectious agents (32), dental care-acquired infectious agents (32), and airborne coronaviruses (12,33), which the EPA List N has listed 36 hypochlorous acid-derived disinfectants (i.e., 24 hypochlorous acid derived disinfectant products in October 2020) with proven effectiveness against COVID-19 to date (i.e., 13 November 2023) (12,33). Specifically, these chlorinated compounds (i.e., hypochlorite ion and hypochlorous acid) are known to pose household bleach- and quaternary ammonium-comparable (34) detrimental damages to macromolecules (32), such as membrane lipids, protein (i.e., aggregation of protein) (28), and non-nuclear membrane-bound genetic materials (35), and enzymatic function (28) in which the EW hypochlorite ion is responsible for cell membrane permeability enabling hypochlorous acid entry (i.e., passive diffusion) and elicitation of metabolic potency, which renders the antimicrobial capability of EW (28,36-39). Samara et al. (24) noted that the EPA-approved COVID-19 bleach, alcohol, hydrogen peroxide, acid compound, and quaternary ammonium produced naturally or artificially are relatively unsafe compared to EW. At the same time, many other reports advocated its environment- and human-friendliness (1,21).

EW generation, antimicrobial mechanism, and optimization: FDA approved the use of EW as a contact surface disinfectant in food processing plants in 2017 (40). EW is increasingly embraced by various entities, including the food industry (21,41), healthcare facilities (42), and households, as an effective antimicrobial disinfectant, attributed to its broad-spectrum (19,32), human- (1,43), environment-safe (1,44), and ease-to-acquire (41,42,45,46) as compared to its counterpart, hypochlorite ion (i.e., corrosive bleach). EW is generated in an electrolysis chamber with reaction mixtures containing tap water and NaCl, in which the positive electrode converts Cl anions into collaboratively potent antimicrobial products, including different levels of hypochlorite ion and hypochlorous acid (2,21,47). EW users, including food processors (i.e., control microbial food quality and safety) (48), healthcare practitioners (i.e., tool disinfection) (49), and household members (12,33,45), can easily and readily self-generate EW on-site (42) on a need basis, using a commercially available portable electrolysis chamber (41,45), or acquire commercially available ready-to-use EW; thereby rendering EW application inclusiveness to all professions, attributed to its generator scalability, affordability, and mobility. EW can be applied in three forms, including acidic EW (AEW), basic EW (BEW), and neutralized EW (NEW) (1). Improved EW antimicrobial activity/efficacy (differential EW activity) is dictated by the NaCl concentration, shelf-storage, environmental pH (50,51), target of application, infectious agent targeted (i.e., bacteria) (52,53), available/free chlorine concentration (ACC/FCC, part per million, PPM), mode of application (i.e., spraying vs suspension/dipping) (53), an EW generator

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pre-determined oxidation/reduction potential (ORP) (45), and treatment duration (21,54,55). Additionally, combined hurdle conditions, such as thermal and EW, have exhibited synergistic effects against bacteria, including *E. coli* (56). In general, Gram-negative bacteria, including *Campylobacter* species (53), *E. coli*, and *Salmonella* species (48), have been consistently more susceptible than their Gram counterpart, such as *L. monocytogenes* (53).

Objective: The objective of this study was to evaluate homemade EW (HEW) for its commercially comparable antimicrobial activity (i.e., vs. bleach) against foodborne bacteria and human/environmental friendliness, attributed to HEW under experimentation and Zhang's bacterial strain-restricted exploration (45). HEW used in this study was prepared using easy-to-use, ready-to-use, quick-to-make, inexpensive electrolyzers, edible salt, and tap water. These HEW solutions were evaluated, for the first time, for their antibacterial and toxicity activities, with various testing parameters, such as the preparation conditions (i.e., NaCl concentration, pH, types of acidifier, generator types) and application mode (i.e., spraying vs dipping/suspension, treatment duration, shelf-life). Foodborne infectious agents of public concern, such as *E. coli*, *L. monocytogenes*, *Salmonella* sp, and *Staphylococcus aureus*, were used in this study. Commercial-free chlorine testing strips and animal erythrocytes were employed to examine HEW friendliness.

2. MATERIALS AND METHODS

2.1 Bacterial culture and storage conditions: Antibacterial assays were carried out with two groups of Gram-negative and Gram-positive bacteria. All ATCC strains of *E. coli* (25922), *Salmonella* sp. (14028, *S. enterica* subsp. enterica serovar Typhimurium strain), and *S. aureus* (25923) were Keener's generous contributions. The Muriana's pathogenic *L. monocytogenes* CW35 was acquired from ready-to-eat (RTE) retail frankfurters (57,58). Bacterial strains from thawed frozen stocks were cultured (1/100 v/v) in sterile fresh Brain Heart Infusion (BHI) broth (Difco; Becton-Dickinson, Franklin Lakes, NJ, USA), incubated (35 °C, 16-20 h) and sub-cultured once prior to experimental assays. Storage bacterial strains were kept in sterile fresh BHI broth supplemented with 10% sterile glycerol at -70 °C.

Table 1: List of test bacteria used in this study

Bacterium	Strain ID	Source
Escherichia coli	25922	ATCC
Salmonella sp.	14028	ATCC
Listeria monocytogenes	CW35	57
Staphylococcus aureus	25923	ATCC

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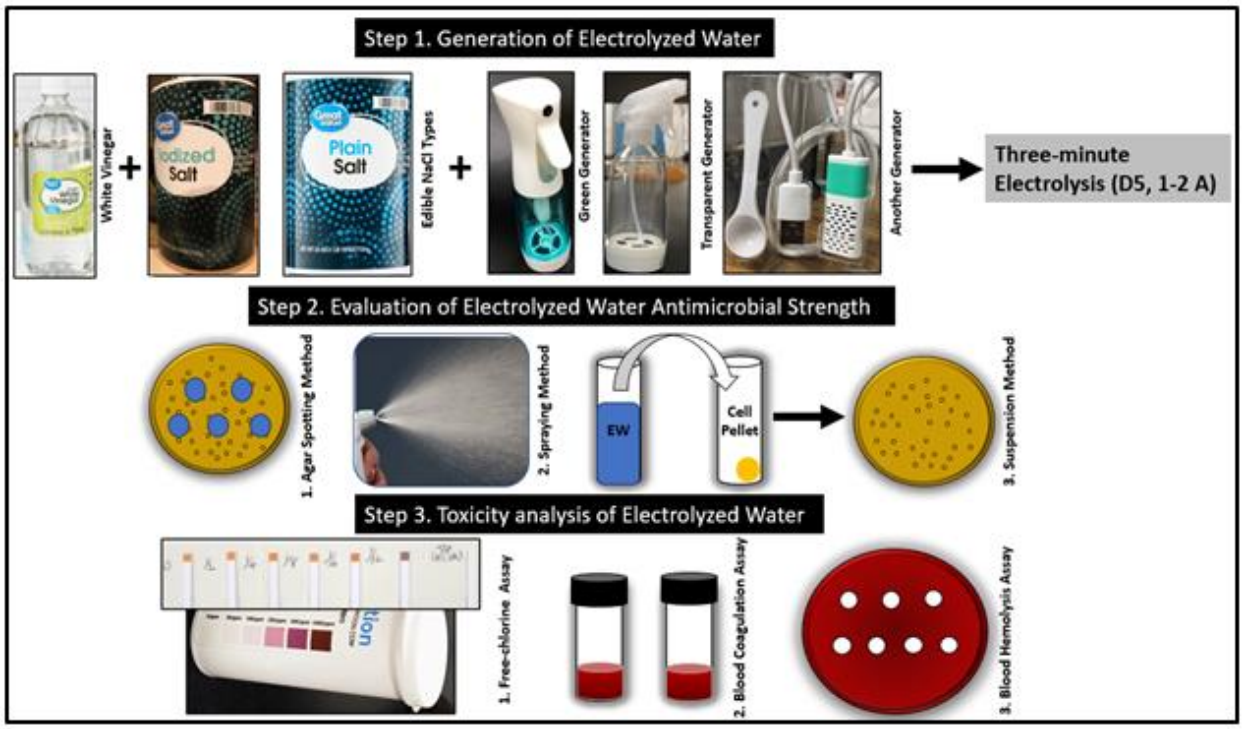
2.2 Homemade electrolyzed water generators: Portable household EW generators from various manufacturers were acquired through an online commercial source (Figure 1). All generators possess comparable physical features, including a polycarbonate- and/or polypropylene (PP)-made (PC) bottle (volume, 70-200 mL) with built-in spray and EW generating functions, DC5V-1A or 5V-2A power supply voltage, USB-C operating port/cable, NaCl pre-measured spoon, and operating and maintaining instructions.

Figure 1: EW testing scheme



2.3 Homemade electrolyzed Water (HEW): Fresh and shelf-stored (weekly) electrolyzed water was prepared in commercially available EW generators (i.e., the green, transparent, and another/hanging generators) (Figure 1) according to the manufacturers' (i.e., generation parameters) and Veasey and Muriana's (i.e., NaCl concentration) (59) instructions, with minor modifications. Briefly, a solution mixture containing edible NaCl (i.e., 23% of iodized or plain NaCl; unless otherwise specified, HEW was prepared with 23% NaCl) and tap water was acidified with edible vinegar (i.e., unless otherwise specified, HEW was acidified with vinegar) to a pH of ~5.5 (i.e., 23% NaCl, vinegar, pH ~5.5) and transferred into an EW generator prior to 3-min electrolysis followed by immediate applications (<10 min after generation, unless otherwise specified) or ambient storage in the dark before applications.

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2.4 Antimicrobial efficacy testing methods

2.4.1 Spraying method: HEW was prepared (i.e., unless otherwise specified, HEW was prepared in the green generator) and applied as instructed by the EW generator manufacturers' (i.e., 0.6% NaCl used for EW generation) and Veasey and Muriana (59) (i.e., 23% NaCl used for EW generation) with modifications. Briefly, tap water was supplemented with 0.6% or 23% NaCl and adjusted to specific pHs (i.e., using HCl or vinegar) prior to 3-min electrolysis. The control spray was prepared with sterile buffered peptone water (BPW) (0.1% *w/v*, pH 7.4), filter-sterilized solution containing tap water supplemented with 0.6% or 23% NaCl and adjusted pH (i.e., pH adjusted to 8.41 using NaOH or 5.52 using HCl or vinegar), or no spray. For spray application, various spray numbers (i.e., 0, 2, 4, 6 sprays) were applied to BHI agar plates pre-inoculated with fresh test bacteria (i.e., 10^7cfu/mL),~16 h cultures), incubated at room temperature for specific times, 2 – 10 min, and the inoculated agars were removed into stomacher bags, homogenized in the stomacher machine and serially diluted for viable cell enumeration after 48 h incubation at 35 °C.

2.4.2 Spot plating assay: Fresh bacterial culture (17 h, 10^7cfu/mL) was spread inoculated on agar media supplemented with Brain-Heart infusion (BHI, Difco, Detroit, MI, USA) or Luria Bertani (LB, Difco, Detroit, MI, USA) and spot treated with serially diluted concentrations of freshly homemade electrolyzed water (i.e., unless otherwise specified, HEW was prepared in the green generator), as previously described by Wang et al. (60). Inoculated and treated plates were incubated at 35 °C for 48 h, and visible inhibition indicative clearing zones were rankingly recorded. The control solutions were prepared using tap water only, tap water only with adjusted pH using HCl, vinegar or NaOH (i.e., to mimic the HEW pH that increased post-electrolysis), or sterile media broth (i.e., LB or BHI broth). All tap water-generated controls were filter-sterilized (0.2 µm, VWR ® Syringe Filters, Atlanta, GA, USA) prior to applications.

2.4.3 Suspension/dipping method: Briefly, tap water was supplemented with 0.6% or 23% NaCl and acidification (i.e., HCl, vinegar, or lactic acid) prior to three minutes of electrolysis (i.e., unless otherwise specified, HEW was prepared in the green generator). The control spray was prepared with sterile buffered peptone water (BPW, 0.1%*wt/v*, pH 7.4), filter-sterilized solution containing tap water supplemented with 23% NaCl and pH adjustment (i.e., pH adjusted to 8.41 using NaOH or 5.52 using HCl or vinegar), or no spray. HEW serial dilutions (i.e., 1/2, 1/4, 1/8, 1/16, 1/32) were prepared using tap water (i.e., non-filter-sterilized). Bacterial suspension (*Salmonella*, 10^7cfu/mL; *E. coli*, *L. monocytogenes*, *S. aureus*, 10^8cfu/mL) in HEW was incubated (i.e., 0, 30, 60, 90, 180 sec) at room temperature, processed (i.e., centrifugation at 11,000 RPM and resuspension in sterile BPW or further dilution in sterile BPW) to remove/eliminate HEW residues/activity prior to viable cell plating for enumeration.

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2.5 Cell enumeration assay: Bacterial cell suspensions were serially diluted prior to plating on BHI agar plates. Inoculated agar plates were incubated at 35 °C for 48 h or until visible colonies emerged.

2.6 Toxicity assays

2.6.1 Available/free chlorine concentration (ACC/FCC) assay

2.6.1.1 Strip-based assay: The commercially available chemical-treated paper strip of Bartovation (i.e., chlorine 0 – 1000 PPM test strips) was deployed to measure the ACC in HEW and bleach comparatively. The strip color formed at post-HEW or -bleach exposure was recorded in PPM as recommended by the product's color-PPM interpretative chart.

2.6.1.2 Automation-based assay: The experimental procedures of the Hach Free Chlorine TNTplus 866 Vial Test method 10231 (free chlorine) were followed with modifications. Briefly, HEW solution was serially diluted, subject to free-chlorine calorimetry (Hach DR 3900, select program 866) (Hach, Loveland, CO, USA) with the light shield (i.e., model LZV849) pre-installed, and the readings were recorded (i.e., in the maximum range of 0.05 – 2 mg/L of the test kit) and converted into HEW original concentrations in mg/L.

2.6.2 Blood coagulation assay: Reaction mixtures containing sheep erythrocytes and HEW or bleach dilutions were serially prepared (i.e., 1:1 serial dilutions in tap water; 1/2, 1/4, and 1/8) in sterile Eppendorf tubes and incubated at 35 °C or ambient temperature until blood coagulation and/or colorless formed; the reaction mixtures containing non-diluted blood and bleach solution exhibited visible clotting within 5 sec.

2.6.3 Hemolysis assay: The agar spotting method of Wang et al. (60) was adapted to analyze the effect of HEW, prepared with tap water supplemented with 23% NaCl, vinegar acidification (pH 5.53), and 3-min electrolysis, and bleach solutions on animal erythrocytes prepared with BHI agar supplemented with sheep blood (5% *v/v*). HEW or bleach dilutions (i.e., 1:1 serial dilutions; 0, 1/2, 1/4, 1/8, 1/16, and 1/32 dilutions in BPW) were distantly spotted (10 µL) on BHI blood agar and incubated at 35 °C until visible blood bleaching/clearing formed around dilution spots. The control was prepared using filter-sterilized tap water.

2.6.4 Significant variation statistics: Comparison tests within bacteria or between bacteria of the same Gram-property, different genera, or species yielded multiple mean bars with respective standard deviation (i.e., error bars).One-way analysis of variance (ANOVA) was used to call variation significance at $P<0.05$.

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3. RESULTS

3.1 HEW antibacterial efficacy

3.1.1 Qualitative efficacy of antibacterial HEW (HEW spotting dilutions). Both control solutions containing 0.6% NaCl-supplemented solution acidified using vinegar (pH 6.5) (Table 2) and 23% NaCl-supplemented solution modified with NaOH (pH 8.41) (Table 3) did not exhibit visible antimicrobial activity against all bacteria strains tested (Table 1). However, visible anti-listeria activity (i.e., visible colonies formed on spotted bacterial lawns) (Figure 2) was exclusively determined with vinegar-acidified control solution (pH 5.75) containing an increased amount of NaCl (23%) compared to 0.6% NaCl-supplemented solution acidified with vinegar (pH 5.75) (Table 4). Subsequently, bacterial lawns (i.e., all bacteria tested) spotted with HEW solution (23% NaCl, HCl, pH 5.52) exhibited antibacterial activity against all bacteria tested (Table 5), and its intensified activity was demonstrated in *L. monocytogenes* lawn spotted with HEW (Figure 2).

Table 2: Agar spotting with control solution (i.e., tap water supplemented with 0.6% NaCl, pH 6.5) and their antibacterial activity against various bacteria

	Control dilution[a]						
Bacterium	0	1/2	1/4	1/8	1/16	1/32	**- control[b]**
E. coli	—	—	—	—	—	—	—
Salmonella	—	—	—	—	—	—	—
L. monocytogenes	—	—	—	—	—	—	—
S. aureus	—	—	—	—	—	—	—

[a]HEW was prepared using tap water, 0.6% NaCl, vinegar (i.e., 0.6% NaCl,vinegar, pH 6.5), and 0-min electrolysis. The solution and dilutions were made in filter-sterilized tap water.Inhibition levels of acidified saline water against each bacterium are reported as +++, highly inhibitory; ++, moderately inhibitory; +, slightly inhibitory; -, no inhibition.

[b] Sterile BHI or LB broth, pH ~7, was used.

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Table 3: Agar spotting with control solution (i.e., tap water supplemented with 23% NaCl, pH 8.41) and their antibacterial activity against all bacterial strains used in this study

	Antimicrobial activity[a]			
Control dilution	*E. coli*	*Salmonella*	*L. monocytogenes*	*S. aureus*
0	—	—	—	—
1/2	—	—	—	—
1/4	—	—	—	—
1/8	—	—	—	—
1/16	—	—	—	—
- control	—	—	—	—

[a] Control solution was prepared using tap water, 23% NaCl, NaOH (i.e., 23% NaCl, NaOH, pH 8.41), and 0-min electrolysis. The solutions and dilutions were made in filter-sterilized tap water.

- control was prepared using filter-sterilized tap water.

Inhibition levels of acidified saline water against each bacterium are reported as +++, highly inhibitory; ++, moderately inhibitory; +, slightly inhibitory; -, no inhibition.

The tap water with pH adjusted to 5.75 using vinegar was filter-sterilized prior to application.

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Table 4: Agar spotting of control solution (i.e., tap water supplemented with 0.6% or 23% NaCl, vinegar, pH 5.75) and their antibacterial activity against *L. monocytogenes*

	[NaCl] and its antimicrobial activity	
Control dilution[a]	0.6% NaCl	23% NaCl
0	—	+++
1/2	—	+++
1/4	—	++
1/8	—	+
1/16	—	—
1/32	—	—
'-' control (pH 5.75)	—	—

[a] Control solution was prepared using tap water, 0.6% or 23% NaCl, vinegar (i.e., 0.6 or 23% NaCl, vinegar, pH 5.75), and 0-min electrolysis. The solution and tap water diluent were filter-sterilized.

'-' control was prepared using vinegar-acidified tap water (pH 5.75) followed by filter-sterilization.

Inhibition levels of acidified saline water against each bacterium are reported as +++, highly inhibitory; ++, moderately inhibitory; +, slightly inhibitory; -, no inhibition.

The tap water with pH adjusted to 5.75 using vinegar was filter-sterilized prior to application.

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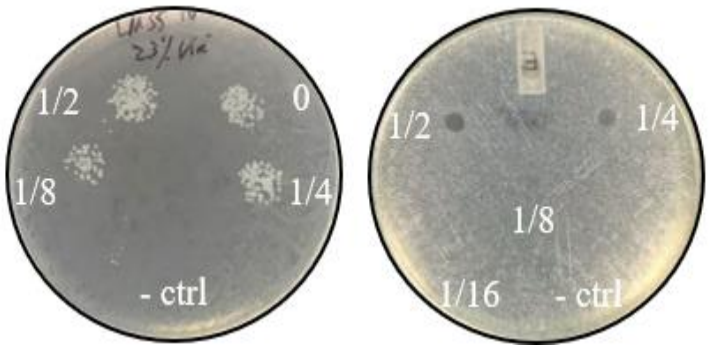
Table 5: Agar spotting of HEW solution (i.e., 23% NaCl, HCl, pH 5.52) and their antibacterial activity against bacteria

	Antimicrobial activity			
Control dilution	*E. coli*	*Salmonella*	*L. monocytogenes*	*S. aureus*
0	+++	+++	+++	+++
1/2	+++	+++	+++	+++
1/4	++	++	++	++
1/8	+	+	+	+
1/16	—	—	—	—
- control	—	—	—	—

[a] Control solution was prepared using tap water, 23% NaCl, HCl (23% NaCl, HCl, pH 5.52), and 3-min electrolysis in a green HEW generator.

- control was prepared using filter-sterilized tap water.

Inhibition levels of acidified saline water against each bacterium are reported as +++, highly inhibitory; ++, moderately inhibitory; +, slightly inhibitory; -, no inhibition.



A **B**

Figure 2: Control solution (i.e., containing 23% NaCl and vinegar acidification, pH 5.75) (A) and HEW solution (i.e., containing tap water, 23% NaCl, and HCl acidification, pH 5.52) (B) antibacterial effects on *L. monocytogenes* lawns. - control was prepared using

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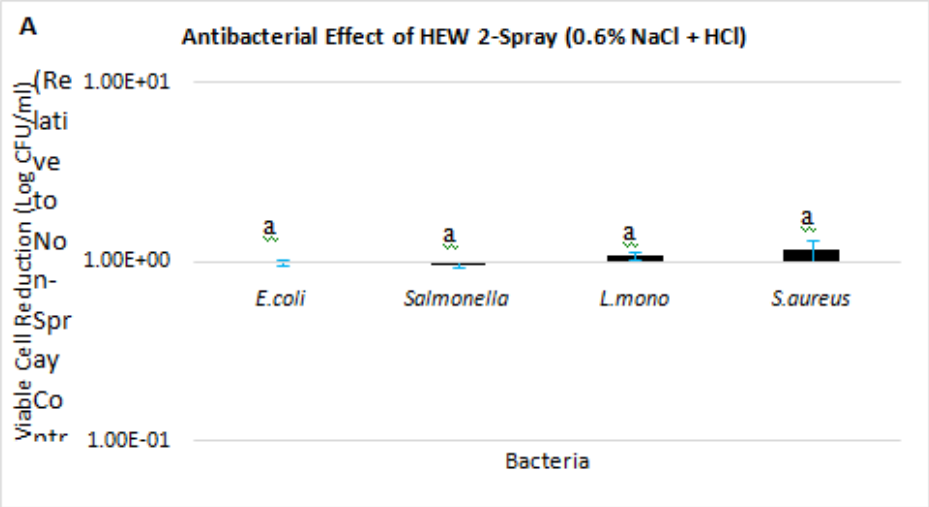
vinegar-acidified tap water (pH 5.75) followed by filter-sterilization (A) or filter-sterilized tap water (B).

3.1.2 Viable count efficacy (spraying with 0 and 2sprays of 100% HEW): The antimicrobial activity of HEW (pH 5.52) supplemented with different NaCl concentrations (i.e., 0.6% vs. 23%) and HCl acidification were evaluated using a spraying method (i.e., two-spray) Both exhibited no significant viable cell reduction (P>0.05) relative to controls, including non-spray control and non-electrolysis solution spray control (Figure 3). Relative to the control spray (i.e., 0 spray), increased spray numbers (i.e., 2, 4, and 6 sprays) did not pose immense viable count variation (i.e., <1-fold) (Figure 4).Subsequent spray examinations using an increased NaCl concentration (23%), vinegar acidification (pH 5.53), increased spray numbers (i.e., 2-6 sprays) (Figure 5), and increased incubation times (i.e., 2-10 min) (Figure 6) exhibited in consistent moderate HEW effectiveness (i.e., 3-log reduction, cfu/mL, against *E. coli*) (Figures 5,6) against bacteria strains tested (i.e., *E. coli*, ~5 min post-HEW spraying, were more susceptible relative to *L. monocytogenes*, 2-10 min post-HEW spraying) (Figures 5,6), and improved HEW activity (i.e., two sprays against *E. coli*) with 23% NaCl and vinegar acidification (pH 5.53) supplementations (Figures 3,5) as opposed to HEW supplemented with 23% NaCl and HCl acidification (pH 5.52).



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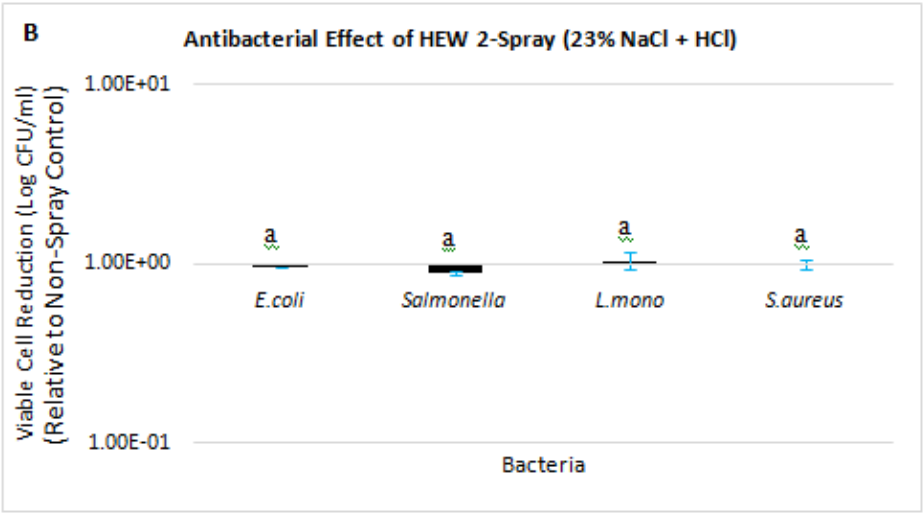

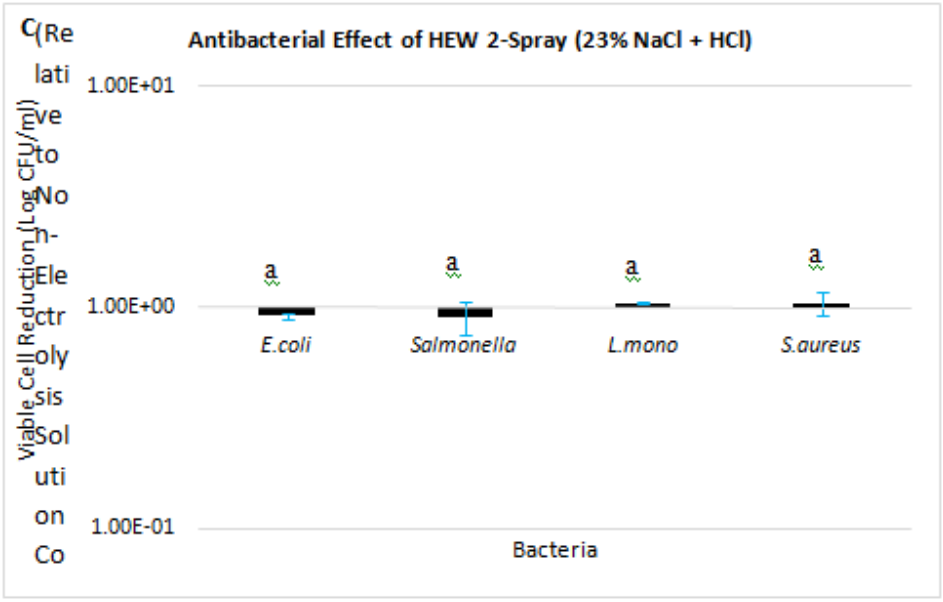

Figure 3: Two-spray antimicrobial activity of HEW, 0.6% NaCl + HCl (pH 5.52) (i.e., 0.6% NaCl, HCl, pH 5.52) (A) and 23% NaCl + HCl (pH 5.52) (i.e., 23% NaCl, HCl, pH 5.52) (B,C), relative to non-spray control (A,B) and non-electrolysis solution control (i.e., containing 23% NaCl + NaOH, pH 8.41) (C), respectively. All data represent the means of triplications. Means with the same lowercase letter are not significantly different; means with different lowercase letters are significantly different (P<0.05). Error bars indicate standard deviation from the mean.

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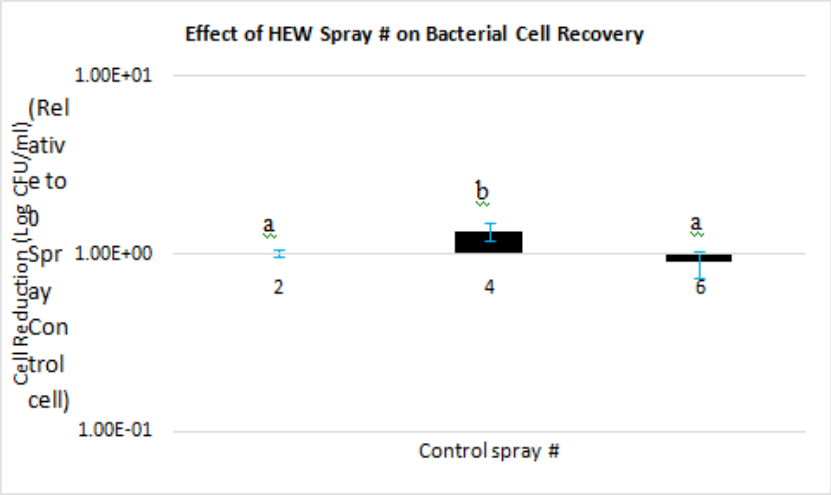

Figure 4: Effect of control spray (i.e., non-HEW sterile BPW) number on *E. coli* cell recovery. All data represent the means of triplications. Means with the same lowercase letter are not significantly different; means with different lowercase letters are significantly different (*P*<0.05). Error bars indicate standard deviation from the mean.

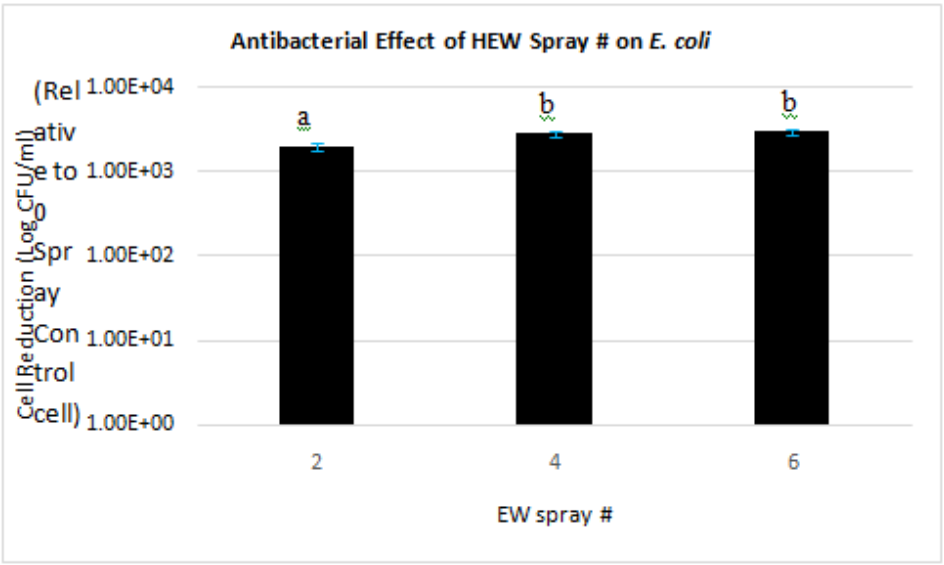

Figure 5: Antibacterial effect of different HEW (23% NaCl, vinegar, pH 5.53) spray numbers on *E.coli* (i.e., 10^8cfu/mL cells) post <5-min treatment incubation. All data represent the means of triplications. Means with the same lowercase letter are not significantly different; means with different lowercase letters are significantly different (*P*<0.05). Error bars indicate standard deviation from the mean.

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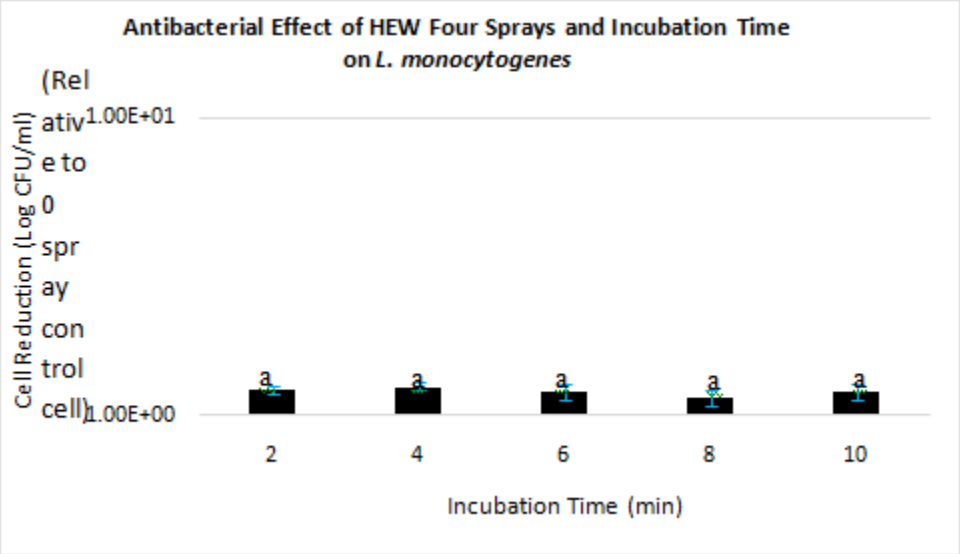

Figure 6: Antibacterial effect of HEW (23% NaCl, vinegar, pH 5.53) four sprays and different treatment incubation times (i.e., 2 – 10 min) on *L. monocytogenes* (i.e., 10^8cfu/mL cells). All data represent the means of triplications. Means with the same lowercase letter are not significantly different; means with different lowercase letters are significantly different (P<0.05). Error bars indicate standard deviation from the mean.

3.1.3 Viable count efficacy (dipping with 100% HEW): Further work examining the antibacterial activity of HEW prepared with 23% NaCl, vinegar acidification (i.e., pH 5.52, prior to electrolysis), and 3-min electrolysis in a HEW generator was conducted with a dipping/suspension method. Bacterial suspension in freshly made HEW (i.e., 0-10 min incubation at room temperature) exhibited greater than 4-log (*E. coli*, *Salmonella*, *L. monocytogenes*, *S. aureus*) – 7-log (i.e., *E. coli*, others not determined) viable cell reduction (cfu/mL) < 1 min (i.e., 0-time examination) – 10 min post-HEW-suspension exposure of *E. coli* (i.e., 5-log, <1 min; 7-log, 10 min), *Salmonella* (i.e., 4-log, <1 min), *L. monocytogenes* (i.e., 5-log, <1 min), and *S. aureus* (i.e., 5-log, <1 min) (Figure 7).

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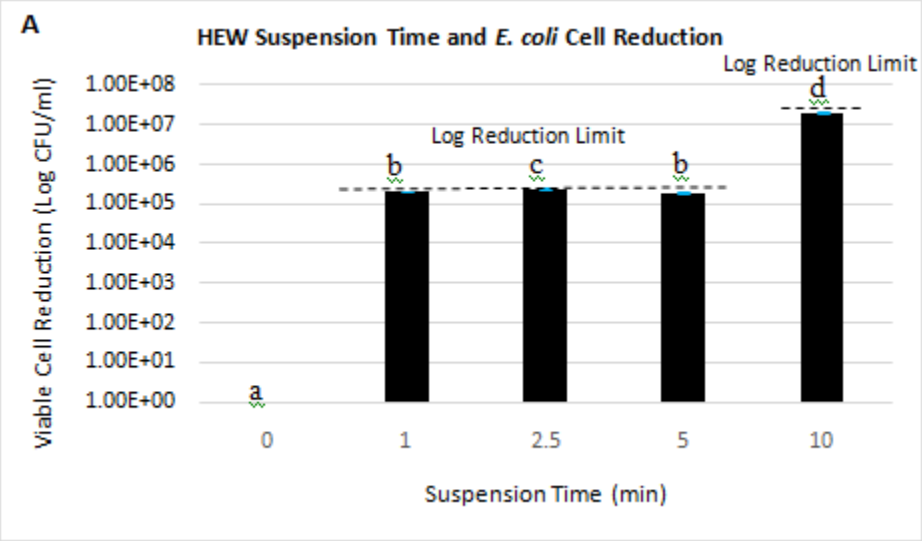

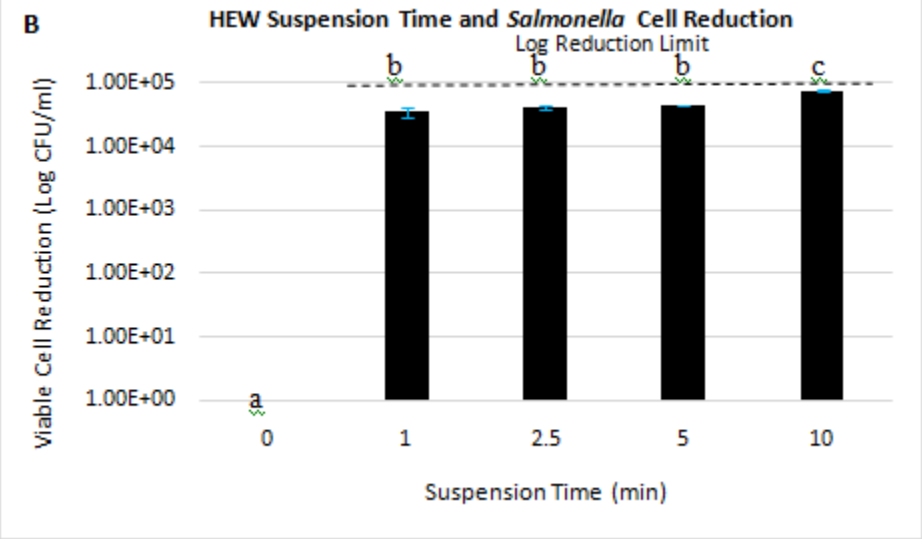

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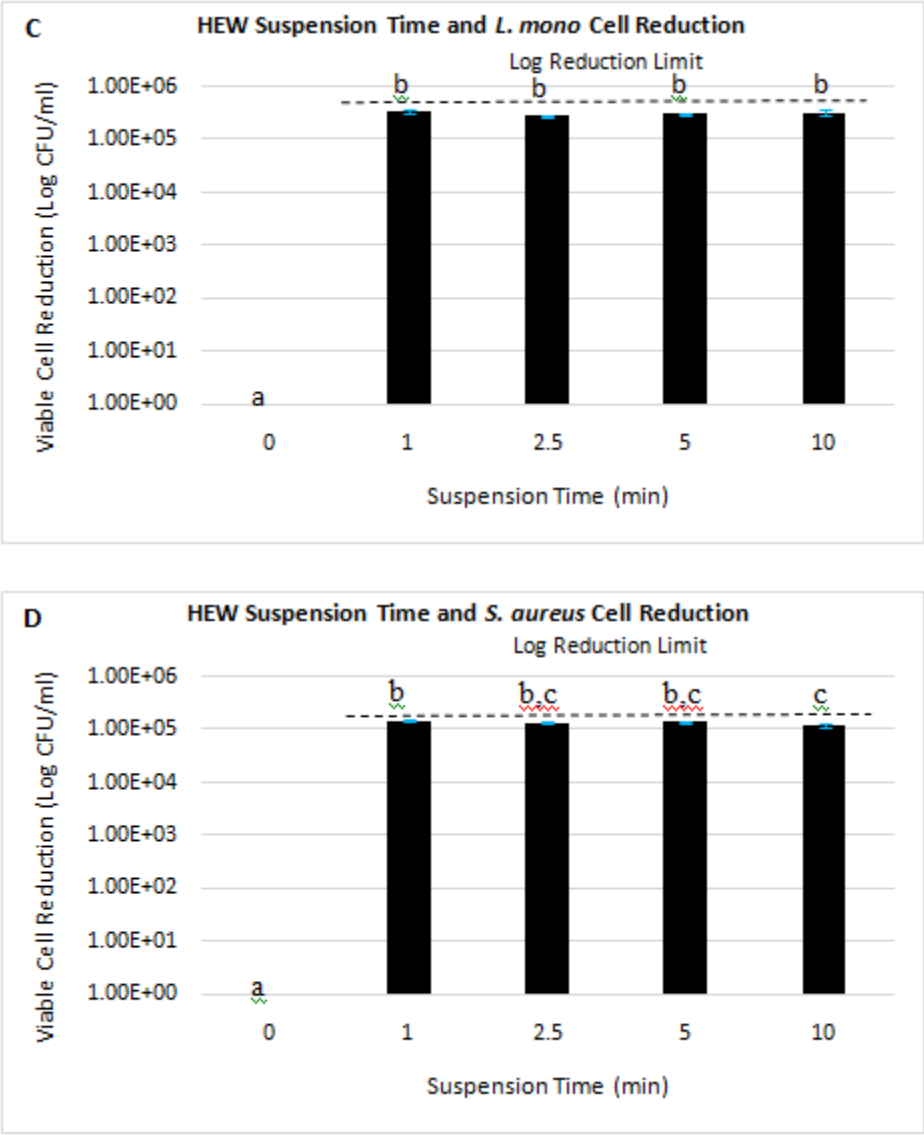

Figure 7: Antibacterial effect of HEW (23% NaCl, vinegar, pH5.52) dipping/suspension on *E. coli* (A), *Salmonella* (B), *L. monocytogenes* (C), and *S. aureus* (D). All data represent the means of triplications. Means with the same lowercase letter are not significantly different; means with different lowercase letters are significantly different ($P<0.05$). Error bars indicate standard deviation from the mean.

3.2 Bacteria resilience strength to HEW dilutions: Bacteria sensitivity (i.e., *L. monocytogens*) to HEW was examined by timely suspension of bacterial cells in serially diluted HEW solution (i.e., 1/2 – 1/32). AHEW dilution ≥ 1/8 (i.e., 1 mLHEW into 7 mL diluent/tap water) exclusively exhibited noticeable bacterial differential sensitivity between 0 and 180-sec post-suspension

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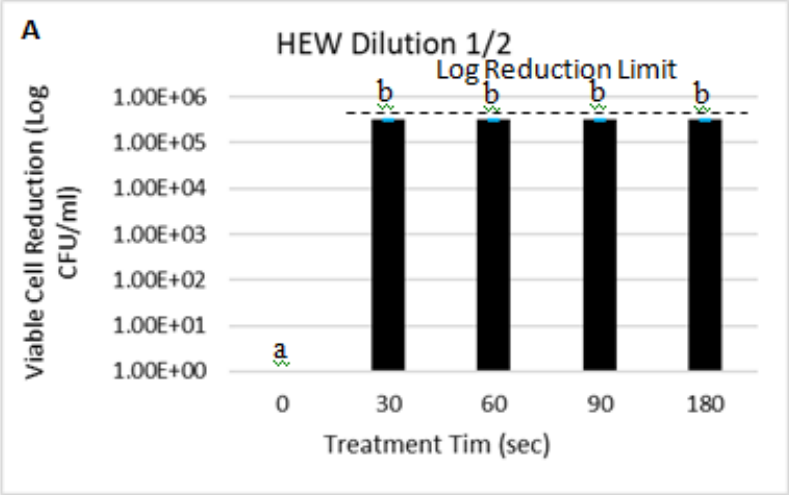
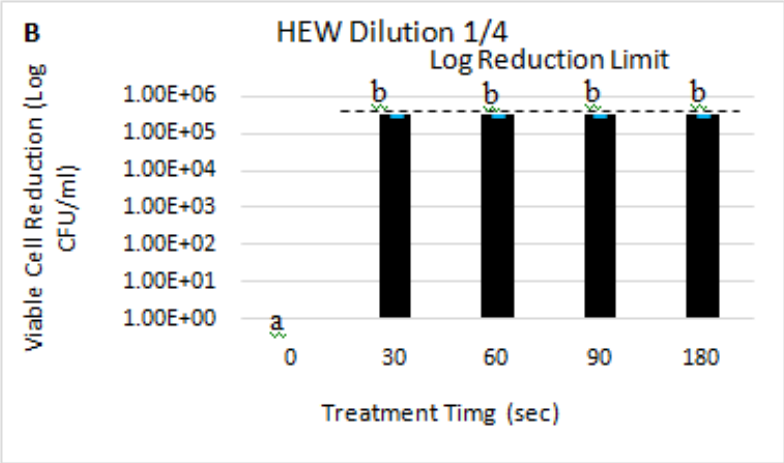
exposure as opposed to 1/2 or 1/4 HEW dilutions (i.e., no discernible sensitivity based on their viable counts at all incubation/treatment times tested, 30 – 180 s) (Figure 8). Subsequent resilience examination of various bacteria to 1/8 HEW and 180-sec incubation was conducted with Gram-negative and Gram-positive bacteria, in which both Gram-negative bacteria posed zero resilience (i.e., ≥5-log reduction, cfu/mL) to the combination of HEW dilution solution and suspension incubation (i.e., 1/8 dilution, 180-sec incubation) (Figure 9). Interestingly, the Gram-positive bacterium *S. aureus* was more susceptible (i.e., ~4-log vs ~2-log reduction, cfu/mL) than its Gram-similar kind (i.e., *L. monocytogenes*) (Figure 9).HEW full-strength (i.e., zero dilution HEW) antimicrobial activity reduced with increasing serial dilutions (i.e., 1/2 – 1/32 *v/v*), with 1/16 and 1/32 dilutions completely neutralized HEW activity.





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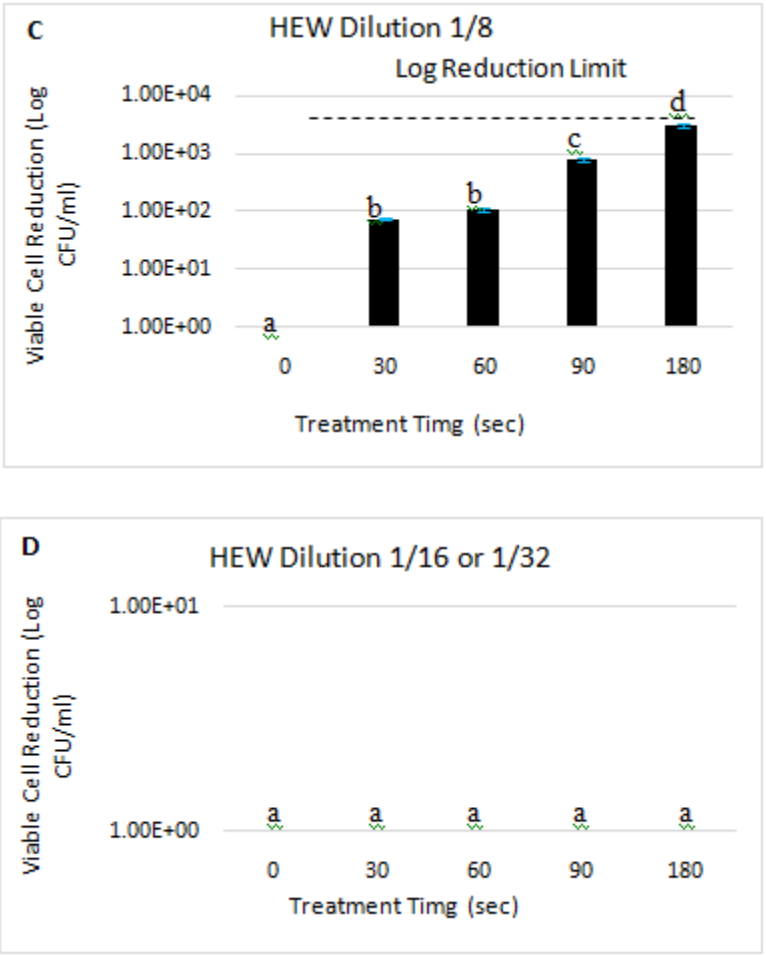

Figure 8: Effect of 1/2 (A), 1/4 (B), 1/8 (C), 1/16 (D), and 1/32 (D) HEW dilutions (i.e., 23% NaCl, vinegar, pH 5.53) on *L. monocytogenes* (i.e., 10^5cfu/mL cells) relative to non-treated control. Means with the same lowercase letter are not significantly different; means with different lowercase letters are significantly different (P<0.05). Error bars indicate standard deviation from the mean.

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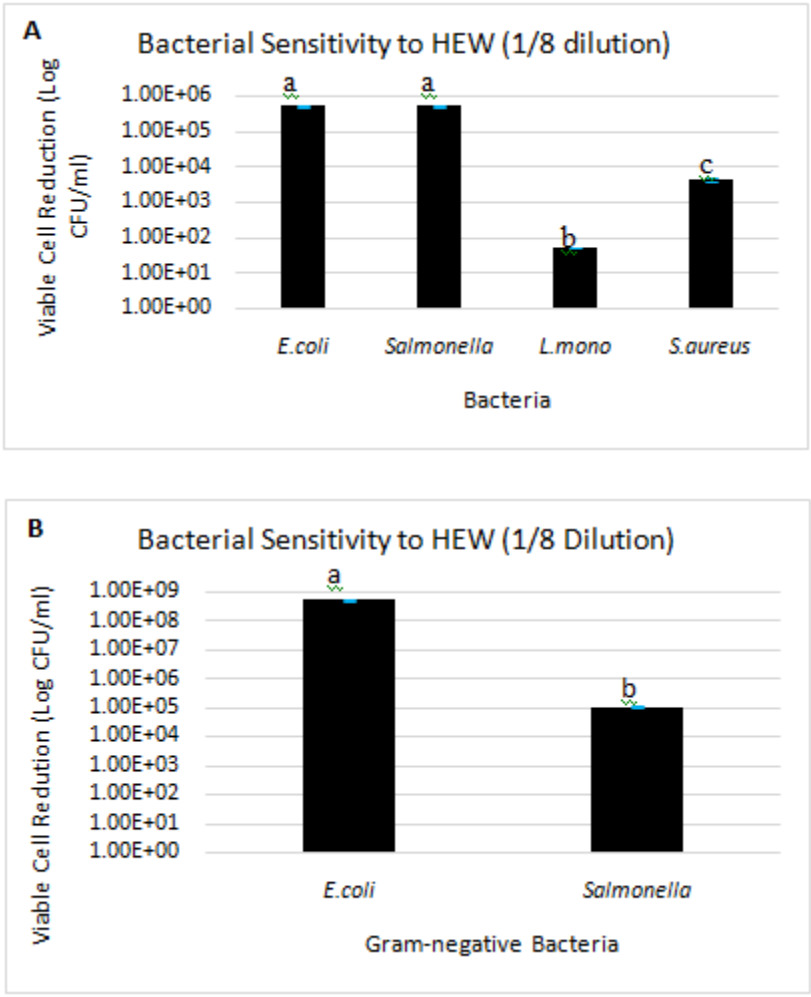

Figure 9: Differential bacteria sensitivity to HEW (1/8 dilution, 3-week-old). Treated bacterial cells were plated at 10^5cfu (i.e., *E. coli, Salmonella, L. monocytogenes,* and *S. aureus*) (A), 10^8 (i.e., *E. coli*) (B), and 10^7 (i.e., *Salmonella*) (B). Means with the same lowercase letter are not significantly different; means with different lowercase letters are significantly different (P<0.05). Error bars indicate standard deviation from the mean.

3.3 Antibacterial efficacy of HEW prepared using various commercial generators (1/8 EW dilution and 180 s suspension time): Subsequently, a comparison of antibacterial strengths of HEW (i.e., 1/8 dilution HEW; 180 s treatment/suspension incubation) generated using generators from various manufacturers exhibited differential antimicrobial strengths against bacteria, including Gram-negative and Gram-positive bacteria (Figure 10). The green generator-generated HEW remained comparably potent (>5-log reduction, cfu/mL) against *E. coli* (i.e., 0-3-week-old HEW), *Salmonella* (i.e., 0-2-week-old HEW), *L. monocytogenes* (i.e., 1-3-week-old HEW), and

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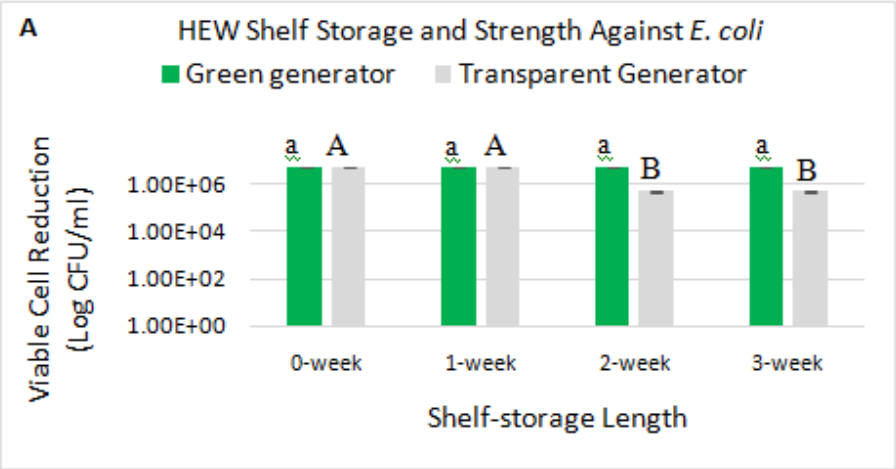
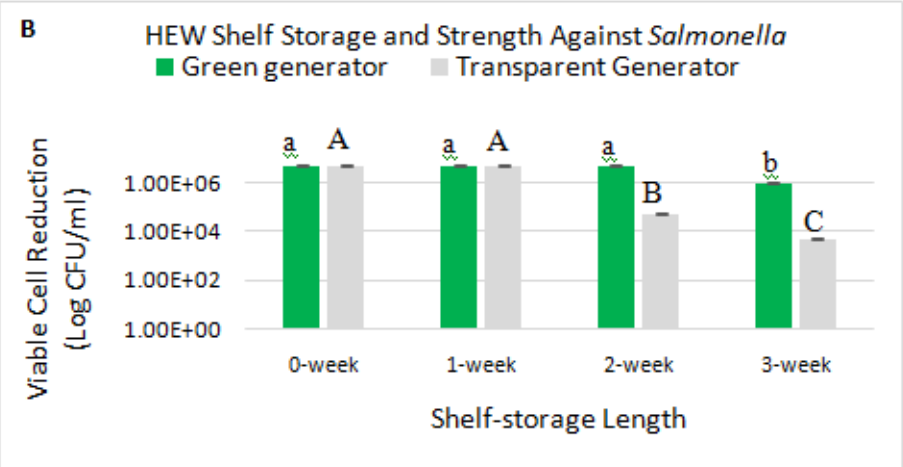
it was interesting that the 3-week-old HEW was remarkably more potent to *S. aureus* than its 0-2-week-old HEW (Figure 10). Gram-negative bacteria (i.e., maximum reduction of ~7-log cfu/mL) were relatively susceptible to HEW compared with Gram-positive bacteria (i.e., maximum reduction of 5-6-log cfu/mL). However, *E. coli* (i.e., vs. *Salmonella* treated with 2-3-week-old HEW) and *L. monocytogenes* (i.e., vs *S. aureus* treated with 1-2-week-old HEW) were more sensitive (i.e., >1-log reduction, cfu/mL) to HEW than their Gram-negative (i.e., *E. coli*) and Gram-positive (i.e., *S. aureus*) counterparts tested in this study, respectively. Differential antibacterial efficacy of ~1-log – 3-log (cfu/mL) was evident among HEW dilutions (i.e., 1/8) prepared using various generators, in which the green generator exhibited consistently improved efficacy throughout all bacteria strains and incubation times tested as opposed to the transparent generator (Figure 10) or another generator (i.e., hanging generator) (data not shown) (Figure 1).





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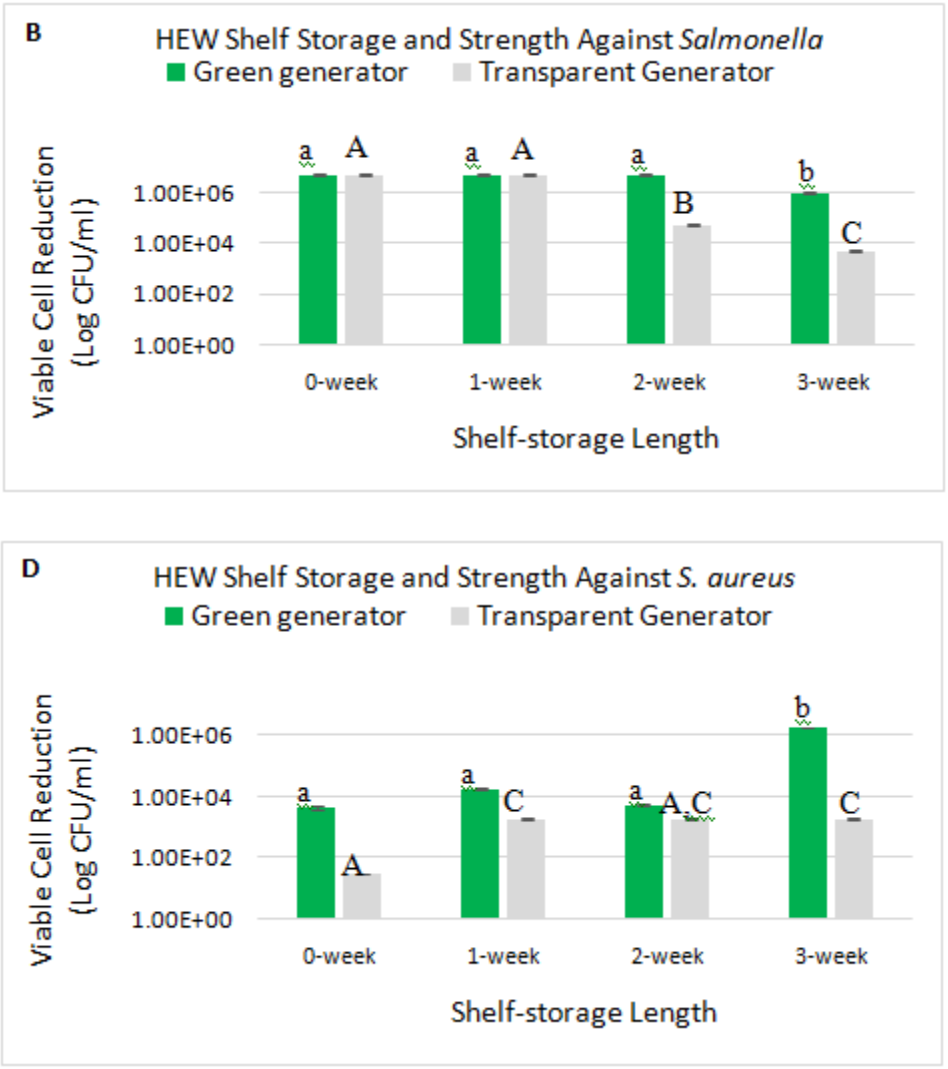

Figure 10: HEW generators and antibacterial strength against *E. coli* (A), *Salmonella* (B), *L. monocytogenes* (C), and *S. aureus* (D). Means with the same lowercase/uppercase letters are not significantly different; means with different lowercase/uppercase letters are significantly different (*P*<0.05). Error bars indicate standard deviation from the mean.

3.4 HEW toxicity: Using free-chlorine measuring paper strips, bleach and freshly made HEW (i.e., within 24 h) serial dilutions (i.e., 1:1 serial dilutions in tap water) of 0-1024 (bleach) and 0-32 (HEW) were analyzed for ACC (Figure 11). Bleach exhibited >1000-fold more free chlorine (PPM) than HEW prepared with tap water supplemented with NaCl (23% *w/v*) and vinegar acidification (pH 5.53) and 3-min electrolysis (Figures 11,12). The free-chlorine PPM of two-week-shelf storage and ~four-week-shelf storage (i.e., 25 days) HEW remained comparable, as

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demonstrated by the strip color-PPM (Figure 11).Automated quantification of specific HEW ACC PPM (i.e., using the Hach Free Chlorine TNT plus 866 Vial Test method) exhibited non-parallel PPM and serial dilutions (i.e., 1:1) (data not shown), and hence, non-comparable with the ACC color-PPM paper strip determined PPM. Further evaluation of chlorinated compounds (i.e., bleach and HEW) revealed that HEW did not render erythrocyte clotting as opposed to bleach (Figure 13), and the bleach clotting effect alleviated with blood dilutions (i.e., blood was diluted in BPW prior to bleach or HEW exposure). Subsequently, a study evaluating bleach and HEW hemolytic activities revealed visible blood-clearing formation only on/around blood agar inoculated with bleach spots, and the clearing size alleviated with bleach dilutions (Figure 14).

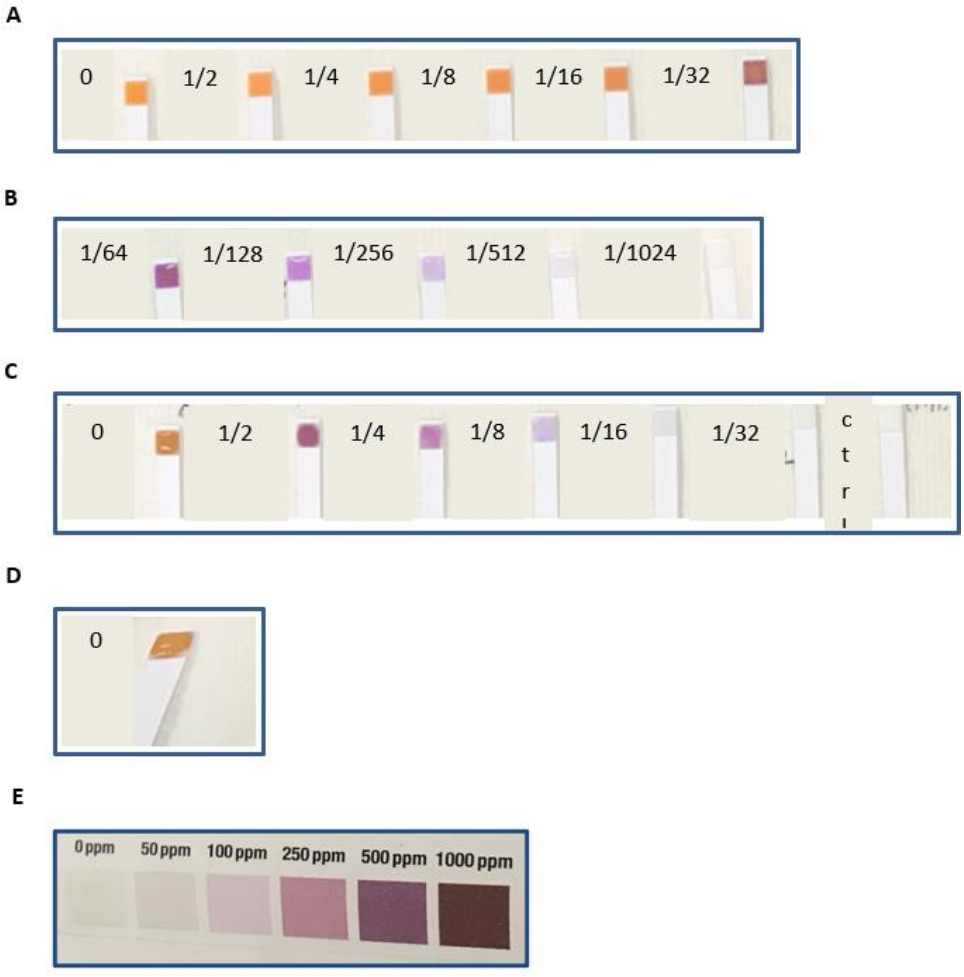

Figure 11: Available/free chlorine concentration (ACC/FCC) (in reference to the test kit color chart, E).Bleach (A,B), ~1-month-old homemade HEW (C), and 2-week-old homemade HEW (D) dilutions (i.e., bleach, 0-1/1024; ~1-month-old HEW, 0-1/32) and their free-chlorine strip color-PPM determination. Ctrl, control, prepared with tap water.

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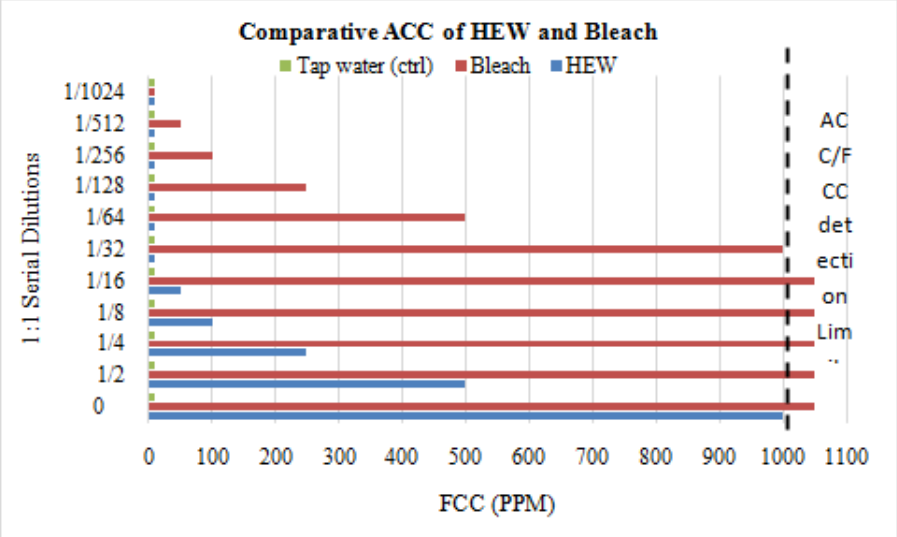

Figure 12: Comparison of ACC of bleach and EW using ACC PPM-color testing strip

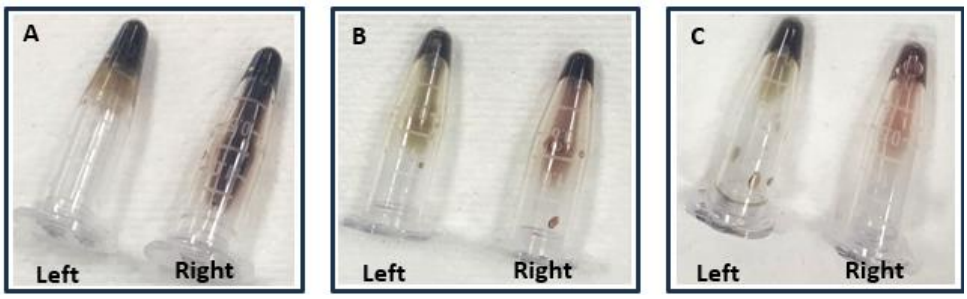

Figure 13: Comparison of the effect of bleach (left panel) and HEW (right panel) coagulation on blood dilutions, 1/2 (A), 1/4 (B), and 1/8 (C)

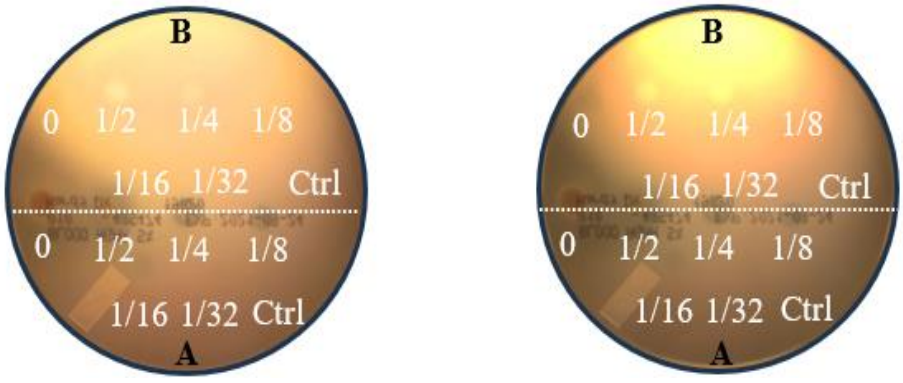

Figure 14: Comparison of the effect of bleach (panel A) and HEW (panel B) on blood hemolysis. The same hemolytic results were observed in at least two separate replications.

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4. DISCUSSION

4.1 Antibacterial testing mode

4.1.1 Synergistic antimicrobial effect of control solution combining increased NaCl (i.e., 23%) and acidity (pH 5.75) levels on bacteria (in this study) (Tables 2-4) was observed, agreeing with the reports on the effectiveness of hurdle technologies (i.e., combining multiple antimicrobial conditions), including organic acid (13), pH, and osmotic stress preservatives (61,62). Visible, ineffective antimicrobial activity was observed at a higher NaCl supplementation (Tables 2,3), agreeing with the increased antimicrobial effectiveness of the solution at post-electrolysis described in prior studies (41,42).

4.1.2 The spraying method of EW application (i.e., in this study) was moderately effective against *E. coli* (Figure 5) relative to *L. monocytogenes* (Figure 6), suggesting the EW antimicrobial selective effectiveness against Gram-negative bacteria, attributed to their thin cell wall nature (63). Increased vinegar-acidified EW potency could be attributed to the permeability-efficient nature of organic acids, including vinegar (13), as opposed to HCl strong acid used to acidify HEW (Figures 3,5) (64,65).

4.1.3 HEW (23% NaCl, vinegar, pH 5.52-5.53) dipping (Figure 7) exhibits comparatively immensely effective antibacterial efficacy on bacterial strains tested (i.e., *E. coli* and *L. monocytogenes*) than the spraying method (Figures 5,6). This could be attributed to the attached/sessile bacterial cell (i.e., agar-plate-attached cells) reduced exposure surface area to HEW than their counterpart planktonic cells (59) and their adherently variant property, where *E. coli* could detach easier and become more susceptible (Figures 5,6) to EW spray (i.e., wet agar containing HEW spray solutions) than the documented adherent strain of *L. monocytogenes* CW35 (57,66).

4.1.4 Bacterial differential sensitivity between two Gram property groups (i.e., Gram-positive bacteria were more resilient than Gram-negative bacteria) were exclusively discernible/noticeable with an increased HEW dilution (i.e., 1/8) applied by suspension followed by 180-sec incubation (Figure 9) prior to viable cell enumeration. This could be attributed to the presence of the effective protective thick cell wall of Gram-positive bacteria than the outer membrane of Gram-negative bacteria (63). In comparison, no visible *L. monocytogenes* growth inhibition was detected with 1/8 HEW spots (i.e., HEW dilution) (Figure 2) acidified with HCl (i.e., pH 5.52) as opposed to 1/8 HEW dipping acidified with vinegar (i.e., pH 5.53), which posed ~2-3-log viable cell reduction (cfu/mL) (Figure 8); thereby further confirming the HEW (i.e., 23% NaCl, pH ~5.53) anti-bacteria activity improving capability of vinegar (i.e., vs HCl acidified EW) (in this study) (Figures 3,5).

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4.1.5 HEW generators of various manufacturers (Figure 1), including green generator, transparent generator (Figure 10), and another generator (i.e., hanging generator) (data not shown), posed differential HEW antibacterial strengths (i.e., with differential antibacterial activities against bacteria tested (i.e., both Gram-positive and Gram-negative bacteria). Their potent strength (i.e., generated with both green and transparent HEW generators) against Gram-positive bacteria (i.e., both *L. monocytogenes* and *S. aureus*) differentially increases with HEW shelf-storage time (i.e., transparent generator, 0 vs. 1week; green generator, 0 vs. 1 or 3 weeks) (Figure 10), thereby disagreeing with the manufacturers' user instructions for effective HEW application (i.e., they require the use of freshly made EW immediately or within seven days post-production). These findings (in this study), Vahabi et al. (54), Xuan et al. (67), and Busch et al. (68) reports agreeably suggest the need for standardized production, application, and quality evaluation requirements to warrant consistent and optimized HEW efficacy.

4.1.6 Both bleach and HEW contain free chlorine compounds (i.e., ACC) (Figure 11), presumably the detectable sensory gas, hypochlorous acid (HOCl), and hypochlorite (OCl^-) that are sanitizing- and cleaning-capable, as this study validated (i.e., homemade EW potency) (Figures 7-10). Hence, HEW antibacterial/disinfection(i.e., acidified HEW, dilution 1/8, ≥100 ACC PPM; 3-7-log viable cell reduction, cfu/mL) (Figures 2,5,7-12) and corrosiveness (i.e., dictated by ACC PPM) (Figures11,12) strengths are positively and negatively (i.e., HEW ACC PPMis >1000X much lesser than 5% bleach ACC PPM) comparable with household bleach application-recommended dilution (i.e., 5%; 1:20 dilution; equivalent to >1000 PPM ACC in this study) (69-72) (Figures 11,12), respectively. It's worth noting that the ≥100 ACC PPM(i.e., estimated using strip color-PPM assay; dilution 1/8) (Figures 11,12)in this study marked effective antibacterial efficacy as opposed to the FDA 60 ACC PPM (40) required for food contact surface sanitation, indicating the need to re-investigate the EW ACC PPM for effective applications by all users. Further, the dark visibility of bleach-exposed erythrocytes could be attributed to bleach-rendered erythrocyte lysis, iron release, hypochlorite oxidizing (73) of oxygenated iron, and iron direct-oxygen exposure, which agrees with Ledford's report on bleach's capability of rupturing living cells, including blood cells (72) (Figure 13).

Discussion summary: HEW production using a commercially available ready-to-use USB-operated EW generator can be easy, quick, and conditionally antibacterial effective, attributed to the simple manufacturing (i.e., 3-min electrolysis, table NaCl, vinegar) and application instructions. However, HEW (i.e., non-diluted EW, 23% NaCl, acidified with vinegar, electrolyzed for 3 min) spray application (i.e., an accustomed/universal application mode) is immensely less effective than the dipping/suspension application mode as demonstrated in *E. coli* (i.e., 3-log vs. 5-7-log viable cell reduction, cfu/mL) (Figures 5,7) and *L. monocytogenes* (i.e., <1-log vs. 5-log viable cell reduction, cfu/mL) (Figures 6,7), suggesting its most likely

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suspension/dipping application (i.e., non-spray application purpose) as an effective, economic cleaning aqueous disinfectant. HEW supplemented with vinegar is much more effective than the HCl counterpart (i.e., the HEW spraying results in this study) (Figures 3,5), and their antimicrobial activity is enhanced with sitting time (i.e., immediate use of freshly made HEW is less effective than incubated HEW) (Figure 10). Differential HEW effectiveness is expected in HEW generators as they possess shelf-life and effectivity variations posed by different homemade EW generator manufacturers (in this study) (Figure 10). Additionally, bacterial Gram property and genera variations could pose an unparalleled EW sensitivity among bacteria (Figures 5,6,7). Comparatively, HEW doesn't pose visible cytotoxicity (i.e., 0 – 1/32 dilutions, 1000 – 0 ACC/FCC) (Figures 13,14).Together, the findings suggest the need to investigate and standardize HEW generators and their effective antibacterial dose to ensure consistent antibacterial efficacy (i.e., >5-log reduction, cfu/mL) of HEW.

5. CONCLUSION

Homemade EW (HEW) production and application is increasingly gaining interest among households and health care settings, attributed to its easy-to-make, quick-to-make, cheap-to-make, environment-friendly natures and persistent emergence of infectious agents (12,33,45, 48,49). Comparatively (in this study), HEW (i.e., prepared with 23% NaCl) can be an effective, human-safe (Figures 13,14) antibacterial disinfectant for cleaning (i.e., suspension application; ≥100 ACC PPM) applications, and its activity can be enhanced with vinegar supplementation (i.e., used as an EW shelf-life enhancer) (50,67,68), with incubated EW (i.e., sit at room temperature) as opposed to applying freshly made EW immediately, and with select commercially available homemade EW generators. Together, these results and others (21,59,67) indicate the need to re-investigate the EW ACC PPM (i.e., in this study, ≥100 ACC PPM vs FDA, 60 ACC PPM) (Figures 11,12) (40)for broad, consistent, effective antibacterial applications, attributed to differential EW effectiveness dictated by the generation parameters (in this study, 67) (Figures 3,5), application mode (i.e., spraying vs dipping/suspending/washing) (in this study, 59) (Figure 7 vs Figures 5,6), applied bacterial target (i.e., Gram property and genus) (in this study, 21) (Figure 9), and generator type (in this study, 67) (Figure 10) demonstrated in this study and previous reports (21,59,67).

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