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RESUSCITATION OF VIABLE BUT NON-CULTURABLE (VBNC) STAPHYLOCOCCUS *AUREUS* AS A RESULT OF ELECTROLYZED WATER (EW) ON FOOD CONTACT SURFACE

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ABSTRACT

The use of electrolytically generated acidic water (SAcEW) as a standalone sanitizer for food contact surfaces, or in sequence with electrolytically generated alkaline water (SAlEW) as a potent cleaning agent due to its sodium and potassium hydroxide content, effectively eliminated *Staphylococcus aureus* contamination and demonstrates promise as a broad-spectrum, high-performance bactericide in the food production industry. This study investigated the disinfection efficacy of SAlEW, SAcEW, and their combined application against *S. aureus*, including quantification of dead cells and Viable but Non-Culturable (VBNC) cells post-resuscitation. SAcEW treatment significantly reduced *S. aureus* populations by 4–6 log CFU/g and achieved a 100% reduction on stainless steel and plastic surfaces. Sequential treatments with SAlEW followed by SAcEW resulted in a 100% reduction across all three tested surfaces (stainless steel, glass, and plastic), whereas SAlEW alone eliminated a portion, but not all, of the *S. aureus* population. Post-resuscitation analysis revealed the following percentages of VBNC and dead cells, respectively: SAlEW—40.65% and 22.58% (SS), 38.95% and 22.42% (glass), 34.68% and 29.5% (Plastic); SAcEW—9.68% and 74.19% (SS), 24.22% and 46.16% (Glass), 21.1% and 78.9% (Plastic); and the combined SAlEW/SAcEW treatment—0.0% and 100%, 19.64% and 80.36%, and 0.0% and 100%, respectively. The public health implications of the VBNC state of *S. aureus* are discussed.

KEYWORDS: Viable but Non-Culturable *Staph. aureus* (VBNC), Electrolyzed water (SAlEW & SAcEW), Resuscitation, RT-PCR, Propidium iodide (PI).

INTRODUCTION

Staphylococcus aureus is a Gram-positive bacterium capable of producing several enterotoxins involved in staphylococcal intoxication (**Din *et al.*, 2016).** The public health risks associated with consuming foods contaminated with *Staph. aureus* has garnered significant attention from governmental and International organizations and bodies concerned with food safety worldwide in recent years. *Staph. aureus* infections in humans have been reported in Japan, with approximately 32.5% of contaminated food samples tested being positive for *Staph. aureus*, with a notably higher contamination rate. In this regard, the authors found that 76.3% were contaminated with the organism. In the United States and Canada, *Staph. aureus* is estimated to be responsible for 33% and 45% of bacterial food poisoning cases, respectively. Similar outbreaks have occurred in China in 2008, where over 100 children were infected after consuming contaminated milk **(Xia and Wolz, 2014).**

Under stress conditions, many bacterial species enter a Viable but Non-Culturable (VBNC) state. Several human pathogenic bacteria have been reported to transition into this VBNC state when subjected to environmental stresses. While pathogenic bacteria in the VBNC state cannot be cultivated using conventional culture methods, they retain viability and the capacity to express virulence factors. The VBNC state can be induced both in vitro and in vivo, and resuscitation from this state is possible under appropriate conditions. The VBNC state represents a survival strategy employed by bacteria, with significant implications for environmental monitoring, food technology, and infectious disease management. Therefore, investigating the association between bacterial pathogens in the VBNC state and water/foodborne outbreaks is crucial. (**Ramamurthy *et al.*, 2014).**

The Viable but Non-Culturable (VBNC) state represents a unique survival strategy adopted by certain bacteria. In adverse environmental conditions, some bacteria enter a dormant state where they cannot be cultured using standard laboratory methods, despite retaining minimal metabolic activity. This state allows them to evade routine testing, leading to false negative results and potentially posing a significant risk to food safety. These "hidden contaminants" can still be revived under favorable conditions, regaining their ability to grow and multiply, thus presenting a potential threat to human health. **(Li *et al.*, 2024).**

In the food industry, food and beverage processing, improper pasteurization, and the use of low and high temperatures and CO_2 pressure during packaging can induce a Viable but Non-Culturable (VBNC) state in bacteria. In clinical settings, pathogens in the VBNC state have been implicated in numerous latent infections, which may manifest after months or even years (e.g. tuberculosis). (**Colwell and Huq, 1998).**

The Viable but Non-Culturable (VBNC) state is a prevalent microbial stress response to external stimuli, including oxidative stress, temperature fluctuations as well as storage temperature (chilling & freezing), nutrient deprivation, food additives and high osmolarity **(Zhao *et al.*, 2017).** This condition characterizes a unique physiological condition where microorganisms fail to proliferate on standard specific culture media while maintaining specific metabolic activities (**Zhao *et al.*, 2013).** A significant challenge posed by VBNC microorganisms stems from their detectability using conventional plate count or detection methods, despite their potential to retain virulence and exhibit resistance to various stressors. Consequently, the presence of VBNC microorganisms within the food processing environment constitutes a substantial threat to food safety and public health.

Viable but Non-Culturable (VBNC) condition of microorganisms has garnered significant attention owing to its distinctive characteristics, namely the difficulty of detection using traditional specific culture-based methods, the retention of virulence, and high resistance to various stressors. As a prominent foodborne pathogen, *Staph. aureus* exhibits a widespread distribution and has been documented to transition into the VBNC state under certain environmental stressors, thereby posing a potential threat to human health **(Yan *et al.*, 2021).**

Viable but Non-Culturable (VBNC) state is a survival strategy employed by bacteria when exposed to unfavorable environmental conditions. Upon encountering favorable conditions, such as nutrient supplementation, removal of external stressors, or the introduction of resuscitation-promoting substances, bacteria can recover from the VBNC state. The successful resuscitation of a bacterium from the VBNC state is crucial for confirming its actual existence, as it necessitates excluding the possibility of regrowth from a small population of culturable cells that may have remained viable. The resuscitation of VBNC cells has been extensively studied to assess and mitigate the risks associated with the recovery of pathogenic or spoilage bacteria. Furthermore, the resuscitation of bacterial function holds significant promise as an area for further exploration **(Pan and Ren, 2022).**

Contamination of food with foodborne pathogens arising from contaminated cutting boards, knives, and food preparation surfaces poses a significant risk to food safety. Effective methods for reducing or inactivating pathogens from these sources are crucial for establishing a safe food supply. Electrolyzed water (EW) is gaining increasing popularity as a cleaner and sanitizer in the food industry due to its demonstrated effectiveness in reducing or eliminating various pathogens **(Tolba, 2020 & 2023).**

Viable but Non-Culturable (VBNC) bacteria are ubiquitous in diverse environments, including water, air, soil, food products, medical settings, food processing facilities, and elsewhere **(Dong *et al.*, 2020).** A significant proportion of bacteria capable of entering the VBNC state are pathogenic, potentially expressing toxins even in this state or regaining infectivity and pathogenicity upon resuscitation, leading to human illness or food spoilage **(Oliver, 2005 and Yoon *et al.*, 2021).** Resuscitation of VBNC cells in food can occur during storage or through contamination of food contact surfaces and utensils, potentially contributing to foodborne outbreaks **(Ferro *et al.*, 2018).** Consequently, the risks posed by VBNC bacteria and particularly their resuscitation, have become a critical area of focus in food safety risk assessment. While researchers have long investigated these risks to human health, understanding and controlling resuscitation can also be crucial in various ecological processes and/or hold significant value within the food industry **(Zhang *et al.*, 2021).** Regardless of the specific context, exploring and elucidating the mechanisms of resuscitation are both necessary and highly significant. This research aims to investigate the bactericidal efficacy of electrolyzed water against *Staph. aureus* on food contact surfaces. Specifically, the study will determine whether electrolyzed water effectively eliminates the bacteria or induces a Viable but Non-Culturable (VBNC) state. Furthermore, the research will explore the potential for resuscitation of VBNC *Staph. aureus* cells, which were determined by using RT-PCR in conjunction with a PI dye to distinguish between live and dead cells. Following standard laboratory techniques and the resuscitation attempts will be conducted following established laboratory protocols.

MATERIAL AND METHODS

1-Bacterial strain and culture conditions

1- *Staph. aureus* ATCC 25923 strain was cultured and activated before applying the experiment. The

activation procedure was referred to the method described by **Bai *et al.* (2019) and İplikçioğlu *et al.* (2020).** The stocked strain was streaked onto tryptic soy agar (TSA) plate and incubated at 37 ◦C for 24 h. Then a single colony was inoculated into tryptic soy broth (TSB) and cultured at 37 ◦C for 24 h. All strains are adjusted to a cell count of 10^6 CFU/mL using a spectrophotometer (Thermo Scientific, TM1000). This standardized inoculum was then used to contaminate food contact surfaces comprising stainless steel, glass, and plastic, serving as experimental substrates in the present study.

2- Preparation and application of Electrolyzed water according to Tolba *et al.* (2023)

Electrolyzed water is a natural, organic, non-toxic, environmentally, and ecologically safe cleaning, sanitizing, and disinfecting agent. It is produced through the electrochemical reaction of potable water, salt (NaCl), and a source of electricity (Cathode and Anode). The reaction products include:

A- Alkaline solution

Sodium hydroxide (NaOH), Potassium hydroxide (KOH), Sodium hypochlorite (NaClO) and Potassium hypochlorite (KClO) Formation

During this process, positively charged sodium ions (Na^+) attracted to the negatively charged electrode (cathode). At the cathode, they react with oxygen and hydrogen, undergoing electrochemical conversion to form sodium hydroxide (**NaOH**). Simultaneously, sodium ions (Na^+) combine with hypochlorite ions (ClO^-) and hydroxide ions (OH^-) to produce sodium hypochlorite (**NaClO**). Potassium ions (K^+), were added to the separating membrane as a soaking solution, also react with hydroxide ions (OH^-) to form potassium hydroxide (**KOH**), as well as with ClO^- to form KClO. Both sodium hypochlorite and potassium hypochlorite exhibit greater stability in alkaline solutions compared to acidic environments, and possess potent antibacterial properties, thereby rendering alkaline solutions effective as both detergents and antibacterial agents. Potassium hypochlorite and potassium hydroxide demonstrate superior stability relative to their sodium counterparts, suggesting that the introduction of potassium enhances the dispersive effect of sodium, contributing to its efficacy as a detergent and antibacterial agent. The chemical reactions as shown in the following equations

$$2\,NaCl\,(aq) + 2\,H_2O\,(l) \rightarrow 2\,NaOH\,(aq) + Cl_2\,(g) + H_2\,(g)$$

$$K^+ + OH^- \rightarrow 2\,H_2O\,(l) \rightarrow 2KOH\,(aq) + Cl_2\,(g) + H_2\,(g)$$

$$ClO^- + OH^- \rightarrow ClO^- + H_2O$$

$$Na^+ + ClO^- + OH^- \rightarrow NaClO + H_2O$$

$$K^+ + ClO^- + OH^- \rightarrow KClO + H_2O$$

Therefore, the alkaline solution containing **NaOH** and **KOH** is considered a powerful cleaner and degreaser, but Sodium and potassium hypochlorite (**NaOCl & KClO)** is primarily a disinfectant due to its ability to kill a wide range of microorganisms.

B- Acidic solution

Hypochlorous acid (HOCl), Hypochlorite ions (OCl) & Hydrochloric acid (HCl): Chloride ions, being negatively charged, are separated from sodium ions and attracted to the positively charged electrode (Anode). Later, they combine with oxygen and hydrogen arises from water hydrolysis, forming Hypochlorite ions (**OCl**), Chlorine gas (Cl_2) and hypochlorous acid (**HOCl**), a potent sanitizer and disinfectant.

$$2H_2O\,(l) + 2e^- \rightarrow H_2\,(g) + 2OH^-(aq)$$

$$Cl_2\,(g) + H_2O\,(l) \rightleftharpoons HOCl\,(aq) + HCl\,(aq)$$

$$HOCl\,(aq) + OH^-(aq) \rightleftharpoons OCl^-(aq) + H_2O(l)$$

3- Food contact surfaces preparation

Three replicates of three different 10x10 cm² food contact surface materials (stainless steel, plastic, and glass), were used in this study to assess the antibacterial activity of electrolyzed water (EW) on new, unscarred surfaces.

Before experimentation, the food contact surfaces underwent a decontamination procedure involving a 60-minute immersion in a 5% surfactant solution, followed by manual cleaning with detergent, a coarse rinse with warm tap water (30.0–35.0 °C), and a final rinse with heated distilled water (85.0–90.0 °C). The surfaces were then air-dried in a biosafety cabinet. Immediately before each experiment, the test surfaces were exposed to UV light for 30 minutes within a biosafety cabinet **(Hillig *et al.*, 2023).** Glass contact surfaces were sterilized by dry heat at 160 °C for 1–2 hours, after being wrapped in aluminum foil.

4- Surface Inoculation (Attachment)

A 100 cm² sampling area was delineated using a template (Technical Service Consultants Ltd) according to **Hillig *et al.* (2023).** One milliliter of each bacterial culture, containing the selected pathogen at a concentration of 10^6 CFU/ml, was then inoculated onto each separate food contact surface and spread evenly across the designated area at room temperature (22–23 °C). Following inoculation, the surfaces were allowed to air dry for 30 min in a laminar flow cabinet under aseptic conditions to facilitate bacterial attachment **(İplikçioğlu *et al.*, 2020).**

5- Sanitization treatment

Following the attachment, each surface covered by EW treatment solution at room temperature for 30 min. After the treatment, the surviving population of the pathogens on the surface and in the soaking solution was determined by sampling. One surface from each type, which is inoculated but not immersed to the EW, was evaluated as a control group for the used pathogen to control the efficiency of inoculation. After immersion, by using a sterile tong each food surface was aseptically removed and water was drained from the plate **(Venkitanarayanan *et al.*, 1999)**.

6- Sampling Procedure

Before sampling, the sterile swab was moistened for 5 minutes with a sodium chloride peptone solution. Surface sampling was performed following **ISO 18593:2018**. Each surface area was swabbed using a meandering pattern, first with the moistened swab and then with a dry swab, swabbing horizontally, vertically, and diagonally **(Fig. 1).** The swab heads were then aseptically excised and vortexed in a sterile test tube containing 9 ml of sterile peptone water. Subsequently, 0.1 ml aliquots from appropriate dilutions were plated onto selective Baird Parker agar media (Oxoid) for *Staph. aureus* count. All plates were incubated at 37°C for 24–48 hours. The number of colonies was counted and recorded as viable *Staph. aureus* count. **(FDA, 2001 & İplikçioğlu *et al.*, 2020).**

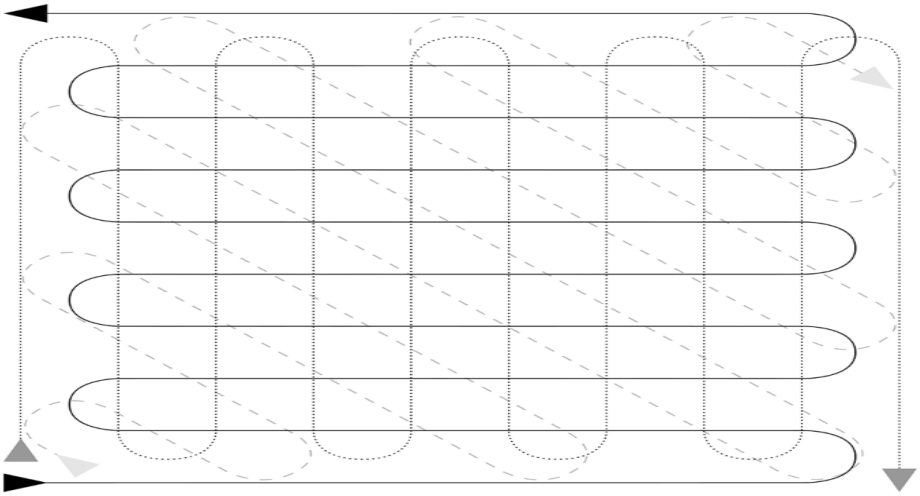

Fig. 1: Illustration of sampling procedure. Wet swab was first swept across the surface in meandering pattern horizontally (black arrowheads), vertically (dark grey) and diagonally (light grey), followed by the dry swab in the same way.

7- Real time (RT-PCR) VBNC DNA Extraction Method

7. A- DNA extraction

Was applied for the Samples that tested negative for *Staph. aureus* by using the traditional culture method were subsequently analyzed using RT-PCR. In conjunction with Propidium Iodide (PI). DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56 °C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

7. B- Materials

1- Material used for Propidium iodide staining
2- Propidium Iodide (PI) (Sigma-Aldrich), part No. P4170
3- DMSO (Sigma-Aldrich), part No. 276855
4- Halogen lamp 500W
5- Oligonucleotide Primers and probes. Primers and probes used were supplied from Willowfort (UK)
6- Oligonucleotides. Primers and probes used were supplied from Metabion (Germany)

Table A: Primers and probe sequence of 16S rRNA gene of *Staph. Aureus.*

Gene	Primer/ probe sequence 5'-3'	Ref
***S. aureus* 16S rRNA**	**CCG TCA CAC CAC GAG AGT TT**	**Ablain *et al.*, 2009**
	TTA CGA CTT CAC CCC AAT CA	
	FAM- CCG AAG CCG GTG GAG TAA CCT T -BHQ1	

7. C- Propidium iodide staining (Taskin *et al.*, 2011) for all protocol and (Deng *et al.*, 2016) for 500W lamp

1- Propidium iodide was dissolved in 20% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) to get a stock solution of 10 mM and stored at -20°C.
2- The PMA stock solution was transferred to 500 µl culture mixtures at a final concentration of 100 µM. All manipulations of PMA solution were performed under minimal light to prevent any potential chemical change in PMA structure.
3- Following 10 min of incubation in the dark, samples were exposed for 5 min to a 500-W halogen light source at a distance of 15 to 20 cm from the light source. Tubes were placed on ice during the light exposure to avoid excessive heating.

7. D- DNA amplification

PCR amplifications were performed in a final volume of 25 µl containing 3 µl of DNA template, 12.5 µl of 2x QuantiTect Probe RT-PCR Master Mix, 8.875 µl PCR grade water, 0.25 µl of each primer (50 pmol conc.) and 0.125 µl of each probe (30 pmol conc.). Primary denaturation was performed at 94^{o}C for 15 min, followed by 40 cycles of denaturation at 94^{o}C for 15 s, annealing at 58^{o}C for 45 sec. The reaction was done in the stratagene MX3005P real time PCR machine.

Samples exhibiting positive PCR results, indicative of the presence of Viable but Non-Culturable (VBNC) *Staph. aureus* cells underwent resuscitation attempts. Following resuscitation, standard microbiological assays were employed to enumerate the previously VBNC *S. aureus* population.

8- Resuscitation and counting of VBNC *Staph. aureus*

Recovery of *S. aureus* was carried out according to **Tallent *et al.* (2016),** all the swab samples were prepared and enriched into Brain Heart Infusion (BHI) broth with 1% sodium pyruvate as antioxidant to mitigate oxidative stress on bacterial cells, which arises from the accumulation of reactive oxygen species (ROS), including free radicals, and leads to damage of DNA, proteins, and lipids, resulting in a viable but non-culturable (VBNC) state, then swabs were incubated at 37°C for 24 h to allow for resuscitation of stressed as well as injured *S. aureus*. After resuscitation, plating on Baird Parker Medium (Oxide, Basingstoke, England), incubated inoculated plates aerobically at 37 ^{O}C for 24 h. The direct plating method using BPA is the standard method against other direct plating media and methods for recovering and counting of sub-lethally injured *Staph. aureus* cells according to **FDA (2001).**

9- Identification of resuscitated *Staph. aureus* strains morphologically (Gram stain), biochemically [catalase, coagulase and sugar fermentation (glucose, sucrose, lactose and mannitol)] according to **Bennett and Lancette (2001), ISO (2003b) and FSSAI, Foods Safety and Standards Authority of India (2015)**

RESULTS

Table (1) and Fig. (2) showed that SAcEW and the Mix treatment effectively reduced mean *Staph. aureus* count on stainless steel surfaces to below the detection limit (<1 $\log_{10}$cfu/g). On plastic surfaces, also both SAcEW and Mix treatment were effective on *Staph. aureus* count (<1 $\log_{10}$ CFU/g). For glass surfaces, both SAcEW and Mix treatments exhibited significantly (P<0.05) lower log counts (1.81±0.10 and <1 $\log_{10}$cfu/g) respectively, compared with control (6.11a±0.05), SAlEW (2.36±0.11 $\log_{10}$cfu/g) and tap water (2.87±0.21 $\log_{10}$ CFU/g). Overall, SAcEW and Mix treatments achieved a greater than 4-6 log reduction compared with control, as well as more than 3 log reductions in *Staph. aureus* as matched with tap water. In general, there are significant differences in *Staph. aureus* count between all treatments and control as well as within the treatments, except the difference didn't exist between SAcEW & mix treatment in stainless steel and plastic contact surface as both treatments could completely eliminate contamination with *Staph. Aureus.*

Table 1: Statistical analysis of *Staph. aureus* count ($\log_{10}$ CFU/g) in different water types using different food contact surfaces.

Type of contact surface	Water type				
	Control	Tape water	SAcEW	SAlEW	Mix (SAlEW + SAcEW)
Stainless Steel	6.20^{a}±0.05	2.86^{b}±0.11	$<1^{c}$	2.28^{d}±0.24	$<1^{c}$
Glass	6.11^{a}±0.05	2.87^{b}±0.21	1.81^{c}±0.14	2.36^{d}±0.11	$<1^{e}$
Plastic	6.17^{a}±0.04	2.69^{b}±0.09	$<1^{c}$	2.21^{d}±0.08	$<1^{c}$

There are significance differences between means having different superscripted small letters within the same raw

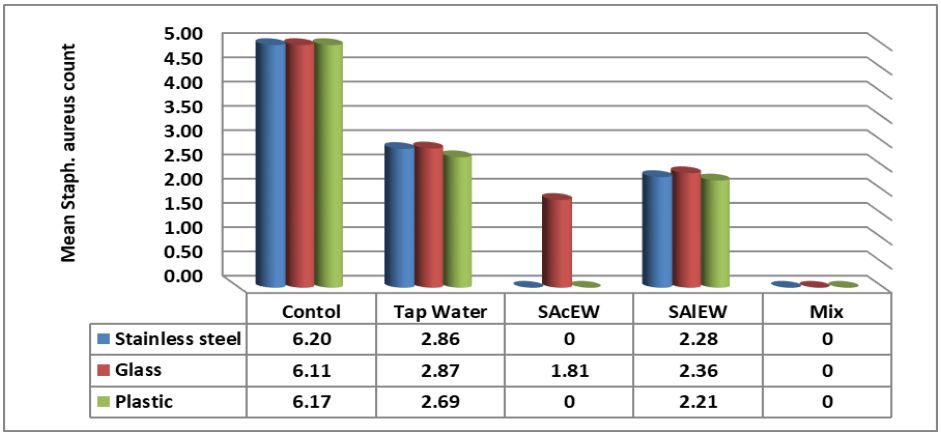

Fig. (2). Variation between the effect of different types of EW on *Staph. aureus* count.

Table 2: Reduction % of *Staph. aureus* on different food contact surfaces using different water types.

Surface type	Reduction %			
	Tap Water	SAcEW	SAlEW	Mix
Stainless steel	53.87	100	63.23	100
Glass	53.2	70.38	61.38	100
Plastic	56.4	100	64.18	100

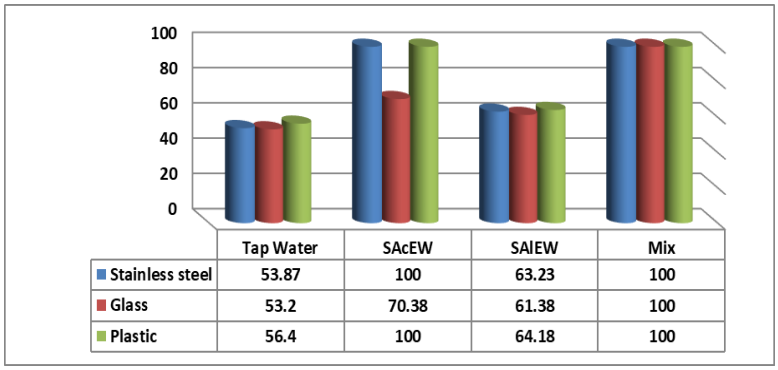

Fig. 3: Reduction % of different treatments in relation to control.

Tab le (2) and Figure (3) demonstrated that the *Staph. aureus* count (log_{10} CFU/g) was completely eliminated (100% reduction) on stainless steel, glass, and plastic surfaces when a combined treatment (SAlEW followed by SAcEW) was applied. Similarly, SAcEW alone exhibited 100% reduction on both stainless steel and plastic surfaces, but only recorded a 70.38% reduction on glass. These findings suggest that stainless steel and plastic surfaces may inherently possess better antimicrobial properties compared to glass contact surfaces. Furthermore, SAlEW alone demonstrated a reduction in *Staph. aureus* by 63.23%, 61.38%, and 64.18% on stainless steel, glass, and plastic food contact surfaces, respectively. This indicates that SAlEW was most effective on plastic surfaces, followed by stainless steel, and finally glass.

Tables (3) and Figures (4–6) demonstrated the complete inhibition of *Staph. aureus* on stainless steel (SS) and plastic food contact surfaces. Neither viable nor Viable but Non-Culturable (VBNC) cells were detected after resuscitation following treatment with a combination of SAlEW and SAcEW. On glass, this combined treatment resulted in a VBNC bacterial load of 1.2 log_{10}cfu/g (19.64%), indicating incomplete cell inactivation. In contrast, SAcEW alone failed to achieve complete *S. aureus* inhibition on any of the three surfaces after resuscitation. VBNC cells were observed, constituting 9.68%, 24.22%, and 21.1% of the initial population on SS, glass, and plastic, respectively, while corresponding dead cell percentages were 74.19%, 46.16%, and 78.9%. SAlEW, classified as a detergent rather than a disinfectant due to its composition and properties, exhibited the lowest antimicrobial efficacy. Following SAlEW treatment, VBNC and dead cells represented 40.65% and 22.58% (SS), 38.95% and 22.42% (glass), and 34.68% and 29.5% (plastic) of the initial population, respectively.

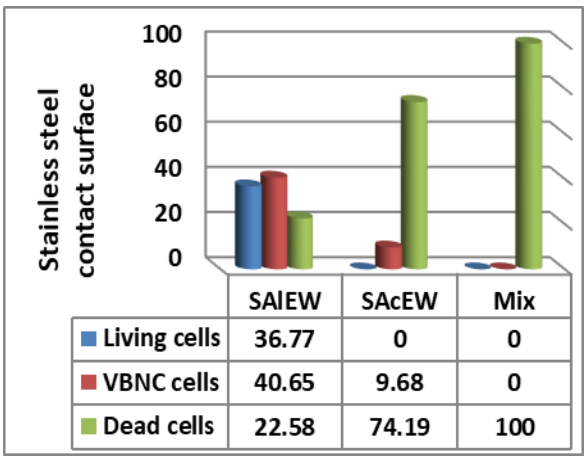

Fig 4. Incidence of Living, VBNC and dead cells of aureus cells on stainless steel contact surface.

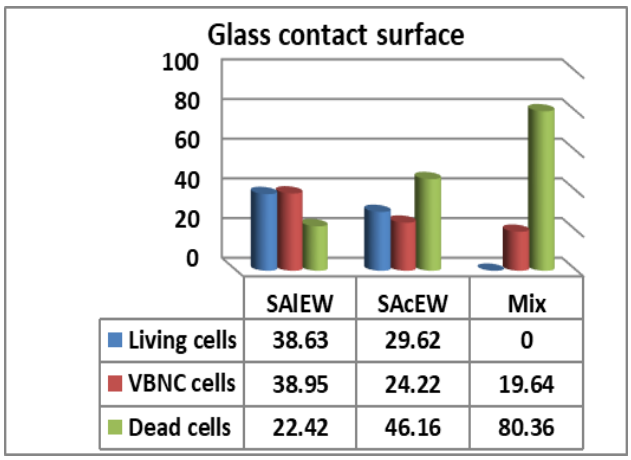

Fig 5. Incidence of living, VBNC & dead Staph. Staph. aureus cells on glass food contact surface.

Table (3). Incidence of viable, VBN-C and Dead cells of *Staph. aureus* on different food contact surfaces.

Log count & %	Surface type								
	Stainless steel			Glass			Plastic		
	SAlEW	SAcEW	Mix	SAlEW	SAcEW	Mix	SAlEW	SAcEW	Mix
Viable before resuscitation									
• Log	2.28	<1	<1	2.36	1.81	<1	2.21	<1	<1
• %	36.77	0.0	0.0	38.63	29.62	0.0	35.82	0.0	0.0
VBNC after resuscitation									
• Log	2.52	1.6	<1	2.38	1.48	1.2	2.14	1.3	<1
• %	40.65	9.68	0.0	38.95	24.22	19.64	34.68	21.1	0.0
Dead cells									
• Log	1.40	4.60	6.2	1.37	2.82	4.91	1.82	4.87	6.17
• %	22.58	74.19	100	22.42	46.16	80.36	29.5	78.9	100
Total									
• Log	6.20	6.20	6.2	6.11	6.11	6.11	6.17	6.17	6.17
• %	100	100	100	100	100	100	100	100	100

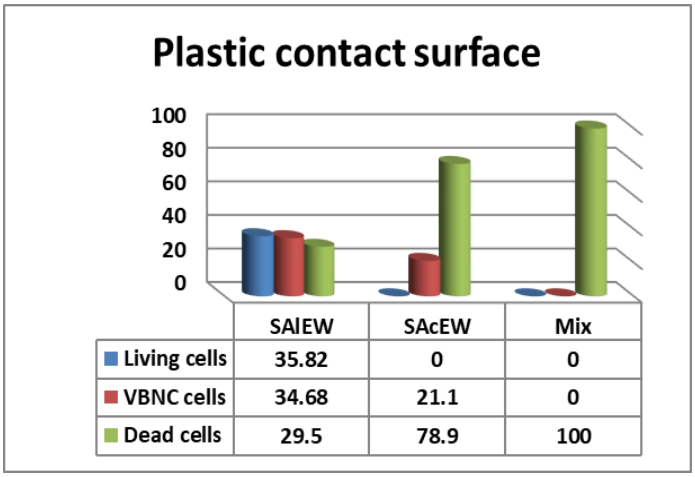

Fig 6. Incidence of living, VBNC & dead *Staph. aureus* cells on Plastic food contact surface.

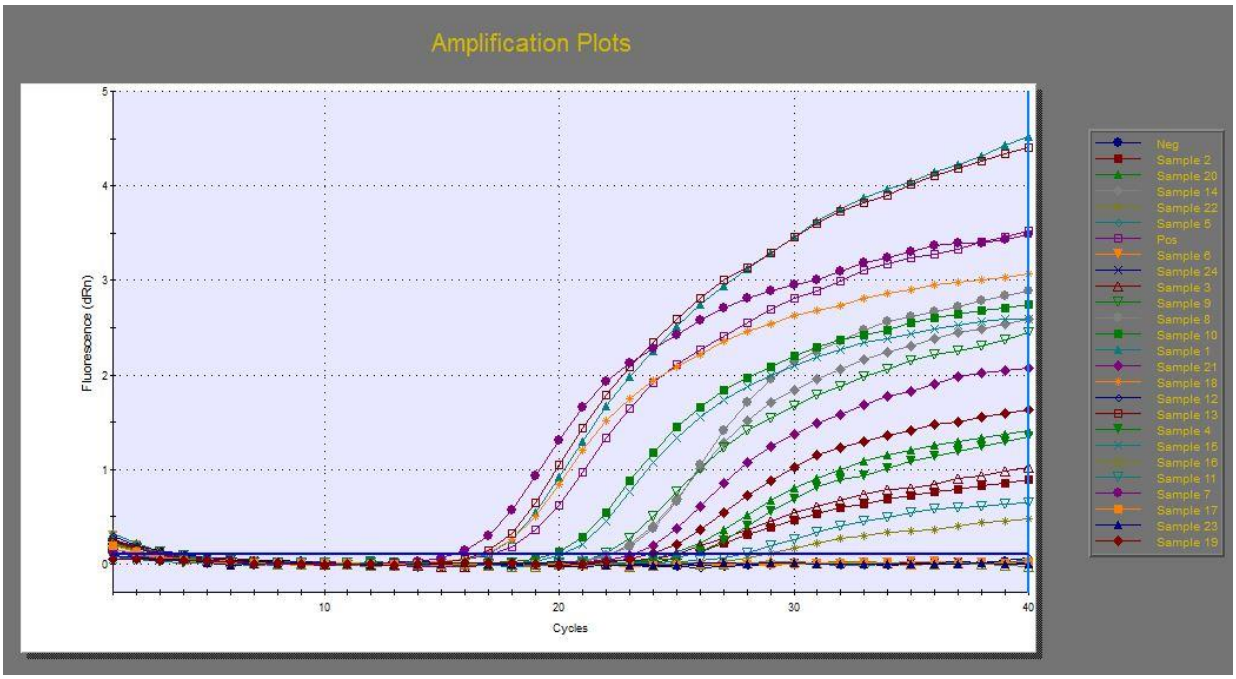

Fig. 7: Amplification curve of randomly collected swabs (n=24).

Figure 7 illustrated the results of 24 swab samples tested for the presence of Viable but Non-Culturable (VBNC) *Staph. aureus*. Seventeen samples tested positive, while seven tested negative. This determination was made by detecting the amplified *16S* rRNA gene using RT-PCR in conjunction with Propidium iodide (PI). PI selectively quantifies DNA from viable cells, including VBNC bacteria with intact cell membranes and cell walls. The RT-PCR results indicated the presence of VBNC S. *aureus* in 17 samples, while the other remaining 7 samples were negative. To resuscitate these strains, positive swabs were inoculated into Brain Heart Infusion broth and incubated at 37°C for 48 hours followed by applying the standard method for counting, isolation and identification of VBNC *Staph. aureus.*

DISCUSSION

Viable but Non-Culturable (VBNC) state and resuscitation of *Staph. aureus*

Staphylococcus *aureus* is capable of entering a Viable but Non-Culturable (VBNC) state. In this state, the organism cannot be cultured on standard media, leading to false-negative results in culture-based detection methods **(Xu *et al.*, 2011b & 2019; Liu *et al.*, 2018 a & b)**. **This is what we found out in the current research.** This issue is not confined to food safety; culturing is also the routine detection method for clinical strains **(Wang *et al.*, 2014).** Therefore, the formation of the VBNC state poses a significant challenge for both food safety and clinical settings.

Regarding VBNC formation and identification, rapid and reliable detection of VBNC MRSA is crucial and urgently needed, particularly in light of the increasing prevalence of this condition **(Liu *et al.*, 2016c, 2017c).** In this context, **Xinyu *et al.* (2020)** stated that when the storage time extended to 80 h, the culturable counts of *S. aureus* were undetectable on the medium, while the viable counts of S. *aureus* remained high (7.56 $\log_{10}$ CFU/ml) as determined by the PMA-qPCR method. This VBNC *Staph. aureus* count was more than that in the present study for any of the three surfaces used in the study. Moreover, **Viable but Non-Culturable (VBNC)** cells are characterized by reduced metabolic activity, diminished or absent pathogenicity, cell shrinkage, and/or aberrant morphology **(Signoretto *et al.*, 2002; Pinto *et al.*, 2013 and Yoon *et al.*, 2019**). These cells, induced by a variety of environmental, biological, or chemical stressors, can resuscitate, regaining their capacity for cell division, increasing metabolic activity, and restoring pathogenicity and typical morphology (Figure 7). The restoration of normal morphology involves cellular remodeling. Furthermore, the recovery of replicative ability during resuscitation from the VBNC state requires the resynthesize of cytoplasmic proteins and cell wall peptidoglycan. **These findings corroborate the results observed in the present study.**

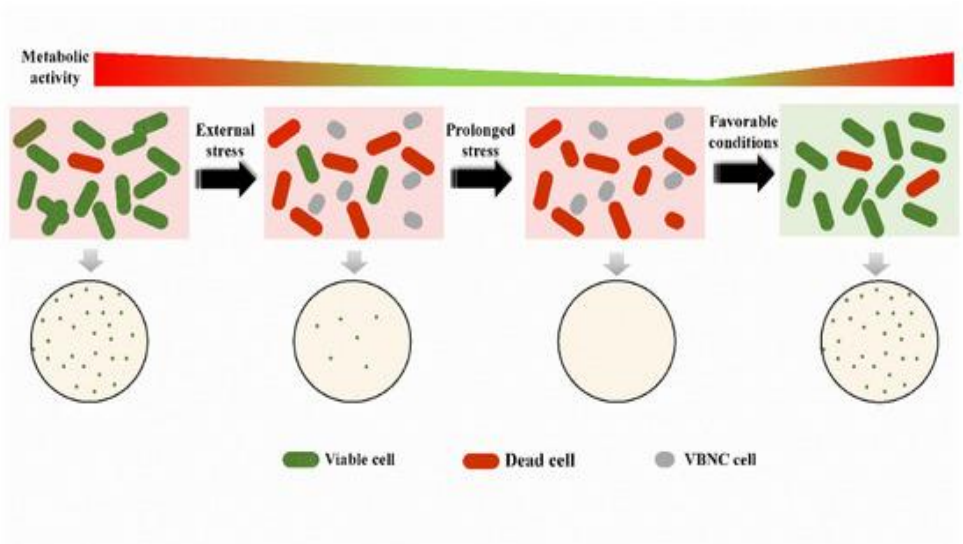

Figure 7: VBNC State Formation and Resuscitation when the conditions are favorable.

Effect of EW treatment of food contact surfaces contaminated with *Staph. aureus*

The sanitizing effects of SAcEW and the combined treatment demonstrated a reduction in Staphylococcus *aureus* counts, likely attributable to damage to the cell membrane structure and intracellular components of the bacterium. This finding aligns with the observations reported by **Hao *et al.* (2017).**

The findings of this study **also comply with the observations** of **Tian *et al.* (2016),** who reported a 5.8 log cfu/ml reduction in Staphylococcus *aureus* count using SAcEW. The authors concluded that SAcEW exhibits superior overall disinfection efficacy, attributing this to the strong oxidizing properties of hypochlorous acid (HClO), the primary active chlorine compound. Their research further suggested that the disinfection mechanism of SAcEW involves disrupting the cell membrane permeability.

In a previous study conducted by **İplikçioğlu *et al.* (2020)**, they found that immersing wooden cutting boards in 500 ml of EW at room temperature for 10 minutes significantly reduced populations of *Salmonella typhimurium*, *Escherichia coli*, *Listeria monocytogenes*, and *Staph. aureus*. All pathogens were reduced by at least 1 $\log_{10}$ CFU/100 cm² within the first minute of treatment. Notably, *Staph. aureus* and *S. typhimurium* were not detected after 8 hours (100% reduction) , while *E. coli* and *L. monocytogenes* were not detected after 2 hours of treatment**.** These findings support the use of sequentially applied Slightly Acidic Electrolyzed Water (SAEW) followed by Slightly Alkaline Electrolyzed Water (SAlEW) in the present study. This sequential application resulted in a 100% reduction of Staphylococcus *aureus* contaminating food contact surfaces. Furthermore, SAcEW alone was effective in completely eliminating Staphylococcus *aureus* from contaminated stainless steel and plastic surfaces.

Surena *et al.* (2020) found that Acidic Electrolyzed Water (AcEW) reduced Staphylococcus *aureus* counts from 2.4 x 10^9 to 4.7 x 10^7cfu/g (98.04%). **This finding aligns with the results obtained in the current study**. Furthermore, Mildly Basic Electrolyzed Water (MBEW) reduced the count from 2.40 x 10^9 to 2.70 x 10^8cfu/g (88.75%). Additionally, Basic Electrolyzed Water (BEW) resulted in a Staphylococcus *aureus* reduction from 9.10 x 10^9 to 6.00 x 10^4 cfu/g (99.99%). Electrolyzed water exhibited efficient antibacterial activity. The antibacterial properties demonstrated were significantly stronger than those observed in the negative control group (EW non-treated food contact surfaces). Previous studies have documented strong and efficient antibacterial activities of electrolyzed water across various pH values against both aerobic and anaerobic pathogenic bacterial species (**Shimada *et al.*, 2000; Okajima *et al.*, 2011; Xue *et al.*, 2014; Gomez-Espinosa *et al.*, 2017; Han *et al.*, 2017; You *et al.*, 2017**). **These data align with the results obtained in this research.** Another study carried by **Athayde *et al.* (2017)** revealed a comparable bactericidal activity at different pressures, temperatures, and pH values, which was further enhanced by combining acidic and basic electrolyzed water types to improve the microbiological quality of pork meat. **These findings agree with the current research.** However, some of these studies utilized electrolyzed water immediately or shortly after its preparation (**Okajima *et al.*, 2011)** and reported bactericidal activity within just under 2 hours of production (**Athayde *et al.*, 2017).** This is consistent with our practice of using electrolyzed water immediately after its preparation.

Alkaline Electrolyzed Water (SAlEW) act as a cleaner for both food and non-food contact surfaces and effectively degreases these surfaces. Conversely, Acidic Electrolyzed Water (SAcEW) is utilized as a disinfectant for both food and non-food contact surfaces, such as kitchen equipment, sinks, and bathroom fixtures. It also serves as an effective hand sanitizer and it is approved by the **Merged Food Code "US-FDA" (2013); Centers for Disease Control & Prevention (CDC, 2008 & updated May 2019) and USDA (2015).**

Detection of VBNC cells using Real Time (RT-PCR) in conjunction with Propidium iodide (PI)

Since bacterial cells in the VBNC state are challenging to cultivate and verify their viability (whether they are in the VBNC state or dead), real-time PCR with Staph. *aureus*-specific 16S rRNA-directed primers were employed. This was performed using total DNA extracted from the cultured samples. Propidium Iodide (PI) was used to selectively quantify DNA from viable cells, including VBNC bacteria with intact cell membranes/cell walls **(Inoue *et al.*, 2008).** This was used in conjunction with RT-PCR to distinguish between dead and live bacterial cells. Real-time PCR was utilized in this study because it is not possible to differentiate between dead and live bacteria using conventional PCR. VBNC cells maintain high levels of rRNA, similar to normal cells, and retain reductase activity, both of which are crucial for all living cells. Consequently, 16S rRNA quantification has been employed in numerous RT-PCR assays. **This approach aligns with the methodology used in the current study**. In this context, the development of simple, rapid, and highly specific and sensitive methods is crucial for the detection of pathogenic bacteria. In recent decades, molecular detection methods, such as polymerase chain reaction (PCR), have become widely adopted for pathogen detection due to their faster turnaround times and simpler workflows compared to traditional culture methods **(Wei *et al.*, 2019).** Among PCR-based methods, real-time PCR has emerged as a valuable tool for detecting and quantifying bacterial species associated with food, owing to its superior sensitivity and efficiency (**Kim *et al.*, 2021; Yang *et al.*, 2021; Xie and Liu, 2021).** Currently, real-time PCR is utilized for monitoring staphylococci in food processing environments (**Zhou *et al.*, 2022; Rodríguez *et al.*, 2016)**. Given its high sensitivity and specificity, RT-PCR was selected for this study to accurately detect and quantify the target gene (16S rRNA) of tested *Staph. aureus.*

CONCLUSION AND RECOMMENDATIONS

The present study demonstrated efficient antibacterial activities of electrolyzed water (EW) on *Staph. aureus* contaminating the food contact surface forming a VBNC state as well as the Resuscitation from the VBNC state which is crucial in the field of food processing and storage as well as in medicinal branch and significant importance for various industrial processes. Considerable research should be conducted in this area, the specific mechanisms underlying the transition of bacteria to the VBNC state or their subsequent revival to culturability remains elusive. There is an urgent need for further research that integrates both traditional cultural methods and molecular approaches to elucidate this phenomenon, as the formation of the VBNC state in *Staph. aureus* or

any other pathogenic bacteria and its potential for resuscitation pose a substantial threat to public health. Consequently, there is an urgent necessity to further refine detection methods, incorporating techniques. 16S rRNA is a highly conserved gene present in all bacteria. It serves as a molecular clock for phylogenetic analysis and can be used to identify bacterial species while Real-Time PCR considered a sensitive technique for detecting and quantifying specific DNA sequences, including 16S rRNA. Furthermore, it is imperative that established reference isolation and identification methodologies for foodborne pathogens, as standardized by international organizations such as ISO, APHA, and FDA, incorporate a step for the detection of viable but non-culturable (VBNC) pathogenic bacteria, particularly those transmitted through food. In cases where these specifications mandate the complete absence of such pathogens, the pre-enrichment broth should be replaced with a resuscitation broth. This resuscitation broth, rich in antioxidants, effectively neutralizes and controls the formation of free radicals a well as stop the damage effect on the microbial protein, fat and DNA, while simultaneously providing a nutrient-dense environment conducive to the revival and reactivation of VBNC bacteria.

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