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Resistance of detached-cells of biofilm formed by *Staphylococcus aureus* to ultra high pressure homogenization

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ABSTRACT

Staphylococcus aureus is one of the main pathogens contributing to foodborne outbreaks, owing in part to its ability to form biofilms on food-contact surfaces. Cells that can detach from mature biofilms are a source for microbial cross-contamination in liquid food systems. The study was to evaluate and compare the resistance of detached-cells of biofilm formed by *S. aureus* and planktonic cells to Ultra High Pressure Homogenization (UHPH), a non-thermal technology applied in food processing. The results showed that the survival of both detached-cells and planktonic cells was dependent upon the applied pressure ranging from 30,000 PSI to 40,000 PSI, and cycle numbers with 1 and 3. A significant difference in UHPH resistance was observed at pressures of 35,000 PSI to 40,000 PSI whereby planktonic cell numbers were reduced about 2.0 log CFU/mL compared to a 0.5 log CFU/mL reduction of detached-cells. Cell resistance was further evaluated following UHPH by measuring membrane integrity and potential, as well as observing the cells using scanning electron microscopy (SEM). SEM images revealed more scattered exopolysaccharides in the biofilm after UHPH treatment compared to the control. Additionally, UHPH treatment resulted in planktonic cells having a greater shift to smaller cell size and a wider cell size distribution compared with detached-cells; this indicated a higher resistance of detached-cells to UHPH. This finding suggests that although UHPH has great potential application in food sterilization, the resistance of detached-cells cannot be ignored.

1. Introduction

Staphylococcus aureus is a pathogenic gram-positive, facultative aerobic bacterium ubiquitous in the environment (da Silva-Candido et al., 2020). It is also widely distributed in various foods including meat, dairy, and poultry (Can, Elmali, & Karagoz, 2017). *S. aureus* have been responsible for confirmed foodborne illnesses associated with the consumption of foods contaminated by *S. aureus* toxins. This constitutes an obstacle to public health and a source of food waste (Amoako et al., 2020).

In the last few years, microbial-biofilm contamination described as an adherent "matrix-enclosed" bacterial colony on the interface (Hoveida, Halaji, Rostami, & Mobasherizadeh, 2019), has drawn attention to and within the food industry, especially biofilms formed by *S. aureus*. Mature biofilms such as described above usually need several days or weeks to grow and form. A prevailing concept is that detached-cells of biofilm are generally more resistant to chemical and physical forces, for example, antibiotics sanitizers, environmental stresses, high temperatures and sloughing than their free-living counterparts (Arce-Miranda, Sotomayor, Albesa, & Paraje, 2011; Chen, Zhang, & Yang, 2020). Liquid foods such as fruit beverages, sauces, and meat batters, which require pipeline transportation during processing, are more likely to support the formation of biofilms due to improper cleaning of pipe walls and their blind angles. There is great potential for pathogenic and spoilage bacteria from biofilms matrix to cause food cross-contamination in the liquid food industry. For these reasons, the formation and persistence of bacterial biofilms contribute to the difficulty in guaranteeing food safety (Kamble & Pardesi, 2020).

Traditionally, the pasteurization of liquid foods has been guaranteed by thermal processing such as microwave pasteurization and ultra-high temperature instantaneous sterilization (Mendes-Oliveira, Deering, San Martin-Gonzalez, & Campanella, 2020; Chen, Ren, Grandison, & Lewis, 2019). However, thermal treatment can reduce the sensory and nutritional properties of food products as well as provide the opportunity for heat-resistant bacterial spores to survive. Consequently, non-thermal technology processing conditions have been proposed in recent years

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including high voltage electric field cold plasma, high hydrostatic pressure, high pressure homogenization, and ultra high pressure homogenization (UHPH). Non-thermal techniques are effective at sublethal or ambient temperature; hence, they reduce or avoid thermal damage and deterioration of sensory quality of food (Tiwari, O'Donnell, & Cullen, 2009). UHPH, as a combined treatment of pasteurization and homogenization, has great potential for the inactivation of microorganisms in liquid foods. It is a continuous process where fluid is forced through a narrow pipe under high pressure achieving rapid acceleration (more than 200 m/s at 340 MPa), leading to a combination of high pressure, shear, cavitation, and impingement. Then the mechanical forces result in molecular conformational changes and the inactivation of bacteria, molds, and spores (Georget et al., 2014). Thus, UHPH can provide liquid foods with high quality and a long shelf-life (Ferragut, Valencia-Flores, Perez-Gonzalez, Gallardo, & Hernandez-Herrero, 2015; Balasubramaniam, Martinez-Monteagudo, & Gupta, 2015).

Most recently, researchers have focused on the effect of UHPH on planktonic bacteria demonstrating that the process has a strong bactericidal effect on planktonic bacteria in a liquid food matrix. Ferragut et al. (2015) found that homogenization condition at 300 MPa was capable of complete inactivation of microorganisms in vegetable-based beverages. The study reported by Roig-Sagues et al. (2015) provided the evidence that UHPH can led to inactivate spores of *Alicyclobacillus spp*. in orange juice. Reduction of 3.2 log CFU/mL of *Salmonella* in liquid whole egg has been observed after treatment of UHPH (Velazquez-Estrada, Hernandez-Herrero, Lopez-Pedemonte, Guamis-Lopez, & Roig-Sagues, 2008). However, food products moving through pipes are easily contaminated due to the impact force of the pressurized fluid on bacteria adhering to the pipe walls in biofilms (Kumar & Anand, 1998; Whitehead & Verran, 2015). Furthermore, it is unclear if UHPH has a lethal effect on the detached-cells.

The objective of this study was to investigate the effects of UHPH on detached-cells of *S. aureus* and to compare differences in the levels of resistance between planktonic cells and detached-cells. This work may provide better insight into the resistance of detached-cells to UHPH and form the basis for the development of novel sterilization approaches for liquid foods.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacteria were isolated from chicken carcasses in 2015 and stored at the National Center of Meat Quality and Safety Control (NCM) in China. Five *S. aureus* isolates (numbered NCM 15100, 15101, 15102, 15103, 15104) were stored in Tryptic Soy Broth (TSB) with 40% glycerin (v/v) at −70 °C until tested. All tested isolates were streaked onto tryptic soy agar (TSA) and suspensions were prepared by transferring a single colony into 7 mL sterile TSB medium for 20 h at 37 °C. The suspensions of 5 isolates were mixed to prepare the planktonic cells and biofilm formation.

2.2. Biofilm formation assay

Growth measurements of planktonic cells and biofilm were performed according to previous studies (Jia et al., 2017). Stainless-steel coupons (50 × 20 × 1 mm, grade 304, 2B finish, Shunfen Stainless Steel Material Co. Ltd., Tongnan, China) were soaked in acetone for 24 h before use. The initial concentration of *S. aureus* was adjusted to about 10^2 CFU/mL with TSB and incubated in a sterile glass box with the stainless-steel coupons. Half of coupons were submerged in TSB, and the remainder were exposed to air; Then, the coupons were incubated at 20 °C for 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days to promote biofilm development. The detached-cells were enumerated after a specific incubation time. The coupon was removed to sample and a 0.9% (m/v) NaCl solution was used to rinse it three times to remove non-attached cells (Wang et al., 2016). To detach the biofilm cells, a violent water-flapping approach with shaking (200 oscillations/min for 2 min) using a bag mixer (BagMixer 400VW, Interscience) was applied (Wang, Zhang, Dong, Xu, & Zhou, 2015). Detached-cells were then harvested by a 0.22 μm pore diameter filter, and were determined by inoculating 0.1 mL serial dilutions suspensions on TSA plates incubated at 37 °C for 24 h in six trials with triplicate samples. The incubation of planktonic cells was the same with biofilm cells but no stainless-steel coupon in TSB medium, and then the cells were harvested by centrifugation (10,000*g*, 15 min) at 4 °C.

2.3. UHPH treatment

Planktonic and detached-cells were incubated as above for 5 days and collected into 0.9% saline (m/v) and their initial concentration adjusted to about $10^{7\text{-}8}$ CFU/mL. The UHPH equipment was carried out by using a system of ultra high pressure homogenizer (Mini DeBee, Bee International, USA) with a single pressure intensifier and a 130-μm opening Y-type diamond nozzle (Genizer™, Los Angeles, USA) in a modular homogenization cell (Chen, Xu, & Zhou, 2016). Cultures of *S. aureus* (resuspended in 0.9% saline (m/v)) with an inlet temperature of 25 °C were passed through the homogenizer at the pressure of 10,000 PSI (69 MPa), 20,000 PSI (138 MPa), 25,000 PSI (172 MPa), 30,000 PSI (209 MPa), 35,000 PSI (241 MPa) and 40,000 PSI (275 MPa) with one to three homogenization cycles respectively (1PSI = 0.00689 MPa). A heat exchanger immediately downstream of the chamber was implemented to maintain the outlet temperature of 20 °C. Treatment without UHPH was used as the control. Viable cell counts were determined for each sample plated on TSA after incubating for 24 h at 37 °C. Six replicates were measured for each condition.

2.4. Measurement of membrane integrity

The cell membrane integrity assay was measured by the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The biofilm and planktonic cells of *S. aureus* were treated by UHPH treatment (40,000 PSI, 1 and 3 circles) and their concentration were adjusted to 10^6 CFU/mL with TSB (Cai et al., 2019). For 1 mL of cells, 3 μL of a mixture of SYTO 9 and propidium iodide (PI) was added and the suspension was incubated for 15 min at 37 °C without light. Flow cytometric analysis (BD AccuriC6; BD Accuri Cytometers, Ann Arbor, MI, USA) was carried out and the results were expressed as the ratio of green/red fluorescence.

2.5. Measurement of membrane potential

Membrane potential was measured by the BacLight Bacterial Membrane Potential Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The biofilm and planktonic cells were treated by UHPH (40,000 PSI, 1 and 3 circles), and their concentration were adjusted to about 10^6 CFU/mL with TSB (Cai et al., 2019). For 1 mL suspension, 10 μL of 3 mM 3,3′-diethyloxacarbocyanine iodide ($DiOC_2$) was added and the suspension was mixed and stored for 15 min at 37 °C without light. The ratio of red/green $DiOC_2(3)$ fluorescence reflected the membrane potential.

2.6. Determination of bacterial diameter

The particle size and distribution of *S. aureus* cells were measured using a particle size distribution analyzer (Malvern Instruments, Worcestershire, UK) and the dynamic light scattering (DLS) principle (Chen et al., 2016; Vargas, Millan-Chiu, Arvizu-Medrano, Loske, & Rodriguez, 2017). Suspensions of planktonic and detached-cells were prepared as the description in method Section 2.3, and were added to 1-cm optical path quartz cuvettes and analyzed at 25 °C with a detection angle of 90°. The mean particle size was estimated as a measurement of a single

exponential fit of the auto-correlation function using the Cumulants method. The breadth of the size distribution was measured to determine the polydispersity index (PDI) value.

2.7. Visualization of S. aureus cells by scanning electron microscopy (SEM)

SEM analysis was to obtain images of *S. aureus* cells treated with UHPH and untreated cells (Wang, Ding, Wang, Xu, & Zhou, 2013). Cells were fixed with 2% (v/v) glutaraldehyde for 12 h. After 90 min, 1% (v/v) osmic acid was added and the cells were dehydrated by using 50%, 70%, 80%, and 95% ethanol for once, and 100% ethanol for twice, and coated with gold. A Hitachi S-3000 N SEM instrument (Hitachi, Tokyo, Japan) was used to obtain images.

2.8. Statistical analyses

Biofilm and planktonic cells that had not been treated by UHPH were used as controls. All assays were repeated for six times and the results were showed as the mean ± standard deviation. Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison using SPSS to assess significant differences ($P < 0.05$).

3. Results

3.1. Biofilm formation

The dynamic growth of five *S. aureus* isolates was obtained by plotting the Log10 population against incubation time. The biofilm formation is a dynamical process including initial attachment, development, maturation, and dispersion, which was observed in Fig. 1. The cell counts in biofilms increased to more than 3.0 log CFU/cm^2 after 1 day, with a significant rise through day 4. After 5 days, the growth of biofilms reached a stationary phase, indicating the maturation stage of biofilm formation. Then the cell counts always maintained more than 7.0 log CFU/cm^2 from 6 to 7 days incubation, and finally the number of cells reached the maximum in the 8th day.

3.2. Cell viability following UHPH treatment

The viable number of *S. aureus* planktonic cells and detached-cells remained stable after UHPH treatments at pressures between 0 PSI and 30,000 PSI; the impact of cycle number (1 vs. 3) was minimal under these conditions (Fig. 2). Furthermore, no difference in cell reduction was found in two kinds of cells. When the pressure was increased from 30,000 PSI to 40,000 PSI, a sharp increase in planktonic cells inactivation was observed for both the 1- and 3-cycle treatments (1.56 log CFU/mL and 2.91 log CFU/mL) compared to the control group. The number of detached-cells reduction also increased but only in the 3-cycle treatment (0.95 log CFU/mL). As a result, the pressure of 40,000 PSI and the cycle number (1 vs. 3) were chosen as the key parameter condition to detect below four indexes as a great physiological state and quantity change happened in two types cells. Overall, the reduction in planktonic cell numbers was greater than that of detached-cells indicating a higher resistance of detached-cells to UHPH treatment.

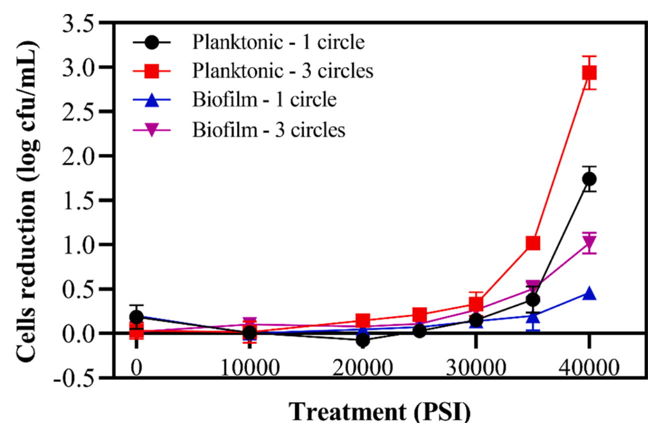

Fig. 2. Effect of different pressure and cycle number (1 vs. 3) on the survival of *S. aureus* planktonic and detached-cells. The data represent the mean ± S.D. (n = 6).

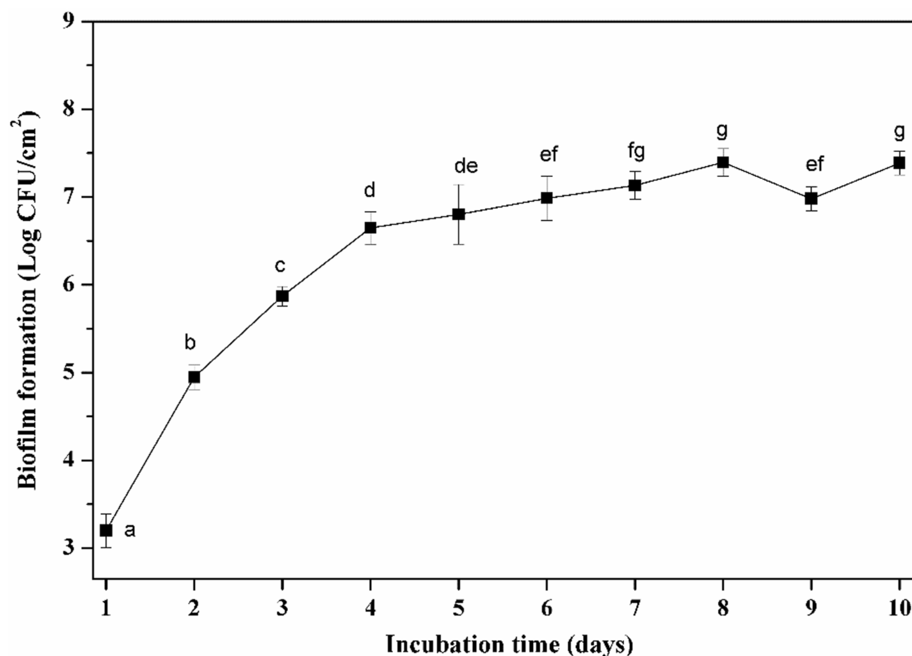

Fig. 1. Formation of *S. aureus* biofilm during a 10-day incubation period. The data represent the mean ± S.D. (n = 6). a–b: for treatments with different incubation time, different lowercase letters represent significantly different ($P < 0.05$).

3.3. Membrane integrity

Changes in the red/green ratio of the fluorescing SYTO 9/PI dyes in treatment and control groups were measured to estimate changes in membrane integrity in biofilm and planktonic cells following UHPH treatment. A lower red/green ratio indicated more intact cell membranes because only the red fluorescing PI dye could enter cells with a broken membrane. An increase in the red/green ratio was observed in both cell types when they were exposed to 1 cycle of UHPH treatment at 40,000 PSI (Fig. 3). After three cycles, more planktonic cells exhibited damaged membranes, whereas there was no apparent increase in damaged detached-cells compared to the single cycle treatment. The maximal change in fluorescence values for the control and 1- or 3-cycle treatment within each cell type were a 2-fold change for planktonic cells (3 cycles) and a 1.6-fold change for detached-cells (1 cycle).

3.4. Membrane potential

The effect of UHPH on membrane potential was evaluated using the dye $DiOC_2(3)$ and measuring the red/green fluorescence ratio. $DiOC_2(3)$ fluoresces green in all bacterial cells; at higher membrane potentials the dye molecules associate and the emission shifts to the red. Following UHPH treatment at 40,000 PSI, the red/green fluorescence ratios of both the planktonic and detached-cells significantly decreased relative to the untreated controls (Fig. 4). Compared to the planktonic cells, a sharper decrease in the fluorescence ratio was found in the biofilm group (1.5-fold change vs. a 5-fold change after 3 cycles). However, no significant difference in the fluorescence ratio of detached-cells was observed between 1 and 3 cycles.

3.5. Bacterial size

The mean size of untreated detached-cells and planktonic cells was approximately 700 nm and 1300 nm, respectively (Fig. 5). Detached-cells showed a smaller size than planktontic cells probably due to the slower growth rate. The size distribution of detached-cells was more concentrated than that of planktonic cells. After UHPH treatment with 40,000 PSI, all particle size peaks of both cells were shifted to the left (smaller cell size). The changes of cell size between detached-cells and planktonic cells were 240 nm and 500 nm respectively after one cycle compared to the control group, whereas the corresponding values for 3 cycles were 300 nm and 600 nm, respectively. From the above data, it is clear that the peak intensity of biofilm cell size was stronger than that of planktonic cells. Meanwhile, both cell types showed a unimodal distribution.

3.6. SEM observations

The characteristic morphology of *S. aureus* biofilm and planktonic cells with and without UHPH treatment (40,000 PSI, 1 and 3 cycles) was observed by SEM (Fig. 6). Planktonic *S. aureus* cells were spherical in shape and adhered in grape-like structures with less EPS on the contact surfaces. Compared to planktonic cells, a larger number of detached-cells were held together with more obvious EPS secretions forming three-dimensional cell aggregates. Following 1 cycle of UHPH, a small number of detached-cells were freed by impact force; more scattered EPS was found after 3 treatment cycles. The planktonic cells exhibited surface indentations and cell distortions with increased treatment cycles.

4. Discussion

As the key factor of cross-contamination in food industry, biofilms have been the safety risk for food problems. One thing in common among biofilms is the resistance to chemical sanitizers and physical mechanical forces, because of the strong structure of the biofilms. Detached-cells disruption is considered to be a continuous process, including biofilm dispersion, cell membrane damage, the release of intracellular metabolites, and complete cell fragmentation. A comprehensive characterization of the UHPH disruption process of *S. aureus* biofilm and planktonic cells can be attained through the use of different methods including cell counting to achieve a quantitative description, cell membrane viability testing using SYTO 9/PI staining, membrane potential testing using the fluorescent dye $DiOC_2(3)$, and scanning electron microscopy to visualize the microstructure of cell suspensions.

In the present study, mature *S. aureus* biofilm formed after 5 days of incubation. UHPH treatment at 40,000 PSI (3 cycles) reduced the number of planktonic and detached-cells 2.91 and 0.95 log CFU/cm^2,

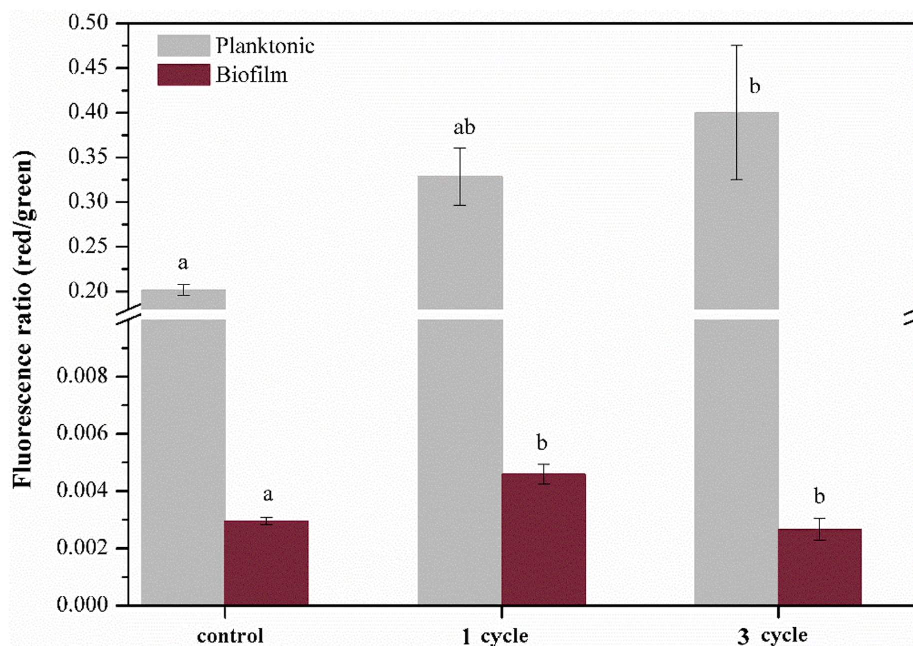

Fig. 3. Effect of UHPH treatment (40,000 PSI) with 1 and 3 treatment cycles on membrane integrity of *S. aureus* planktonic and detached-cells. Error bars represent standard deviations (n = 6). a-b: for different cycle treatments with one kind of cell, different lowercase letters represent significantly different ($P < 0.05$).

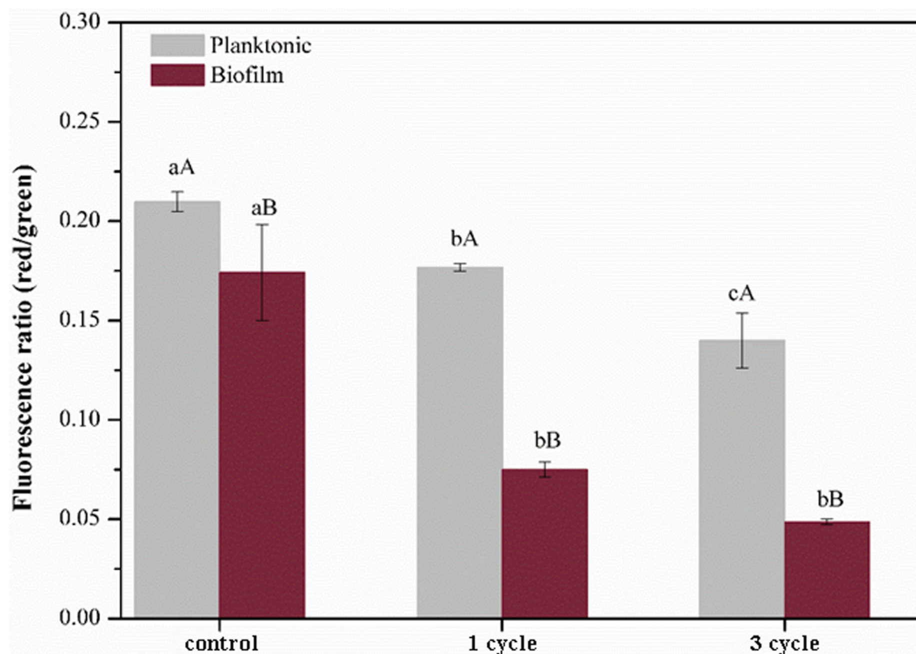

Fig. 4. Effect of UHPH treatment (40,000 PSI) with 1 and 3 treatment cycles on membrane potential of *S. aureus* planktonic and detached-cells. Error bars represent standard deviations (n = 6). a–b: for different cycle treatments with one kind of cell; A–B: for treatments with the same cycle to two kinds of cells. Different lowercase/uppercase letters represent significantly different ($P < 0.05$).

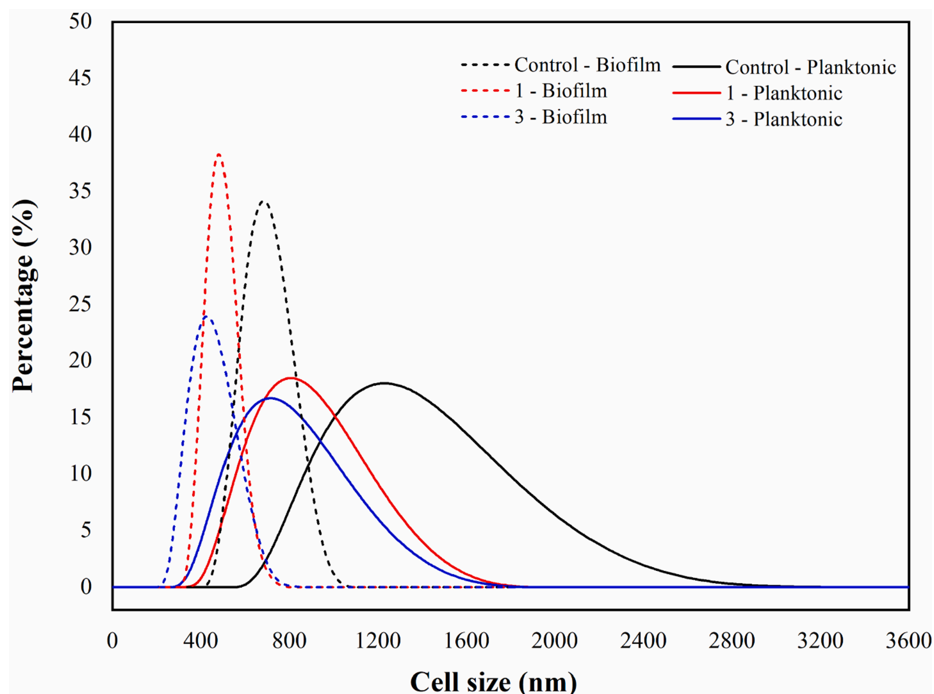

Fig. 5. Effect of UHPH treatment (40,000 PSI) with 1 and 3 treatment cycles on *S. aureus* planktonic and biofilm cell size. The data represent the mean ± S.D. (n = 6).

respectively (Fig. 2), which suggested that 1) the detached-cells of biofilm may be more resistant to UHPH than the planktonic cells; 2) although the resistance of biofilm cell to UHPH cannot be ignored, UHPH has potential as an emerging technology for pasteurization of liquid foods. A tendency that an increase in process pressure following a higher inactivation rate in both two cells was noted, which was verified by Vachon et al. (2002). Additionally, the successive cycles of UHPH can increase the death of *S. aureus*, which was also demonstrated by Wuytack, Diels and Michiels (2002). Inactivation or loss of viability as a result of UHPH was not linearly dependent on pressure or cycle number. This was most likely due to the distribution of cell resistance (Patrignani & Lanciotti, 2016), which is probably decided by the amount of peptidoglycan of cell wall. As for detached-cells wrapped by a large number of tightly EPS, which greatly reduces the damage of UHPH to cell membrane.

Pasteurization of UHPH was achieved mainly through mechanical destruction of the cell membrane. Therefore, the factors that influence microbial inactivation also include microbial factors that affect the

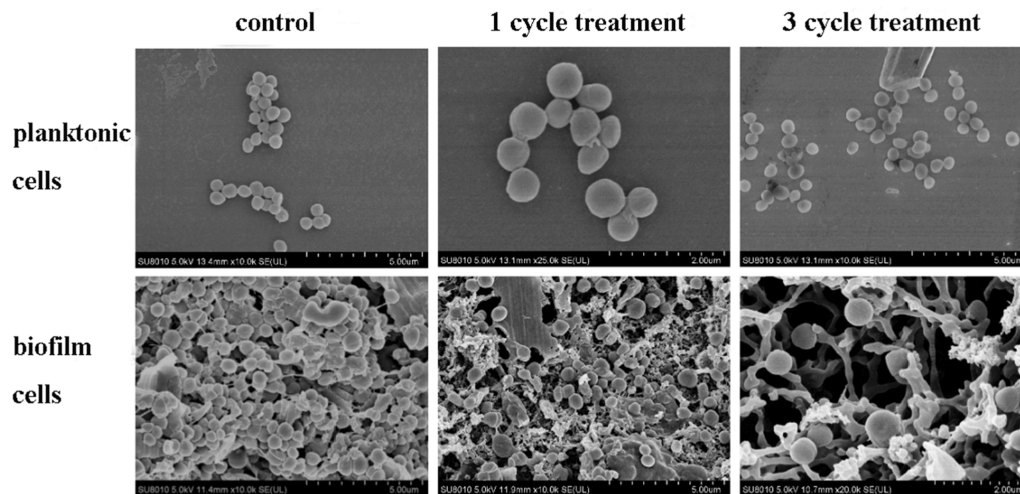

Fig. 6. Scanning electron microscopy images of *S. aureus* planktonic and detached-cells after treatment with 1 and 3 cycles of UHPH under a pressure of 40,000 PSI.

bacterial strength (Van-Impe et al., 2018). Detached-cells of biofilm produce EPS to enable cell aggregation and cohesion. The resistance of such organism in biofilm is more due to the protective effect of the biofilm matrix than the structural differences in the detached-cells. The spatial pressure, velocity gradients, turbulence, and impingement caused by a sudden pressure drop after the valve lead to an 'opening hole' of the planktonic cell envelope and induce alterations in cell membranes, and causing the disturbance of detached-cells EPS structure and internal bacterium outflow (Maresca, Donsi, & Ferrari, 2011; Foladori, Tamburini, & Bruni, 2010). Membrane potential and membrane integrity were measured to observe the physiological state of cell membrane, and to determine whether UHPH treatment exhibited similar destructiveness to isolated detached-cells and planktonic cells. The proportions of cells with damaged cell membranes estimated by two methods above obviously confirm the results in Fig. 2. However, one thing that cannot be ignored is that the relationship between membrane integrity and treatment cycle number appeared to be non-linear in the range of 0 to 3 cycles, at least for the detached-cells. This may be due to the heterogeneity of the microbial populations and the presence of resistant bacteria that can survive repeated passages despite losing the protection of EPS (Donsi, Ferrari, Lenza, & Maresca, 2009). The membrane potential, as the fundament in live cell physiology processes, it is strongly related to bacterial viability. UHPH treatment reduced the membrane potential by apparently increasing the electrical depolarization of the target cells (Fig. 4). Compared to the detached-cells, the planktonic cells do not show a large change in the ratio. It is presumable that microbial response mechanisms to microbial adaptation and survival such as cell membrane repair will be triggered the longer incubation after the UHPH exposure (Braschi et al., 2018). It is likely that the planktonic cells have better self-repairing ability after undergoing physical therapy completed to the detached-cells.

UHPH treatments also resulted in morphological changes of the bacterial cells (Fig. 6). The quantity of the deformed cells and the membrane debris of detached-cells increases following UHPH treatment and the percentage of this heterogeneous population was enhanced with increasing cycle number. After 3 treatment cycles, the visual appearance of the aggregates observed showed that more EPS fell off and scattered, detached-cells with broken membranes were more increased, which coincide with the smaller changes of cell size of processed detached-cells (Fig. 5). The difference in cell disruption mechanism between the detached-cells and planktonic cells is probably related to the protection of the biofilm matrix, demonstrating a higher rigidity of the biofilm-embedded bacteria (Kubota, Senda, Nomura, Tokuda, & Uchiyama, 2008).

5. Conclusion

In summary, the results indicated that UHPH treatment led to cell reduction and damage of cell membrane and morphology of both planktonic and detached-cells of *S. aureus*. The detached-cells of *S. aureus* were more resistance to UHPH treatment than planktonic cells. In addition, although the resistance of biofilm cells to UHPH, we still cannot ignore the potential of UHPH as a pasteurization method applied in liquid food systems, since its ability to reduce biofilm. The sterilization parameters employed in this study could provide a basis for commercial sterilization process.

CRediT authorship contribution statement

Lingling Zhang: Writing - original draft, Visualization, Writing - review & editing. **Changqing Zhu:** Resources, Visualization, Writing - review & editing. **Xiaojing Chen:** Investigation, Methodology. **Xinglian Xu:** Project administration. **Huhu Wang:** Conceptualization, Funding acquisition, Writing - review & editing, Supervision.

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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