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Effect of neutral electrolyzed water (NEW) on *Salmonella* Typhimurium, Enteropathogenic *Escherichia coli* (EPEC) and *Staphylococcus aureus*

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ABSTRACT

In this study, Neutral electrolyzed water (NEW) bactericidal efficacy and inactivating capacity as a promising novel antimicrobial agent, green sanitizer and natural alternative to conventional decontamination techniques as chemical disinfectants was evaluated by using NEW with free available chlorine (FAC) at concentrations of 7.5 ppm (NEW 1%), 24 ppm (NEW 12%) and 49 ppm (NEW 25%) against food-borne related microorganisms and their biofilms. Our Results revealed that by 10 min of exposure to the sanitizer (NEW) with different concentrations, changes to the bacterial count, bacterial cell morphology, biofilms were evaluated by plate counting, minimum inhibitory concentration method (MIC), Transmission Electron microscope (TEM) examination for the isolated *Salmonella* Typhimurium, Enteropathogenic *E. coli* (EPEC), and *S. aureus*, respectively. Beside, modulation of bacterial gene expression by real time Polymerase Chain Reaction screening (RT-PCR) for methicillin-resistant gene (*mec*A) and Enterotoxin gene (*seb*) of *S. aureus*. It was shown from the results that *Salmonella* Typhimurium highest reduction percentage achieved was 99.9% after 10 min exposure to 12% and 25% NEW concentrations, while EPEC and *S. aureus* highest reduction percentages achieved were 99.8%, and 99.95% after 10 min exposure to 25% NEW concentration, respectively. The lowest reduction was detected by 59.5% for *S. aureus* treated with NEW 1% for 5 min. While MIC for NEW 25% was 12.25 ppm for EPEC and 24.5 ppm for each of *Salmonella* Typhimurium and *S. aureus*. TEM photos revealed that NEW has achieved a broad-spectrum bactericidal activity by causing changes and destruction in cell envelope and cytoplasm of all strains. RT-PCR showed downregulation of enterotoxin (*seb*) and resistant genes (*mec*A) of *S. aureus*. In conclusion, the study demonstrated that NEW as a green sanitizer is significant in reduction and elimination of the most food-borne bacterial contamination.

Introduction

Foodborne outbreaks are more relevant in developing countries where it is harder to apply food safety management (WHO, 2015). *Salmonella* Typhimurium, *Escherichia coli*, and methicillin resistant *Staphylococcus aureus* (MRSA) are considered the most common food poisoning bacteria related to food outbreaks (Cho *et al*., 2016).

Electrolyzed water (EW) was reported as an arising, recent, effective, and broad-spectrum sanitizer because of its main effective forms of free available chlorine compounds (FAC) as hypochlorous acid (HOCL) which kills wide range of pathogenic microorganisms within a short period of time introducing it as a safe substitute to chemical sanitizers (Hsu *et al*., 2019).

One From EW advancement's in food industry is that it provides bacterial control and reduction without changing the organoleptic parameters of the food (Hsu, 2003).

Thus, EW is widely used in food sterilization, including chicken, shrimp, meat, fish, eggs, and other types of foods (Hao *et al*., 2013).

There are 2 types of EW producing devices widely used in the market. The first type is EW electrolyzer without a diaphragm that produces neutral EW, known as single cell chambers, whereas the second type is an electrolyzer with a diaphragm that produces acidic and alkaline EW, known as two cell chambers (Yan *et al*., 2021).

Mainly, Neutral electrolyzed water (NEW) is prepared by electrolyzing the dilute salt solution (NaCl) using a single cell chamber without diaphragm, resulting of NEW with high sterilization efficiency from a neutral pH (7-8) to a strongest oxidant, with an oxidation-reduction potential (ORP) of +800 to +900 mV (Iram *et al*., 2021).

From advantages of NEW is it's less corrosion effect on metals, food or skin irritation of workers compared with Acidic electrolyzed (AEW) water with similar strong antimicrobial mechanism (Iram *et al*., 2021).

The main bactericidal factor in NEW is the free active chlorine species (FAC), it contains nearly 95% hypochlorous acid (HOCl), 5% hypochlorite ion (ClO^-), and little amounts of Cl_2. The bactericidal efficiency of HOCL is 80 times than that of ClO^- which suggests that it is the main agent responsible for microbial inactivation (Deng *et al*., 2019).

Basically, these highly oxidative compounds in NEW lead to destruction of bacterial enzymes and bacterial biomolecules including DNA, proteins, and RNA which results in the final bacterial death (Yan *et al*., 2021).

Besides, high ORP of NEW results in the destruction of layers of bacteria, disturbing the metabolic pathways and oxidation of sulfhydryl mixtures of cells and affects the certain intracellular enzyme systems by changing the electron flow in the cells. The result could accelerate the inactivation of bacterial cells (Liao *et al*., 2007).

Therefore, the main properties of EW including FAC (Cl_2, −OCl and HOCl), pH and ORP, directly influence its sanitizing efficacy, while properties of EW is directly affected by different electrolytic parameters such as salt concentration, electrode materials, water temperature, and storage environments (Ding *et al*., 2016).

The pH of a solution determines the amount of HOCl and hypochlorite ion (OCl^-) generated and their distribution which affects NEW biochemical characteristics. As at higher pH, OCl^- is generated, But at a lower pH the solution is a mixture of chlorine (Cl_2) and HOCl. But at neutral pH between (pH 5 -7), HOCl is the main species found, HOCl concentration is optimum and dissociation is minimal within this neutral pH range (Hsu *et al*., 2019; Nurul Aniyyah *et al*., 2022).

The ORP and FAC of electrolyzed water decline sharply with the upsurge in pH from acidic (2.5) to alkaline (9.0). At pH 9, the antimicrobial

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efficiency ceased. The effect of FAC is also assessed in multiple researches and more than 20 ppm is correlated with effective inactivation (Quan *et al*., 2010).

Numerous studies have proved the broad spectrum of EW as an antimicrobial agent in the food industry that is capable of total elimination or logarithmic reduction of pathogens such as *E. coli* and *S.* Typhimurium in different food contaminated samples as egg shell and chicken meat (Reihane *et al*., 2020).

Interestingly, there are difference in the sensitivity and susceptibilities of some bacterial strains than other to EW, it may be attributed to different bacterial properties such as cell wall in the resistance to the action of EW (Rahman *et al*., 2016).

Biofilms are polymer matrix (glycocalyx) enclosing community of bacterial cells and self-produced by bacteria which support it against different environmental stress factors, such as preservatives, antibiotics, disinfectants and sanitizers. Thus, eradicating of biofilm is important for food safety, and public health. EW has been recorded as a significant method for eradicating the biofilm (Yan *et al*., 2021).

Not only food borne bacteria are hazardous but also their toxins, so more research are needed to demonstrate the ability of EW in the removal of microbial toxins (Escobedo-González *et al*., 2016).

This work targeted the Neutral electrolyzed water as NEW satisfies many properties of an ideal disinfectant and sanitizer, as effectiveness against a broad spectrum of pathogens even at low concentrations, environmental friendly, and low operational cost because of expendable ion-selective membrane is not utilized during electrolysis (Hsu *et al*., 2019).

Electrolyzed water can replace conventional chemical decontamination methods in the food industry and household. However, the NEW has not yet been applied to inactivate food-borne bacteria in Egypt and more research is needed to know its actual mechanism of antimicrobial action.

Thus the objectives of this study were firstly to evaluate the antimicrobial effect of Neutral electrolyzed water (NEW) on *S.* Typhimurium, EPEC, and *S. aureus*. Secondly, to study the effect of NEW on *S. aureus* enterotoxins and resistant genes using RT-PCR. Thirdly, to study antibiofilm effect of NEW on *S.* Typhimurium, EPEC, and *S. aureus* biofilms. Finally, to study morphological changes on *S.* Typhimurium, EPEC, and *S. aureus* using Transmission electron Microscope (TEM).

Materials and methods

Preparation of NEW disinfectant solution

Neutral electrolyzed water (NEW) with different conc of NaCl (w/v) (1%, 12% and 25%), pH and FAC values were obtained as in Table 1 by using portable hand-made electrochemical cell without diaphragm, which consists of 2 titanium electrodes (Tio2) (cathode and anode) installed in glass container with 0.7 gap between electrodes. Obtained NEW were labelled and stored in closed glass containers at refrigerator temp. (4ºC) (Seham and EL-Amawy, 2020). FAC and pH of the EW generated from the portable sanitizing unit were determined by a Iodometric method for chorine detection and a pH meter (Biochemistry Unit at Animal Health Research Institute, Dokki, Egypt).

Table 1. Chemical properties of tested Neutral electrolyzed water solutions (NEW) with different concentrations.

Electrolyzed water samples	pH	FAC (ppm)
NEW 1%	8.45 ± 0.1	7.55 ppm
NEW 12%	7.51 ± 0.1	24 ppm
NEW 25%	7.29 ± 0.1	49 ppm
Control Sterilized DW	6.9±0.5	ND

ND: not detectable.

Bacterial inoculum preparation

Three different bacterial strains *S.* Typhimurium, EPEC, and *S. aureus* (MRSA) isolated previously from different food stuffs .,were activated by transferring them into test tubes contained 10 ml of sterile tryptic soya broth (TSB; Oxoid, UK) and incubated at 37ºC for 24 h. After two consecutive transfers, the harvested cell pellets were collected by centrifugation (4000×g for 10 min), washed and suspended in 10 ml of phosphate buffer saline (PBS) to obtain a working culture at a concentration of around 10^8 CFU/ml for subsequent experiments (USFDA, 2001).

Antimicrobial activity and resistance testing

The antibacterial activity of NEW25% against inoculated agar with *S.* Typhimurium, EPEC and *S. aureus* strains was examined by agar-well diffusion method (CLSI, 2021). As 100 µL of NEW 25% were filled into wells of 6 mm diameter, compared with well filled with sterile DW then incubated at 37ºC for 24 h. Diameters of the inhibition zones were measured in mm.

In addition, antibiotic resistance identified for *S.* Typhimurium, EPEC and *S. aureus* against 14 antimicrobials according to the disk diffusion method and the results interpreted and determine MDR and MAR by the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2021).

Molecular Detection of virulence (*seb* and *sea*) genes and antibiotic resistance (*mec*A) genes in identified *S. aureus* strains by conventional PCR (cPCR)

Molecular detection of virulence and resistant genes was accomplished according to Sambrook *et al*. (1989), the used cPCR Oligonucleotide primers are shown in Table 2.

Antimicrobial analysis of NEW in broth

For each treatment, 1 mL of each bacterial suspension of *S.* Typhimurium, EPEC, and *S. aureus* (approximetly 10^8 CFU/mL) was mixed with 9 mL of each NEW (1%, 12%, and 25%) solution for 5 min. After treatment, 1 ml of each treated sample was mixed with 9 mL of neutralizing buffer solution (0.5%) (w/v) which contained Na thiosulphate, $Na_2S_2O_3$ (El-Ghomhoriya company, Egypt) for 5 min to stop the sanitizing process. The mixtures were serially diluted (1:10) in sterile 0.1% peptone water and spread on EMB, XLD, and BP media (Oxoid, UK) and incubated at 37°C, for

Table 2. Target genes, Primers sequences and cycling conditions for cPCR and real time quantitative -PCR.

Gene	Primer sequence (5'-3')	Reverse transcription	Primary denaturation	Amplification (40 cycles)			References
				Secondary denaturation	Annealing (Optics on)	Extension	
mecA	F- GTA GAA ATG ACT GAA CGT CCG ATA A R- CCA ATT CCA CAT TGT TTC GGT CTA A	50ºC/30 min.	94ºC/5min	94ºC/15 sec	50ºC/30sec	72ºC/30sec	McClure *et al*. (2006)
Seb	F- GTATGGTGGTGTAACTGAGC R- CCAAATAGTGACGAGTTAGG				57ºC/30sec	72ºC/30sec	Mehrotra *et al*. (2000)
16sRNA	F- CCTATAAGACTGGGATAACTTCGGG R-CTTTGAGTTTCAACCTTGCGGTCG				55ºC/40sec	72ºC/70 sec	Mason *et al*. (2001)

24 h for EPEC, *S.* Typhimurium, and *S. aureus* bacterial counts. The group treated with sterilized DW water was used as the control group. All the experiments were performed in triplicate.

Minimum inhibitory concentration (MIC) of NEW 25%

The MIC was determined using a broth microdilution technique in a 96 -well microplate and Resazurin microtiter assay (REMA) in accordance with standard guidelines of CLSI (2021) and Martin and Palomin's instructions Martin *et al.*(2003) against identified *S.* Typhimurium, EPEC and *S. aureus*. Briefly, 20 microliters from the stock solutions of NEW 25% (49 ppm FAC) was two-fold serially diluted to 0.047 ppm, control positive, and negative wells were prepared. The microplates were incubated at 37ºC for 24 h. Then, each well received 30 µl of resazurin solution and the plates were re-incubated for 1-2 h. When the color shifts from blue to pink, it indicated bacterial growth. The MIC was determined to be the lowest dose of NEW that keep blue color, which indicates bacterial inhibition.

Quantitative evaluation of biofilm formation

A 96-well sterile flat -bottomed microtiter plate (Greiner Bi One, Germany) was used to assay biofilm production ability of *S.* Typhimurium, EPEC and *S. aureus* as documented by Hashem *et al*. (2017) and Coffey and Anderson (2014). Interpretation of results was done according to Stepanović *et al*. (2007) depending on the optical density (OD) using ELISA reader (Clindiag MR-96) at 570nm. Bacterial isolates were categorized into four degrees of biofilm formation relaying on the optical absorbance of negative control (ODc) as follows: non-biofilm producer (OD ≤ ODc), strong biofilm producer (4×ODc < OD)), moderate biofilm producer (2×ODc < OD ≤ 4×ODc),) and weak biofilm producer (ODc < OD ≤ 2×ODc). All procedures were carried in triplicate.

Antibiofilm assay

Investigations of NEW (12% and 25%) effects on strong biofilms of *S.* Typhimurium, EPEC, and *S. aureus* was carried out by the broth microdilution test (Raja *et al*., 2011). As, each well containing 180 µl of tryptic soya broth (TSB) filled with 20 µl of each bacterial suspension (10^6 cells/ml) and incubated for 24h, formed biofilm aspirated and wells washed with PBS. Then, 200 µl of different NEW concentrations (12% and 25%) were added and plates were incubated at 37ºC for 24 h. Biofilm inhibition percentages were calculated by following equation: 1- (A570 of the treated isolate / A570 of control isolate) ×100, where A is the absorbance results at 570 nm using the Clindiag MR-96 ELISA reader. All procedures were carried in triplicate.

Transmission Electron Microscope (TEM)

Transmission Electron microscope analysis helped in visualization of bacterial samples and observe the morphological changes in *S.* Typhimurium, EPEC and *S. aureus* before and after treatment with NEW at concentrations (12% and 25%) and incubation at 37ºC for 30 min (Feliciano *et al*., 2012; Li *et al*., 2016). Then examined and photographed using JEOL-JEM-1400 (electron microscope, Tokyo, Japan) electron microscope unit- CURP Agriculture Faculty, Cairo University, Egypt.

Reverse transcriptase - quantitative Polymerase chain reaction (RT-qPCR) of MRSA (Sambrook et al., 1989)

Pure colonies of MRSA strain of a dose of 10^8 (experiment challenge dose) was mixed with NEW 25% (49 ppm) broth and incubated at 37ºC overnight, 2 mL aliquots of the samples were collected by centrifugation for 4 min at 5000 × g, to which 1mL of RNA protector was added. RNA extraction from samples (treated with NEW and untreated MRSA) was done following QIAamp RNA mini kit (Qiagen, GmbH, Germany) instructions.

The relative expression levels of virulence gene (*seb*) and methicillin-resistance gene (*mec*A) in MRSA isolates was examined by qRT-PCR using QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH) and Stratagene MX3005P real-time PCR machine according to manufacture instructions. Each sample was normalized to16S rRNA expression. Oligonucleotide primers sequences used in RT-qPCR are listed in Table 2.

Variation in gene expression and fold changes in the transcript levels of examined genes in treated MRSA isolates relative to their levels in untreated ones were calculated according to $2^{-\Delta\Delta ct}$ method (Yuan *et al*., 2006). Each assay was performed simultaneously in triplicates.

Statistical analysis

The experiments were replicated three times for each NEW treatment. The data were analyzed by ANOVA using SPSS Statistics 20 software (IBM Co., USA) and expressed as mean value ± standard deviation. The confidence level for statistical significance was set at a probability value of 0.05.

Results

In-vitro bactericidal analysis of NEW in broth

The viable cell count reduction for *S.* Typhimurium, EPEC, and *S. aureus* after 5 and 10 min exposure to various concentration of NEW are presented in Figure 1 and Table 3.

For *S.* Typhimurium the highest reduction percentage was 99.9±3 after 10 min exposure to NEW 12% and 25% concentrations.

For EPEC and *S. aureus* the highest reduction percentages were 99.8±2.69, 99.95±3.39, respectively, after 10 min exposure to NEW 25% concentrations.

NEW 25% demonstrated moderate antimicrobial action against analyzed isolates with inhibition zones diameters up to 10 mm, 41 mm, and 21 mm for *S.* Typhimurium, EPEC, and *S. aureus*, respectively (Fig. 2).

Antimicrobial Susceptibility pattern

Several multidrug resistance (MDR) profiles to three or more antimicrobial classes were detected in 100% of *S.* Typhimurium, EPEC and *S. aureus*. Methicillin resistant (MRSA) was detected in 100% of *S. aureus*

Table 3. Reduction percentage of *S.* Typhimurium, EPEC and *S. aureus* after treatment with different concentration of NEW for 5 and 10 min.

NEW Conc	After 5 min treatment			After10 min treatment		
	Salmonella Typhimurium reduction (%)	*EPEC* reduction (%)	*S. aureus* reduction (%)	*Salmonella* Typhimurium *reduction (%)*	*EPEC* reduction (%)	*S. aureus* reduction (%)
1%	60.00±0.39^b	85.00±0.82^b	59.56±0.392^b	99.71±2.53^a	99.50±2.3^a	99.29±2.10^a
12%	95.00±1.30^a	90.00±1.00^b	89.19±0.96^b	99.90±3.00^a	99.50±2.3^a	99.40±2.226^a
25%	97.60±1.60^a	95.50±1.34^a	99.73±2.56^a	99.90±3.00^a	99.80±2.69^a	99.95±3.39^a

Initial (control) bacterial count 2×10^8.

Results are presented as means ± standard error of three trials. Values with different superscripts letters in the same row are significantly different ($p<0.05$).

isolates (Table 4).

MAR index for each bacterial isolate was calculated as : Number of antibiotics to which the isolate showed resistance / Total number of antibiotics to which the isolate has been tested.

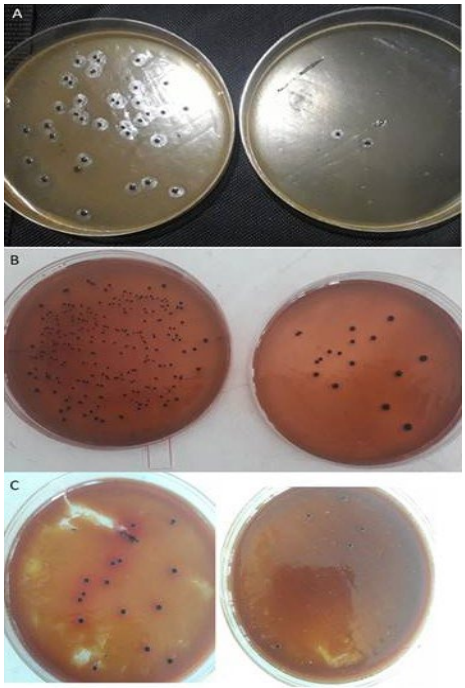

Fig. 1. Reduction percentage of bacterial count (A-C) after application of NEW 25%. A) *S. aureus* TPC, B) *E. coli*, C) *S.* Typhimurium.

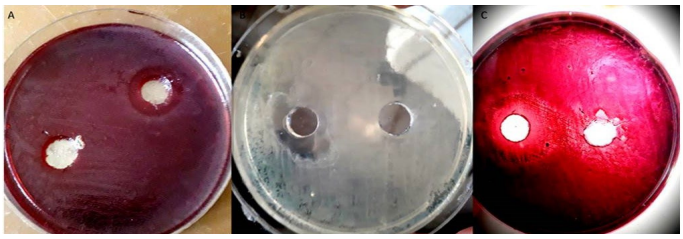

Fig. 2. Agar well diffusion results (A-C) after application of NEW 25%. A) *S.* Typhimurium. B) *S. aureus*. C) EPEC.

Molecular identification, virulence and resistant determinants of S. aureus

Using PCR, out of tested five *S. aureus* isolates that underwent enterotoxin gene investigation, (*seb*) gene was detected in four isolates (n. 2, 3, 4, 5) (90%) as shown in Fig. 3, while (*sea*) gene was not detected at any of the examined isolates. Out of the tested two *S. aureus* isolates that underwent resistance gene investigation, *mec*A gene was detected in two isolates (n. 3, 5) (100%) as shown in Fig. 3.

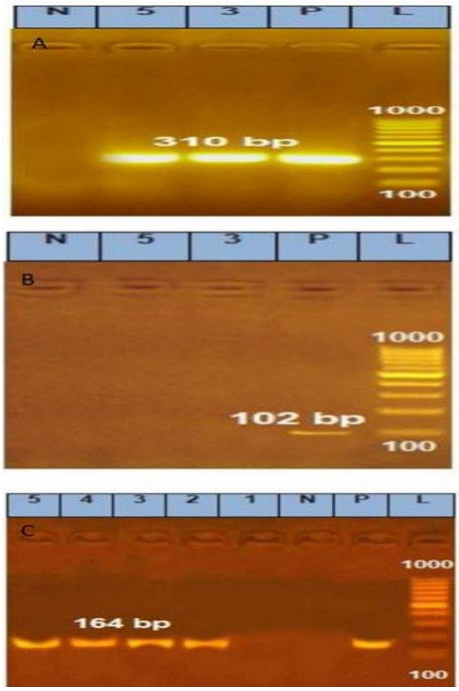

Fig. 3. Grouping of 3 separate images for (A) Agarose gel electrophoresis for PCR amplification of *mec*A resistant gene at (310 bp) detected in (2) isolates, (B) *Sea* virulence gene at (102 bp) not detected at any isolate, (C) *Seb* virulence gene at (164 bp) detected in (4) isolates: Lane L ladder (100-1000bp), Lane "Pos" (control positive), Negative Lan (control negative), Lane 1 -5 *S. aureus* isolates.

Results of the MIC using NEW 25%

Bacterial growth at different concentrations of NEW was assessed after 24 h, the MIC of EPEC was observed maximum for NEW 25% at FAC concentration of 49, 24.5, and 12.25 ppm while MIC of *S.* Typhimurium and *S. aureus* was observed. Maximum for NEW 25% at FAC concentration of 49 and 24.5 ppm as shown in Fig. 4 indicating that NEW has both bacteriostatic and bactericidal activity.

Biofilm formation ability of S. Typhimurium*, EPEC, and S. aureus*

Assaying the biofilm formation by 1 MDR *S.* Typhimurium, 2 MDR EPEC, and 5 MDR *S. aureus* isolates using crystal violet staining method resulted in all tested isolates turned out to be biofilm producers; among them 100% isolates were categorized as Strong biofilm producers. All were resistant to at least 3 antimicrobial agents (MDR) (Table 4).Anti-Biofilm Effect of NEW

The effect of NEW 12% and NEW 25% on preexisting biofilms produced by *S.* Typhimurium, EPEC, and *S. aureus*. Our results proved a good

Table 4. Antimicrobial resistance patterns, MAR index and biofilm forming ability in *S. aureus* , *S.* Typhimurium and EPEC isolated from different food stuffs.

Isolate number	Antimicrobial resistance pattern	MAR Index	Biofilm formation		Virulence and resistant gene	
			Mean OD570 .	Degree	*mecA*	*Seb*
S. aureus isolates						
1 (Meat)	AMP, AMC, ATM, AZM, CN, C, FEP, DA, E, CFP	0.77	0.29	Strong	NT	-
2 (Kariesh cheese)	AMP, AMC, ATM, AZM, C, CN, E, DA, FEP	0.77	0.29	Strong	NT	+
3 (Kariesh cheese)	AMP, AMC, AZM, CN, NOR, LEV, CIP, C, FEP	0.77	0.32	Strong	+	+
4 (Milk)	AMP, AMC, AZM, ATM, C, FEP, DA, TE, E, CFP, LEV	0.85	0.31	Strong	NT	+
5 (Milk)	AMP, AMC, AZM, ATM, FEP, DA, E, CFP, LEV	0.69	0.35	Strong	+	+
S. Typhimurium (Chicken)	AMP, AMC, AZM, ATM, CN, C, FEP, DA, TE, E, CFP	0.85	0.29	Strong		
E. coli (Meat) O 86:K61	CIP, AMC, AMP, FEP,	0.44	0.33	Strong		
O119:K69	DO, C, FEP, NOR, AMP, CIP, AMC	0.78	0.31	Strong		

AMP: Ampicillin, AMC: amoxicillin -clavulanic acid, ATM: aztreonam, AZM: azithromycin, FEP: cefepime, C: chloramphenicol, CN: gentamicin, DO: doxycycline, TE: tetracycline, E: erythromycin, CFP: cefoperazone, CIP: ciprofloxacin, NOR: norfloxacin, LEV: levofloxacin. NT: not tested.

antibiofilm activity as NEW 25% on *S.* Typhimurium, EPEC, and *S. aureus* are shown in Table 5.

Transmission electron microscope findings were shown in Figures 5, 6, and 7.

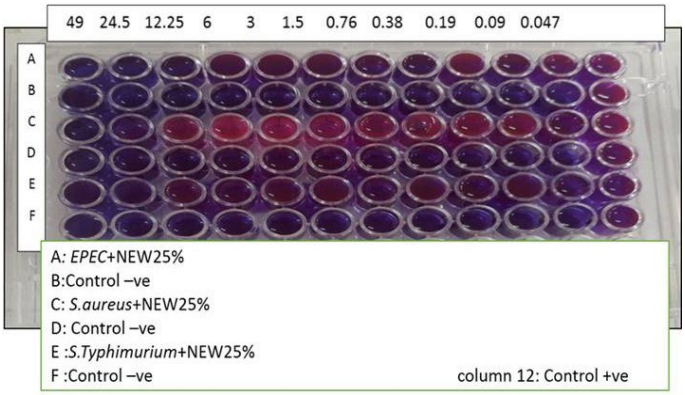

Fig. 4. MIC of NEW25% was 12.25 ppm for EPEC and 24.5 ppm for each of *Salmonella* Typhimurium and *S. aureus*.

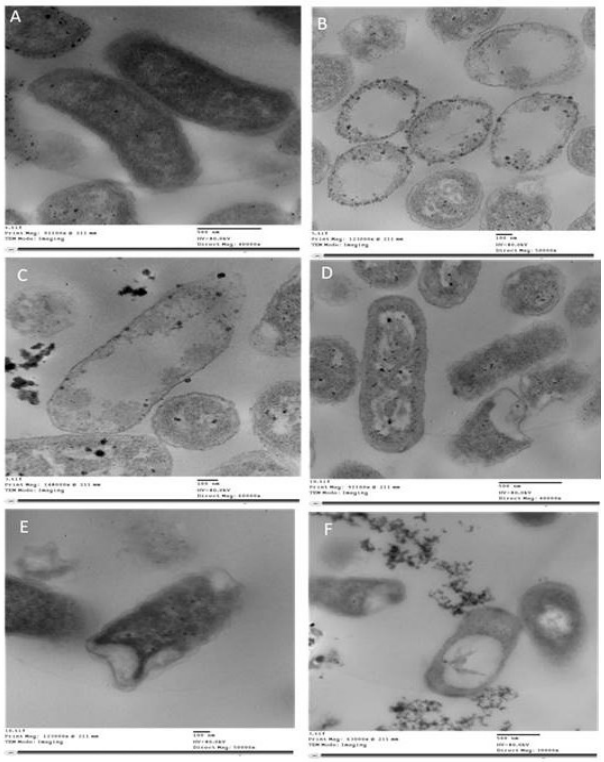

Fig. 5. A) Micrographs of untreated *Salmonella* cell culture without exposure to NEW showed continuous thin, smooth cell walls and other defined cellular structures. B-D) TEM images of *S.* Typhimurium cells grown in the media containing NEW 25% after treatment for 30 min showed elongation of bacterial cell and particles around bacterial surface, disruption of cell membrane, damage to cell surface, cavity formation (bite like pattern), cell wall roughness, disorganization of cytoplasm and Ghost appearance of cell. E-F) TEM images of *S.* Typhimurium cells grown in the media containing NEW 12% after treatment for 30 min, *Salmonella* showed less damage. Vacuolation within cytoplasm, Disruption to cell membrane and bite like structures and Ghost like appearance.

Effect on Microbial Toxins

Efficacy of NEW 25% on target virulence (enterotoxin) gene expression and methicillin – resistant gene expression of *S. aureus* by -quantitative real time- PCR (qRT-PCR)

The expression of virulence gene (*seb*) and resistant gene (*mec*A) were significantly downregulated in the treated *S. aureus* with NEW 25% (49 ppm T.C) compared to the control non – treated sample. The *seb* gene was nearly down regulated with 0.3737, while the *mec*A gene was nearly down regulated with 0.2679 as shown in Fig. 8.

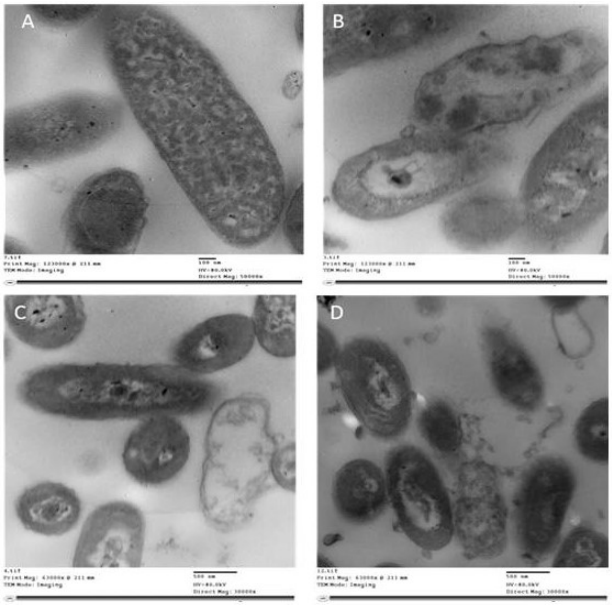

Fig. 6. A) Micrographs of untreated *E. coli* cell culture without exposure to NEW showed continuous thin, smooth cell walls and other defined cellular structures. B-D) TEM images of EPEC cells grown in the media containing NEW 25% after treatment for 30 min showed elongation of bacterial cell, shedding -disorganization of the cytoplasm and in some, areas there was a lack of cytoplasm, and developing of vacuoles within cytoplasm, ghost like appearance, presence of bacterial filaments within the cytoplasm in response to DNA damage and finally destruction to cell envelope.

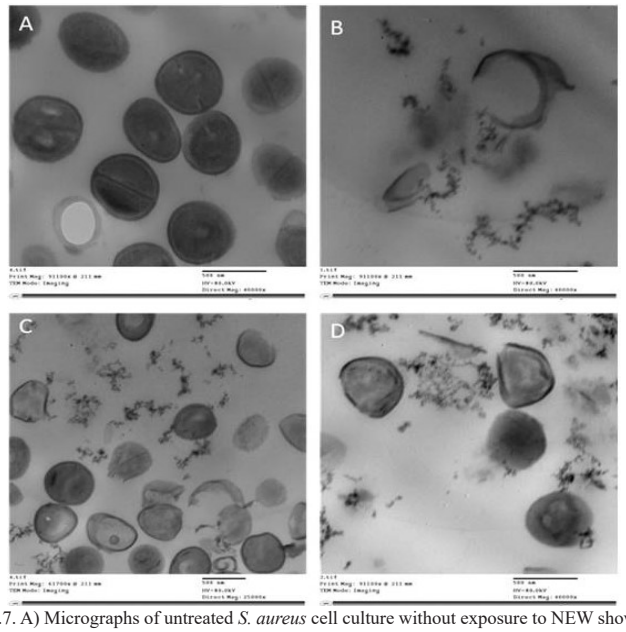

Fig.7. A) Micrographs of untreated *S. aureus* cell culture without exposure to NEW showed continuous thin, smooth cell walls and other defined cellular structures. B-D) TEM images of *S. aureus* cells grown in the media containing NEW 25% after treatment for 30 min showed adulterated morphology, where cell walls had irregularities, less smoothness, less uniformity, and degenerative changes leading to wall ruptures and subsequent cellular lysis in some cases. An unequal cytoplasm distribution caused by the clumping and agglomeration of intracellular material was observed in the treated cell. Furthermore, the cells lacked cytoplasm in certain regions due to the loss of membrane functionality.

Table 5. Reduction percentage (R%) of NEW (12 and 25%) on by *S.* Typhimurium, EPEC and *S. aureus* biofilms.

NEW Conc	*S.* Typhimurium		*EPEC*		*S. aureus*	
	Mean OD650	R%	Mean OD650	R%	Mean OD650	R%
NEW 12%	0.25	83.20%	0.20	54.90%	0.31	57.40%
NEW 25%	0.28	94.60%	0.26	80.10%	0.32	76%

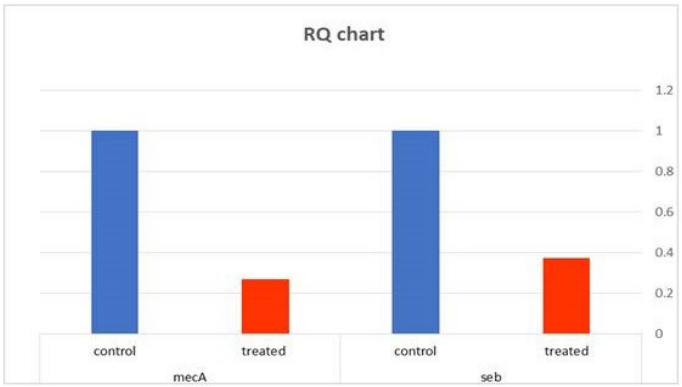

Fig. 8. Effect of NEW25% on mRNA expression levels of *S. aureus* enterotoxin gene (*seb*) and resistant gene (*mec*A). The relative expression was determined by the ΔΔCT method, and the relative expression ratio (R¬) was calculated. Showed downregulation of *mec*A and *seb* genes after treatment with NEW 25%.

Discussion

Electrolyzed oxidizing water (EOW) is considered as chlorine based oxidizing product generated by electrolysis of sodium chloride. In our study, electrolyzing dilute sodium chloride solution in a diaphragm – less electrolytic cell, resulted in a sterilizing aqueous solution with a pH of 6-8 called Neutral electrolyzed water (NEW), its major sterilizing component is hypochlorous acid (HOCL) (Kurahashi *et al*., 2021). Hypochlorous acid (HOCL) in its unprotonated form, which is produced by EOW at neutral pH, has the highest oxidizing potential which can penetrate and destruct bacterial cell barriers (Yan *et al*., 2021). Thus, electrochemically free active chlorines (HOCl, OCl^- and dissolved Cl_2 gas) and the corresponding high oxidation–reduction potential (ORP) are the main factors of the strong bactericidal activity of EW (Park *et al*., 2004).

Nowadays, antimicrobial efficacy of electrolyzed water is in a continuous assessment, and the use of electrolyzed water is increasing in food industry, veterinary and medical fields because of its antimicrobial activity and relative safety (Boyce, 2016).

In the present study, we used the homemade electrolyzer at which EW generator used two titanium electrodes in non-separating chamber at 9V, 30 minutes electrolyzing time is similar to Khalid *et al*. (2018) and Seham and EL-Amawy (2020) who used titanium anode for 20 min.

In this study, the characteristic properties of NEW were pH and free available chorine (FAC) by using NEW 1%, NEW 12% and NEW 25% we obtained pH values of 8.45, 7.52 and 7.2, respectively. These results agree with Yan *et al*. (2021) who reported pH values of 7-8. These slightly alkaline pH is due to the presence of hypochlorite ions, which are basic anions, these values are considered neutral and eligible in food industry.

By using NEW 1%, NEW 12% and NEW 25% we obtained FAC values 7.5 ppm, 24 ppm and 49 ppm, respectively. Value of FAC from 20-49 ppm promote stability and potency of bactericidal agents. Our results agree with previous reports by Yan *et al*. (2016) who generated NEW with 50 ppm, Yan *et al*. (2021) who found characteristic FAC as 30-200 ppm and Elena *et al*. (2022) who used NEW with 50 ppm, 100 ppm, and 200 ppm.

Also, the distribution of the relative fractions of FAC (HOCL) in EW is dependent on pH values as HOCl dissociates to Cl_2 at low pH values and change to −OCl in alkaline pH (Park *et al*., 2004). Thus, by maintaining neutral pH value HOCL is more effective.

Our results recorded that increasing NaCl concentration did not change the stabilized values of pH, but significantly increased ($p<0.05$) TC, which agree with Park *et al*. (2004).

Salmonella, *E. coli* and *S. aureus* are common food contaminants that cause human illness. Their prevalence in different food stuffs in this study were 2%, 30% and 9%, respectively. Nearly similar to Ishaeeb *et al*. (2023) and Liu *et al*. (2022) who isolated *Salmonella*, and coagulase positive *S. aureus* by16.25%, 6.25%.

Thus, food sterilization especially at local poultry and meat processing plants before consumption is a must to prevent infection. NEW is also a highly effective sterilization method to prevent *Salmonella*, EPEC, and *S. aureus* contamination.

In this study, the antimicrobial effect of NEW on Gram-negative bacteria (*S.* Typhimurium and EPEC) and Gram positive bacteria (*S. aureus*) was recorded. NEW exhibited significant bacterial inhibition in broth. Besides, NEW demonstrated effect on bacterial cell characteristics.

In this study, antibacterial analysis assay of NEW in broth displayed the effect of different NEW concentrations and (bacterial suspension-NEW) contact time on the viability of bacteria to achieve the best bactericidal activity, which increases with higher concentrations and prolonged time. The bactericidal effect reached 99% with 49 ppm FAC (NEW 25%) and 10 min. treatment.

Generally NEW application on all bacterial suspensions for 10 min was more bactericidal than 5 min, and higher FAC (49ppm) was more bactericidal than 7ppm this agrees with Yuan *et al*. (2023) who stated that electrolyzed water effect increases with time and FAC ($P < 0.05$).

Our in vitro EOW studies on bacterial suspensions have shown good results in their ability to reduce and eliminate MDR food pathogens.

As shown about 99.7%, 99.7% and 99.9% reduction Log CFU/ml of *S.* Typhimurium in the bacterial suspension was recorded by NEW treatment after 10 min for three concentrations of 1%, 12% and 25%, with FAC 7.5, 25, 49 ppm, respectively.

Results of bacterial count reduction agree with Hamidi *et al* .(2021) who found that NEW achieved total elimination (100%) on *Salmonella* pure culture when treated for 10 min with different concentrations (50,100 and 200 ppm of FAC) and similar to Hernández-Pimentel *et al*. (2020) who found that NEW produced ten times higher antimicrobial activity than sodium hypochlorite on *Salmonella* pure culture.

As shown about 99.5%, 99.5% and 99.8% reduction Log CFU/ml of EPEC in the bacterial suspension was recorded by NEW treatment after 10 min for three concentrations (1%,12% and 25%), respectively. This result agrees with Hsu *et al*. (2019) and Hernández-Pimentel *et al*. (2020) as shown that bactericidal activity of NEW against EPEC using T.C 100mg and 50 mg for 10,30 second was very effective and rapid.

As shown in Table 3, about 99.2%, 99.4% and 99.7% reduction log CFU/ml of *S. aureus* in the bacterial suspension was recorded by NEW treatment after 10 min for three concentrations (1%, 12%, and 25%), respectively, which agree with Kim *et al*.(2019) who recorded complete inactivation of *S. aureus* after 10 min treatment with NEW.

In developing countries, chicken meat is one of the most important sources of protein. With increasing of chicken demand and distance of markets from production unit, the demand of using effective sanitizers increase to maintain chicken meat safety and quality during storage and transportation chain (Patricia *et al*., 2023).

NEW treatments were significant in decreasing initial count of *S.* Typhimurium and EPEC than *S. aureus*. These results suggest that there is a difference in susceptibility between Gram-ve bacteria and Gram +ve bacteria using NEW 25%.

In our findings, NEW exhibited a strong inhibitory effect against *S.* Typhimurium, EPEC and *S. aureus* (inhibition zone diameter ≤10 mm, ≤ 41 mm and ≤ 21 mm) using agar well diffusion test and MIC values was 12.25 ppm for EPEC, 24.5 ppm for *S.* Typhimurium and *S. aureus*.

Transmission electron microscope analysis (TEM) of the bacterial cellular -morphology before and after treatment with solutions (NEW) showed that *S.* Typhimurium, EPEC and *S. aureus* cells were significantly deformed and destructed after exposure to NEW with free available chlorine concentration within (24- 49 ppm) which is relatively similar to Li *et al*. (2016) and Kim *et al*. (2019) who observed damage to bacterial cell at free chlorine between 10 and 60 ppm

In this study, TEM images show that NEW treatment with its free chlorine substances (HOCL), exerted a destructive effect on bacterial barrier (cell wall and cell membrane) leading to increase the permeability and leakage of intracellular components (DNA, protein and k^+) and inhibiting of enzyme activity, which is considered a main mode of action for NEW, thus, the bacterial morphology appeared rough, shrunken and even dissolved, the same was documented by Otter *et al*. (2015) and Hernández-Pimentel *et al*. (2020).

In the present study, NEW sanitizer exhibited a significant visible impact on structural changes of Gram negative bacteria (*S.* Typhimurium and EPEC) when compared to Gram positive bacteria (*S. aureus*). *S.* Typhimurium and EPEC exhibited elongation of bacterial cell, wrinkling, and developing of vacuoles in the cytoplasm and finally bacterial destruction with ghost appearance. In contrast to this, *S. aureus* showed better structural integrity but also, developed vacuoles in the cytoplasm, these results agree with previous reports. This variation in bacterial response to NEW treatment in TEM images between Gram -ve (*Salmonella* and *E. coli*) and Gram +ve (*S. aureus*) bacteria may be because of the variation in their cell- wall composition, structure, and thickness (Feliciano *et al*., 2012). It is proposed that DNA damage is the main cause of appearance of filaments in the central region of the bacterial cytoplasm (Fukuzaki., 2006). Staphylococcal enterotoxins (SEs) are the main virulence factor that causes foodborne intoxications by *S. aureus* leading to severe clinical conditions in the public health (Ghaemi *et al*., 2013).

Results of uniplex PCR demonstrated that *seb* gene was detected in 4 out of 5 examined *S. aureus* isolates (80%), while *sea* gene was absent in all *S. aureus* examined isolates (0%) similar to Abolghait *et al*.(2020) who found that most MRSA isolates harbored the *seb* gene (75%, 6/8). It is interesting to note that this finding is different than that reported in some other countries (Ghaemi *et al*., 2013).

Thus, RT-qPCR technique was used to target the modulation in relative expression levels of virulence gene (SEB toxin encoding- gene) of

S. aureus before and after treatment with NEW 25% (49 ppm FAC) for 30 min.

The obtained findings revealed that SEB toxin-encoding genes were significantly down regulated after treatment. Obviously, the significant inhibition of bacterial toxin gene expression by NEW is useful in preventing outbreaks of food-borne disease. Our findings agree with Shimamura *et al*. (2015) and Escobedo-González *et al*. (2016) who eliminated *SEA* enterotoxins by 100%.

The antimicrobial susceptibility test (AST) revealed that all isolated bacteria from Gharbia, Egypt showed multiple drug resistant for more than 3 antimicrobials, similar to findings reported in Iraq, where MRSA incidence was 100% (Saber *et al*., 2022; Naeim *et al*., 2023). MRSA has been found in a growing number of food products. Besides, it has been defined by WHO as one of the 12 major pathogens that threaten human health (Craft *et al*., 2019).

Findings of uniplex PCR in this study demonstrated that *mec*A gene was detected in 2/2 (100%) of the examined *S. aureus* isolates. So, our work suggests that veterinarians should pay more attention to antimicrobial misuse to face antimicrobial resistance problem. In our study, RT-qPCR was used to target the modulation in relative expression levels of methicillin resistant gene (*mec*A) in *S. aureus* before and after treatment with NEW 25% (49 ppm FAC) for 30 min. Our study explored that resistant *S. aureus* gene (*mec*A) were significantly down regulated in the treated *S. aureus*. According to the obtained findings, the inhibitory effect of NEW 25% on the methicillin – resistant *mec*A gene was higher than that of the enterotoxin *seb* gene.

Biofilm formation is associated with a protective mechanism compared to floating planktonic cells as it resist adverse environmental conditions, maintain bacterial growth and results in a greater resistance to disinfectants, preservatives, antibiotics and immune defenses (Lister and Horswill, 2014; Hussain *et al*., 2019).

Thus, Electrolyzed water was recorded as a significant method for destructing bacterial biofilms which is important for food safety and public health (Yan *et al*., 2021)

In our study, biofilm forming activity of all *S.* Typhimurium, EPEC, and MRSA isolates was recorded using a microtiter plate assay. These polystyrene microtiter plates similar to plastic materials widely used in chicken processing plants and poultry farms (Borges *et al*., 2018).

Our findings revealed that at all *S.* Typhimurium, EPEC and MRSA isolates formed a strong biofilm on microtiter plates. These results agree with Borges *et al*. (2018) who reported the attachment of bacterial biofilms to hydrophobic surfaces such as polystyrene.

Moreover, our results showed that there is a relationship between drug resistance and biofilm, in agreement with Hamad *et al*. (2019) who detected that misuse of low doses of antibiotics enhance bacterial biofilm formation ability.

A 5/5 (100%) of MDR *S. aureus* were biofilm producers; 100% of them were strong biofilm producer, this finding agrees with Hamad *et al*. (2019) who reported that all isolated biofilm producing bacteria were MDR bacteria.

In this study, the antibiofilm activities of NEW in 96-well flat bottom microtiter plate were investigated. As a novel method to eradicate bacterial biofilms of *S.* Typhimurium, EPEC and *S. aureus* which are major pathogens forming biofilm on the surfaces of food and food equipment.

Our findings revealed that a good antibiofilm activity (> 50% inhibition of bacterial biofilm formation) with NEW 25% (49ppm F.A.C) treatment for S.Typhimurium EPEC and *S. aureus* biofilms resulting in inhibition value of 94.6%, 80.1% and 76%, respectively. However, lower results were obtained with NEW 12% (24.5 ppm FAC) treatment for *S.* Typhimurium, EPEC and *S. aureus* biofilms resulting in inhibition value of 83.2%, 54.9%., and 57.4 %, respectively. These results agree with Hamad *et al*. (2019) who recorded that higher chlorine concentration (200 ppm) generated a biofilm reduction by 94.8%, 93.53% and 71.38% for *S.* Typhimurium, EPEC and *S. aureus* biofilms, respectively. Which assumes that NEW antibiofilm activity is concentration dependent. NEW solutions had good stability, during the 4 days storage, they keep chlorine concentration, pH and ORP values approximately constant, as reported by Rahman *et al*. (2010). Thus, NEW also has a longer shelf life than different types of EOW.

These findings support novel direction of using Neutral electrolyzed water (NEW) as a potent sanitizer and antimicrobial agent against MDR and strong biofilm - producing bacteria.

Conclusion

The present study has displayed that neutral electrolyzed water significantly reduced bacterial count in vitro, even destroyed bacteria using TEM analysis which suggests the potential use of NEW as a safe natural alternative of antimicrobials and sanitizers in reducing bacterial contamination with *Salmonella*, *E. coli*, and *S. aureus*, owing to environmentally friendly, easy production, safe handling, low toxicity because of no by-product generation and low corrosion capacity on food surfaces and handlers. Besides the significant use of Neutral electrolyzed water in eradicating biofilms produced by *Salmonella*, *E. coli* and *S. aureus*. Furthermore, this study is the first to target transcriptional expression patterns of the toxin-associated genes and resistance -associated genes after neutral electrolyzed water treatment on MRSA in Egypt. More research is needed to understand the mechanism of antimicrobial action of EOW and discuss method of its application in the processing of different food products to control food poisoning bacteria.

Conflict of interest

The authors declare that they have no conflict of interest.

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