Combined an acoustic pressure simulation of ultrasonic radiation and experimental studies to evaluate control efficacy of high-intensity ultrasound against *Staphylococcus aureus* biofilm

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**A B S T R A C T**

This study evaluated efficacy of high-intensity ultrasound (HIU) on controlling or stimulating *Staphylococcus aureus* biofilm. Acoustic pressure distribution on the surface of glass slide cultivated *S. aureus* biofilm was first simulated as a standardized parameter to reflect sono-effect. When the power of HIU was 240 W with acoustic pressure of \( \sim 1.38 \times 10^5 \) Pa, a reasonably high clearance rate of *S. aureus* biofilm was achieved (96.02%). As an all-or-nothing technique, the HIU did not cause sublethal or injury of *S. aureus* but inactivate the cell directly. A further evaluation of HIU-induced stimulation of biofilm was conducted at a low power level (i.e. 60 W with acoustic pressure of \( \sim 6.91 \times 10^4 \) Pa). The low-power-long-duration HIU treatment promoted the formation of *S. aureus* biofilm and enhanced its resistance as proved by transcriptional changes of genes in *S. aureus*, including up-regulations of rbf, sigB, lrgA, icaA, lcaB, and down-regulation of icar. These results indicate that the choice of input power is determined during the HIU-based cleaning and processing. Otherwise, the growth of *S. aureus* and biofilm formation are stimulated when treats by an insufficiently high power of HIU.

1. Introduction

*Staphylococcus aureus* is a gram-positive pathogen that produces a wide variety of exoproteins contributing to its ability to colonize and cause disease [1]. Among the predominant bacteria involved in foodborne outbreaks, *S. aureus* is a leading cause of gastroenteritis resulting from the consumption of contaminated foods [2]. The majority of the staphylococcal food poisoning was attributed to the absorption of staphylococcal enterotoxins preformed in the foods by improper handling and then storage at elevated temperatures [3]. In general, the biofilm of *S. aureus* in the food industry are distributed in raw materials, processing equipment and environment, as well as commodity circulation [4].

Extracellular polymeric substances (EPS) of biofilm consist of polysaccharides, proteins, and extracellular DNA [5]. The biofilm is regarded as a common strategy employed by bacteria to survive in adverse conditions [6]. The biofilm formation is detrimental in the food industry which may lead to the food contamination, fouling of heat exchangers and pipes, as well as colonization of many harborage sites (e.g. crackers, dead ends, crevices, joints, etc.) in which leads to repeated pollution of food products. As long as the matured biofilm structure is formed, the biofilm becomes extremely hard to be removed due to the strong adhesion [7].

Cleaning by using chemical disinfectants and high-pressure flushing is commonly used in the food industry for controlling *S. aureus* biofilm. However, the efficacy of conventional cleaning is relatively poor, which is mainly attributed to a strong adhesion of biofilm on food contact surfaces and low penetration of disinfectants into the biofilm structure [8]. It is also possible to induce the exchange of certain resistance genes among inner cells in the biofilms after the improper treatment of chemical disinfectants leading to reinforcement of their resistances [9,10]. Previous studies have indicated that some of the commonly used disinfectants (e.g. chlorine, quaternary ammonia, peracetic acid) were capable of successfully inactivating planktonic bacteria instead of

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controlling biofilm in *Listeria monocytogenes*, *S. aureus*, and *Clostridium jejuni* [8,11,12]. Hence, it is necessary to develop new and practical methods for achieving the biofilm control formed on food contact surfaces.

An extremely high pressure and temperature environment is created due to cavities generated and kept growing throughout ultrasonic treatment [13]. High-intensity ultrasound (HIU, 1–1000 W/cm²) can expand the cavities faster than the low-intensity ultrasound (<1 W/cm²) and meanwhile, strong agitation, shear stress, and turbulence are also characterized as distinctive actions throughout the HIU treatment [14,15]. For now, the HIU treatment has gained much attention as a mean of controlling foodborne microorganisms and their biofilms [6]. The residues of amino acid on protein chains were degraded, and the polysaccharides in EPS were destroyed trough mechanical oscillation generated by HIU. As indicated by Oulahal-Lagsir, Martial-Gros, Boistier, Blum and Bonneau [16], more than 80% of *Bacillus starotherophilus* biofilm formed on a stainless-steel surface was removed at the end of a 40 kHz ultrasonic treatment for 10 s. Another design of flat ultrasonic transducer would completely remove *S. aureus* and *Escherichia coli* biofilm in milk, gathered on hard surfaces in 10 s with a frequency of 40 kHz. It is noticeable that bactericidal effect of HIU reported in previous studies were inconsistent. For example, about 55% of *L. monocytogenes* biofilm was inactivated in 1.0 min after the HIU treatment (20 kHz, 120 W) as reported by Baumann, Martin and Feng [17]. However, a 100 min-HIU treatment at a frequency of 37 kHz and power of 1200 W only reduced 1.4 Log_{10} CFU/mL of viable cells in *L. monocytogenes* biofilm [18]. Since very limited bactericidal effect of HIU on the biofilm has been noticed, adverse effects brought by HIU treatment, such as the HIU-stimulated growth of biofilm are needed to be evaluated. The HIU-induced stimulation of planktonic cell has been indicated in previous studies, whereas cases of stimulated biofilm for HIU on the biofilm has been noticed, adverse effects brought by HIU treatment were rarely reported [19–21]. Contrary to the common knowledge that HIU would only detach the biofilm from surface materials, the initial adhesion of bacteria was basically unaffected by HIU. The net growth of *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* biofilms formed on a polyethylene surface was stimulated after an HIU treatment at 70 kHz and 2 W/cm² [19]. Therefore, the efficacy of HIU on the biofilm control highly depends on ultrasonic conditions (e.g. intensity and frequency of ultrasound) and meanwhile, microbial strains, biofilm stage, and surface conditions should be considered [22].

*S. aureus* as an adaptable microorganism has a strong ability to form biofilm on surfaces making it continuously contaminated foods. Therefore, a systematic study was conducted to evaluating efficacy of HIU treatment on controlling the biofilm of *S. aureus*. Since ultrasound cavitation affects biofilm removal and bacterial response, it is necessary to predict the performance of ultrasonic treatment. Moreover, the sound field distribution inside the vessel would significantly influenced by the location of ultrasonic probe where high acoustic pressure occurs mainly in the solution around the probe while other parts respond more slowly, especially the surface of container where grows the biofilm. Considering many factors would influence ultrasonic treatment, a computer-based simulation of acoustic pressure distribution was first to conduct as a standardized parameter to reflect sono-effect. Different input powers of ultrasound were to be chosen for achieving either inhibition or stimulation of the *S. aureus* biofilm. Changes of viability and ultrastructure, as well as sono-influences on biofilm-related gene were to be studied.

2. Materials and methods

2.1. Preparation of *S. aureus* biofilm

The bacterial strain, *S. aureus* ATCC 6538, was obtained from Beina Science & Technology Co., Ltd. (Beijing, China). Glass was chosen as a typical food contact surface that formed *S. aureus* biofilms. The glass slide was first washed by acetone followed by soaking in a sodium hypochlorite solution (3.5%, w/v) for 5 min at 75°C followed by rinsing by water, soaking for 5 min in a phosphoric acid solution (7.0 g/L), and rinsing again by distilled water. Lastly, the glass slide was autoclaved at 121°C for 15 min. The glass slide was transferred to a microtiter plate followed by filling 2 mL of nutrient broth (NB) into each well. The 40 µL suspended bacteria (about 10^6 CFU/mL) was transferred to the well and then incubated at 37 °C for 48 h until the maturity stage.

2.2. High-intensity ultrasound (HIU) treatment

Planktonic bacteria were gently washed 3 times by phosphate-buffered saline (PBS) buffer for cultivating *S. aureus* biofilm. The glass slide with *S. aureus* biofilm was put into a glass tank with 0.85% sterile saline solution and then ultrasonicated by using an ultrasonicator with 6 mm probe (SCIENTZ-II, Scientz Biotech, China). The treatment duration was set to 10 min at 2.5 min interval. Ultrasonic power was set to 60 to 240 W at 60 W interval and frequency was maintained at 20 kHz. During the ultrasonication, temperature was controlled under 20°C. For the test of HIU-induced stimulation of the *S. aureus* biofilm, the HIU at 60 W was continuously treated the biofilm for up to 18 h at 3 h interval. The rest conditions were the same as described previously.

2.3. Simulation of acoustic pressure distribution during ultrasonic treatment

Numerical simulation was conducted to evaluate acoustic pressure distribution in the beaker and that on the glass cultivated *S. aureus* biofilm at the four input power levels. The 3D model of glass beaker, the glass cultivated *S. aureus* biofilm, and ultrasonic probe was established by using COMSOL Multiphysics (V5.2, Stockholm, Sweden). As shown in Fig. 1A, diameter of glass beaker (*D_b*) was 50 mm and its height (*H_b*) was 71 mm. It was also considered to thickness of beaker (*T_b*) as 0.2 mm. The diameter (*D_s*) and thickness of glass slide (*T_s*) was 20 mm and 0.2 mm, respectively. Furthermore, the diameter of ultrasonic probe (*D_p*) was 6 mm, and the depth immersed into the water (*H_p*) was measured to be 36 mm. In this 3D model, acoustic impedance boundaries were assigned to water-air, water-probe, and water-glass. The mesh of 3D model (Fig. 1B) was first automatically generated followed by refining the mesh until no further noticeable change of the predictions was observed.

Pressure acoustics in frequency domain (ACPR) model was chosen to simulate acoustic pressure according to the Helmholtz equation Eq. (1):

\[
\nabla \cdot \left( \frac{\rho}{\rho} \nabla p \right) = 0
\]

Where

\[
\nabla = \frac{\partial}{\partial x} + \frac{\partial}{\partial y} + \frac{\partial}{\partial z}
\]

and

\[
\omega = 2\pi f
\]

where \( \rho \) refers to density of material (kg/m³), \( p \) refers to pressure field (N/m²), \( c \) refers to sound velocity of material (m/s), \( \omega \) refers to angular frequency (rad/s), which was derived from frequency of ultrasound (f, rad/s).

During the ultrasonication, normal acceleration (\( a_n \)) on the surface of ultrasonic probe is calculated based on Eq. (4):

\[
a_n = \frac{\omega}{\rho c} \sqrt{\frac{I}{\rho c}}
\]

where \( I \) (W/m²) refers to intensity of ultrasound, which is calculated from ultrasonic power (\( P_u \)) and radium of ultrasonic probe (r) according
3. Parameters adopted in the COMSOL simulation.

The equation for calculating the power density of ultrasound is given by:

\[ P \rho \pi r^2 \]

All parameters presented in the above-mentioned equations are summarized in Table 1.

### Flow cytometric (FCM) analysis

The FCM analysis was largely followed by Yu, Liu, Yang, Xie, Guo, Cheng and Yao [10] with slight modification. The cells from *S. aureus* biofilm were first suspended into a sterile saline solution (0.85%, w/v). Propidium iodide (PI) was prepared in distilled water at the concentration of 1.5 mM (Sigma-Aldrich Co., USA). The membrane compromised cells were stained by PI through mixing 30 μL PI working solution and 1 mL of cell suspension following by incubating for 15 min at 4°C. At the end of incubation, PI residue was removed by using the sterile saline solution. Similarly, 1 mM carboxyfluorescein diacetate (cFDA) working solution was prepared by dissolving cFDA into acetone. The 1 mL of cell suspension was stained by cFDA and then incubated for 20 min at 37°C. At the end of incubation, cFDA residue was removed by using the sterile saline solution. The double staining was conducted by using both PI and cFDA staining which followed the same procedures as described before. All the stained *S. aureus* cells were stored in ice water. FCM analysis was conducted using a flow cytometer (FACSAriaII, BD Co., USA). The sheath fluid was sterilized water. Data acquisition was set to 10,000 events, and flow rate was 200–400 events/s. All data were analyzed in the list mode as area signals. FlowJo software (BD Co., USA) was utilized to process collected data.

### Scanning electron microscopy (SEM) analysis

The *S. aureus* biofilm was analyzed by SEM (SU1510, Hitachi, Japan). The *S. aureus* biofilm on glass slide was analyzed by SEM (SU1510, Hitachi, Japan). The biofilm was then stained by the SYTO 9 and PI reagents (1:30) to obtain staining solution. The glass slide of *S. aureus* biofilm was then stained by the solution and then stored in dark for 15 min at room temperature. At the end of staining, the stained *S. aureus* biofilm on glass slide was analyzed by CLSM (LSM 710, Carl Zeiss Microscopy GmbH, Germany). The images were captured by using Zen 2012 microscopy software. The fluorescence intensity of PI and SYTO 9 was determined through analyzing the CLSM images through ImageJ software.

### qRT-PCR analysis

Trizol reagent was utilized to extract RNA of the *S. aureus* biofilm cells following the manufacturer’s instructions (Comwin Biotech, Beijing, China). The concentration and purity of the RNA were determined by measuring absorbance values at 260 nm and 280 nm through an ultraviolet spectrophotometer (UV-1800, Shimadzu Co., Ltd., Japan), and its integrity was confirmed by using an agarose gel electrophoresis. The transcriptional levels of genes in *S. aureus* were determined by qRT-PCR, including *aur*, *agrA*, *clp*, *cIDA*, *icaA*, *icaD*, *hla*, *icaR*, *nuc1*, *sarA*, *rfb*, *spa*, *sigB*, and *sea*. One mg of total RNA was reverse-transcribed by using a PCR kit purchased from Vazyme Biotech (HiScript™ II, China) to get cDNA. The forward and reverse primers were summarized in Table 2. The PCR was initiated using a qRT-PCR System (StepOnePlus, Applied Biosystems, USA) with the PCR kit (ChamQ™, Vazyme Biotech, China). The sequence included a holding stage for 1 cycle (95°C for 30 s), a

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**Table 1** Parameters adopted in the COMSOL simulation.

| Parameters          | Description          | Value          |
|---------------------|----------------------|----------------|
| \( \rho_{\text{water}} \) | Density of water    | 998 kg/m³      |
| \( \rho_{\text{silica glass}} \) | Density of silica glass | 2500 kg/m³ |
| \( \rho_{\text{air}} \) | Density of air      | 1.2 kg/m³      |
| \( \rho_{\text{titanium}} \) | Density of titanium (the surface of ultrasonic probe) | 4510 kg/m³ |
| \( c_{\text{water}} \) | Sound velocity in water | 1500 m/s |
| \( c_{\text{silica glass}} \) | Sound velocity in silica glass | 5639 m/s |
| \( c_{\text{air}} \) | Sound velocity in air  | 343 m/s        |
| \( c_{\text{titanium}} \) | Sound velocity in titanium | 6100 m/s |
| \( f \) | Frequency of ultrasound | 20 kHz |
| \( r \) | Radius of ultrasonic probe | 3 mm |
| \( P_{\text{ultrasound}} \) | Power of ultrasound  | 60 W           |
| \( P_{\text{air}} \) | Power of ultrasound  | 120 W          |
| \( P_{\text{water}} \) | Power of ultrasound  | 180 W          |
| \( P_{\text{glass}} \) | Power of ultrasound  | 240 W          |
cycling stage for 40 cycles (95 °C for 10 s and 60 °C for 30 s). At the end of each run, a melt curve analysis was conducted for verifying PCR products. The levels of target genes expression in the S. aureus was normalized and taken a housekeeping gene 16S rRNA as reference.

### 2.8. Statistical analysis of data

Analysis of each test was repeated three times. In order to make statistical valid comparisons, a one-way ANOVA with Duncan’s multiple range test as a Post-hoc analysis was performed to determine significant difference ($p < 0.05$) using SPSS software (V22, IBM, USA).

### 3. Results and discussion

#### 3.1. Acoustic pressure modeling

Simulations of acoustic pressure distribution in the beaker and that on the glass slide cultivated S. aureus biofilm are shown in Fig. 2. In general, the high acoustic pressure region was concentrated around the ultrasonic probe where the position right below the probe had the highest acoustic pressure level. With the increased distance, isosurfaces of acoustic pressure was distributed around the probe and acoustic pressure level was decreased, which is attributed to the reflection of sound waves on the boundaries [23]. When the input power of ultrasound was 60 W, the simulated result of highest acoustic pressure was $3.10 \times 10^5$ Pa. If the input power was increased to 120 W, 180 W, and 240 W, highest acoustic pressure reached to $4.36 \times 10^5$ Pa, $5.36 \times 10^5$ Pa, and $6.20 \times 10^5$ Pa, respectively. Furthermore, the acoustic pressure distribution on the glass slide cultivated S. aureus biofilm was simulated as shown in Fig. 2a-d. Since the glass slide was located at the bottom of beaker with the distance of 35 mm, the acoustic pressure level on the glass slide was relatively low. Data acquisition was conducted at the central point ($X = 0, Y = 0, Z = 0.2$), the half position ($X = -2.5, Y = 0, Z = 0.2$), and the edge of glass slide ($X = -5.0, Y = 0, Z = 0.2$). The difference of acoustic pressure among the three positions was relatively small and therefore, averaged acoustic pressure on the glass slide was derived as follows: $-6.91 \times 10^4$ Pa, $-9.77 \times 10^4$ Pa, $-1.20 \times 10^5$ Pa, and $-1.38 \times 10^5$ Pa when the input power of ultrasound was set to 60 W, 120 W, 180 W, and 240 W.

There is no doubt that frequency and power of ultrasound are the two determined factors during ultrasound treatment. Many other factors would also significantly influenced control efficacy of biofilm such as position of ultrasonic probe, shape of probe, etc. As reviewed by Yu, Liu, Li, Guo, Xie, Cheng and Yao [6], the authors claimed that almost all the research papers did not reported these factors simultaneously and therefore, bactericidal effect and cleaning efficacy of biofilm brought by HIU became incomparable. In this study, the APCR model was chosen to simulate acoustic pressure, which considered all factors simultaneously [24]. Therefore, acoustic pressure distribution on the glass slide cultivated biofilm is recommended to be simulated since it is a standardized parameter for evaluating sono-effect.

#### 3.2. Inactivation and clearance of S. aureus biofilm at different HIU input powers

The four levels of HIU power were chosen for testing inactivation and clearance of S. aureus biofilm as shown in Fig. 3A and B, respectively. When the HIU treatment was conducted at 60 W for 10 min. The proportion of viable cells in the S. aureus biofilm was only decreased from $4.56 \pm 0.06$ to $4.48 \pm 0.09 \log_{10}$ CFU/cm$^2$ without significant different. Inactivation rate, however, was only 12.90% after the HIU treatment. With the increased input power of HIU to 120, 180, and 240 W, the concentration of viable cells was significantly decreased to $4.39 \pm 0.06$, $4.30 \pm 0.05$, and $4.21 \pm 0.10 \log_{10}$ CFU/cm$^2$, respectively, and the inactivation rate was significantly increased to 29.21%, 42.46%, and 53.23%, respectively. Results of the bactericidal effect of HIU was emphasized that the usage of HIU alone may not be a promising approach for achieving a sufficiently high inactivation rate of the S. aureus biofilm at the four input powers of HIU. A previous study on a HIU-treated L. monocytogen biofilm indicated that a 100 min-HIU treatment at 1200 W reduced viable cells in the biofilm from 6.9 to 5.5 $\log_{10}$ CFU/mL. The bactericidal rate was 96.02% due to a higher bacterial density that made mechanical and chemical energy generated by ultrasonic waves acted on a higher proportion of bacteria [17,25]. Although the inactivation rate of viable cell was higher than 95% after the long-duration-high-power HIU treatment, the remained
Fig. 2. Simulation of acoustic pressure distribution in Y-Z plane and X-Y plane (where Z = 0.2 mm indicated the surface of glass slide cultivated S. aureus biofilm) at the four input power levels, i.e. (A, a) 60 W, (B, b) 120 W, (C, c) 180 W, and (D, d) 240 W. Units for the colored labels and axes are Pa and mm, respectively.
concentration of viable cells was still at a high level. Additionally, such a HIU treatment was very hard to be practical in the food industry due to high energy consumption and long duration [26]. As recommended by Piyasena, Mohareb and McKellar [27], the usage of HIU coupled with heat, high pressure, and/or chemical disinfectants would be more effective and energy-efficient approaches for achieving the microbial and biofilm control.

The clearance rate of biofilm was 12.8% ± 4.2% and 21.1% ± 6.4% with the HIU power of 60 W and 120 W, respectively. When the power of HIU was increased to 180 W and 240 W, the clearance rate was reached to 52.9% ± 7.3% and 87.4% ± 5.8%, respectively. As indicated by Yu, Liu, Li, Guo, Xie, Cheng and Yao [6], the destruction of fundamental biofilm structure was mainly attributed to breaking down polysaccharides and degrading amino residues on protein chains in EPS through mechanical oscillation and HIU-induced oxidation. In addition, the localized heat, high pressure, shear stress, and free radicals generated during the cavitation contributed to the alteration of cytoplasmic membrane permeability and direct inactivation of viable cells in the biofilm [28].

3.3. Multiparametric analysis of S. aureus through FCM

The multiparametric analysis of S. aureus was conducted through FCM as shown in Fig. 4. The double-staining method by using PI and cFDA was adopted to stain the samples. There were four quarts in the FCM images which represented dead in Q1, sublethal/injured in Q2, viable in Q3, and debris/lysed S. aureus cells in Q4 [29,30]. The dead cells with damaged membranes and inactivated esterase were stained by PI and located in Q1. The sublethal or injured S. aureus cells were double-stained in Q2 with compromised membranes and residual activity of esterase. In Q3, the viable cells were only stained by cFDA but not PI. The unstained cells in Q4 were attributed to debris/lysed S. aureus cells.

With the increase of HIU power from 0 to 240 W, the highest proportion in Q3 was observed as viable S. aureus cells. The fluorescence in Q3 was decreased from 99.1% to 87.7% with the increased input power of HIU. Meanwhile, the significantly increased fluorescence in Q4 was observed, which increased from 0.8% to 12.0%. It is noticeable that the fluorescence in both Q1 and Q2 was almost remained unchanged with the increased input power levels of HIU. This phenomenon indicated that the HIU treatment would not lead to sublethal or injury of S. aureus but inactivate the cell directly. Under such a high power of ultrasound (i.e. 180 W or 240 W), the inactivated cells would be further lysed to fractions instead of keeping their original forms due to a physical action, namely mechanical oscillation during the HIU treatment, which would explain the increased proportion in Q4 but no change in Q1. This finding is also consistent with the study reported the HIU as an all-or-nothing technology since the cells were ruptured and disintegrated by the HIU that could not be revived [25].

3.4. Impacts of ultrasound on ultrastructure of S. aureus biofilm

3.4.1. CLSM analysis

Bactericidal effect of S. aureus was visualized using CLSM after the HIU treatment at different input powers. The cells with either intact or damaged membrane were penetrated by SYTO 9 which would bind to nucleic acid and emit a green fluorescent color as an indicator of live cells. The cells with the damaged membranes were stained by PI into red fluorescent color since PI has a higher affinity to nucleic acids than SYTO 9 [31]. As shown in Fig. 5, both untreated and HIU treated (20 kHz, 60 W, 10 min) biofilms emitted the green fluorescence which indicated the viable S. aureus cells. With the increased input power of HIU, a higher
proportion of red fluorescence but less green fluorescence appeared in the CLSM images. When the HIU power reached the highest level (20 kHz, 240 W, 10 min), the dominant fluorescent color was shifted from green to yellow that indicated an increased proportion of damaged cells. Therefore, CLSM results are consistent with the inactivation rate and FCM analysis.

Average thickness, roughness, and porosity as apparent characteristic values of S. aureus biofilms are summarized in Table 3. The average thickness of S. aureus biofilm was significantly decreased from 18.27 ± 1.96 μm to 1.41 ± 0.49 μm with the increase of HIU power from 0 to 240 W. The roughness and porosity showed an opposite trend and both were significantly increased with the increase of HIU power. These phenomena were attributed to the destruction of fundamental structure of biofilm leading to intact surfaces (i.e., increased roughness), and more holes (i.e., increased porosity) when treated by the HIU. Alteration of biofilm adhesion was triggered by the HIU simultaneously that decreased the thickness and detached the biofilm from surface materials. The localized heating, mechanical oscillation, as well as reactive H⁺ and OH⁻ radicals generated by the caviation during the HIU treatment would trigger the sono-degradation of amino residues on protein chains and polysaccharides in the EPS [6,27,32,33]. A different finding was reported in [34], which ultrasonics and sonics alone did not influence the structure of the biofilm or the spatial arrangement of the cells. The authors proposed that the bioacoustic effect may be related to the enhancement of disinfectant transport to deeper layers of the biofilm or through the cell membrane so that enhanced bactericidal effects of ultrasound and chlorhexidine. It is also suggested to further investigate metabolic activities of bacteria before and after HIU treatment, which could give more insights through NMR analysis [35].

### 3.4.2. SEM analysis

Morphological and ultrastructure changes of S. aureus were presented in SEM images after the HIU at different power levels. The SEM image of untreated S. aureus showed elliptical or spherical shapes and most of them gathered together as grape-like clusters as shown in Fig. 6A. When the input power increased to 60 W, the overall shapes of S. aureus cells largely remained unchanged. Although a grape-like cluster still appeared in the middle of SEM image (Fig. 6B), a part of the cluster was dispersed to single cells. In addition, almost all the cells were dispersed without gathering as the grape-like cluster when treated by the HIU at 120 W (Fig. 6C) and 180 W (Fig. 6D). Most of them remained intact shapes and only a few were lysed and leaked out inner contents. The SEM image of HIU treatment at 240 W was shown in Fig. 6E. More than half of S. aureus lost the elliptical or spherical shapes, lysed, and leaked inner content. Moreover, only a few cells appeared in the SEM image that mainly caused by detachment of biofilm from the surface materials and dispersion of cells because of the HIU [36]. The membrane permeability change might be caused by the loss of a permeability barrier due to sono-effect [37,38].

### 3.5. Stimulation of S. aureus biofilm by using continuous HIU at a low power

As indicated in the previous sections, the HIU treatment (20 kHz, 60 W) would not significantly cause the bactericidal effect and detachment of S. aureus biofilm. Therefore, a further test on extending the HIU treatment up to 18 h was conducted to evaluate whether the HIU stimulated the growth of biofilm. As shown in Fig. 7A, no significant different change of relative S. aureus biofilm mass was observed within a 12 h-HIU treatment. When the duration of HIU treatment was extended to 15 and 18 h, the biofilm mass of S. aureus was 1.18 and 1.21 times higher than the untreated one, respectively. These findings clearly indicated a stimulated growth of S. aureus biofilm after the HIU treatment. As summarized in Yu, Liu, Li, Guo, Xie, Cheng and Yao [6], the HIU may activate some inner channels and meanwhile, promote the transportation of nutrient, oxygen, and waste products in the biofilm community leading to the promoted growth of microorganisms in the deeper layers of biofilms. Another potential reason is activation of mechanosensitive channels in the cells after the HIU treatment (e.g. MsS and MscL), followed by evoking microorganisms located at the deep layer of biofilm [21]. As consequences of HIU-induced stress at transcriptional and posttranscriptional levels, the synthesis of intracellular RNA and total protein as constituents of EPS in the biofilm would be promoted simultaneously.

To further investigate the molecular response after the HIU-induced

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**Table 3**

Changes of the average thickness, roughness, and porosity of S. aureus biofilm treated by HIU at the power of 0, 60, 120, 180, and 240 W.

| Power of HIU (W) | Average thickness (μm) | Roughness (Ra/μm) | Porosity (%) |
|------------------|------------------------|-------------------|--------------|
| 0                | 18.27 ± 1.96<sup>a</sup> | 0.23 ± 0.024<sup>d</sup> | 2.58 ± 0.32<sup>d</sup> |
| 60               | 13.97 ± 0.86<sup>b</sup> | 0.26 ± 0.011<sup>d</sup> | 8.54 ± 0.41<sup>d</sup> |
| 120              | 10.36 ± 0.67<sup>c</sup> | 0.48 ± 0.028<sup>f</sup> | 26.53 ± 2.66<sup>c</sup> |
| 180              | 6.52 ± 1.32<sup>e</sup>  | 0.67 ± 0.08<sup>b</sup>  | 40.43 ± 2.96<sup>e</sup> |
| 240              | 1.41 ± 0.49<sup>f</sup>  | 0.88 ± 0.06<sup>a</sup>  | 72.72 ± 3.21<sup>g</sup> |

<sup>a</sup> The different lowercase letters indicate significant differences of apparent characteristic values of biofilms among the power of HIU at 0, 60, 120, 180, and 240 W (p < 0.05).
stimulation, differential gene expression in the *S. aureus* biofilm was evaluated. Fifteen biofilm-related genes in the *S. aureus* were selected and then classified them into five groups. Eight genes were first investigated as shown in Fig. 7B, which regulated biofilms and surface proteins. The *rbf* gene in *S. aureus* regulated the development of biofilm in the presence of NaCl or glucose; the *sigB* gene involved the resistance of various stress during biofilm formation [39,40]. Compared with the control group, *rbf* and *sigB* genes in *S. aureus* were significantly up-regulated after the HIU stimulation. The up-regulation of *rbf* and *sigB* genes indicated the promoted formation of biofilm with enhanced resistance after the HIU stimulation. The *lrgA* and *cidA* genes regulate holin-antiholin system and encode antiholin- and holin-like proteins, respectively [41]. In the *S. aureus*, *lrgA* and *cidA* operons involve in antibiotic tolerance as well as cell death and lysis in an opposing manner [42]. The *lrgA* promotes antibiotic tolerance and shows an inhibitory effect on murein hydrolase activity [43], whereas *cidA* decreases tolerance to various antibiotics and shows a positive effect on murein hydrolase activity [44]. After the HIU stimulation, the *lrgA* gene was significantly up-regulated in *S. aureus* and meanwhile, no significant difference on the *cidA* gene expression was observed between the untreated and HIU-stimulated *S. aureus* cells. The up-regulation of *lrgA* after the HIU-stimulation indicated a promotion of antibiotic tolerance and an enhanced inhibitory effect on murein hydrolase activity in *S. aureus*.

In general, the biofilm formation has two steps: 1) adhesion of bacteria to a substrate surface, and 2) cell-cell adhesion forming the multiple layers of the biofilm which is associated with the polysaccharide intercellular adhesion (PIA). The intercellular adhesion (*ica*) locus mediated the PIA production and cell-cell adhesion as reported in Cramton, Gerke, Schnell, Nichols and Götz [45]. The gene expression of *ica* group in untreated and HIU-stimulated *S. aureus* is shown in Fig. 7C. Both *icaA* and *icaD* expression were significantly up-regulated; meanwhile, a significant down-regulation of *icaR* gene expression clearly indicated the promoted biofilm formation in *S. aureus* after the HIU stimulation compared with those in the control. In addition, there was no significant change of *nucl1* and *seb* gene expression after the HIU stimulation which indicated that the HIU stimulation might not influence the secretion of enterotoxin in *S. aureus*.

4. Conclusions

The control efficacy of HIU against the *S. aureus* biofilm was evaluated in this study. Acoustic pressure distribution on the surface of glass slide cultivated *S. aureus* biofilm was first simulated as a standardized parameter to reflect sono-effect using COMSOL. With the increased input power of ultrasound from 60 W to 240 W, acoustic pressure on the glass slide was changed from $-6.91 \times 10^4$ Pa to $-1.38 \times 10^5$ Pa. The clearance of biofilm and inactivation of viable cells in the *S. aureus* biofilm were promoted with the increased input power of HIU. The highest clearance rate of biofilm reached to 96.02% although the inactivation rate remained 53.23% when treated by HIU at 240 W. The changes of ultrastructure through the CLSM and SEM analysis clearly indicated that the HIU at high power levels would disperse the cluster of *S. aureus* and let them lose the elliptical or spherical shapes, lyse, and leak inner content. As an all-or-nothing technique, the HIU would not cause sublethal or injury of *S. aureus* but inactivate the cell directly. When the input power was maintained at 60 W, the stimulated growth of *S. aureus* biofilm was observed after incubating for longer than 15 h. The up-regulated gene expressions of *rbf*, *sigB*, *lrgA*, *icaA*, and *icaD* as well as down-regulation of *icaR* indicated a promoted formation and enhanced resistance of *S. aureus* biofilm after the HIU stimulation.

Results of this study may inspire the food industry when the HIU is utilized as an approach of biofilm control as follows: 1) acoustic pressure distribution on the biofilm needs to be regarded as a standardized parameter when evaluated sono-effect; 2) effective detachment of biofilm is one of the advantages by using the HIU at a high power level; 3) bactericidal effect of HIU is very limited even at the high power; therefore, the combination of HIU and other strategies (e.g. heat, high pressure, chemical disinfectant, etc.) is recommended to enhance the control of viable cells; 4) the stimulated growth of biofilm may happen when treated by HIU with insufficiently high input power, which should be avoided during cleaning and food processing. Future studies on developing ultrasound-based emerging strategies for achieving biofilm control are recommended. More insights into mechanisms of ultrasound-induced biofilm stimulation are also needed to be revealed in the further.

**Fig. 6.** SEM images of (A) untreated *S. aureus* biofilm and those undergone 10 min-HIU treatment at (B) 60 W, (C) 120 W, (D) 180 W, and (E) 240 W.
interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Hang Yu: Conceptualization, Writing – original draft, Writing – review & editing, Supervision. Yang Liu: Investigation, Data curation. Fangwei Yang: Formal analysis. Yunfei Xie: Supervision. Yahui Guo: Resources. Yuliang Cheng: Visualization. Weirong Yao: Validation, Project administration.

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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