

Research Article

Efficacy and Mechanism of Ultrasound Combined with Slightly Acidic Electrolyzed Water for Inactivating *Escherichia coli*

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In the present study, the synergistic effect and mechanism of ultrasound (US) and slightly acidic electrolyzed water (SAEW) on the inactivation of *Escherichia coli* (*E. coli*) were evaluated. The results showed that US combined with SAEW treatment showed higher sanitizing efficacy for reducing *E. coli* than US and SAEW alone treatment. US and US combined with SAEW treatments resulted in smaller particle size of *E. coli* compared to the control and SAEW treatment. In addition, US combined with SAEW treatment induced the highest potassium leakage. However, the highest protein leakage was recorded in US treatment. Moreover, scanning and transmission electron microscopy analysis revealed that the greatest damage of the appearance and ultrastructure of *E. coli* was achieved after US combined with SAEW treatment. The synergistic effect was also confirmed by CLSM analysis. Fluorescence spectroscopy suggested that treatments of US, SAEW, and US combined with SAEW changed protein conformation of *E. coli*. Overall, the present study demonstrated that the sterilization mechanism of US combined with SAEW treatment was decreasing the particle size and disrupting the permeability of cell membrane and the cytoplasmic ultrastructure as well as changing protein conformation of *E. coli*.

1. Introduction

Escherichia coli (*E. coli*), a Gram-negative rod-shaped bacterium, is widely recognized as one of the major food-borne pathogens often from the consumption of contaminated foods [1, 2]. Therefore, a great variety of sanitization strategies, including heating, ultrasound, ultraviolet-C, sodium hypochlorite, sodium benzoate, potassium sorbate, chlorine dioxide, and so on, have been used to reduce the microbial population and prolong the shelf life of food products by the food industry [3–5]. Even so, a more effective and much safer sterilizing technology is crucial for environmental conservation and food preservation.

Ultrasound, as a promising non-thermal sterilization technique, can cause physical effects and/or chemical effects, thus decontaminating microorganisms from the surfaces of foods. Koda et al. [6] reported that inactivation of microorganisms by high frequency ultrasound was mainly dependent on the chemical effects. On the other hand, reactive oxygen species generated by cavitation also assisted sterilization during ultrasound processing [7]. In recent years, many literatures reported that sterilization effect of combined treatment of ultrasound with chemical sterilant was more effective than each used alone [8]. Our results showed that ultrasound produced micro-cracks in the bacterial cell membranes, allowing NaOCl into the cells, and thus

deactivated *E. coli* and reduced the usage amount of sodium hypochlorite [9]. In China, non-thermal sterilization has not been widely used in food industry. NaOCl also has been widely used in sterilization in our country, but its security is a concern.

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 [10–13]. Hypochlorous acid (HClO) produced by SAEW can inactivate the microbial cell via improving the oxidation of lipids and protein compounds as well as resulting in a modification in the electron transfer mechanism of microorganisms [14]. The results of Naka et al. [12] indicated that, at the same chlorine concentration, SAEW is more effective than NaOCl in reducing or eliminating bacterial count. Recently, some studies investigated the synergistic action of electrolyzed water and ultrasound on microbial inactivation. José Cichoski et al. [14] reported that the combination of US and SAEW could effectively reduce some microorganisms, including enterobacteria, mesophilic bacteria, lactic acid bacteria, and psychrotrophic bacteria, and thus has a great potential to improve the prechilling of poultry carcasses. In addition, SAEW simultaneous with US treatment at 40°C for 3 min showed the synergic effects against *B. cereus* on potato [10]. Scanning and transmission electron microscopy analysis revealed that combined ultrasound-SAEW treatment resulted in greater damage of *Staphylococcus aureus* than either treatment alone [15]. However, little information is available on the effect of US combined with SAEW against *Escherichia coli* and the related mechanism.

Therefore, in this study, the effects of US combined with SAEW treatment on the antibacterial activity, membrane permeability, membrane integrity, cell morphology, intracellular organization, and protein conformation of *E. coli* were investigated to the antibacterial mechanism of US combined with SAEW.

2. Materials and Methods

2.1. Microbial Inoculation. *Escherichia coli* CICC 10899 was obtained from Chinese Center of Industrial Culture Collection). The stock cultures were transferred to 50 mL of nutrient broth (NB) (Hiabo Bio-Tech Co., Qingdao, China) and incubated at 37 °C in an air bath incubator with a reciprocal shaker for 16 h at 150 rpm. Following incubation, the microbial culture was sedimented by centrifugation at 6,000 × g for 10 min at 4°C. The supernatant was discarded and the bacterial cells were washed twice with 0.90% sterile saline solution and resuspended for following use. The final population in bacterial suspension of *E. coli* was approximately 10⁶ CFU/mL.

2.2. SAEW Preparation. SAEW was produced by electrolysis with a continuous supply of dilute NaCl solution (0.9%) in a chamber without a membrane using an electrolysis device (Anywhere-320W, Rui Andre Environmental Equipment

Co., Ltd., Beijing, China). SAEW pH and ORP values were determined immediately before sample treatment using a pH meter (Starter 300, Ohaus Co., USA) with pH and ORP electrodes. A colorimetric method with a digital chlorine test kit (RC-3F; Kasahara Chemical Instruments Corp., Saitama, Japan) was used to measure ACC. In this study, SAEW with a pH of 6.18, ORP of 827 mV, and available chlorine concentration (ACC) of 30 mg/L was used to sterilize.

2.3. Single or Combined Treatments with US and SAEW. US treatment was applied using a probe-style ultrasonic processor (Scientz-II D; Ningbo Scientz, Zhejiang, China). A total of 27 mL of cell suspension was added with 3 mL 0.90% sterile saline solution, and then the ultrasonic emitter was immersed 2.0 cm into the solution and ultrasonically treated for 10 min at a frequency of 20 kHz and 10 W/cm³ energy density.

For SAEW treatment, the inoculated samples of 27 mL were mixed with 3 mL SAEW in a sterile glass beaker for 10 min. For combined treatment, after mixing 3 mL SAEW in 27 mL cell suspension, the US treatment followed immediately for 10 min under the above ultrasonic conditions. In this study, a thermostatic water bath (DC-1006, Safe Corporation, Ningbo, China) was used to maintain the temperature at 20°C in order to prevent a lethal thermal effect after US treatment.

2.4. Microbiological Analysis. Following treatments, microbiological analysis was conducted using plate counting method according to the previous procedure [9]. After incubation, microbial colonies were counted with an automated plate counter (ProtoCOL, Synoptics, Cambridge, UK). All analyses were conducted in duplicate with 3 replicates for each experiment.

2.5. Microbiological Size Measurement. The Malvern Mastersizer 2000 (Malvern Instruments, UK) was used to measure the particle size measurement of bacterial suspensions according to the method described by Gao et al. [16].

2.6. Measurement of the Intracellular Protein Leakage and Potassium Leakage. After treatments, the suspension was centrifuged at 10000 g for 10 min at 4°C. The protein content in the supernatant was according to the method of Bradford [17], using bovine serum albumin as standard.

The intracellular potassium leakage of the supernatant was determined using flame atomic absorption spectrophotometry (AAS) (AAnalyst 100, PerkinElmer Co., USA) as previously described by Tang et al. [18]. A linear relationship between potassium concentration and emission was obtained using potassium standards (analytical grade, Sigma-Aldrich, Poole, United Kingdom). The content of potassium was measured by AAS and calculated by the calibration.

2.7. Scanning and Transmission Electron Microscopy Analysis.

Scanning electron microscopy (SEM) was used to observe the morphological changes in *E. coli* cells according to the method of Li et al. [15]. After centrifugation at 10000 g for 10 min at 4°C, the precipitates were collected and rinsed twice with 0.85% sterile saline solution. The samples were fixed with 2.5% glutaraldehyde for 24 h and then were washed three times with phosphate buffer solution (pH 7.2) and post-fixed with 1% osmium tetroxide for 2 h. Afterwards, the samples were dehydrated using a graded ethanol (30, 50, 70, 80, 90, 95, and 100%) series and transferred to a mixture of ethanol and tertiary butanol (*v:v*=1:1) for approximately 30 min. They were then placed in pure tertiary butanol. Finally, the dehydrated samples were coated with gold-palladium and observed using a JSM-7500F scanning electron microscope (JEOL Ltd., Tokyo, Japan).

For TEM analysis, the cells were infiltrated and embedded in Epon-812 after washing and dehydration. The prepared specimens were sliced to thin sections 70 nm and stained with uranyl acetate and alkaline lead citrate for 10 min. A HT7700 transmission electron microscope (Hitachi Ltd., Tokyo, Japan) was used to examine at 80 kV.

2.8. Confocal Laser Scanning Microscopy (CLSM) Analysis.

To assess the damage to *E. coli* cell membranes following treatments with single and combination of US and SAEW, CLSM analysis was performed using the method of Kang et al. [7], with some modifications. Cell suspensions were incubated with dye buffer (LIVE/DEAD® BacLight Bacterial Viability Kits, L7012, ThermoFisher) and stained with 20 µM propidium iodide (PI) in the dark for 30 min at room temperature. The mixture was washed with 1 mL sterile HEPES buffer (pH 7.0) and then observed in a fluorescence microscope (TCS SP5, Leica, Germany).

2.9. Fluorescence Spectroscopy Experiments. All recordings of fluorescence, synchronous, and resonance light scattering spectra were carried out on a FL2700 luminescence spectrometer (Hitachi High-Technologies Corporation, Japan) with a quartz cell of 10 mm path length. The excitation and emission wavelength, excitation and emission bandwidths intervals, and scanning wavelength range were in accordance with our previous study [9].

2.10. Statistical Analyses. All experiments were performed in triplicate. Data were expressed as the mean ± standard deviation (SD). Significant differences were determined using one-way analysis of variance (ANOVA) and Duncan's multiple range tests (SPSS 19.0, SPSS Inc., Chicago, IL, USA) at $p < 0.05$.

3. Results and Discussion

3.1. Effect of US Combined with SAEW Treatment on the Microbicidal Efficiency and Particle Size Distribution. Microbial reduction values resulting from different treatments are shown in Figure 1(a). US treatment for 10 min

decreased the number of *E. coli* by 0.48 log CFU/mL, indicating that the US treatment alone was not effective for inactivating *E. coli* CICC 10899. The similar phenomenon was also found in decontamination of *S. aureus* [15], *E. coli* ATCC 10536, and *V. Parahaemolyticus* KCTC 2471 [19]. The action of the US and SAEW is related to the species of bacteria. Park et al. [19] reported that SAEW treatment (chlorine 30 mg/L) showed the higher sterilizing effect for *V. Parahaemolyticus* KCTC but showed the lower sterilizing effect for *E. coli* ATCC 10536 than US treatment for 50 min. SAEW treatment also was not efficient in reducing *Staphylococcus* spp. [14]. However, in the present study, SAEW treatment led to 7.01-fold reduction of *E. coli* CICC 10899 when compared with US treatment for 10 min. This result indicated that SAEW was an effective disinfectant for inactivating *E. coli* CICC 10899. The different phenomena of US and SAEW treatments on *E. coli* ATCC 10536 and *E. coli* CICC 10899 might have resulted from the difference of ultrasonic time. It also needs further confirmation.

Cichoski et al. [14] reported that SAEW combined with the application of US at 25 kHz showed the synergistic effect on inactivating of enterobacteria, mesophilic bacteria, lactic acid bacteria, and psychrotrophic bacteria; however, SAEW combined with the application of US at 130 kHz had no synergistic effect on inactivating of all bacteria. Moreover, for *Staphylococcus* spp., SAEW combined with US treatment did not increase and even decreased the inactivation efficacy compared to single US and SAEW treatment [14]. These results suggested that the synergistic effect of US combined with SAEW was also related to ultrasonic frequency and microbial species. In addition, SAEW combined with US treatment also significantly improved the reductions in the populations of inoculated *S. aureus*, *B. cereus*, *E. coli* O157:H7, and *A. fumigatus* in kashk compared to SAEW alone [20]. In the present study, US and SAEW treatment exhibited the synergistic effect in sterilization of *E. coli* and presented the highest reduction of *E. coli* with value of 3.64 log CFU/mL. The reason might be that cavitation resulted from US disrupted cell membrane, accelerating SAEW into microbial cells, and thus inactivated *E. coli* CICC 10899.

Monomodal was observed in control and SAEW treated samples (Figure 1(b)). However, a small volume distribution at 100–500 nm was found after US and US + SAEW treatments. The average Sauter diameters of control and SAEW treated samples were 1688 nm and 1436 nm, respectively. US and US + SAEW treatments caused significant reduction in particle size of *E. coli*, which was 718 nm and 762 nm, respectively. These results indicated that the small distribution and decrease in particle size of *E. coli* were mainly attributed to cavitation of ultrasound rather than SAEW. The similar phenomenon was also observed by combined treatment of US and NaOCl [9].

3.2. Effect of US Combined with SAEW Treatment on the Intracellular Protein and Potassium Leakage. The protein and potassium leakage can be used to investigate the damage of the cell membranes [21, 22]. As shown in Figure 2, all treatments led to the leakage of intracellular protein and

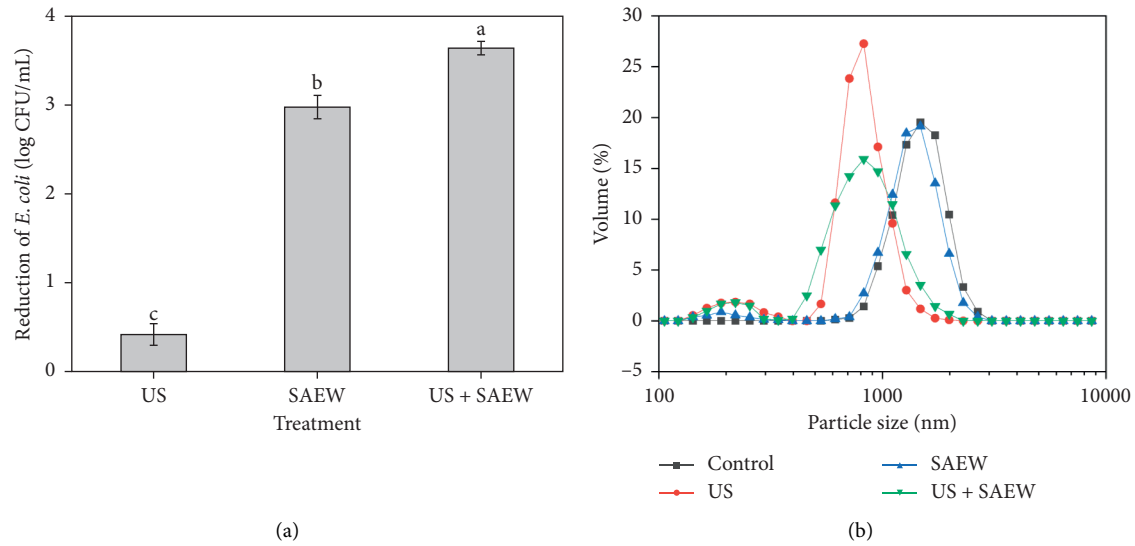


FIGURE 1: Reduction of *E. coli* (a) and particle size distribution (b) after the single and combined treatment of ultrasound and slightly acidic electrolyzed water. Values not sharing the same letter are significantly different at $p < 0.05$. US: ultrasound, SAEW: slightly acidic electrolyzed water, and US + SAEW: ultrasound combined with slightly acidic electrolyzed water.

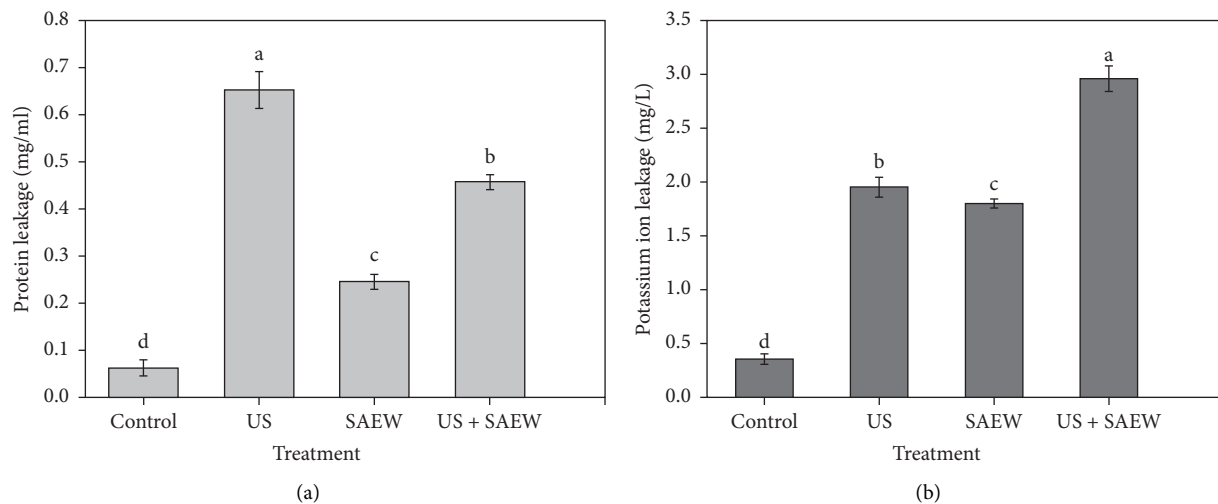


FIGURE 2: Protein leakage (a) and potassium ion leakage (b) of *E. coli* after the single and combined treatment of ultrasound and slightly acidic electrolyzed water. Values not sharing the same letter are significantly different at $p < 0.05$. US: ultrasound, SAEW: slightly acidic electrolyzed water, and US + SAEW: ultrasound combined with slightly acidic electrolyzed water.

potassium of *E. coli*. After US, SAEW, and US + SAEW treatments, the protein concentrations in suspension increased to 0.65, 0.25, and 0.46 mg/mL, respectively. On the other hand, US + SAEW treatment caused the highest potassium leakage, which was increased by 44.2% and 64.3% compared to US and SAEW treatment, respectively, indicating that US + SAEW treatment led to the most serious damage of the cell membranes of *E. coli*.

3.3. Morphological Changes Revealed by Electron Microscopy. Morphological changes of *E. coli* induced by US and SAEW were observed using SEM and TEM. SEM micrographs revealed that the cells of control samples maintained intact

shapes, but with markedly deformation after SAEW treatment. *E. coli* was found markedly shrunk and cell wall was collapsed (Figure 3). This phenomenon could be due to the oxidative damage and the permeability of the cell membrane, thus resulting in the leakage of intracellular protein and potassium, and throwing the osmotic pressure out of balance [15, 23]. While US and US + SAEW treatments resulted in more serious damage compared to SAEW treatment, in addition to shrink and collapse, cell membrane and cell wall of *E. coli* were also damaged.

The TEM micrographs of *E. coli* after treatments with US, SAEW, and US + SAEW are shown in Figure 4. For control samples, cell wall and membrane of *E. coli* were continuous and intact and well defined. SAEW treatment

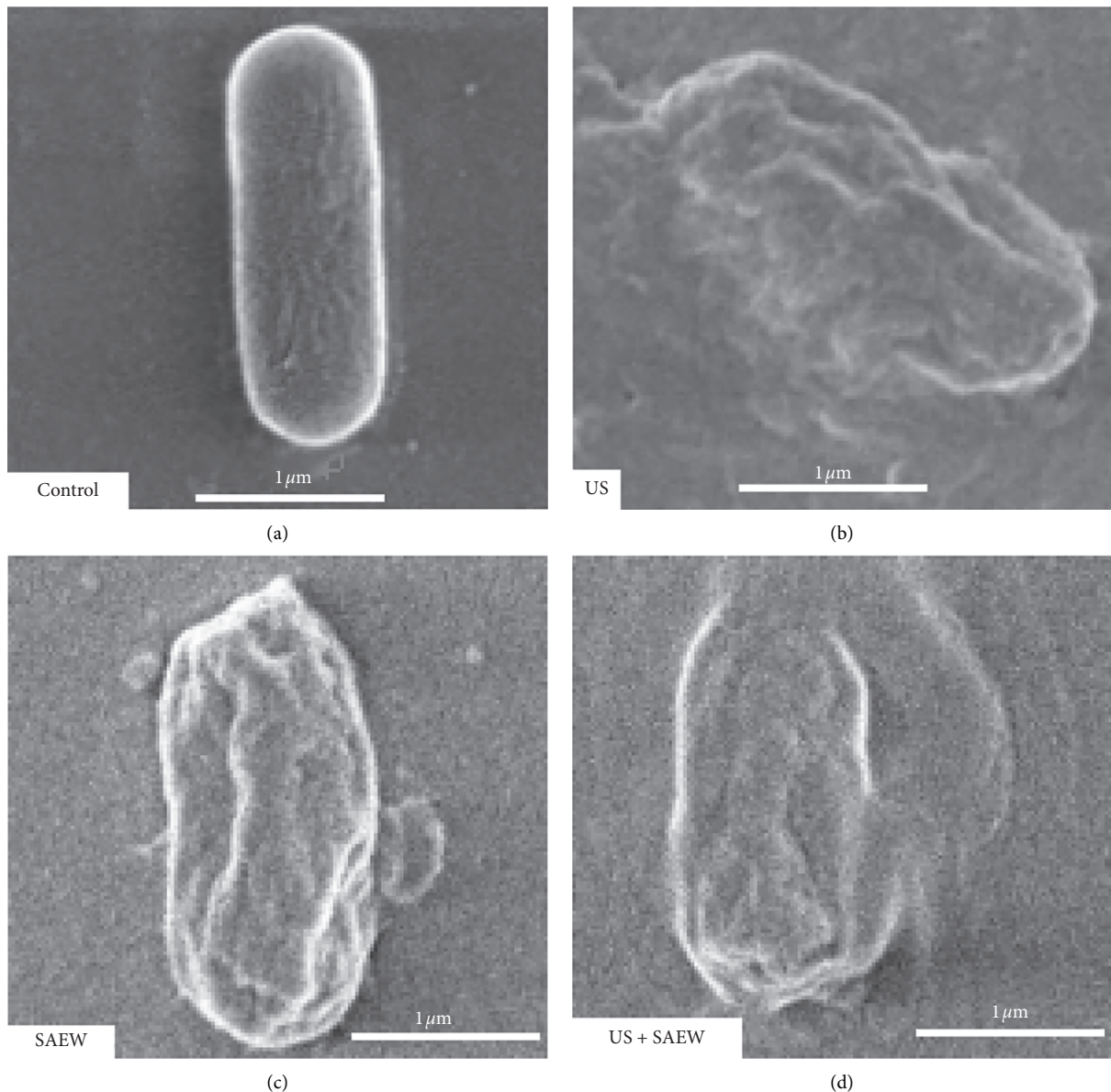


FIGURE 3: Scanning electron microscopy of *E. coli* after the single and combined treatment of ultrasound and slightly acidic electrolyzed water. Images were taken at magnification of $\times 20$ K. US: ultrasound, SAEW: slightly acidic electrolyzed water, and US + SAEW: ultrasound combined with slightly acidic electrolyzed water.

resulted in slight damage of cell wall. However, after US treatment, cell wall and membrane of *E. coli* were damaged and indefinite. US + SAEW treatment led to the most serious damage; meanwhile, a plenty of intracellular compounds also leaked. US could rupture the chemical bonds between molecular components in cell membranes, thus accelerating SAEW into bacteria [15, 24]. Hence, the release of cell contents and disintegration of the cell wall was mainly attributed to the action of ultrasound on the damaged cells.

3.4. CLSM Analysis of *E. coli* Under US Combined with SAEW Treatment. The cells of *E. coli* with intact cell membranes were stained with fluorescent green, whereas cells with a damaged membrane were stained by red PI [25]. Figure 5

shows the live and dead population of *E. coli* after different treatments. A small fraction of dead cells was found after US and SAEW alone treatment. However, after treatment of US combined with SAEW, nearly all cells of *E. coli* showed red, indicating cytoplasmic membrane of most treated cells was injured, which was in accordance with results of microbial reduction values, SEM, and TEM analysis (Figures 1, 3, and 4).

3.5. The Effect of US Combined with SAEW Treatment on the Membrane Protein of *E. coli*. The conformational changes of proteins of *E. coli* can be successfully investigated by fluorescence spectroscopy [26, 27], since the intrinsic fluorescence of indol chromophores in Trp residues is sensitive to

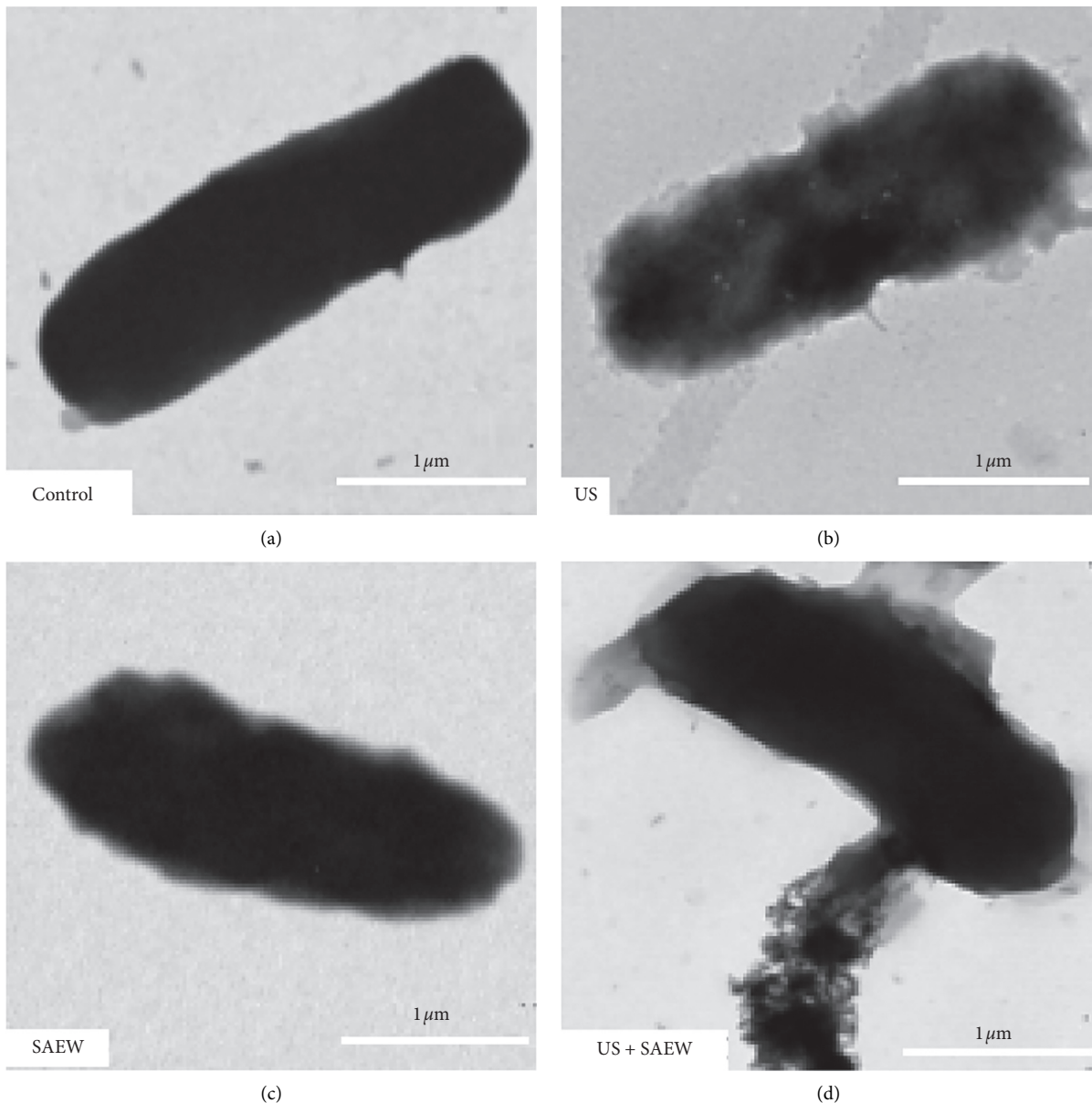


FIGURE 4: Transmission electron microscopy of *E. coli* after the single and combined treatment of ultrasound and slightly acidic electrolyzed water. Images were taken at magnification of $\times 5K$. US: ultrasound, SAEW: slightly acidic electrolyzed water, and US + SAEW: ultrasound combined with slightly acidic electrolyzed water.

microenvironment particularities [27], which can provide information about the molecular microenvironment in the vicinity of the chromophores [28]. The spectrum of *E. coli* with different treatments is shown in Figure 6. Under the excitation wavelength of 278 nm, the maximum emission wavelength of protein in control and US treated samples was 332 nm. Nevertheless, the maximum emission wavelength of protein fluorescence was decreased after SAEW and US + SAEW treatments, which was 330 nm, which suggested that SAEW could result in a blue shift of the maximum emission peak. In addition, it has been reported that US treatment could increase the fluorescence intensity of *E. coli*, thus improving the hydrophobicity of *E. coli* protein [29]. In the present study, the similar result was also observed after

US treatment. Nevertheless, SAEW and US + SAEW treatments reduced fluorescence intensity. The reduction of fluorescence intensity and the blue shift of the maximum emission peak implied the Trp residues transfer to a polar environment after SAEW and US + SAEW treatments [30].

The microenvironment of amino acid residues of biomolecules can be evaluated by synchronous fluorescence spectroscopy. The maximum emission wavelength of Tyr ($\lambda = 15$ nm) was 283 nm under control and US treatments. However, it was decreased to 281.5 nm after SAEW and US + SAEW treatments (Figure 7(a)). The similar change of Trp ($\lambda = 60$ nm) was also observed. After SAEW and US + SAEW treatments, the maximum emission wavelength of Trp shifted from 278 nm to 276 nm

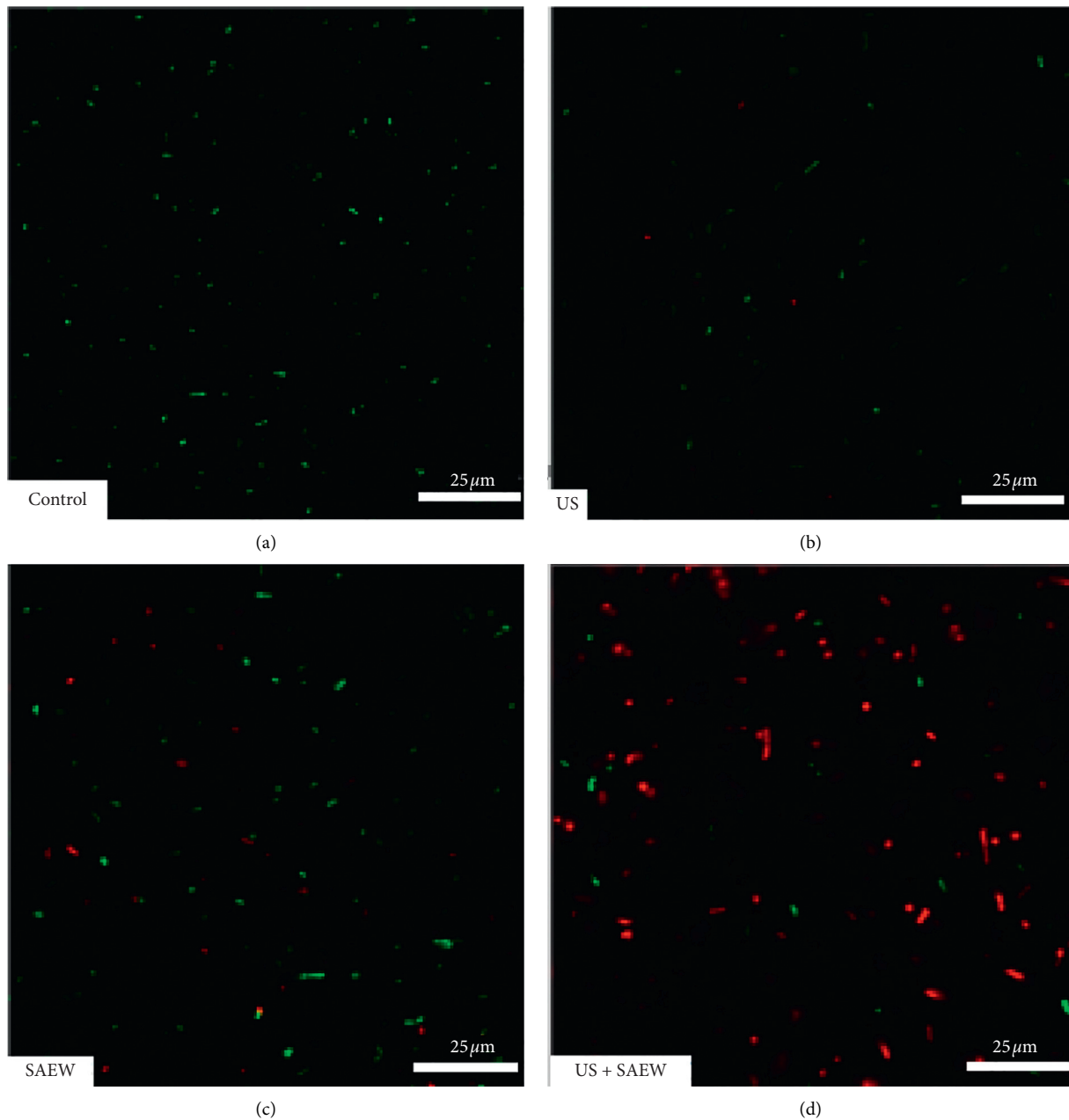


FIGURE 5: Confocal laser scanning micrographs of *E. coli* after the single and combined treatment of ultrasound and slightly acidic electrolyzed water. Images were taken at magnification of $\times 630$. US: ultrasound, SAEW: slightly acidic electrolyzed water, and US + SAEW: ultrasound plus slightly acidic electrolyzed water.

(Figure 7(b)). These results indicated that the polarity around the Tyr residues and Trp residues of *E. coli* decreased and the hydrophobicity increased after SAEW and US + SAEW treatment [27]. Additionally, the enhancement of fluorescence intensity was detected in US-treated *E. coli*, whereas the decrease of fluorescence intensity was found in

SAEW and US + SAEW treated samples. However, there was no significant difference between SAEW and US + SAEW treatment. The identical changes in resonance intensity of *E. coli* were also observed after US, SAEW, and US + SAEW treatments compared to the control (Figure 8). In the present study, US treatment enhanced the resonance

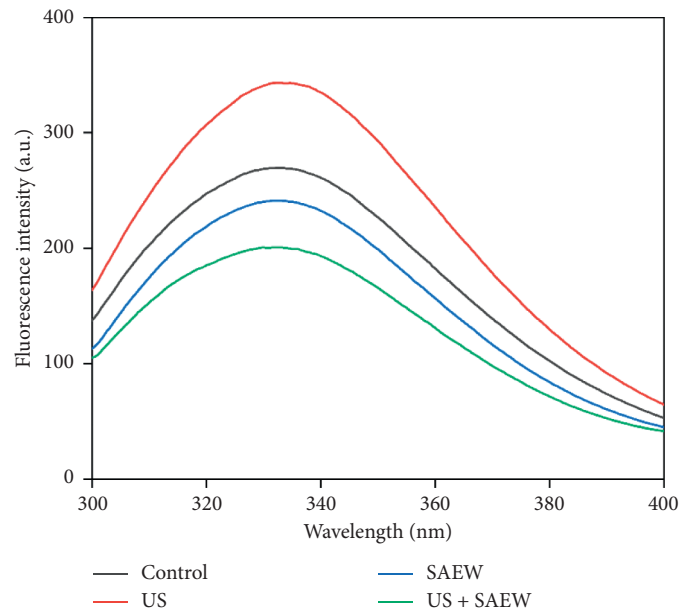


FIGURE 6: Endogenous fluorescence spectrometry of *E. coli* after the single and combined treatment of ultrasound and slightly acidic electrolyzed water. US: ultrasound, SAEW: slightly acidic electrolyzed water, and US + SAEW: ultrasound plus slightly acidic electrolyzed water.

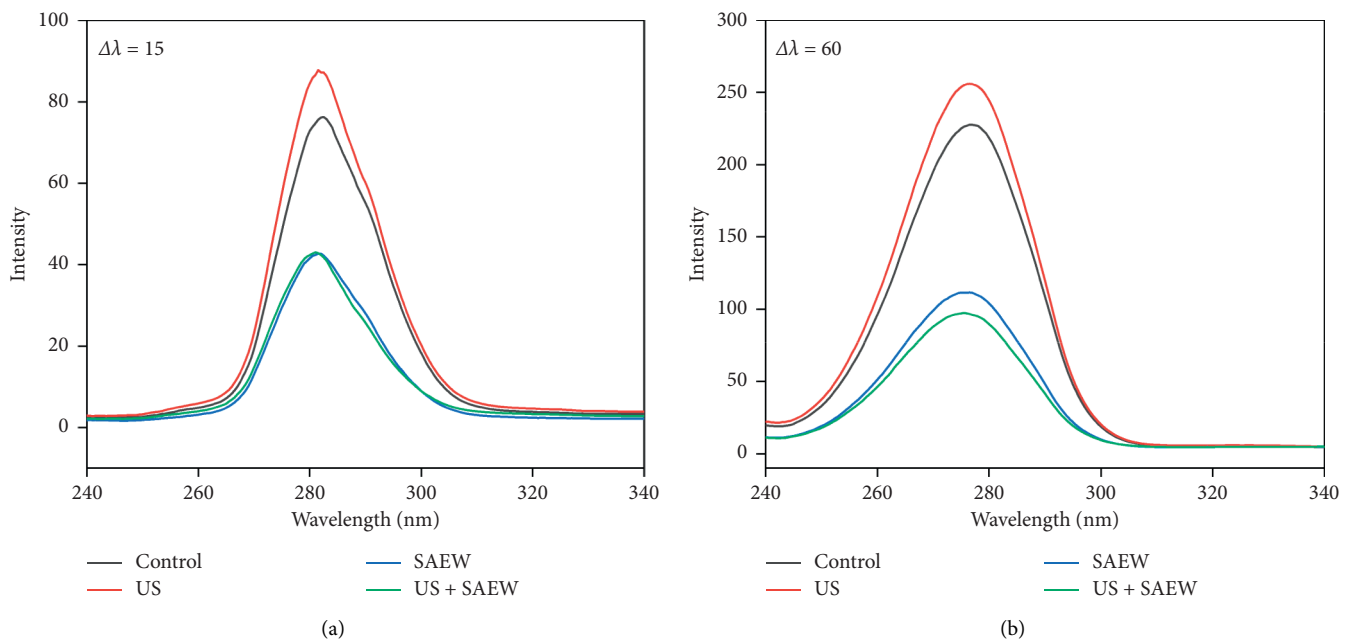


FIGURE 7: Synchronous fluorescence spectrometry of *E. coli* after the single and combined treatment of ultrasound and slightly acidic electrolyzed water. US: ultrasound, SAEW: slightly acidic electrolyzed water, and US + SAEW: ultrasound plus slightly acidic electrolyzed water.

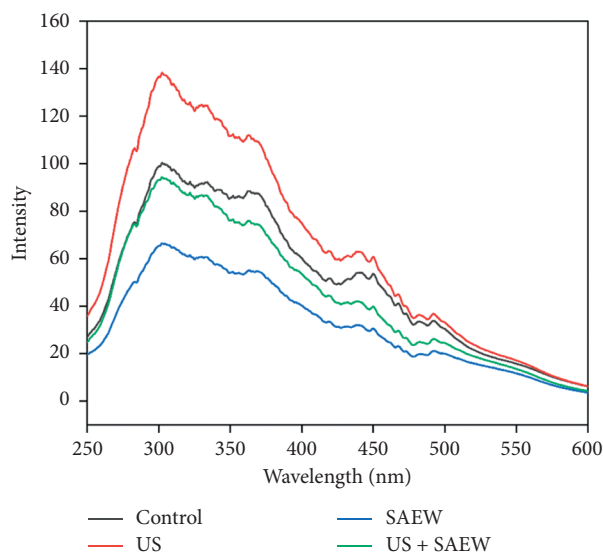


FIGURE 8: Resonance light scattering fluorescence spectrometry of *E. coli* after the single and combined treatment of ultrasound and slightly acidic electrolyzed water. US: ultrasound, SAEW: slightly acidic electrolyzed water, and US+SAEW: ultrasound plus slightly acidic electrolyzed water.

intensity of *E. coli*, indicating proteins of *E. coli* assembled. On the other hand, the decrease in resonance intensity of *E. coli* after SAEW treatment might be due to breakdown of protein [31].

4. Conclusion

US combined with SAEW showed the best sterilizing efficacy, with a significant reduction of survival cells compared with single US and SAEW treatments. Protein and potassium leakage tests as well as the morphologies of *E. coli* and CLSM analysis showed visible change under the combined treatment of US and SAEW. Fluorescence spectroscopy analysis found US and SAEW treatment changed membrane integrity and protein conformation of *E. coli*. In short, US treatment disrupted the cell membrane of *E. coli* and facilitated SAEW into the cells, thus improving the sterilizing effect. These results showed that US in combination with SAEW, as an environment friendly and safety sterilizing technology, could be developed as an effective and practical sterilizing method for food industry.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

Xuecong Zhang and Liping Guo are the co-first authors.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Liping Guo and Xuecong Zhang contributed equally to this work.

Acknowledgments

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