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Impact of electolyzed water on *B. cereus* contaminating milk collection utensils

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ABSTRACT:

In the dairy industry, cleaning and disinfection of surfaces are important issues, and the development of innovative strategies in this matter may improve food safety. Biofilm formation on food-contact surfaces can lead to recurrent contamination. To find an environmental friendly and energy-efficient alternatives to acidic detergent for a milking system clean-in-place (CIP) process, this study was planned to investigate the feasibility of applying Electolyed Water (EW) alone to clean and sanitize the soiled stainless steel (304) pipes system as well as study the effect of EW on biofilm formation process on stainless steel containers used in the dairy industry and to clarify the synergistic action between electrolyzed reduced water (ER) and Acidic oxidized water (EO) to optimize the effect as alternative cleaners and disinfectants to unsafe human health chemical cleans and disinfectants on stainless steel plates (SSP) and examined the disinfection efficacy and mechanism of electrolyzed water (EW) on *Bacillus cereus* biofilms. Acidic (EO) with a pH ranging from 2 to 3 , alkaline electrolyzed water (ER) with a pH ranging from 10 to 13 is regarded one of the most applicable in the antimicrobial treatment of milk collecting containers and utensils. Both ER and EO achieved a >5 log CFU/cm2 reduction of *B. cereus* to a non-detectable level (< 1 log CFU/cm2) . The optimal effect was achieved by using ER as cleaner followed by using EO as sanitizer (temperature 40 °C, contact time 10 min). and therefore, rendered EW as a promising cleaner and sanitizer to be applied in the food industry. EW can be advantageous for environmentally friendly, it considered also one of the promising novel antimicrobial agents recently proposed as an alternative to conventional decontamination methods such as heat and chemical sanitizers.

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INTRODUCTION:

Milk from the mammary glands of healthy animals is initially sterile, but post-harvest handling like the milking personnel and milk handling containers; remain to be the major sources of bacterial contamination of raw milk (**Coorevits et al. 2008; Reta et al. 2016).** Therefore, milk should be produced in hygienic conditions so as to meet set standards **(Ahmad et al. 2015)** which are <106 colony forming units/ml in the case Kenya (KEBS 2010).

Bacillus cereus is one of the most important endospores-forming spoilage microorganisms in the dairy environment, which is not only associated with foodborne outbreaks but also its growth may result in various dairy defects **(Guinebretiere et al. 2013)**. The milk secreted fom a healthy animal's udder is sterile by nature, but it becomes contaminated by *Bacillus spp.* either through unhygienic milking practices, poor personal hygiene, and unsanitary utensils **(Reta et al. 2016)**. *B. cereus* can negatively affect product quality. It produces various extracellular enzymes which can be responsible for undesirable effects in the organoleptic quality of milk and dairy products **(Lücking et al. 2013)**. These enzymes are thermostable which resist the milk pasteurization pocess, leading to degradation of milk components, spoilage, and reduction of the shelf life of milk and dairy products **(Kumari & Sarkar 2014b)**. Spoilage is related to various flavors, taste, smells, and textural defects such as bitter, rancid, acidic, or sour taste, curdling or thickening, and ropy texture in milk and dairy products **(Lücking et al. 2013).** Also, *B. cereus* is involved in serious foodborne illnesses including diarrheal and emetic syndromes, depending on the ingested produced toxin. These toxins are highly resistant to heat treatments, pH extremes, and proteolytic degradation in the digestive system **(Jovanovic et al. 2021).**

One of the biggest safety problems with these organisms is their adherence to stainless steel equipment surfaces in dairy plants and form biofilms resulting in serious hygienic problems and economic losses due to spoilage of dairy products and equipment damage **(Gopal et al. 2015).** The hydrophobic properties of *B. cereus* endospores and their resistance to heat, desiccation, and disinfectants allow strong biofilm formation on dairy utensils **(Kumari and Sarkar 2014b).** Biofilms formed from exopolysaccharides of mucous substances produced by *B. cereus* protect bacterial cells from adverse environmental stresses, such as chemical disinfectants, antimicrobial agents, heat, and acid challenges, and act as a reservoir for recurrent contamination of dairy products **(Yuan et al. 2020).** The most commonly used disinfectants include sodium hypochlorite and quaternary ammonium compound **(Peng et al. 2002)** ,glutaraldehyde ***(*Simões et al. 2011*)***, and peroxyacetic acid **(Ryu and Beuchat 2005),** However, these disinfectants failed to eliminate *B. cereus* biofilms. The inability of these disinfectants to penetrate the biofilm's matrix and their undesirable by-product residues in milk constitutes a considerable challenge to the dairy industry **(Gil et al. 2009).**

Electrolyzed water (EW) represents a green cleaning alternative, colorless, odorless, highly efficient, and inexpensive with a high broad bactericidal action with less corrosion and no rinsing requirement for dairy utensils **(Wang et al. 2019)**. In the USA, the (Environmental Protection Agency (EPA) has approved the application of EW as an effective food cleaning and sanitizing agent without leaving any traces of chlorine residues (Cl_2) residues in the food manufacturing industry **(Venturini 2013)**.

Electolyzed water contains non-dissociated hypochlorous acid (HOCl) which considered the main component obtained by the electrolysis of water and sodium chloride at the anode side of the electolsis .It has been widely used to inactivate several foodborne pathogenic bacterial spores and biofilm formation in food products, food processing surfaces, and non-food-contact surfaces in a very short time **(Possas et al. 2021)**.

EW was an effective disinfectant for the elimination of biofilms of *Staphylococcus aureus, Candida albicans* and *Streptococ-*

cus mutans **(Ozaki et al. 2012),** and *Listeria monocytogenes* **(Arevalos-Sánchez et al. 2012).** Most disinfectants may work better against the initial attachment of microbes than against developed biofilms so, eliminating the formed biofilms is an essential aspect in the dairy industry. The emergence of multidrug-resistant food poisoning microorganisms and the demand for disinfection of heavily contaminated dairy equipment is expected to continue growing in the future. There are few studies concerning the mechanism of EW disinfection on *B. cereus* biofilms. Therefore, the objective of this study was to investigate the inhibitory effect when spraying EW against *B. cereus* biofilm and its effect on the expression of viulence genes (*tas*A and *sip*W) on dairy utensils.

2. MATERIAL and METHODS

2.1. Preparation of electrolyzed water According to Tolba et al. (2023)

Acidic electrolyzed water (AcEW) of pH 2 - 3 and Alkaline electrolyzed water (AlEW) of pH 10-13 were prepared using a current of 9-10 VA passed through two separate electrolysis cells containing potable water, sodium chloride (NaCl) with two poles; anode (+) and cathode (-). Upon the onset of the electrolysis process, at the anode side, water was oxidized (**EO**) to give Oxygen gas (O_2), Chlorine gas (Cl_2), Hydrochloric acid (HCl), Hypochlorite ions (OCl), and hypochlorous acid (HOCl) according to the following formula:

$2\ H_2O\ (l) \rightarrow 4\ H^{+}(ions) + O_2\ (gas) + 4\ e^{-}$,$2\ NaCl \rightarrow Cl_2\ (gas) + 2\ Na^{+}$, $(2\ Cl^{-} \rightarrow Cl_2 + 2e^{-})$, $Cl_2 + H_2O\ (l) \rightarrow HCl + HOC + Ocl$

While at the cathode side, water was reduced **(ER)** to give Hydrogen gas (H_2), chlorine ions (Cl), and sodium hydroxide (NaOH) according to the following formula:

$2\ H_2O\ (l) + 2\ e^{-} \rightarrow 2\ OH^{-} + H_2\ (gas)$
$2\ NaCl + 2OH^{-} \rightarrow 2NaOH + Cl$

2.2. Preparation of *B. cereus* for experimental inoculation

The target bacterium for this study was *B. cereus* strain, which was obtained from Animal Health Research Institute (AHRI) ,which has the ability to biofilm formation. The strain was streaked onto brain heart infusion (BHI; BD, Heidelberg, Germany) agar plates from stocks solution stored in BHI broth containing 15% glycerol at – 80 °C and was incubated at 30 °C for 24 h without shaking. Single colonies were inoculated into tubes containing 10 mL of BHI broth and left to grow for 18 h at 30°C. This overnight culture was contained an average of 8.0 to 8.5 log_{10} CFU/ml. Seial dilution were made to obtain an initial concentration of 5.5 – 6.0 log_{10} CFU/ml which used for expeimentall biofilm formation. Ethical approval was granted by the Ethical Approval Committee of the Faculty of Veterinary Medicine, Benha University, Egypt (BUFVTM 01-01-24).

2.3. Preparation of Stainless-steel food contact surfaces according to the method recommended by Rosmaninho et al. (2007):

Stainless-steel plates (SSP) of 2 cm X 5 cm dimensions (10 cm^2) were cleaned by immersing in neutral detergent (Hyclin-plus, Hycel, Ciudad de Mexico, Mexico) at 65°C for 5 min, followed by rinsing with distilled water for 5 min. and then sterilized using dry heat at 180 °C for 30 min.

2.4. Design of The Experiment:

2.4.1. Preparation of milk contact sufaces:

Stainless steel plates (SSP) (304) materials used in milking systems were classified into 4 groups; **1st** group was control (SSP with inoculum only without treatment), The **2nd** (SSP with inoculum and sanitized with EO only), while the **3rd** one (SSP with inoculum and cleaned with ER only.), The **4th** one (SSP with inoculum and cleaned with ER followed by sanitized with EO). To contaminate the Stainless-steel plate sample, 0.1 mL of *B. cereus* culture was evenly soiled on the whole surface of each Stainless-steel plate with a sterile glass -coated rod. Then, the plates were dried using laminar flow for 30 minutes to evaporate all visible liquid. The initial concentrations of bacteria on control stainless steel plates were 5.57 log_{10} CFU/cm^2 and EW treatments were applied for 10 min. (temp. of EW water was 40° C) based on the method of **Yu Liu et al (2020).**

2.4.2. Cleanliness Assessment and *B. cereus* Counting:

Control plates of SSP were checked for their initial contamination levels, while the other treated plates were checked for effects of EW of both types (EO & ER). The plates were swabbed for microbiological analysis using sterilized cotton swabs soaked with 0.1% peptone water, The *B. cereus* counting process was calculated according to **ISO/ 7932 (2004) AMD 1 (2020).**

2.5. Nucleic Acid extraction:

Following the manufacturer's instructions, mRNA was extracted using the FastPure® DNA/RNA Mini kit. Using a HERA SYBR® Green RT-qPCR Kit (Willowfort) and the Applied Real-Time PCR Detection System (Applied Biosystem), were used to determine the *B. cereus* biofilm genes (*tas*A and *sip*W) expression (Table 1). 10 µL reaction volumes with 0.5 µl of each primer and 1 µL of RNA were used for the amplification process. The subsequent thermal cycling parameters were used: 30 minutes of reverse transcription at 55 °C, 5 minutes of activation at 95 °C, 40 cycles of denaturation at 95 °C for 10 s, 30 seconds of annealing (60 °C for 16srRNA and *tas*A, and 54 ° C for *sip*W), and 30 seconds of extension at 60 °C. The synthesized oligonucleotide primers (Oligo™) were used in this study (Table A).

Table A. Primer used for determine the *B. cereus* taget genes.

Gene	Primer sequence (5' to 3')	Reference
16S *rRNA*	F- TCG AAA TTG AAA GGC GGC	Priha et al. 2004
	R- GGT GCC AGC TTA TTC AAC	
*tas*A	F- AGC AGC TTT AGT TGG TGG AG	Caro-Astorga et al. 2015
	R- GTA ACT TAT CGC CTT GGA ATTG	
*sip*W	F- AGA TAA TTA GCA ACG CGA TCTC	
	R- AGA AAT AGC GGA ATA ACC AAGC	

2.6. Statistical Analysis

The bacteria population was expressed as $\log_{10}$ CFU/cm^2. The mean values for *B. cereus* were calculated from the independent triplicate trials. Significant differences in mean values of bacteria counts and reduction rates & percentages were analyzed using least significant differences with analyses of variance (ANOVAs) and a 95% confidence interval in SPSS 21.0 (SPSS, Inc., Chicago, IL, USA).

Microsoft Excel was used to perform the statistical and imaging analyses. Using the $2^{(-\Delta\Delta Ct)}$ technique, the relative expression of target genes was analyzed and compared with that of the distilled water (d.d. H_20) group. The average cycle threshold (Ct) of the target genes was deducted from those of the endogenous control gene 16srRNA to obtain the ΔCt value

RESULTS:

Table 1. Mean *B. cereus* count (log_{10} CFU/m^2), reduction rate, and % of control and treated plates

B. cereus	Control	ER	EO	ER & EO
Initial count	5.57^{A}	1.3^{ab}	1.1^{ab}	$<1^{aB}$
Reduction rate (log_{10} CFU/m^2)	----	4.27	4.47	5.57
Reduction %	0.0	76.66	80.25	100

Significance difference between small and capital litters in the same raw
<1 log is represented by zero in estimating the significant difference

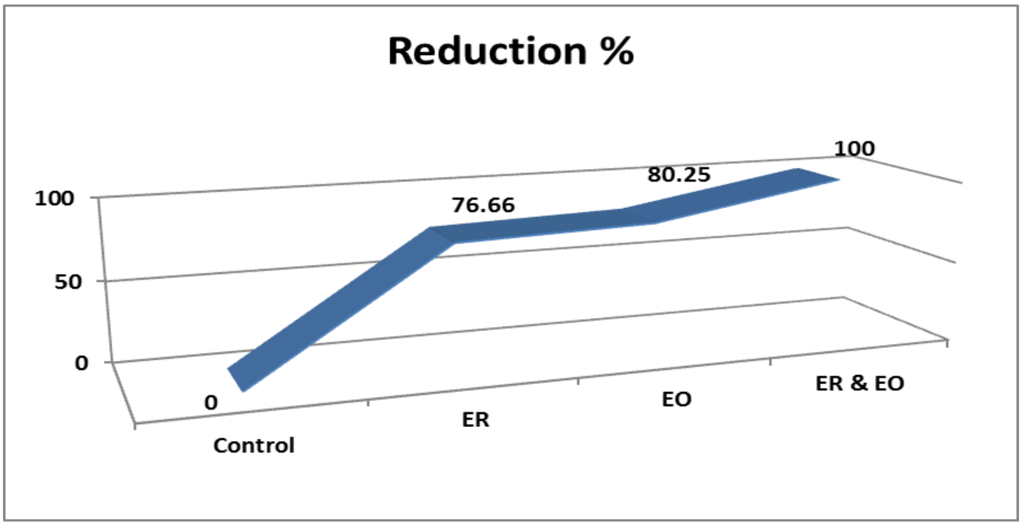

Fig 1. reduction percentage of different treatments as compared with the control plates

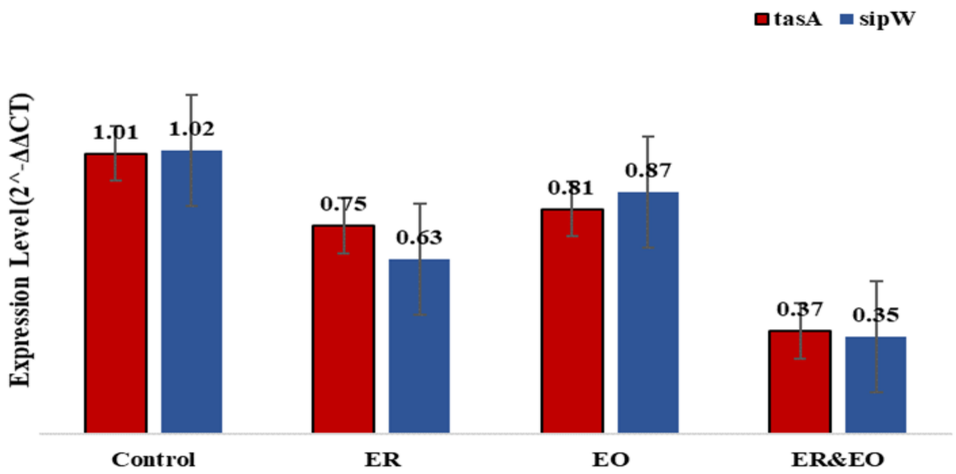

Fig (2): Relative *tas*A and *sip*W genes expression of *B. cereus* contaminated stainless steel surfaces after cleaning by EW (ER, EO, and mix of ER&EO) compared to the control. Values are expressed as the mean ±SD. Total RNA was extracted. Biofilm gene expression levels were measured by the 2−ΔΔCt method with relative quantification by real-time quantitative reverse transcription polymerase chain reaction (Real-time qRT-PCR).

DISCUSSION:

Biofilm formation *by Bacillus* spp. on milk equipment is a most common phenomenon and it has caused contamination, substantial economic loss and safety hazards with about 60% of foodborne outbreaks. 78.5% of dairy products such as milk, ice cream and cheese contaminated with *B. cereus* strains from dairy utensils **(Ibrahim et al. 2022)**. There is evidence that electrolyzed water has been introduced to food industries as a novel disinfecting agent can work better than water and chlorine solutions as a sanitizer of cutting boards and utensils.**Table (1) and Fig (1)** illustrate EW efficacy of both types (ER & EO) in eleminating *B. cereus* contaminating the milk contact surfaces. The results cleared that the highest reduction rate and % of EW were observed when using ER followed by EW (5.57 log_{10}/100 %, followed by EO (4.47 log_{10}/ 80.25%) and finally, ER which recorded a reduction rate of 4.27 log_{10} (76.66%) as compared with the control plates . There were significant differences ($P<0.05$) between the control on one hand and all othe treatments on the other hand, such differences were also clear between both ER and EO separately as well as between their combination together , while no significance difference was observed between using ER and EO ($P>0.05$). The obtained results in the current study were in line with **Vorobjeva et al. (2004)** who reported that five minutes was sufficient to complete the decontamination of *B. cereus* from food contact surface. Moreover, **Kim et al. (2000)** obtained the same log reduction of *B. cereus* within two minutes by using EO water. In this respect, **AOAC (2000)** recommended the use of EW containing 10 ppm available chlorine (AC) for 30 s to reduce > 5 log cfu of aerobic plate count(APC). Similar results were recorded by **Al-Qadiri et al. (2019)** who revealed that the exposure of food contact surfaces to the reactive chlorine (60 mg/L) in acidic elyctrolyzed water (AEW) for 5 min could reduce *B. cereus* by 2.11 log CFU/cm^2. Furthermore, the inhibitory effect of EW against different microorganisms was previously investigated by **Arevalos et al. (2012)** against *L. monocytogenes* and **Rahman et al. (2016)** against *E. coli* and *L. monocytogenes* (reduction rate of 6.9 log_{10} cfu/ml. at > 2 ppm (AC) and 30s exposure time. **Bremer et al. (2002)** and **Parkar et al. (2004)** also stated that the cleaning efficiency of Clean-In-Place (CIP) systems significantly depends on the exposure time, temperature, and cleaning agent concentration. Meanwhile, stainless steel food contact materials treated with EW achieved a 5-log reduction in *B.cereus* species, which corresponded with the definition of sanitization recommended by the **Food and Drug Administration (2005).** Strong oxidizing effect of EO due to the presence of HClO, ClO, and Cl2. HClO can kill bacteria by destroying the membrane, leading to leakage of the cytoplasmic content **Mokgatla et al. (2002),** protein denaturation, and stopping cellular metabolism **Chen et al. (2001).** Bactericidal Activities of EW was greater than common sanitizers used in dairy industry **(Jiménez-Pichardo et al. 2016).**

Fig. (2) Cleared the assay results of the relative expression of *B. cereus tasA* and *sipW* genes contaminating stainless-steel surfaces after cleaning by using different types of electrolyzed water (EW)**.** The expression of both genes was significantly reduced ($p < 0.05$) as for the relative expression of the *tas*A gene. It was recorded as 0.75, 0.81, and 0.37 after using ER, EO, and a mix of ER & EO respectively, when matched with control one (1.1). In the same way, *sipW* gene expression was recorded at 0.63, 0.87, and 0.35 by the same aforementioned water types, while the control one recoded 1.02. The potential way in which EW affects gene expression is by suppressing enzymatic activity, causing the cell wall to become less permeable, and allowing intracellular components to escape **(Rahman et al. 2016).** In this respect, **Park et al. (2019**) concluded that there are multiple factors involved in biofilm formation by emetic toxin-producing *B. cereus*. Furthemore, **Hussain & Oh (2017)** stated that *B. cereus* can form several types of biofilms such as air-liquid, submerged, and floating pellicles**.** Bacterial attachment to a surface is influenced by several factors, including the physicochemical properties of the substratum surface and the surface characteristics of the strain used (**Whitehead & Verran, 2009).** In addition, bacterial motility is an important factor that triggers biofilm formation or attachment of cells to a surface (**Abee et al. 2011**).

However, mechanisms involved in biofilm formation of *B. cereus* such as genes that control biofilm formation are less known compared to those of *Bacillus subtilis*. SinR is known to play a central regulatory network for the biofilm formation of *B. subtilis* (**Caro-Astro et al. 2015).** This is what we would like to point out through this research point, the importance of conducting more research to find out the effect of different genes of *B. cereus* on the formation of biofilm.

CONCLUSION :

In conclusion, electrolyzed water is an excellent way to clean and sterilize food and food contact surfaces, which hinders the growth and multiplication of foodborne pathogens as well as the rapid spoilage of food. It was also successful in eliminating *B. cereus* completely from contact surfaces. Additionally, further research is advised to protect the environment's health and enhance the quality and safety of food supplied to consumers, as there are other potential uses for electrolyzed water that have not been thoroughly explored in scientific studies.

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