The combination of ultrasound and chlorogenic acid to inactivate *Staphylococcus aureus* under planktonic, biofilm, and food systems

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**ABSTRACT**

This study aimed to investigate the mechanism of different treatments, namely, ultrasound (US), chlorogenic acid (CA), and ultrasound combined with chlorogenic acid (US plus CA) on the inactivation of *Staphylococcus aureus* planktonic and biofilm cells. Results showed that the combined treatment of US and CA exhibited remarkable synergistic antibacterial and antibiofilm effects. Scanning electron microscopy images indicated that the combined treatment of US and CA caused the most serious damage to the cell morphology. Confocal laser scanning microscopy images revealed that the combined treatment led to sharp increase and severe damage to the permeability of the cell membrane, causing the release of ATP and nucleic acids and decreasing the exopolysaccharide contents in *S. aureus* biofilm. Finally, the combined treatment of US plus 1% CA for 60 min inactivated *S. aureus* cells by 1.13 lg CFU/g on mutton. Thus, the combined treatment of US and CA had synergistic effect against *S. aureus* under planktonic, biofilm, and food systems.

1. Introduction

Mutton is well received because of its low fat and high protein contents, abundant vitamins, and presence of trace elements [1,2]. However, mutton is easily perishable during preservation because its high protein content provides nutrients for the growth of bacterial cells [1]. *Staphylococcus aureus* is a Gram-positive zoonotic pathogen that causes lower respiratory tract site infections, surgical site infections, cardiovascular infections, and pneumonia in humans and animals [3,4]. Meat products, such as mutton, chicken, pork, and beef, are easily contaminated by *S. aureus* [5,6,7]. Additionally, *S. aureus* has strong ability to form biofilm on surfaces of food, food processing equipment, and water [8,9,10]. Biofilm is a three-dimensional dense network structure that comprises, proteins, nucleic acids, and polysaccharides [3,7,11]. Bacterial cells in biofilm would be protected by the extracellular matrix from the interference of external environment challenges [9,12,13]. Therefore, developing an effective and efficient bactericidal technology to inactivate *S. aureus* planktonic and biofilm cells is of great significance in the food industry.

Ultrasound (US) is an environment-friendly, non-thermal, and non-destructive bactericidal technology used in the food industry. However, US treatment alone exhibits weak antibacterial and antibiofilm activities for the inactivation of bacterial cells [14]. Therefore, many researchers considered synergistic sterilization as a substitutable method to improve the efficiency of sterilization. Guo et al., (2020) reported that the combined treatment of US and sodium hypochlorite had synergistic effect against *Escherichia coli* planktonic cells [15]. Bi et al., (2019) found that ultrasound combined with lysozyme effectively inactivated *Salmonella typhimurium* [16]. Huu et al., (2021) revealed that the combined treatment of ultrasound and propyl gallate had higher bactericidal efficiency to inactivate *Listeria innocua* and *E. coli* O157:H7 cells than single treatment [17]. Chlorogenic acid (CA) is an ester abundant in fruits and vegetables and has a wide range of antibacterial activities [18]. CA exhibits strong antibacterial activity against many kinds of microorganisms, including *E. coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *S. aureus* [19]. Lou et al., (2011) reported that CA effectively inhibited the growth of bacterial cells by destroying the integrity of the cell membrane [20]. However, the synergistic effect of US and CA on the inactivation of *S. aureus* planktonic and biofilm cells has not been reported yet.

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This study aimed to (1) determine the effect of chlorogenic acid (CA), ultrasound (US), and ultrasound combined with chlorogenic acid (US plus CA) on *S. aureus* planktonic and biofilm cells and (2) investigate the bactericidal activity and mechanism of these treatments against *S. aureus* under planktonic, biofilm, and food systems.

2. Materials and methods

2.1. Cultivation of microorganisms

The strain of *S. aureus* was obtained from the Institute of Agricultural Products Processing, Jiangsu Academy of Agricultural Sciences (Nanjing, China). Prior to the assay, a loopful of *S. aureus* cells were streaked on brain–heart infusion (BHI) agar (Qingdao Hope Bio-Technology Co., Ltd.) and incubated at 37 °C for 24 h. One single colony was transferred into 5 mL of BHI broth (Qingdao Hope Bio-Technology Co., Ltd.). *S. aureus* cells were cultured to exponential phase under shaking at 200 rpm (37 °C) for 24 h. The concentration of *S. aureus* cells in the exponential phase reached approximately 9.1 g CFU/mL [21,22].

2.2. US, CA, and combined US and CA

The bacterial suspension and biofilm were subjected to US (400 W, 50 kHz), CA (0.5%, 1%, and 2%), and US plus CA. CA powder was diluted in deionized water to produce 0.5%, 1%, and 2% solutions. Treatments with US and US plus CA were carried out in an ultrasonic cleaning machine (50 kHz, 800 W) (Kunshan Ultrasonic, Inc, Suzhou, China). The untreated bacterial suspension and biofilm were placed at room temperature and used as negative control.

2.3. Inactivation of *S. aureus* planktonic cells

*S. aureus* cells in the exponential phase were harvested by centrifugation at 5000 g and 4 °C for 10 min. The bacterial pellets were washed with 0.85% NaCl solution. *S. aureus* planktonic cells were treated with control, US, CA, and US plus CA. For US treatment, the bacterial pellets were mixed with 0.85% NaCl solution and placed in the ultrasonic cleaning machine for 5, 10, 20, 30, and 60 min. For CA treatment, 0.5%, 1%, and 2% CA was added into the bacterial pellets for 5, 10, 20, 30, and 60 min. For combined treatment, the bacterial pellets were mixed with 0.5%, 1%, and 2% CA and then placed into the ultrasonic cleaning machine for 5, 10, 20, 30, and 60 min. After treatment, 1 mL of the bacterial suspension was neutralized with 9 mL of 0.1 mol/L PBS to terminate the antibacterial action of CA. The neutralized solution was serially diluted tenfold with 0.85% NaCl solution. Microbiological analysis was conducted according to our previous paper [23].

2.4. Inactivation of *S. aureus* biofilm cells

For biofilm formation, approximately 10^9 CFU/mL of *S. aureus* cells (1 mL) were inoculated into 100 mL of BHI to obtain the final concentration of *S. aureus* cells (10^7 CFU/mL). Biofilms were formed on the 24-well polystyrene microtiter plates (Costar, Corning, USA) after incubation for 72 h at 37 °C in an incubator. BHI broth was replaced with fresh broth every 24 h. After incubation, the broth was discarded, and the biofilm was washed twice with 0.01 mol/L PBS buffer. The *S. aureus* biofilm cells were treated with control, US, CA, and US plus CA and subjected to similar steps in section 2.3 [23].

2.5. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) analyses

SEM and CLSM analyses were conducted according to our previous paper [23–25]. *S. aureus* planktonic and biofilm cells were treated with US, 2% CA, and US plus 2% CA for 30 min prior to analyses. Additionally, *S. aureus* planktonic and biofilm cells without any treatment were placed at room temperature and used as negative control.

In the SEM analysis, the treated and untreated bacterial suspensions were centrifuged at 5000 g and 4 °C for 10 min, and the bacterial pellets were left. The pellets were fixed with glutaraldehyde (2.5%, v/v) at 4 °C for 12 h. Approximately 10^7 CFU/mL of *S. aureus* cells (400 μL) were added to each well of eight-well chamber slides (Nunc™ Lab-Tek™, Thermo Fisher Scientific), and the biofilm was incubated according to Section 2.4. After different treatments, the plates were cut into small squares and fixed with 2.5% glutaraldehyde at 4 °C for 12 h. Finally, the bacterial cells were observed by EVO-LS10 SEM (Carl Zeiss AG, Jena, Germany).

In the CLSM analysis, the treated and untreated bacterial suspensions were centrifuged at 5000 g and 4 °C for 10 min. The bacterial pellets were dyed by the LIVE/DEAD BacLight viability kit (Molecular Probes; Life Technologies, Eugene, OR). *S. aureus* biofilm used for CLSM analysis was prepared according to the methods of SEM. After different treatments, the *S. aureus* biofilm was dyed by the LIVE/DEAD BacLight viability kit. Finally, the CLSM images were observed under a Leica Ultra View VOX CLSM (Leica Microsystems, Ltd., Wetzlar, Germany).

2.6. Exopolysaccharide (EPS) content analyses

The *S. aureus* biofilm was incubated according to Section 2.4, and the contents of EPS were determined according to our previous paper [25]. The *S. aureus* biofilms were treated by US, 0.5% CA, 1% CA, 2% CA, US plus 0.5% CA, US plus 1% CA, and US plus 2% CA for 30 min. The treated and untreated biofilms were collected into different tubes. The biofilm was centrifuged at 5000 g and 4 °C for 30 min, and the precipitate was collected and resuspended in 10 mL of 0.85% NaCl solution (including 0.22% formaldehyde) for determination of the content of insoluble polysaccharides. The supernatant was determined to determine the content of soluble polysaccharides. The contents of soluble and insoluble polysaccharides were measured using phenol–sulfuric acid method [26].

2.7. Release of intracellular ATP and nucleic acids

The release of intracellular ATP and nucleic acids in *S. aureus* planktonic and biofilm cells was determined according to our previous paper [27]. The bacterial suspension and biofilms were treated with US, 0.5% CA, 1% CA, 2% CA, US plus 0.5% CA, US plus 1% CA, US plus 2% CA, and US plus 2% CA. The supernatant was collected by centrifugation at 3000 g and 4 °C for 10 min and used to measure the release of nucleic acids. The concentration of nucleic acids was determined at 260 nm on a UV–VIS Spectrophotometer (Mapada, Shanghai, China).

For determining the concentration of extracellular ATP, 1 mL of untreated or treated *S. aureus* planktonic and biofilm cells were mixed with 9 mL of 0.1 mol/L PBS buffer. After that, the suspension was centrifuged at 10,000 × g at 0 °C for 1 min, the supernatants were used to measure the release of intracellular ATP by following the instructions of the ATP detection kit (Beyotime, Shanghai, China).

2.8. Inactivation of *S. aureus* cells on mutton

2.8.1. Inoculation of mutton

Mutton was bought from a supermarket (Nanjing, China) and cut into 10 g sample in the laboratory. About 1 mL of each 10^−9 CFU/mL *S. aureus* suspension was inoculated into the mutton. The concentration of *S. aureus* in mutton was approximately 6 lg CFU/g.

2.8.2. US in combination with CA treatment

Inoculated mutton was treated by US plus 1% CA for different durations of 5, 10, 20, 30, and 60 min. The mutton was immediately mixed with 90 mL of 0.1 mol/L PBS to neutralize the pH. About 1 mL of neutralizing solution was serially diluted in 9 mL of 0.01 PBS. Appropriate dilutions of bacterial suspension (1 mL) were added into plate
containing 15 mL of *S. aureus* Chromogenic Medium (Qingdao Hope Bio-Technology Co., Ltd.). All of the plates were incubated at 37 °C for 24 h.

2.9. Statistical analysis

All samples in the experiment were prepared in triplicate. ANOVA in SPSS version 26.0 was used to analyze significant difference (p < 0.05) between the control and experimental groups.

3. Results and discussion

3.1. Inactivation of *S. aureus* planktonic and biofilm cells

Tables 1 and 2 show the inactivation of *S. aureus* planktonic and biofilm cells with different treatment times of 5, 10, 20, 30 and 60 min in the presence of US or CA alone and their combination (US plus CA). US by itself was not effective to inactivate *S. aureus* planktonic and biofilm cells. US treatment alone for 30 min only inactivated 0.31 and 0.22 lg CFU/mL of *S. aureus* planktonic and biofilm cells, respectively. Similarly, He et al. [28] reported that US treatment alone for 9 min only inactivated 0.36 lg CFU/mL of *S. aureus* planktonic cells. Yu et al. [29] reported that US alone for 10 min only caused 0.09 lg CFU/cm² reduction in *S. aureus* biofilm cells. Treatment with 0.5% CA, 1% CA, and 2% CA for 30 min inactivated 0.9, 1.78, and 3.4 lg CFU/mL of *S. aureus* planktonic cells, respectively, while treatments with US plus 0.5% CA, US plus 1% CA, US plus 2% CA for 30 min achieved 2.18, 4.52, and 6.9 lg CFU/mL reduction in *S. aureus* planktonic cells. After treatment with 0.5% CA, 1% CA, and 2% CA for 30 min, the viable bacterial counts of *S. aureus* biofilm cells were reduced by 1.08, 1.80, and 2.77 lg CFU/mL, respectively. The combinations of US plus 0.5% CA, US plus 1% CA, and US plus 2% CA for 30 min achieved 2.18, 4.52, and 5.53 lg CFU/mL reduction in *S. aureus* biofilm cells. Hence, the combined treatment (US plus CA) exhibited significantly (p < 0.05) stronger antibacterial and antibiofilm efficacy than US or CA alone to inactivate *S. aureus* planktonic and biofilm cells.

*S. aureus* is a Gram-positive bacterium that has a thick peptidoglycan layer in the cell wall. This layer helps *S. aureus* cells to become resistant to US, so US treatment alone was insufficient to inactivate *S. aureus* planktonic and biofilm cells [28,30]. Therefore, to obtain higher bactericidal efficacy, many researchers explored the synergistic effect of US and chemical agent to inhibit the growth of bacterial cells as an alternative method. Bi et al., (2020) reported that US treatment alone only inactivated 3.31 log CFU/mL of *Salmonella typhimurium* planktonic cells, whereas the combination of lysozyme and US inactivated 4.26 lg CFU/mL of bacterial cells; this finding indicated the synergistic relationship between lysozyme and ultrasound [16]. Zhang et al., (2020) reported that ultrasound in combination with carvacrol, citral, cinnamic acid, geraniol, gallic acid, lactic acid, or limonene had great synergistic effect against *E. coli* K12 and *Listeria innocua* cells [31]. Luo et al., (2016) reported that the combination of slightly acidic electrolyzed water, US, and mild heat had synergistic effect on the inactivation of *L. monocytogenes* and *S. typhimurium* [32]. US passes through the caviation activity to destroy the cell membrane and cell wall, thereby facilitating the penetration of the chemical agent into the cell membrane and cell wall and resulting in the synergistic effect between US and the chemical agent [33].

3.2. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) analyses

SEM was used to analyze the effect of US, CA, and US plus CA treatments on morphological changes in *S. aureus* planktonic and biofilm cells. The untreated *S. aureus* planktonic and biofilm cells possessed a complete and smooth surface. The bacterial cells in the biofilm were closely arranged and surrounded by a large amount of EPS (Fig. 1 A1, B1). After US treatment for 30 min, slight morphological destruction appeared on the surface of *S. aureus* planktonic and biofilm cells, only a small part of the cell surface was wrinkled, and most *S. aureus* planktonic and biofilm cells still had intact surface (Fig. 1 A2, B2). This finding is in accordance with the report of He et al., (2021). After treatment with US alone for 30 min, some pores appeared on the cell membrane, but most *S. aureus* cells still had cell membrane [28]. Yu et al., (2021) revealed that high-intensity ultrasound destroyed the structure of *S. aureus* biofilm, but the bacterial cells in the biofilm still had intact shape [29]. After treatment with 2% CA for 30 min, *S. aureus* planktonic and biofilm cells became shriveled, and many wrinkles and gullies appeared on the cell surface (Fig. 1 A3, B3). As shown in Fig. 1 A4 and B4, the combined treatment of US and slightly acidic electrolytic water could severely damage the cell wall [39]. Guo et al., (2021) reported the complete collapse of *E. coli* O157:H7 cells after the combined treatment of US and thyme essential oil nano-emulsion [34].

CLSM was used to estimate the effect of US, CA, and US plus CA on the permeability of the cell membrane. *S. aureus* planktonic and biofilm cells after treatment with control, US, CA, and their combination (US

Table 1

| Treatments | *S. aureus* planktonic cells (lg CFU/mL) at min: |
|------------|-----------------------------------------------|
|            | 5     | 10       | 20       | 30       | 60       |
| Control    | 9.40±  | 9.36±    | 9.34±    | 9.42±    | 9.38±    |
| US         | 9.27±  | 9.26±    | 9.13±    | 9.11±    | 9.03±    |
| 0.5% CA    | 9.07±  | 8.99±    | 8.77±    | 8.52±    | 6.47±    |
| 1% CA      | 8.97±  | 8.75±    | 8.46±    | 7.64±    | 4.94±    |
| 2% CA      | 8.86±  | 8.67±    | 7.05±    | 6.02±    | 3.55±    |
| US plus 0.5% | 9.00±  | 8.87±    | 8.64±    | 7.24±    | 3.71±    |
| CACA       | 9.05BC | 9.04BC   | 9.03BC   | 9.02BC   | 9.01BC   |
| US plus 1% CA | 8.90±  | 8.75±    | 7.01±    | 4.90±    | 3.00±    |
| US plus 2% CA | 8.80±  | 8.62±    | 8.54±    | 2.52±    | <1.4     |

Different lower cases indicate significant differences among treatments (p < 0.05).

Table 2

| Treatments | *S. aureus* biofilm cells (lg CFU/mL) at min: |
|------------|-----------------------------------------------|
|            | 5     | 10       | 20       | 30       | 60       |
| Control    | 9.43±  | 9.42±    | 9.42±    | 9.43±    | 9.42±    |
| US         | 9.35±  | 9.31±    | 9.28±    | 9.21±    | 9.00±    |
| 0.5% CA    | 9.30±  | 9.21±    | 9.08±    | 8.35±    | 6.75±    |
| 1% CA      | 9.25±  | 9.12±    | 8.34±    | 7.63±    | 4.60±    |
| 2% CA      | 9.09±  | 8.95±    | 6.99±    | 6.68±    | 4.21±    |
| US plus 0.5% | 9.03±  | 8.85±    | 8.20±    | 8.03±    | 3.83±    |
| CACA       | 9.03D  | 9.04E    | 9.05F    | 9.06G    | 9.07G    |
| US plus 1% CA | 9.89±  | 9.69±    | 6.90±    | 5.02±    | 3.57±    |
| US plus 2% CA | 8.86±  | 8.55±    | 6.06±    | 3.90±    | 3.10±    |

Different lower cases indicate significant differences among treatments (p < 0.05).
plus CA) were examined by CLSM (Fig. 2). As shown in Fig. 2 A1 and B1, the control *S. aureus* planktonic and biofilm cells emitted green fluorescence, indicating that all of the cells were alive and the permeability of the cell membrane did not increase. After treatment with US alone for 30 min, few *S. aureus* planktonic and biofilm cells emitted red fluorescence, indicating that the permeability of the cell membrane slightly increased, but most of the cells remained viable (Fig. 2 A2, B2). This finding is in accordance with a published work by Guo et al., (2020). The CLSM images indicated that a small proportion of *E. coli* planktonic cells emitted red fluorescence after treatment with US alone, meaning that most *E. coli* cells were alive [34]. Li et al., (2017) revealed that very few *S. aureus* planktonic cells emitted red fluorescence after US treatment for 15 min [14]. For 2% CA treatment for 30 min, the proportion of *S. aureus* planktonic and biofilm cells emitting red fluorescence significantly increased (Fig. 2 A3, B3), indicating that CA was more effective than US to increase the permeability of the cell membrane. Fig. 2 A4, B4 shows that the proportion of *S. aureus* planktonic and biofilm cells emitting red fluorescence after the combined treatment of US and 2% CA sharply increased, indicating that the combined treatment sharply increased the permeability of the cell membrane. US could damage the integrity of the

Fig. 1. SEM images of *S. aureus* planktonic and biofilm cells treated with control, US, 2% CA, and US plus 2% CA.
cell membrane, helping CA penetrate the membrane and causing the sharp increase in the permeability of the cell membrane.

3.3. Exopolysaccharide (EPS) content analysis

Changes in the EPS content in *S. aureus* biofilms after US or CA alone and their combination (US plus CA) are shown in Fig. 3. The concentration of soluble and insoluble polysaccharides in the control biofilm were 30.44 and 71.95 μg/mL, respectively. After treatment with US, 0.5% CA, 1% CA, and 2% CA, the concentration of soluble polysaccharides in *S. aureus* biofilms were 25.51, 23.78, 15.86, and 14.19 μg/mL, respectively. Meanwhile, the contents of insoluble polysaccharides in *S. aureus* biofilms decreased to 60.22, 48.99, 26.27, and 22.94 μg/mL, respectively. The concentrations of soluble and insoluble polysaccharides in the biofilm decreased to 17.53, 12.18, and 12.81 μg/mL after treatment with US plus 0.5% CA, US plus 1% CA, and US plus 2% CA, respectively. Meanwhile, the insoluble polysaccharide contents in *S. aureus* biofilms decreased to 44.30, 19.60, and 12.81 μg/mL, respectively. Thus, the combined treatment of US and CA was more effective than US or CA alone in decreasing the thickness of *S. aureus* biofilms by reducing the contents of EPS.

*S. aureus* biofilm cells were more difficult to be inactivated than planktonic cells because bacterial cells in the biofilm were protected by the matrix of glycoproteins, EPS, and other compounds [25]. In the cells treated with the combination of US and CA, US could destroy the structure of the extracellular matrix, promoting the penetration of CA into the biofilm barrier; thus, the combined treatment of US and CA was more effective than US or CA alone in inactivating *S. aureus* cells in the biofilm. Yu et al., (2021) reported that US via mechanical oscillation promoted ClO₂ penetration into the *S. aureus* biofilm, thereby enhancing the bactericidal rate to inactivate *S. aureus* biofilm cells [29].

### 3.4. Release of intracellular ATP and nucleic acids

The effects of US or CA alone and their combination (US plus CA) on the leakage of ATP from *S. aureus* planktonic and biofilm cells are shown in Table 3. The intracellular ATP levels of control *S. aureus* planktonic and biofilm cells were 16.71 and 8.96 nmol/OD, respectively. After treatment with US alone for 30 min, the concentration of intracellular ATP in *S. aureus* planktonic and biofilm cells significantly increased to 27.91 and 20.61 nmol/OD, respectively (p < 0.05). After treatment with 0.5% CA, 1% CA, and 2% CA, the concentration of intracellular ATP in *S. aureus* planktonic cells significantly increased to 40.97, 50.87, and 102.97 nmol/OD, respectively (p < 0.05). Meanwhile, the concentration of intracellular ATP in *S. aureus* biofilm cells significantly increased to 38.65, 58.96, and 145.74 nmol/OD, respectively (p < 0.05). After the combined treatment of US plus 0.5% CA, US plus 1% CA, and US plus 2% CA, the concentration of intracellular ATP in *S. aureus* planktonic cells significantly increased to 81.31, 142.4, and 205.5 nmol/OD, respectively.

**Table 3**

| Treatments          | ATP concentration (nmol/OD) |
|---------------------|-----------------------------|
|                     | S. aureus planktonic cells  | S. aureus biofilm cells |
| Control             | 16.71 ± 2.75A               | 8.96 ± 0.85A            |
| US                  | 27.91 ± 2.60B               | 20.61 ± 2.30B           |
| 0.5% CA             | 40.97 ± 3.60C               | 38.65 ± 4.90C           |
| 1% CA               | 50.87 ± 3.13D               | 58.96 ± 9.43D           |
| 2% CA               | 102.97 ± 3.16F              | 145.74 ± 3.29F          |
| US plus 0.5% CA     | 81.31 ± 9.15E               | 55.85 ± 5.52D           |
| US plus 1% CA       | 142.40 ± 9.07G              | 116.21 ± 6.19E          |
| US plus 2% CA       | 205.5 ± 5.53H               | 189.46 ± 6.62G          |

Different lower-case letters indicate significant differences among treatments (p < 0.05).
respectively (p < 0.05). Meanwhile, the concentration of intracellular ATP in S. aureus biofilm cells significantly increased to 55.85, 116.21, and 189.46 nmol/OD, respectively (p < 0.05). Obviously, the combined treatment of US and CA was more effective than US or CA alone in destroying the integrity of the cell membrane, causing the leakage of ATP.

The effects of US or CA alone and their combination (US plus CA) on the leakage of nucleic acids at 260 nm from S. aureus planktonic and biofilm cells are shown in Table 4. The OD260 of the control S. aureus planktonic and biofilm cells were 0.31 and 0.12. After treatment with US, 0.5% CA, 1% CA, and 2% CA, the OD260 of the S. aureus planktonic cells significantly increased to 0.40, 0.57, 0.69, and 1.42, respectively (p < 0.05). Meanwhile, the OD260 of S. aureus biofilm cells significantly increased to 0.17, 0.23, 0.57, and 0.68, respectively (p < 0.05). In addition, the OD260 of S. aureus planktonic cells treated by US plus 0.5% CA, US plus 1% CA, and US plus 2% CA significantly increased to 0.87, 1.56, and 2.24 respectively (p < 0.05). Meanwhile, the OD260 of S. aureus biofilm cells treated by US plus 0.5% CA, US plus 1% CA, and US plus 2% CA significantly increased to 0.55, 0.88, and 1.10, respectively (p < 0.05).

The release of intracellular ATP and nucleic acids in S. aureus planktonic and biofilm cells after the combined treatment of US and CA was significantly higher than US or CA treatment alone, indicating the synergistic relationship between US and CA in destroying the integrity of the cell membrane, resulting in the leakage of nucleic acids and ATP. Li et al., (2021) reported that the leakage of nucleic acids and protein in Rhizopus stolonifer after treatment with US combined with slightly acid electrolytic water was significantly higher than the individual treatment [33]. In fact, US could damage the outer cell membrane, which could help CA penetrate into the membrane and cell wall, thereby enhancing the ability of CA to damage the cell membrane and cause the leakage of ATP and nucleic acids [35,36].

3.5. Inactivation of S. aureus in mutton by the combined treatment of US plus 1% CA

The survival of S. aureus in mutton after treatment by US plus 1% CA for 5, 10, 20, 30, and 60 min are presented in Fig. 4. The count of S. aureus cells in untreated mutton was 6.11 lg CFU/mL. After treatment with US plus 1% CA for 5, 10, 20, 30, and 60 min, the counts of S. aureus cells in mutton significantly decreased to 5.83, 5.63, 5.54, 5.32, and 4.97 lg CFU/mL, respectively. With increasing treatment time, the counts of S. aureus cells in mutton significantly decreased (p < 0.05). In recent years, many researchers reported the application of US combined with agent against bacterial cells in foods. Li et al., (2021) reported that US combined with slightly acid electrolytic water significantly controlled the growth of R. stolonifer in sweet potato [33]. Yoon et al., (2021) reported that the combined treatment of 3% malic acid, 0.1% nisin, and 40 kHz US for 20–30 min significantly decreased the counts of L. monocytogenes in king oyster mushrooms to lower than the detection level (< 1.4 lg CFU/mL) within 30 min [37]. He et al., (2021) reported that US and thyme essential oil nanoemulsions had remarkable synergistic effect on inhibiting the growth of E. coli O157:H7 on cherry tomatoes [38].

4. Conclusion

The bactericidal value of US combined with CA was greater than the sum of US and CA treatment alone, indicating that US combined with CA treatment had great synergistic effects on inactivating the growth of S. aureus planktonic and biofilm cells. Furthermore, the combined treatment of US and CA showed great synergistic effect on decreasing the concentration of polysaccharides in biofilm. The combined treatment of US and CA was also more effective in destroying the integrity of the cell membrane, causing the leakage of ATP and nucleic acids. In addition, the US plus 1% CA could inactivate 1.14 lg CFU/mL of S. aureus counts of 4.97 lg CFU/mL, respectively. With increasing treatment time, the cells in mutton significantly decreased to 5.83, 5.63, 5.54, 5.32, and 5.24 respectively (p < 0.05). In fact, US could damage the outer cell membrane, which could help CA penetrate into the membrane and cell wall, thereby enhancing the ability of CA to damage the cell membrane and cause the leakage of ATP and nucleic acids [35,36].

Table 4
Measurements of nucleic acids released from S. aureus planktonic and biofilm cells after treatment with US, CA, and US plus CA.

| Treatments          | OD260 S. aureus planktonic cells | OD260 S. aureus biofilm cells |
|---------------------|----------------------------------|------------------------------|
| Control             | 0.31 ± 0.022A                    | 0.12 ± 0.022A                |
| US                  | 0.40 ± 0.008B                    | 0.17 ± 0.012B                |
| 0.5% CA             | 0.57 ± 0.010C                    | 0.23 ± 0.010C                |
| 1% CA               | 0.69 ± 0.005D                    | 0.57 ± 0.001E                |
| 2% CA               | 1.42 ± 0.009F                    | 0.68 ± 0.003F                |
| US plus 0.5% CA     | 0.87 ± 0.005E                    | 0.55 ± 0.002D                |
| US plus 1% CA       | 1.56 ± 0.009G                    | 0.88 ± 0.003G                |
| US plus 2% CA       | 2.24 ± 0.043H                    | 1.10 ± 0.030H                |

Different lower-case letters indicate significant differences among treatments (p < 0.05).

Fig. 4. Survival of S. aureus in mutton after treatment with US plus 1% CA. Different lower case letters indicate significant differences among treatments (p < 0.05). S. aureus on mutton after 60 min treatment.

CRediT authorship contribution statement

Jinyue Sun: Methodology, Writing – original draft. Debao Wang: Software, Writing – original draft. Zhilan Sun: Conceptualization, Funding acquisition, Writing – review & editing. Lihui Du: Conceptualization, Methodology, Writing – review & editing. Daoying Wang: Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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