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Multiple action sites of ultrasound on *Escherichia coli* and *Staphylococcus aureus*

Xinyu Liao [a,b,c], Jiao Li [a,b], Yuanjie Suo [a,b], Shiguo Chen [a,b], Xingqian Ye [a,b], Donghong Liu [a,b,c], Tian Ding [a,b,c,*]

[a] *Department of Food Science and Nutrition, National Engineering Laboratory of Intelligent Food Technology and Equipment, Zhejiang University, Hangzhou, Zhejiang 310058, China*

[b] *Key Laboratory for Agro-Products Postharvest Handling of Ministry of Agriculture, Zhejiang Key Laboratory for Agro-Food Processing, Hangzhou, Zhejiang 310058, China*

[c] *Fuli Institute of Food Science, Zhejiang University, Hangzhou 310058, China*

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Abstract

Ultrasound, is thought to a potential non-thermal sterilization technology in food industry. However, the exact mechanisms underlying microbial inactivation by ultrasound still remain obscure. In this study, the action modes of ultrasound on both Gram-negative and Gram-positive microorganisms were estimated. From colony results, ultrasound acted as an irreversible effect on both *Eshcerichia coli* and *Staphylococcus aureus* without sublethal injury. The result in this study also showed that a proportion of bacteria subpopulation suffered from serious damage of intracellular components (e.g. DNA and enzymes) but with intact cell envelopes. We speculated that the inactivated effects of ultrasound on microbes might more than simply completed disruption of cell exteriors. Those microbial cells who had not enter the valid area of ultrasonic cavitation might be injected with free radicals produced by ultrasound and experienced interior injury with intact exterior structure, and others who were in close proximity to the ultrasonic wave field would be immediately and completely disrupted into debris by high power mechanic forces. These findings here try to provide extension for the inactivation mechanisms of ultrasound on microorganisms.
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Keywords: Ultrasound; Inactivation mechanism; Reactive oxygen species (ROS); *Staphylococcus aureus*; *Escherichia coli*

1. Introduction

Ultrasound, pressure wave with a frequency of 20 kHz or more, is known as potential non-thermal technology to ensure food safety without loss of food sensory properties [1,2]. As early as 1929, it has been reported that ultrasound had the ability to kill vegetative microbes [3]. Generally, high power ultrasound with lower frequency (20 to 100 kHz) is employed for microbial inactivation in food industry [2]. The mechanism of microbial inactivation by ultrasound is widely thought to be the responsibility of cavitation phenomenon [4–8]. Under strong ultrasonic field, the bubbles will implode coincident with production of intensive pressure, high temperature or even active species (e.g. free radicals). So far, the inactivation mechanisms on microbes by ultrasound has been studied and investigated in some studies [9–11]. The mechanical effects (e.g. shear force) generated by ultrasonic wave were commonly thought to the primary inactivation mechanism leading to the completed rupture of microbial cell envelopes and the death of microbes [11–18]. However, apart from strong physical effect, increasing temperature and pressure during the collapse of bubbles was enough

* Corresponding author at: Department of Food Science and Nutrition, Zhejiang Key 14 Laboratory for Agro-Food Processing, Zhejiang University, Hangzhou, Zhejiang 15 310058, China.

E-mail address: tding@zju.edu.cn (T. Ding).

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to trigger sonochemical reaction of surrounding medium and produce active compounds [19,20]. According to that, few studies proposed another inactivated mechanism that free radicals produced by the explosion of bubbles might be transported into microbial cells through microjet and react with interior components with intact cell exterior structure [13,14,18,20]. So far, the exact mechanisms underlying ultrasound inactivation still have no consensus and require to fully elucidate.

The aim of this study is to investigate the molecule mechanisms of ultrasound-induced microbial inactivation. Gram-negative *Esherichia coli* and Gram-positive *Staphylococcus aureus* were used the model bacterium in this case. The extracelluar H_2O_2 and intracellular reactive oxygen species (ROS), the membrane integrity and permeabilization, the membrane potentials, ATP production, DNA fragment and morphological properties were measured for the estimation of microbial cells physiological activity during ultrasound treatment in molecular level. We try to provide new insight into the action mode of ultrasound on microbial cells for future research.

2. Materials and methods

2.1. Bacterial strains preparations

Gram-negative *Esherichia coli* (ATCC 25922, Hope Bio-Technology Co., Ltd., Qingdao, Shandong, China) and Gram-positive *Staphylococcus aureus* (ATCC 25923, Hope Bio-Technology Co., Ltd., Qingdao, Shandong, China) were stored with 50% glycerol at −80 °C. The stock culture of each strain was transferred into 100 mL of nutrient broth (NB, Base Bio-Tech Co., Hangzhou, China) and incubated at 37 °C in a well-shaken of 150 rpm to reach stationary phase, which required 18 h for *E. coli* and 24 h for *S. aureus*. The enriched culture was centrifuged at 5000 rpm for 10 min at 4 °C to harvest bacteria cells. The precipitated cells were then washed for three times with 0.85% sterile saline solution. The final bacteria concentration of each strain determined by plating count method was approximately 9 log CFU/mL.

2.2. Ultrasound treatment

Thirty milliliter of cell suspension (about 8 log CFU/mL) was placed in a cylindrical tube with a volume of 85 mL. An ultrasonic probe (diameter = 10 mm) was immersed 2.0 cm into the bacteria solution. In this case, the input power was 198 W, power intensity was 252 W/cm^2, frequency was 20 kHz and treatment times were 0, 3, 5 and 12 min. The temperature was maintained at 20 ± 2 °C with the use of a thermostatic water bath (DC-1006, Safe Corporation, Ningbo, China). The details of the ultrasound equipment (Scientz-II D; Ningbo Scientz, Zhejiang, China) were described in our previous study [21].

2.3. Microbiological analysis

One milliliter of each sample was serial diluted with 0.85% sterile saline solution. One hundred microliter of appropriate dilution was plated on non-selective medium, tryptone soya agar (TSA, Hope Bio-Technology Co., Ltd., Qingdao, Shandong, China) and selective medium, supplemented TSA with 2% (w/w) sodium chloride for *E. coli* and 7% (w/w) sodium chloride for *S. aureus*. The plates were then incubated at 37 °C for 48 h under atmospheric conditions. Both viable and sublethally damaged cells appeared on non-selective medium, while microbial cells with compromised membranes failed to grow on the selective mediums. Therefore, the differences between non-selective and selective medium resulted from the occurrence of sublethal injury on microbes [22,23]. Each experiment was conducted in triplicate independent cultures.

2.4. Membrane integrity

Propidium iodide (PI, Sigma-Aldrich Co., USA) was used to indicate compromised cell membrane. One milliliter of bacteria sample was stained with 10 μL of PI solution (1.5 mM) for 30 min at 37 °C in the dark. The excess PI was removed by centrifugation of 8,000 rpm at 4 °C for 10 min. The pelleted cells were then resuspended with 0.85% sterile saline solution and stored in the dark for no more than 1 h until flow cytometry analysis.

2.5. Membrane permeabilization and esterase activity

Carboxyfluorescein diacetate (cFDA, Sigma-Aldrich Co., USA) diffused freely through viable cell membrane and was converted by intracellular esterase into membrane-impermeable green fluorescent dye, carboxyfluorescein (cF). One milliliter of sample was incubated with cFDA solution (50 μM) for 30 min at 37 °C. After centrifugation 8,000 rpm at 4 °C for 10 min, the pellet cells were resuspended with 0.85% sterile saline solution to remove excess cFDA. The stained samples were stored in dark for no more than 1 h and analyzed by flow cytometry (Beckman Coulter Inc., Miami, FL, USA).

2.6. Membrane potential assessment

Membrane potential was measured with a BacLight^{TM} Bacterial Membrane Potential Kit (B34950, Molecular Probes, Invitrogen, Grand Island, NY). $DiOC_2$ (3) (3,3′-diethyloxacarbocyanine iodide) was a probe, altering from green to red fluorescence as the membrane potential increasing. CCCP (carbonyl cyanide 3-chlorophenylhydrazone) could destroy cell membrane potential through eliminating membrane proton gradient. Specifically, 10 μL $DiOC_2$ (3) (3 mM) was added into 1 mL sample and mixed thoroughly. As for control, 10 μL CCCP (500 μM) was transferred into sample and mixed before the addition of $DiOC_2$ (3). The mixture was then incubated for 30 min at room temperature. The samples were then centrifuged and washed with 0.85% sterile saline solution to remove excess $DiOC_2$ (3). The stained samples were stored at dark measured by flow cytometer (Beckman Coulter Inc., Miami, FL, USA).

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