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Review Article

Microbial Inactivation Mechanism and Properties of Slightly Acidic Electrolyzed Water: A Review

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Abstract: Slightly acidic electrolyzed water (SAEW) obtained by electrolyzing 2-6% dilute hydrochloric acid in a chamber without membrane is novel non-thermal sanitizer widely used in the sterilization of foods such as fruits, vegetables, and meat owing to its effective antibacterial activity and low operating costs. Despite the well-documented and validated antimicrobial and sporicidal properties of SAEW, its precise mode of action against bacteria and bacterial spores remains uncertain and subject to ongoing debate. The primary aim of this review article is to scrutinise the active constituents of SAEW that contribute to its antimicrobial properties. Additionally, the review critically elucidates the mechanisms by which SAEW effectively inactivate vegetative bacteria cells and spores, based on a comprehensive scrutiny of existing literature. It is demonstrated that the application of SAEW can kill vegetative bacterial cells by the disruption of their cellular membrane, disruption of their intracellular reactive oxygen species (ROS) balance, and lowering their ATP levels, deactivation of key enzyme and damaging DNA affecting other bacterial cells vitals. Bacterial spore inactivation by SAEW being achieved through the induction of structural modifications in the spores, including coat damage, mutagenesis, and alterations in the properties of the inner membrane (IM).

Keywords: Slightly Acidic Electrolyzed Water, Microbial Inactivation, Mechanism, Food Pathogens, Bacterial Spores

1. Introduction

Chemical sanitizers are often used in the food industry to create high-quality, microbiologically safe food for human consumption, include chlorine [72], hydrogen peroxide [3], ozone [4], and organic acids [32]. However, due to the possibility for the development of carcinogenic halogenated disinfection byproducts, which are harmful to both the environment and human health, certain chemical sanitizers are prohibited in a number of European nations and other countries [57, 59, 63]. Numerous studies on food decontamination methods have been conducted recently, with the goal of finding substitute sanitizers that would ensure the quality and safety of food. The slightly acidic electrolyzed water (SAEW), as an alternative and novel method with great potential for sterilization, has recently received a great deal of attention for its sanitizing efficacy and environmentally friendly nature [37, 55].

Slightly acidic electrolyzed water (SAEW) obtained by electrolyzing 2-6% dilute hydrochloric acid in a membrane-less chamber is novel non-thermal sanitizer widely used in the sterilization of foods such as fruits, vegetables, and meat owing to its effective antibacterial activity and low operating costs [26, 67, 86]. SAEW is reported as a new ultra-high effect and wide-spectrum disinfectant that is colourless, odourless, and harmless to humans and the environment, is directly used on food [80]. It has been recognized as an antimicrobial functional water. Developed by Japanese companies over two decades [41], SAEW was approved as a food additive by the Japanese Ministry of Health, Labor, and Welfare in 2002 [41, 81]. As a result of SAEW's approval as a control agent in 2014 by the Ministries of Agriculture, Forestry, and Fisheries and the Environment [41], its use in Japan, China and Korea is growing and is gaining popularity, especially in the field of food sanitization. It is viewed as a viable replacement for anti-microbial detergents and a disinfectant that is kind to the environment [6].

Slightly acidic electrolyzed water has a high oxidation reduction potential (ORP) value (800 to less than 1000 mV), a pH near neutrality of pH 5.0-6.5 and low available chlorine concentration (ACC; 10-30 mg/l) in form of hypochlorous acid (HOCl) [5, 7, 23, 34]. Considerable study has been undertaken to investigate the antibacterial effectiveness of SAEW, mostly ascribed to the presence of HOCl and its high ORP, which has repeatedly demonstrated efficacy [7, 56, 61]. According to Al-Haq et al [1], it has been argued that the bactericidal activity of SAEW can be attributed to several key factors including, the abundance of HOCl, a high ORP, a significant content of free available chlorine (Cl_2), and the presence of hypochlorite ions (OCl^-). At its near-neutral pH of 5.5-6.5, available chlorine is predominantly HOCl (>95%) and is the main form of active chlorine compounds thought to be responsible for microbial inactivation [29]. The bactericidal effect of the chlorine-related substances is stronger with non-dissociated HOCl than with dissociated OCl^- against a broad range of microorganisms.

It has been thoroughly investigated and confirmed that SAEW has the ability to sanitize fresh vegetables, fruits, meats, fish and grains by inactivating spoilage and pathogenic microorganisms [19, 33, 37, 44, 53, 71, 85]. SAEW has been emphasized as one of the alternative sanitizers since it can be made easily on-site, its raw ingredients (water, NaCl, and/or HCl) are inexpensive and widely accessible, and it produces little trihalomethane chemical [21, 22]. In recent years, SAEW has been widely used in food preservation, such as eggs, meat products, fruits, and vegetables. In addition, SAEW can effectively remove pesticide residues and promote seed germination [50].

Several investigations have documented a significant bactericidal impact of SAEW on both pathogenic and non-pathogenic bacteria in laboratory [11, 41-42]. Other findings substantiated that the use of SAEW has the ability to effectively eradicate pure cultures of *Escherichia coli*, *Salmonella enterica*, *Typhimurium, Staphylococcus aureus*, and *Bacillus cereus* spores within 1 min [35, 37]. Similar study provided evidence that a pH value of 6.0 and a free chlorine content of 20 ppm resulted in effective eradication of around 8-9 log CFU/mL of several foodborne pathogens when subjected to a 1-min treatment with SAEW [37]. In the study conducted by Ding et al. [11], a disinfection test was performed on a pure culture in which pure culture was exposed to SAEW with an ACC concentration of 33 mg/L, a pH of 6.4, and an ORP of 834.9 mV. The results showed that the exposure to SAEW led to a considerable reduction of *S. aureus* by 5.8 log CFU/mL within a duration of 1 min.

Slightly acidic electrolyzed water, considered as a broad-spectrum and high-performance bactericide are increasingly applied in the food industry. Its broad-spectrum bactericidal action and affordability make it a popular choice for food cleaning [67]. Despite of its distinct anti-bacterial or sporicidal capability, the specific inactivation mechanism of SAEW on bacteria and bacterial spores remains unclear and is still a matter of discussion [84]. In this review article, the mechanisms by which SAEW inactivates bacteria and spores are examined. Both the active components of SAEW and its antimicrobial capabilities, which are responsible for the inactivation of microorganisms, are examined in great detail.

2. Active Ingredients and Antimicrobial Properties of SAEW Responsible for Bacteria Cells and Spore Inactivation

The active ingredients and antimicrobial properties of SAEW responsible for bacteria and spore inactivation are shown in Table 1. Slightly acidic electrolyzed water is reported to contain a high oxidation reduction potential (ORP) value (800 to less than 1000 mV), a pH near neutrality (pH 5.0-6.5) and low available chlorine concentration (ACC; 10-30 mg/l) in form HOCl [5, 7, 23, 34]. Hypochlorous acid (HOCl) exhibits the most potent bactericidal activity against a wide spectrum of microbes among the several forms of free accessible chlorine. The equilibrium between HOCl and the hypochlorite ion (OCl-) in aqueous solutions is influenced by the pH of the solution. At pH 5.0–6.5, the effective form of chlorine compounds in SAEW is mainly HOCl (> 95%), 5% is OCl- and traces of Cl_2 as depicted in Figure 1 [29, 76]. As the pH falls, the concentration of HOCl increases, as illustrated in Figure 1. According to a study done by Cao et al. [7] and Rahman et al. [60], Hypochlorous acid (HOCl) is the strongest form of chlorine, which shows sanitizing power 80 times greater than hypochlorite (OCl-) when the pH is around 5–6.5. At lower pH, HOCl is dissociated to Cl_2 gas, and at higher pH it forms OCl- (Figure 1). The proportion of the HOCl and OCl- in the water depends on the pH (Figure 1). In alkaline conditions (pH7) OCl- is the predominat chlorine type, while at pH below 7, HOCl is the predominant. At very low pH, formation of toxic Cl_2 gas occurs: $HOCl + HCl = H_2O + Cl_2$. Since chlorine in the Cl_2 form can volatilize [9] and lose its efficiency against germs, HOCl plays a more significant role in sanitization than other forms. Therefore, maintaining a HOCl concentration and preventing chlorine loss are both facilitated by a neutral pH of SAEW.

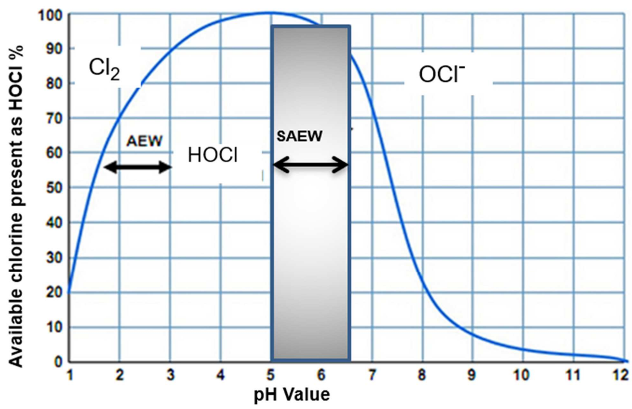

Figure 1. *pH level and equilibrium between HOCl and the hypochlorite ion (OCl-). Referred to Shiroodi and Ovissipour [68].*

The antimicrobial effect of SAEW mainly caused by the presence of HOCl has been extensively studied and proved [7, 56, 61]. According to Al-Haq et al. [1], the primary factors that appear to be responsible for the bactericidal activity of SAEW are the presence of a high hypochlorous acid (HOCl), a high oxidation-reduction potential (ORP), free available chlorine content, and the presence of hypochlorite ions (OCl-). All of these factors appear to be present in SAEW.

Some studies on antimicrobial mechanism of SAEW attributed its available chlorine concentration in mg/L (ACC) as a main factor affecting the disinfection efficacy of SAEW. Recent study by Li et al. [42] showed that the strains of *L. monocytogenes* were killed completely within 30s by SAEW whose available chlorine concentration (ACC) was higher than 12 mg/L, and it was confirmed that ACC is the main factor affecting the disinfection efficacy of SAEW. Li et al. [42] further suggested that as a result of its high ORP value, SAEW could disrupt or break the intracellular ROS balance of *L. monocytogenes* by inhibiting the antioxidant enzyme activity, thus promoting the death of *L. monocytogenes*. Therefore, the application of SAEW with a near-neutral pH and high ORP is very promising since SAEW minimizes human health and safety issues from Cl_2 off-gassing, reduces corrosion of surfaces, and limits phototoxic side effects while maximizing the application of hypochlorous acid species [23].

Table 1. *Active ingredients and properties of slightly acidic electrolyzed water.*

Properties of SAEW	Microbial inactivation charasteristics	Reference
Active Chlorine (HOCl, -OCl and Cl_2) (mg/L)	The active ingredient for sanitization Available chlorine concentration (ACC) 10-30 mg/l Over 95 % of ACC is in this form of HOCl HOCl possess microbial inactivation power 80 to 150 times than that of hypochlorite ion (Ocl-) Does not affect the taste, color, smell, or nutrient value of food Effective against bacteria, fungi, yeast, virus, bacteria spores	[9, 23, 27, 29, 60]
pH 5.0 – 6.5	Slightly acidic, it has a near-neutral pH of 5 – 6.5 minimizes human health and safety issues from Cl_2 off-gassing reduces corrosion of surfaces At this more than 95 % of ACC is in this form of HOCl Because of near neutral pH, SAEW can be used the same way as tap water It is disposed immediately after use; it is environmentally friendly as it does not produce trihalomethane (THMs)	[9, 12, 23, 28, 29]
Oxidation-Reduction Potential (ORP) (mV)	High ORP ranging from 800 to less than 1000 mV Its high ORP value SAEW could disrupt or break the intracellular ROS balance of microbes	[9, 23, 29, 42]

3. Mechanism of SAEW in Killing Microorganism

Understanding the mechanism of action of SAEW would facilitate more precise dosage determination for a wide range of applications within the food industry and other relevant domains. Nevertheless, there is a restricted number of literature available regarding the underlying mechanism responsible for the bactericidal activity of SAEW. The antimicrobial mechanism of electrolyzed water remains ambiguous, while numerous possibilities have been proposed [1, 30, 68, 74, 84, 69]. A summarized antimicrobial mechanism of SAEW based on active chlorine (HOCl, OCl-, Cl_2), high ORP and hydroxyl radical (OH) are listed in Table 2. Several studies have documented that the primary factors responsible for the bactericidal activity of SAEW appear to be the presence of a high hypochlorous acid (HOCl), high oxidation-reduction potential (ORP), free available chlorine content (Cl_2), as well as the presence of hypochlorite ions (OCl-) [1, 41, 45, 58, 60]. Along the same lines, Hricova et al. [30] argued that the antimicrobial activity of electrolyzed water strongly depends on pH, oxidation reduction potential (ORP), and the form and concentration of available chlorine. Electrolyzed water can be regarded as a hurdle technology due to its different parameters which are responsible for its antimicrobial properties as elucidated by Hricova et al. [30] and illustrated in Figure 2.

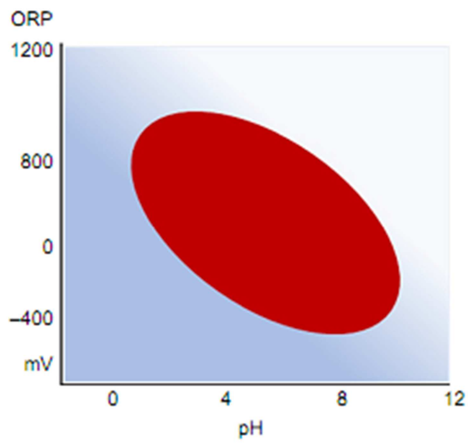

Figure 2. *Biosphere of a bacterium in response to pH and ORP. Referred to Shiroodi and Ovissipour [68].*

Looking at the biosphere of a bacterium in response to pH and ORP shown in Figure 2, it can be observed that microorganisms have their biosphere (red) in which they can survive and grow, while in the blue area, electrolyzed water prevent their growth because of acidic condition, and high ORP. Generally, bacteria can grow in a pH range of 4–9. Aerobic bacteria can grow at the ORP range of +200 to +800mV, and anaerobic bacteria grow between -700 and +200mV [30]. SAEW that is reported to contain a high oxidation reduction potential (ORP) value (800 to less than 1000 mV) and pH (pH 5.0-6.5) appears to align with the biosphere concept provided by Hricova et al. [30].

Table 2. *Antimicrobial mechanisms of SAEW based on active chlorine (HOCl, -OCl, Cl_2) and high ORP.*

Active ingredient	Inactivation action against microbes	Reference
Chlorine (Cl_2)	inhibiting carbohydrates metabolism enzymes that have sulfhydryl groups sensitive to chlorine, and this blocked glucose oxidation.	[15]
HOCl	inactivation of key-enzymes, nucleic acid damage, the wall and other vitals react with enzymes to form N–Cl bonds, cause leakage of proteins and the destruction of bacterial ultrastructure bacteria. inhibit the enzyme activity essential for microbial growth, damaging the membrane and DNA since it can penetrate the walls and membranes of microbial cells HOCl can change bacterial respiration destroying the electron transport chains and affecting adenine nucleotide pool. HOCl exerts its bactericidal effects via protein unfolding and aggregation oxidizing sulfhydryl groups of certain enzymes, disrupting the protein synthesis, and oxidative decarboxylation of amino acids to nitrites and aldehydes	[2, 11, 18, 48, 54, 68, 69]
Ionized OCl-	diffuse through the microbial cell membrane and affect the affect, its diffusion across the microbial cell membrane is impeded, resulting in limited germicidal efficacy	[18]
ORP	cause damage to bacteria cell and attack inner and outer membranes leading to leakage of intracellular ingredients (e.g., proteins and nucleic acids) ultimately necrosis of cells. damage cell membranes, cause the oxidation of sulfhydryl compounds on cell surfaces, and create disruption in cell metabolic processes, leading to the inactivation of bacterial cells. energy metabolism and ATP production in microbes can be severely disrupted A high ORP can kill bacteria by oxidizing sulfhydryl groups on their surfaces and disrupting metabolic pathways inside the cells.	[11, 45, 58, 83]
Hydroxyl radical (OH)	break the intracellular ROS equilibrium suppressing antioxidant enzyme activity damage the normal structure of bacterial cell destabilizing its ionic equilibrium	[78]
Combined active chlorine (HOCl, Cl_2 and OCl-)	destroy membranes of the microorganisms, decarboxylate the amino acids, inhibit oxygen uptake and oxidative phosphorylation coupled with leakage of some macromolecules, inhibit glucose oxidation by chlorine-oxidizing sulfhydryl groups, form toxic N-chlorine derivatives of cytosine, disrupt protein synthesis, react with nucleic acids, purines, and pyrimidines, and unbalance metabolism of key enzymes	[30, 52, 68]

3.1. Inactivation Mechanism via the Active Chlorine Species (HOCl, Cl_2, and OCl-)

The active chlorine species in SAEW, including HOCl, Cl_2, and OCl-, play a significant role in the inactivation of microorganisms. Generally it was reported that active chlorine can destroy membranes of the microorganisms, decarboxylate the amino acids, inhibit oxygen uptake and oxidative phosphorylation coupled with leakage of some macromolecules, inhibit glucose oxidation by chlorine-oxidizing sulfhydryl groups, form toxic N-chlorine derivatives of cytosine, disrupt protein synthesis, react with nucleic acids, purines, and pyrimidines, and unbalance metabolism of key enzymes [30, 38, 52]. The antimicrobial effect of SAEW mainly caused by the presence of HOCl has been extensively studied and proved [56, 61]. According to a study done by Cao et al. [7] and Rahman et al. [60], HOCl is the strongest form of chlorine, which shows sanitizing power 80 times greater than hypochlorite (OCl-) when the pH is around.

Fukuzaki [18] provided an explanation of the mechanism by which chlorine operates to inactivate microorganism. The presence and form of chlorine plays a crucial role in the disinfection capabilities of SAEW, as highlighted by Hao et al. [25]. Fukuzaki [18] developed a model (Figure 3) to explain the microbial inactivation mechanism of HOCl. It was suggested that microbial inactivation by HOCl is attributed to its penetration into microbial cells across the cell walls and membranes. This model elucidates that the primary mechanism responsible for the inactivation of microorganisms is thought to be the penetration of chlorine in the form of hypochlorous acid (HOCl) and hypochlorite ions (OCl-). The germicidal activity of SAEW is governed by the abilities of HOCl and OCl- to diffuse through the microbial cell membrane.

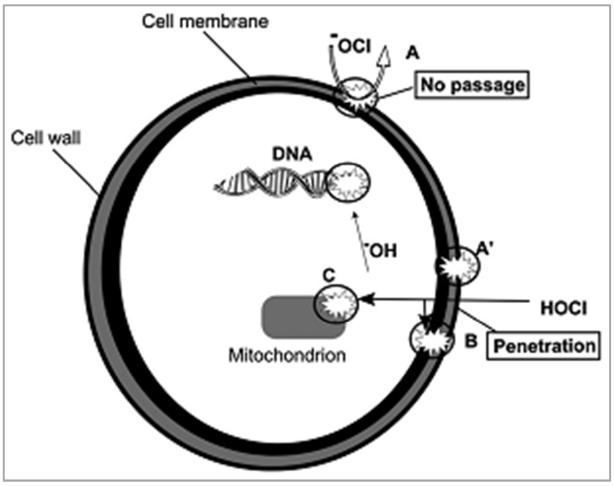

Figure 3. *Model representing the germicidal activity of EW. Referred to Fukuzaki [18].*

Fukuzaki [18] argues that the passage of ionized OCl- across the microbial barrier is impeded, resulting in limited germicidal efficacy. The OCl- only targets the outer membrane of the cell (circle A) in Figure 3 and has limited efficacy in terms of germicidal action due to its inability to permeate the microbial barrier. On contrary, HOCl serves as the active species responsible for the germicidal effect. It possesses a neutral charge and exhibits the ability to diffuse across the cell membrane. Hypochlorous acid has the capability to initiate an attack on both the outer membrane (circle A') and the intracellular regions (circles B and C) in Figure 3.

The cell membrane of a pathogenic bacteria is naturally negatively charged, therefore ionized OCl- cannot permeate the membrane due to the presence of the hydrophobic lipid bilayer and some protective cell wall components. The negative charge of the pathogenic bacteria's cell wall will repel the negative charge of the hypochlorite ions (OCl-), limiting the oxidizing activity to the cell's periphery. Because it is neutral, HOCl can easily pass through the pathogen's cell wall and disinfect both the outside and inside of the bacterium. This was supported by Rahman et al. [60] that HOCl can also penetrate slime layers, cell walls, and protective layers of microorganisms. In addition, Fukuzaki [18] further explained that HOCl could inhibit the enzyme activity essential for microbial growth, damaging the membrane and DNA since it can penetrate the walls and membranes of microbial cells. Further, HOCl can kill the bacteria by oxidizing sulfhydryl groups of certain enzymes, disrupting the protein synthesis, and oxidative decarboxylation of amino acids to nitrites and aldehydes [68, 69]. Active chlorine compounds (Cl_2, HOCl, and OCl-) have also been found to induce damage to the outer membrane [62]. In addition, it was also reported that the destruction of cells membrane caused by SAEW would lead to the inactivation of intracellular enzymes and the death of cells [70]. After SAEW enters the cell, HOCl and the generated active substance would cause a series of complex changes in intracellular metabolites [67].

Previous study reported that chlorine can affect microorganisms by inhibiting carbohydrates metabolism enzymes that have sulfhydryl groups sensitive to chlorine, and this blocked glucose oxidation [15]. Inactivation of key-enzymes, nucleic acid damage, the wall and other vitals can be affected [76]. The concentration of OH- present in SAEW can be one point of fungicidal efficiency, because OH- can damage the normal structure of conidia, destabilizing ionic equilibrium [78]. SAEW activity is attributed to HOCl, indirectly, because after HOCl permeation in bacterial cell, the radical OH- is generated [54]. Additionally, it has been proved that HOCl exerts its bactericidal effects via protein unfolding [13, 48].

3.2. Inactivation Mechanism by High ORP (mV)

Liao et al. [45] and Huang et al. [31] report that high ORP is the primary reason for bacterial inactivation. Others have found that bacterial inactivation can be achieved even at low ORP. For instance, Rahman et al. [61] used electrolyzed water with an ORP of 500–700mV and obtained a 5-log reduction in bacteria. Since ozone also has a high ORP, but its antibacterial qualities are much lower than electrolyzed water, Koseki et al. [40] concluded that ORP is not the primary determinant for inactivating the bacteria. Similarly, Fabrizio and Cutter [16] suggested that the antimicrobial mechanism of ORP is when bacterial cells exposed to extremely high or low ORPs, their cellular membrane become unstable, and then facilitate the penetration of antimicrobial agents to disturb metabolic process there by inactivating them. This was further supported by Liao et al. [45] that oxidation due to the high ORP of SAEW may damage cell membranes, cause the oxidation of sulfhydryl compounds on cell surfaces, and create disruption in cell metabolic processes, leading to the inactivation of bacterial cells. ORP of acidic electrolyzed water can cause damage to *E. coli* O157:H7 on bacterial ORP-reactions and attack inner and outer membranes, causing necrosis of cells [45], with damage verified with microscopy [17]. In a similar vein, Nan et al. [56] argue that the high ORP of SAEW efficiently damaged, destroyed, or caused deformation of the outer membrane of foodborne pathogenic bacteria, such as *S. aureus* and *E. coli* O157:H7. Additionally, the study by Ding et al. [11] demonstrated that the disinfection mechanism of SAEW was disrupting the permeability of cell membrane and the cytoplasmic ultrastructures in *S. aureus* cells. SAEW contain ORP of (+800 to +900 mV) as it was reported earlier, ORP directly and irreparably damages the microbial cell wall. Energy metabolism and ATP production in microbes can be severely disrupted by high ORP [11] due to changes in electron transport within bacterial cells. Recent research by Li et al. [42] and Zhang et al. [83] discovered that SAEW's high ORP value can break the intracellular ROS equilibrium of *L. monocytogenes* by suppressing antioxidant enzyme activity, hastening the bacterium's death. In another study by Liu et al. [49], it was observed that the intracellular ROS generated by SAEW was strengthened significantly with the increase of ACC, and the cells were injured to death accordingly.

4. SAEW Disrupt the Intracellular ROS Balance and Decrease ATP Causing Bacteria Death

It has been shown that cell damage and death as a result of SAEW treatment are associated with the accumulation of reactive oxygen species (ROS) and decrease in ATP [83] as it was previously shown that the combination of UV and SAEW treatment could lead to the collection of ROS in *Salmonella* [83]. Similar findings were reported by Liu et al. [49] who found that SAEW could disrupt the intracellular ROS balance in *Shewanella putrefaciens* and *Staphylococcus saprophytic* further confirming the antimicrobial mechanism of SAEW on pathogens. Previous study reported that HOCl from SAEW could enter the interior of bacterial cells and reduce the relative activity of TTC-dehydrogenase by 65.84%, an essential component of the respiratory chain, its reduction may contribute to ROS production [11]. This was further confirmed by Zhang et al. [83] in which SAEW treatment caused ROS accumulation (821.19 ± 25.99 AU) and its combination with UV caused even more ROS accumulation (982.69 ± 39.24 AU). This could be attributed to the –OH, which can react with bacterial cell membranes for lipid peroxidation, increasing ROS in bacteria. It is reported that when –OH comes into contact with the bacterial cell membrane, it attacks the unsaturated fatty acids on the cell membrane, causing intracellular production of oxygen radicals, which increases intracellular ROS [39]. The reaction of –OH with the bacterial cell membrane, leads to the production of intracellular peroxides. In addition, the stability of the electron respiratory chain within the mitochondria is essential for producing ATP by the mitochondria and its disruption may lead to a decrease in ATP [83]. The bactericidal efficacy and mechanism of slightly acidic electrolyzed water (SAEW) on *L. monocytogenes* were evaluated by Li et al. [42], the findings of their study revealed that SAEW with ACC higher than 12 mg/L could disrupt the intracellular ROS balance of *L. monocytogenes* by inhibiting the antioxidant enzyme activity, thus promoting the death of *L. monocytogenes.* Researchers concluded that the bactericidal mechanism of SAEW on *L. monocytogenes* was explained from two aspects including the damage of the cell membrane and the breaking of ROS balance.

5. SAEW Kills Bacteria by Damaging Bacterial Cell Membrane

The cell membrane is a crucial component of bacteria, serving the vital role of regulating the exchange of materials. Bacterial mortality can occur as a result of cell membrane disruption, which causes the leakage of intracellular substances [8, 77]. The results from Zhang et al. [83] indicated that SAEW treatment caused damage to the membrane function. The results show that although no leakage of nucleic acids and proteins was found in the above study, the bacterial cell membrane was damaged by SAEW. Many studies have found that SAEW attacks bacterial cell membranes via HOCl and OCl- [79] thereby killing bacteria. In addition, it has been reported that nucleic acid and protein leakage decreases with increasing dilution of SAEW, implying that the ACC of SAEW affects the amount of nucleic acid and protein leakage [26]. Moreover, it was further reported that SAEW can damage the cell membranes of *S. aureus*, *Listeria monocytogenes*, and *E. coli*, resulting in the leakage of proteins, DNA, RNA and ATP [26]. Similarly, a previous study reported that SAEW treatment effectively damaged, destroyed, or caused deformation of the outer membrane of foodborne pathogenic bacteria, such as *S. aureus* and *E. coli* O157:H7 [56].

In the study carried by Li et al. [42], it was observed that the strains of *L. monocytogenes* were killed completely within 30 s by SAEW whose available chlorine concentration (ACC) was higher than 12 mg/L, and it was confirmed that ACC is the main factor affecting the disinfection efficacy of SAEW. It was moreover, demonstrated that SAEW could destroy the cell membrane of *L. monocytogenes*, which was observed by SEM and FT-IR, thus resulting in the leakage of intracellular substances including electrolyte, protein and nucleic acid, and DNA damage. In addition, studies on the disinfection mechanism of SAEW on Staphylococcus aureus have concluded that the permeability of the cell membrane and the cytoplasmic ultrastructures are disrupted, resulting in the death of the bacteria [43]. Additional studies analyzing the disinfection mechanism of SAEW found that both cell barriers and intracellular components were involved in the disinfection activity against *E. coli* and *S. aureus* [46].

In a study conducted by Li et al. [42], it was discovered that treatment with SAEW led to a significant elevation in the extracellular levels of potassium (K+) concentration, proteins, and nucleic acids. These findings suggest that the cell membrane of *L. monocytogenes* experienced damage, resulting in the release of intracellular K+, protein, and nucleic acids. The investigation conducted by Li et al. [42] shown that the application of SAEW on *L. monocytogenes* resulted in noticeable cellular shrinkage and partial collapse. The observed phenomena could perhaps be attributed to the extrusion of intracellular material resulting from cellular membrane impairment and the subsequent disruption of osmotic equilibrium [43]. In another study conducted by Kim et al. [37], it was observed that the bactericidal activity of SAEW against *Escherichia coli, Salmonella enterica Typhimurium, Staphylococcus aureus*, and *Bacillus cereus* resulted in an increase in cell permeability at a concentration range of 40-60 mg/l of ACC. This increase in permeability indicated the disruption of cell membrane integrity. The postulation of changes in cell membrane permeability was confirmed through the observed rise in Propidium iodide (PI) fluorescence following to SAEW treatment [37]. Likewise, SAEW disrupted the cytoplasmic and outer membranes of *P. deceptionensis CM2* cells, thereby resulting in the leakage of intracellular ingredients (e.g., proteins and nucleic acids) [50]. Increases in the extracellular proteins and nucleic acids, as well as the fluorescence intensities of propidium iodide and n-phenyl-1-napthylamine, suggest that the cytoplasmic and outer membrane integrity of *Pseudomonas deceptionensis CM2* cells were disrupted after treatment with SAEW [50]. These findings show that SAEW is indeed a promising antimicrobial agent.

SAEW (pH 5.9, ORP of 945 mV, and 64 mg/L of ACC) retreatment for 60 s reduced population of *P. deceptionensis CM2* cells by 5 log CFU/ml and resulted in significant increases in the extracellular proteins and nucleic acids [50]. These findings indicated that SAEW disrupted the extracellular membranes of *P. deceptionensis CM2* cells, which might contribute to the cell death [24]. Studies have found that electrolyzed water with a high ORP can kill bacteria by oxidizing sulfhydryl groups on their surfaces and disrupting metabolic pathways inside the cells [45]. In addition, active chlorine forms (Cl_2, HOCl, and OCl-) are the main crucial substances for the damage to the out membrane [62]. High ORP in electrolyzed water causes modification of metabolic fluxes and ATP production, because of the change in electron flow in cell.

Previous studies reported that active chlorine (Cl_2, OCl-, and HOCl) contribute to microbial inactivation and can destroy membranes of the microorganisms, decarboxylate the amino acids, inhibit oxygen uptake and oxidative phosphorylation coupled with leakage of some macromolecules, inhibit glucose oxidation by chlorine-oxidizing sulfhydryl groups, form toxic N-chlorine derivatives of cytosine, disrupt protein synthesis, react with nucleic acids, purines, and pyrimidines, and unbalance metabolism of key enzymes [30, 52, 68, 69].

The mechanism by which chlorine works was described by Fukuzaki [18]. In a technical sense, the bacteria are killed because HOCl and OCl- are able to penetrate their cell membranes. Since the cell of a pathogenic bacteria is inherently negatively charged, the hydrophobic lipid bilayer and several protective cell wall features prevent ionized OCl- from penetrating the microbial cell membrane. The pathogenic bacteria's negatively charged cell wall will repel the negatively charged hypochlorite ions (OCl-), limiting any oxidizing effect to the cell's periphery. Further, the neutral HOCl may penetrate the cell wall of the harmful bacterium very easily, therefore making it a very efficient disinfectant which can work on both outside and inside of the microorganism. HOCl can also break down the barriers of microorganisms like their slime layers, cell walls, and protective layers [60]. The sulfhydryl groups of certain enzymes are oxidized by HOCl, which interferes with protein synthesis and the oxidative decarboxylation of amino acids to nitrites and aldehydes, ultimately killing the bacterium.

6. Inactivation Mechanisms of SAEW on Bacterial Spores

Inactivation of microbial cells in EW is widely attributed to the presence of active chlorine species (HOCl, OCl-, and Cl_2). In SAEW with a pH of 5.0–6.5, HOCl (~95%) is the main form of active chlorine compounds responsible for microbial inactivation [60]. HOCl could inhibit the enzyme activity essential for microbial growth, damaging the membrane and DNA since it can penetrate the walls and membranes of microbial cells [18]. In recent years, reports regarding the mechanism of the electrolyzed water bactericidal action have been increasing and a number of studies have explored the potential mechanism by which SAEW (with high ORP and HOCl) can inactivate bacterial cells [11, 46]. Nevertheless, it is not justifiable to extrapolate the rationale behind the inactivation of bacterial vegetative cells to their spores treated with SAEW, as the intricate structure and chemical composition of bacterial spores exhibit substantial differences compared to vegetative cells [84]. Unlike vegetative cells, bacterial spores exhibit a unique and complex structure, consisting of an exosporium, a coat, an outer membrane, a cortex, a germ cell wall, an inner membrane (IM), and a central core [66]. The spore coat consists of many insoluble proteins, protecting them against various treatments [20]. As an evidence of spore structure complexity, hypochlorite and chlorine dioxide treatment could not kill *B. subtilis* spores by DNA damage, as reported by Young and Setlow [82], and spore resistance to these chemicals appears to be mostly attributable to their coat.

The inactivation mechanism of SAEW against bacterial spores remains a topic of debate. However, published literature suggests three primary pathways of spore inactivation following EW treatments: (1) Spores are first activated in a treated environment and then germinated as vegetative cells, which the subsequent treatments could quickly kill. (2) Spores are inactivated by directly damaging their structures. (3) Spores are subjected to adverse treatment and are inactivated by inhibiting or hampering their subsequent germination and growth [65, 66]. The inactivation mechanism of the SAEW treatment on the *Bacillus cereus* spores is displayed in Figure 4 according Fukuzaki [18].

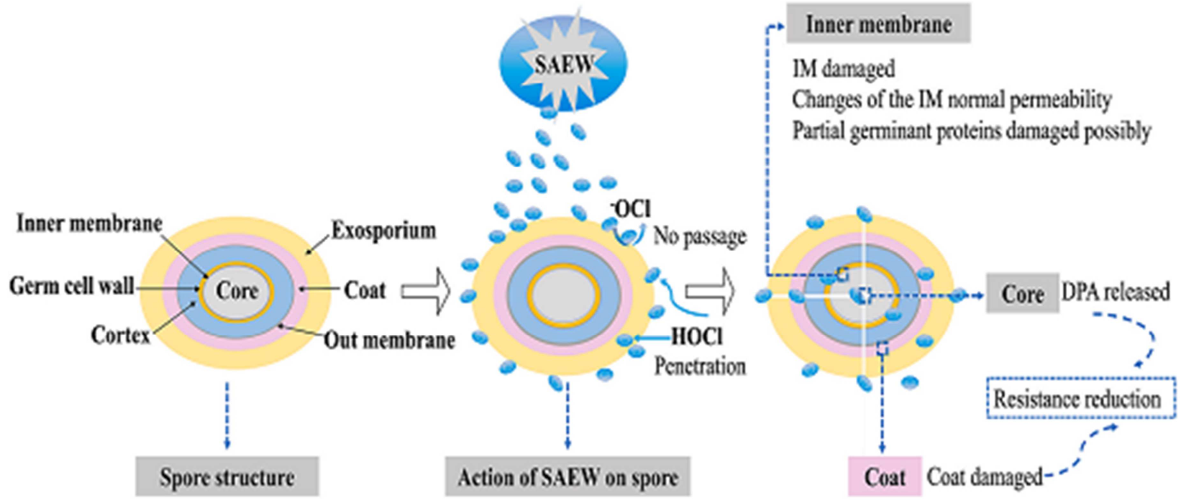

Figure 4. *An outline of the inactivation mechanism of SAEW on the B. cereus spores. Referred to Fukuzaki [18] for the effect of SAEW on spores.*

Zhang et al. [84] investigated the inactivation mechanism for *Bacillus cereus* spores by SAEW by observing structural changes in the spores, coat damage, mutagenesis, and inner membrane (IM) properties. A surface rupture, IM damage, and loss of core contents were all visible in scanning and transmission electron micrographs of *B. cereus* spores after SAEW treatment. SAEW treatment resulted in destruction of the spore structure, showing the detachment of the exosporium and an incomplete structure. Due to the destruction of the spore structure, the membrane permeability was altered, escalating the release of the core contents [84]. Findings suggests that the inactivation of spores by SAEW is directly correlated with structural spore damage, including the coat and the IM and prevention of spore germination by damaging the relative IM Proteins [84]. Wang et al. [75] and Lv et al. [51] also reported that SAEW treatment changed the shape, surface morphology, and ultrastructure of *B. cereus* spores. Unlike bacterial vegetative cells that is inactivated by SAEW via DNA damage [42], Zhang et al. [84] reported that the *Bacillus cereus* spores killed by SAEW were not due to DNA damage. Similarly, Young and Setlow [82] reported that the elimination of *B. subtilis* spores by hypochlorite and chlorine dioxide was not due to DNA damage but the property changes of IM.

Previous investigations have proposed a mechanism by which SAW effectively inactivates bacterial spores. In their study, Tang et al. [70] found that the application of electrolyzed oxidizing water resulted in a decrease in dehydrogenase activity, an upsurge in membrane permeability, an elevation in suspension conductivity, and the leakage of cellular components (such as K+, proteins, and DNA) from *Bacillus subtilis var. niger* spores that ultimately led to the death of the spores. It was further observed by Wang et al. [75] that combining high pressure with SAEW damaged the morphological structure and accelerated the pyridine-2,6-dicarboxylic acid (DPA) release of B. cereus spores. Another study by Young and Setlow [82] found that despite hypochlorite and chlorine dioxide injuring the DNA, *B. subtilis* spores survived. This may have been due to extensive damage to the spores' IM. Despite the importance of these investigations, the mechanisms by which SAEW destroys the B. cereus spore structures remain unclear and require further investigation.

7. SAEW Inactivation Mechanism via Morphological Changes

To present, most studies on SAEW's antibacterial mechanism have examined it's physical and chemical properties such as ORP, OCl-, Cl_2, HOCl and pH. Only few studies have examined the effects of SAEW on the physiological and biological changes of bacteria. While apoptosis and necrosis are more commonly associated with eukaryotic cells, the hallmark phenomena of apoptosis have recently been found in *E. coli* that was caused by a number of bactericidal antibiotic treatment [14]. The bactericidal effect and damage to cell esterase activity produced by SAEW were further demonstrated using a fluorescence-based live-dead experiment. Ye et al. [80] observed that, the cell morphology changed, which was characterized by cell expansion, cell elongation and increased membrane permeability. Simultaneously, the bacterial cells experienced the release of reactive oxygen species (ROS). The inactivation of *E. coli* and the induction of apoptosis were detected as a result of exposure to SAEW. The results demonstrate that the bactericidal properties of SAEW against *E. coli* were observed through cellular and biochemical pathways involving cell necrosis and death. During the bactericidal process, morphological changes often can be induced by antibiotics or disinfectants [80]. The study conducted by Dwyer et al. [14] shown that exposure to SAEW had the potential to alter the morphology of *E. coli* cells while preserving their overall cell shape. This observation suggests that the cells may not undergo disruption or division as a result of SAEW treatment. Nevertheless, the presence of a particular cell shape does not necessarily indicate the continued persistence of the corresponding cellular function. In their study, Dwyer et al. [14] further observed an increase in cell permeability, indicating a disruption in cell membrane integrity. The changes of cell membrane permeability were verified when the PI fluorescence increased following SAEW treatment.

Dimmeler and Zeiher [10] observed that ROS might cause apoptotic cell death in a wide range of cell types. In the study by Ye et al. [80], the relative ROS contents in the *E. coli* increased significantly after treatment with SAEW, which produced free radicals, such as superoxide anion ($O2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH) that could oxidize the lipids, glycolipids, and proteins in the cytomembrane into peroxides [73].

Based on their research on the function of reactive oxygen species (ROS) in electrochemical disinfection, Jeong et al. [36] found out that that hydroxyl radical (.OH) is the primary deadly species responsible for the inactivation of *E. coli* during the chloride-free electrochemical disinfection method. Additionally, electrochemical oxidizing water (EOW) was observed by Tang et al. [70] to increase membrane permeability, conductivity of suspension, and K+ and protein leakage from Bacillus subtilis cells. Damage to the cell membrane and cell wall was also seen, which may account for the observed effects of SAEW.

The antibacterial action and mechanism of SAEW against *Shewanella putrefaciens* and *Staphylococcus saprophyticus* were studied by Liu et al. [49]. SAEW treatment exhibited strong antimicrobial activity against tested bacteria, in which SAEW (60.0 mg/L of ACC) treatment showed that the cell morphology and structure were destroyed by SAEW [49]. The antibacterial mechanisms of SAEW against the tested bacteria was proposed by Liu et al. [49] as presented in Figure 5. In their study, investigation mainly focused on the destruction of membrane integrity by SAEW, which caused the leakage of intracellular compounds, as well as changes in cell microstructure, internal ROS concentration, and changes in antioxidant enzymes.

Generally, SAEW had multiple targets in the antibacterial mechanism (Figure 5). First of all, the surface of the cells treated with SAEW was rough, shrunken, and even dissolved instead of smooth, continuous, and bright. At the same time, the increase of membrane permeability promoted the leakage of intracellular compounds (protein and DNA), which was the consequence of the protective barrier (cell wall and cell membrane) being attacked and destroyed by chlorine substances [49]. Intracellular metabolites undergo a series of complex changes under the action of HOCl and ROS produced by SAEW, which resulted from the diffusing of SAEW in cells. HOCl and produced radicals (such as O, Cl-, and .OH) significantly disrupted the normal physiological functions and destroyed the cellular ultrastructure in different degrees, including: (1) DNA: changing the conformation and structure of DNA; (2) enzymatic: the activities of intracellular oxidative damage-related enzymes were decreased, such as the SOD, CAT, GSH-Px; (3) intracellular micro environment: boosting the release of ROS to induce cell necrosis and apoptosis. Moreover, SAEW presented a wide range of bactericidal properties, which could destroy the structure and change the permeability of the cell membrane. Once interacting with multiple targets at the same time to produce antibacterial effects, the normal cellular functions were disrupted, which lead to cell necrosis and apoptosis.

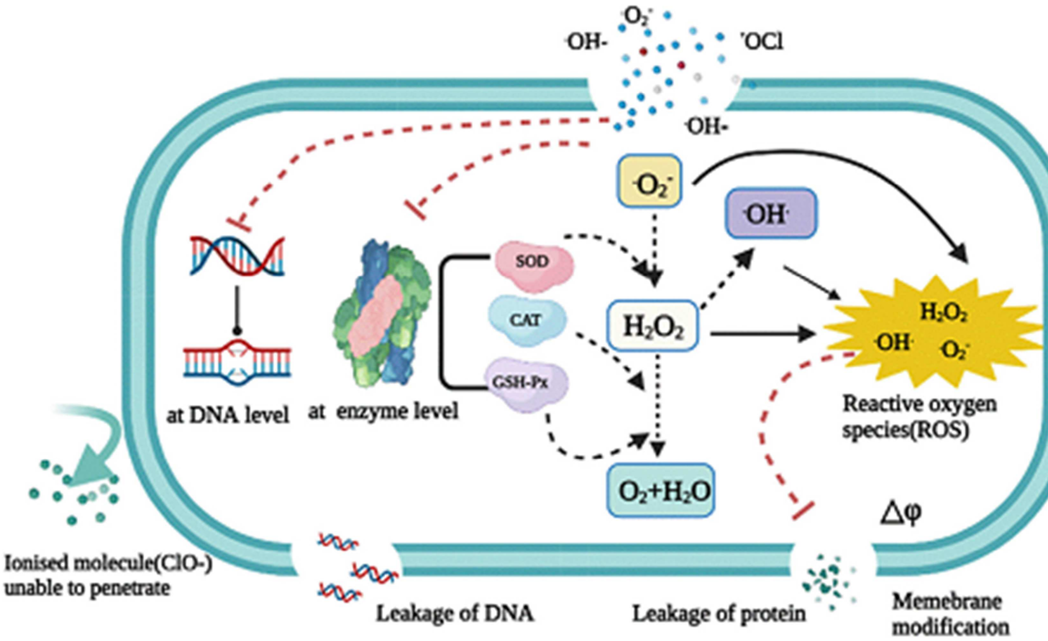

Figure 5. *Proposed antibacterial mechanisms of SAEW against S. putrefaciens and S. saprophyticus. Referred to [49].*

8. Conclusions

In conclusion, the antimicrobial efficacy of SAEW can be principally ascribed to the existence of hypochlorous acid (HOCl), a substantial oxidation-reduction potential (ORP), an available pool of free chlorine (Cl_2), and the presence of pH-dependent hypochlorite ions (-OCl). The application of SAEW might be considered a hurdle technology in light of its various characteristics that contribute to its antibacterial capabilities. This review demonstrates that the application of SAEW can kill vegetative bacterial cells by the disruption of their cellular membrane, disruption of their intracellular reactive oxygen species (ROS) balance, and lowering their ATP levels. SAEW cause key enzyme deactivation, DNA damage, and affect other bacterial cells vitals. It is believed that SAEW has the ability to render bacterial spores inactive through the induction of structural modifications in the spores, including coat damage, mutagenesis, and alterations in the properties of the inner membrane (IM). The findings in this review suggest that SAEW has efficacy in the inactivation of both vegetative bacterial cells and their spores.

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Conflicts of Interest

The authors declare no conflict of interest.

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