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Research Article

Inactivation of *Staphylococcus aureus* by the Combined Treatments of Ultrasound and Nisin in Nutrient Broth and Milk

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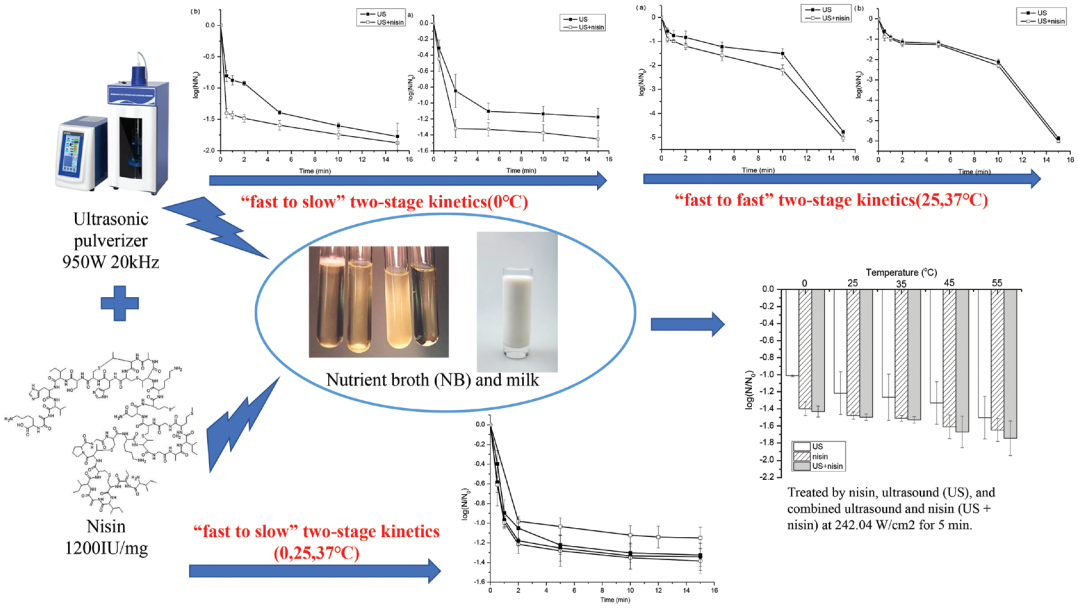
ABSTRACT

In this study, ultrasound (US) and nisin, applied individually or in combination (US + nisin), were investigated to determine their inactivation effect on *Staphylococcus aureus* in nutrient broth (NB) and milk. The inactivation of *S. aureus* by nisin at 0, 25 and 37°C followed "fast to slow" two-stage kinetics, and there was no significant difference in *S. aureus* reduction under these temperatures. A maximum reduction of 1.40 $\log_{10}$ cycles was obtained by nisin at 37°C for 15 min. The inactivation of *S. aureus* by US also followed "fast to slow" two-stage kinetics at 0°C, but it followed "fast–slow–fast" there-stage kinetics at 25 and 37°C. A maximum reduction of 5.87 $\log_{10}$ cycles was obtained by US at 968.16 W/cm^2 and 37°C for 15 min. The reduction of *S. aureus* in milk was similar as in NB when treated at 968.16 W/cm^2 and 25°C for 5–15 min. However, the reduction of *S. aureus* by US + nisin was similar to that obtained by either nisin or US alone, indicating that US and nisin had no added or synergistic inactivation effect on *S. aureus* in both NB and milk under the tested conditions.

GRAPHICAL ABSTRACT



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1. INTRODUCTION

Nisin is an antibacterial peptide produced by *Lactococcus lactis* subspp. *Lactis* [1]. Nisin has been internationally recognized as a food preservative since 1969 because of its relatively long history of safe use and its documented effectiveness in the control of pathogenic bacteria [2]. It is the solely bacteriocin that has been found practical application as a natural food preservative in cheese, milk, dairy products, canned foods, and hot baked flour products [3,4]. Nisin has a broad spectrum of activity against various Gram-positive bacteria [5]. In normal circumstances, nisin does not significantly inhibit yeasts, molds, or Gram-negative (G–) bacteria [6]. The antimicrobial mechanism of nisin is based on the pore formation in the cytoplasmic membrane of the target microorganisms which leads to a loss of small intracellular molecules and a collapse of the proton motive force [7]. Gram-negative bacteria are typically resistant to nisin since the outer membrane of Gram-negative bacteria does not allow the entry of nisin into the cytoplasmic membrane [8]. The effectiveness of nisin depends on growth and exposure conditions, such as temperature and pH, and nisin is more active at lower pH values (pH < 4.0), whereas the influence of temperature on its effectiveness is controversial [4,9]. Alternative methods for pasteurization and sterilization are gaining great attention, which is due to increased consumer demand for new methods of food processing that have a reduced impact on nutritional content and overall food quality. Ultrasound (US) processing is one of the alternative technologies that have shown promise in the food industry. US, in its most basic definition, refers to ultrasonic waves with a frequency of 20 kHz or more [10,11]. Ultrasound can be classified into low- and high-intensity ultrasound. Low-intensity ultrasound is commonly used in the medical science for diagnostic purpose, and can also be used in food science to measure the texture, composition or viscosity of food. Furthermore, the microbial and enzyme inactivation effects of high-intensity ultrasound can also be applied to the food industry as a potential preservation technique [8].

Staphylococcus aureus is a Gram-positive pathogen normally present in milk. A minimal concentration of *S. aureus*, about 10^7 Colony Forming Unit (CFU)/g of food, can produce enough enterotoxin to cause food poisoning [12]. The effect of amplitude levels, duty cycles and time of US on inactivation of *S. aureus* inoculated into trypticase soy broth were previously investigated [13]. Soleimanzadeh et al. [13] found a 1.13 $\log_{10}$ reduction of *S. aureus* was achieved at amplitude 4 μm and 30°C for 14 min and the inactivation could be enhanced by higher amplitude level and longer treatment time. There is an increasing demand for the development of new alternative processing technologies that could be combined with biological preservatives in the food industry [14,15]. US is not very effective in killing bacteria in food when used alone, but the effectiveness of ultrasound waves is enhanced when used in combination with other techniques, such as antibiotics, hypochlorite, mild heat or pressure [16]. Also, the sensitivity of bacteria to nisin was enhanced when it was combined with other treatments [5,17,18]. A synergistic action on inactivation of Gram-positive bacteria has been described for the combinations of nisin and non-thermal technologies such as high hydrostatic pressure [8,15,17], high-intensity pulsed electric fields [19,20] and hyperbaric carbon dioxide [21]. However, few studies have been carried out on the combination of nisin and US on the inactivation of Gram-positive pathogens. The pore formation action of nisin and the cavitation effect of US may enhance the inactivation effect to each other. We assumed that there might also be synergistic action between US and nisin on the inactivation of *S. aureus*. Results of this study might open possibilities for more in-depth exploration into the combination of US + nisin as a hurdle approach to inactivate target bacteria in milk.

2. MATERIALS AND METHODS

2.1. Preparation of Bacterial Strains

Staphylococcus aureus (CGMCC1.1861) was obtained from China General Microbiological Culture Collection Center (CGMCC, Beijing, China) and was maintained on slants of Nutrient Agar (NA, Beijing Aoboxing Biological Technology Co. Ltd., Beijing, China). The strain was inoculated in Nutrient Broth (NB, Beijing Aoboxing Biological Technology Co. Ltd.), and then incubated at 37°C for 12 h, to obtain the initial stationary phase. The stationary phase was determined by timing measurement of Optical Density (OD) values during incubation in NB using a spectrophotometer at 600 nm. A working solution was prepared by diluting the above subculture into NB, with the final bacterial concentration 10^7–10^8 CFU/mL.

To test the influence of cell suspension media on the inactivation effect of US and US + nisin, *S. aureus* at initial stationary phase in NB were centrifuged at 4000 rpm for 10 min at 4°C (Model TD-5M, Sichuan Shu Instrument Co., Ltd, Sichuan, China), the harvested cells were then washed with physiological salt solution (0.85% NaCl) twice, and re-suspended in sterilized skim-milk [20] (pH 6.5). The cell concentration in the resulting suspensions was 10^7–10^8 CFU/mL.

2.2. Preparation of Nisin Solution

Dissolve 0.2 g of commercial nisin (Zhejiang Silver-Elephant Bioengineering Co., Zhejiang, China) with a content of 1200 IU/mg in 100 mL of 0.02M HCl sterile solution with a pH of about 2.0, and then autoclave it in Steam at 121°C for 15 min [22]. Then add 0.5 mL nisin solution to 50 mL of prepared sterile NB or milk, and then immediately undergo US treatment.

2.3. Treatments of US or US + Nisin

Each 50 mL *S. aureus* suspension was treated by US at 20% or 80% maximum power (950 W) (SCIENTZ-IID, Ningbo Xinzhi biological Polytron Technologies Inc., China), with or without nisin in an ice bath. The ultrasound frequency was 20 kHz. The probe had a diameter of 1 cm and the operating immerse depth was 10.0 cm. Accordingly, the output sonic power density of the sonotrode was 242.04 and 968.16 W/cm^2. Pulse intervals of 2 s on and 2 s off were applied for up to 0–15 min. The treatment temperature was 0–55°C. The untreated working solutions with and without nisin were performed as controls. Each treatment was performed in triplicate. The experiment uses alcohol for sterilization after each test before proceeding to the next test.

The intensity of ultrasound power dissipated from the probe tip was calculated by Equation (1) [23].

$$I = \frac{P}{\pi r^2} \quad (1)$$

where r is the radius of the titanium tip (cm), and P is the input power level (W).

2.4. Experimental Design

To evaluate the influence of treatment temperature on the inactivation of US and US + nisin on *S. aureus,* cells were treated at 242.04 W/cm^2 and 0–55°C for 5 min. The kinetic of the inactivation of US and US + nisin on *S. aureus* in NB were carried out at 242.04 W/cm^2 and 0°C, 968.16 W/cm^2 and 0°C, 25°C, or 37°C for 0.5, 1, 2, 5, 10, 15 min. Furthermore, the kinetic of the inactivation of US and US + nisin on *S. aureus* in milk were carried out at 968.16 W/cm^2 and 25°C for 2, 5, 10, 12, 15 min. The untreated cells were used as a control, and the inactivation of cell suspension by nisin at 0, 25, 37°C for 0–15 min were used as a comparison.

2.5. Statistical Analysis

Microcal Origin 8.0 (Microcal Software, Inc., Northampton, MA, USA) was used for analysis of variance. The significance level was set to 0.05. The values in all graphs are expressed as mean ± SD. All experiments were repeated three times.

3. RESULTS

3.1. Effect of US, Nisin and US + Nisin on the Inactivation of *S. aureus* at Different Temperatures

As shown in Figure 1, US alone (242.04 W/cm^2, 5 min) significantly reduced cell counts of *S. aureus* by 1.01, 1.21, 1.26, 1.33 and 1.50 $\log_{10}$ cycles at 0, 25, 35, 45 and 55°C, respectively. But the inactivation of US was not significant changed with increasing treatment temperature from 0 to 45°C ($p > 0.05$). Nisin alone significantly reduced cell counts of *S. aureus* by 1.40–1.65 $\log_{10}$ cycles at different temperatures but there was no significant difference on the inactivation effect among these temperatures ($p > 0.05$). Under the same treatment conditions, the reduction of *S. aureus* cells by US + nisin was significantly higher than by US, but was closed to the reduction obtained by nisin (Figure 1), indicating that under these treatment conditions, ultrasound and nisin had no added or synergistic inactivation effect on *S. aureus*.

3.2. Effect of Nisin on the Inactivation of *S. aureus* at Different Temperatures

The inactivation of *S. aureus* in NB by nisin at 0, 25 and 37°C for 0.5, 1, 2, 5, 10, 15 min is shown in Figure 2. At different temperatures, inactivation of *S. aureus* by nisin all followed "fast to slow"

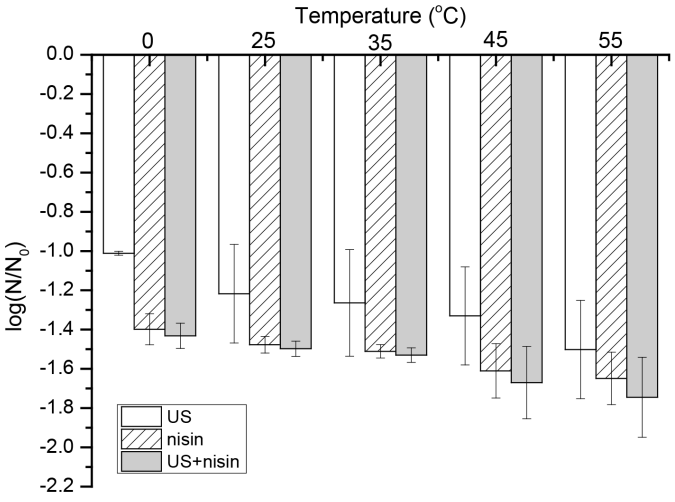

Figure 1 | Inactivation of *Staphylococcus aureus* in nutrient broth treated by nisin, ultrasound (US), and combined ultrasound and nisin (US + nisin) at 242.04 W/cm^2 for 5 min. Error bars represent standard deviation.

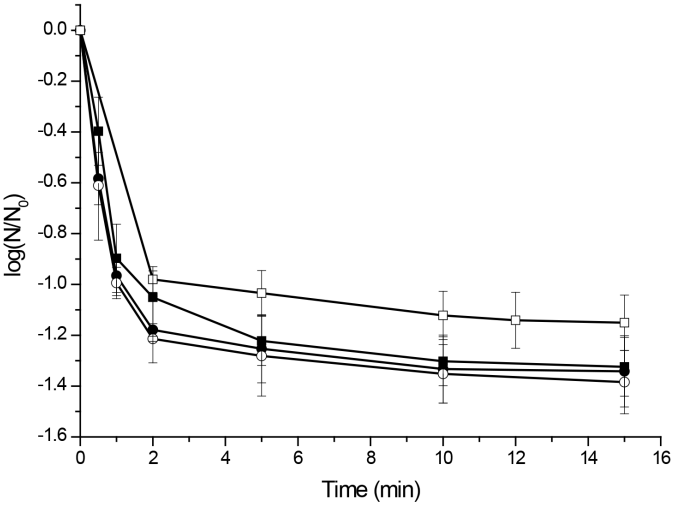

Figure 2 | Inactivation of *Staphylococcus aureus* treated by nisin at different temperatures. (▪), (●), (○) represent 0, 25 and 37°C in nutrient broth. (□) represent 25°C in milk.

two-stage kinetics, a reduction of 1.05–1.21 $\log_{10}$ cycles of *S. aureus* was achieved by nisin at the first 2 min' treatment, and it was gradually increased with prolonging time ($p > 0.05$). However, there was no significant difference in *S. aureus* reduction by nisin under different treatment temperatures. A maximum reduction of 1.40 $\log_{10}$ cycles was obtained by nisin at 37°C for 15 min.

As shown in Figure 2, inactivation of *S. aureus* by nisin in milk at 25°C for 2–15 min also followed "fast to slow" two-stage kinetics. The inactivation of microorganisms is dose-dependent, so the later "slow" stage may because it has been used up. The reduction of *S. aureus* by nisin in milk was similar to that in NB, and the largest reduction of 1.40 $\log_{10}$ cycles was obtained by nisin at 37°C for 15 min.

3.3. Effect of US and US + Nisin on the Inactivation of *S. aureus* at 0°C

The inactivation of *S. aureus* by US and US + nisin at 242.04 W/cm² and 0°C is shown in Figure 3a. The inactivation of *S. aureus* by US and US + nisin followed "fast to slow" two-stage kinetics. *S. aureus* was significantly inactivated by US or US + nisin at the first 2 min' treatments, and the reduction was gradually increased with prolonging time. A maximum reduction of 1.17 and 1.44 $\log_{10}$ cycles was obtained by US and US + nisin at 242.04 W/cm² and 0°C for 15 min, respectively. The inactivation of *S. aureus* by US + nisin was greater than by US, but was close to that obtained by nisin, indicating that the combination of US and nisin had no added or synergistic inactivation effect on *S. aureus* under the above conditions.

The inactivation of *S. aureus* by US and US + nisin at 968.16 W/cm² and 0°C is shown in Figure 3b. The inactivation of *S. aureus* by US and US + nisin at 968.16 W/cm² and 0°C also followed "fast to slow" two-stage kinetics. *Staphylococcus aureus* was significantly inactivated by US or US + nisin at the first 30 s' treatments, and the reduction was gradually increased with prolonging time. A maximum reduction of 1.8 and 1.9 $\log_{10}$ cycles was obtained by US and US + nisin at 968.16 W/cm² and 0°C for 15 min, respectively. The inactivation of *S. aureus* by US + nisin was similar to that obtained by nisin at 0.5–5 min, but it was similar to that obtained by US at 5–15 min, indicating that the inactivation of US + nisin on *S. aureus* only showed the greater values between US and nisin treatments. Under the same temperature and time, the inactivation of *S. aureus* was increased by 0.5 $\log_{10}$ cycles with increasing the power density from 242.04 to 968.16 W/cm². The results showed that the treatment power contributed greatly to the inactivation effect of *S. aureus* by US and US + nisin and the increase in the treatment power density could increase inactivation effect.

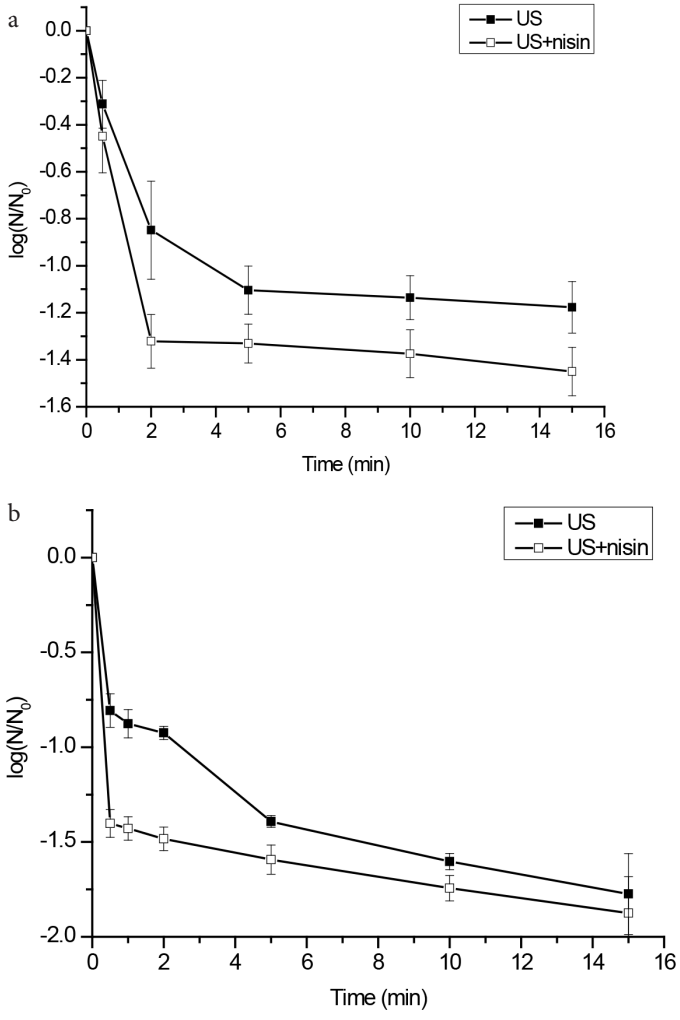

Figure 3 | Inactivation of *Staphylococcus aureus* in nutrient broth treated by US and US + nisin at 0°C. (a) 242.04 W/cm²; (b) 968.16 W/cm².

3.4. Effect of US and US + Nisin on the Inactivation of *S. aureus* at 968.16 W/cm² and Different Temperatures

The inactivation of *S. aureus* in NB subjected to US, US + nisin at 968.16 W/cm², 25 and 37°C is shown in Figure 4a and 4b, respectively. The inactivation of *S. aureus* by US and US + nisin both followed "fast–slow–fast" there-stage kinetics. *S. aureus* was significantly inactivated by US or US + nisin at the first 30 s' treatments, the reduction was gradually increased at 30 s–10 min' treatment, and the reduction was significantly increased at

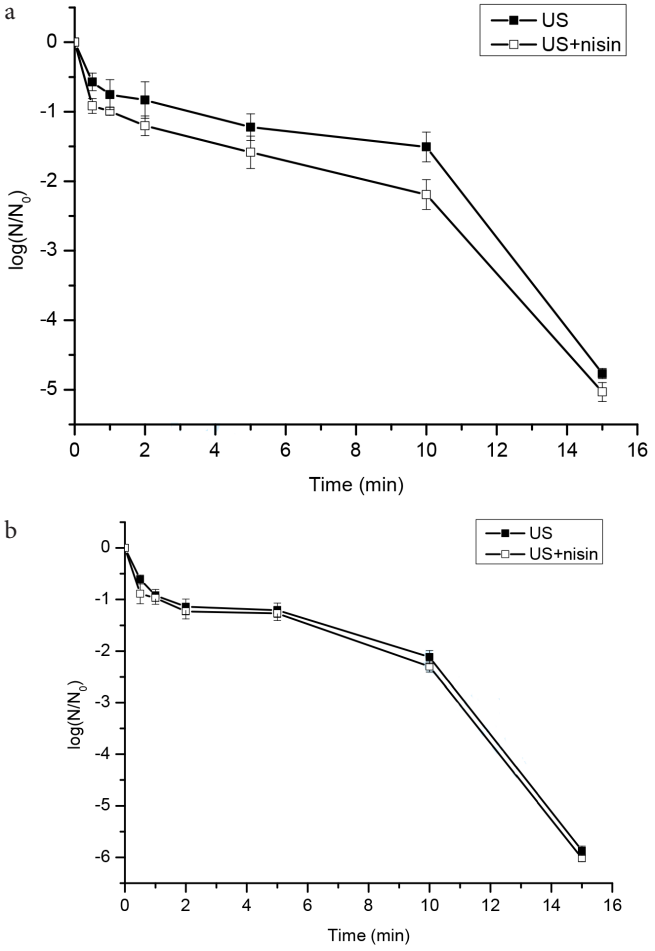

Figure 4 | Inactivation of *Staphylococcus aureus* in nutrient broth treated by US and US + nisin at 968.16 W/cm². (a) 25°C; (b) 37°C.

10–15 min' treatment. A maximum reduction of 4.77 and 5.03 $\log_{10}$ cycles was obtained by US and US + nisin at 968.16 W/cm^2 and 25°C for 15 min, respectively (Figure 4a). When the treatment temperature was increased to 37°C, the inactivation effect was enhanced. A maximum reduction of 5.87 and 6.01 $\log_{10}$ cycles was obtained by US and US + nisin at 968.16 W/cm^2 and 37°C for 15 min, respectively (Figure 4b). Specifically, the reduction of *S. aureus* by US, nisin and US + nisin at 968.16 W/cm^2 and 25°C for 10 min was approximately 1.5, 1.3 and 2.30 $\log_{10}$ cycles, respectively, indicating that US and nisin had added inactivation effect on *S. aureus*. However, this was the only condition under which the added inactivation effect between US and nisin was observed. Under the other conditions, the inactivation of *S. aureus* by US + nisin was similar to that obtained by US.

3.5. Inactivation of *S. aureus* in Milk by US, Nisin, and US + Nisin

As shown in Figure 5, the inactivation of *S. aureus* in milk by US and US + nisin both followed "fast–slow–fast" there-stage kinetics. *S. aureus* was significantly inactivated by US and US + nisin at the first 2 min' treatments, the reduction was gradually increased at 2–5 min' treatments, and the reduction was significantly increased at 5–15 min' treatments. A maximum reduction of 4.8 and 5.0 $\log_{10}$ cycles was obtained by US and US + nisin at 968.16 W/cm^2 and 25°C for 15 min. Moreover, the reduction of *S. aureus* in NB was slightly greater than that in milk by identical treatment before 5 min ($p > 0.05$). After 5 min, the reduction of *S. aureus* in milk was significantly greater than that in NB. However, the inactivation of *S. aureus* in milk by US + nisin was also similar to that by US, indicating that under the treatment conditions of this study, US and nisin had no added or synergistic inactivation effect on *S. aureus* suspended in both NB and milk.

4. DISCUSSION

There was no significant difference among the inactivation effects on *S. aureus* by nisin under different treatment temperatures.

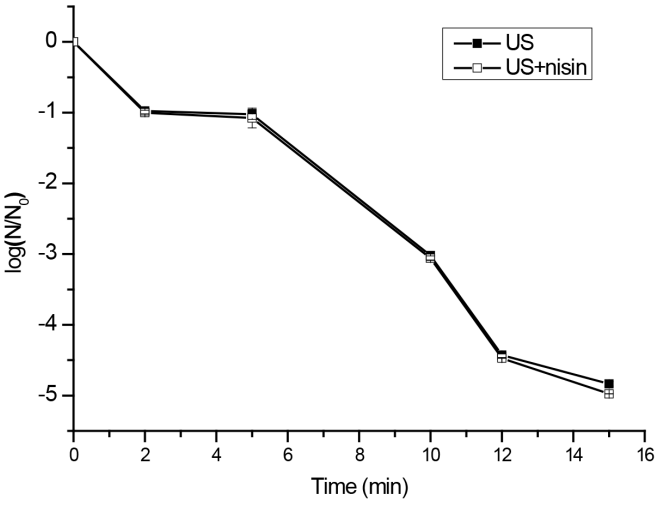

Figure 5 | Inactivation of *Staphylococcus aureus* treated by US and US + nisin at 25°C and 968.16 W/cm^2 in milk.

Moreover, the reduction of *S. aureus* in NB was close to that in milk by nisin. Different results were obtained by others [9,24]. Yu et al. [24] found that the reduction of *S. aureus* cells in litchi juice increased with increasing temperature from 30 to 45°C when exposed to 200 IU/mL nisin for 1 h. Antolinos et al. [9] found the antimicrobial effect of nisin (0.13 μmol/L) on *Bacillus cereus* was more evident at 16°C than at 25°C. Therefore, the influence of temperature on nisin's inactivation effectiveness was controversial. In our study, nisin caused a maximum reduction of 1.40 $\log_{10}$ cycles on *S. aureus*, indicating only part of the *S. aureus* cells were sensitive to nisin while the other part was quite resistant.

The inactivation of *S. aureus* by US increased with increasing the treatment power density. Similarly, the inactivation of *S. aureus* by US was found to be dependent on the amplitude levels [13,25]. This was probably due to the enhancement of damaging the cell wall and membrane under a greater amplitude value [26]. Hence, sensitivity of the *S. aureus* might be increased with higher treatment power, leading to an increase in death of the microorganism. In addition, the inactivation efficiency is also related to the treatment temperature. The inactivation effect on *S. aureus* by US was greatly enhanced with increasing the treatment temperature. The combination of US and heat could achieve a higher degree of bacterial inactivation, which was greater than the additive effect of the two treatments considered separately [25,27]. In our study, application of a mild heat at sublethal temperatures of 25 and 37°C with US at 968.16 W/cm^2 for 15 min could improve the inactivation of *S. aureus* from 1.8 $\log_{10}$ cycles (0°C) to 4.77 and 5.87 $\log_{10}$ cycles, respectively. Therefore, the combination of US and mild heat could possibly meet the Food and Drug Administration's (FDA's) requirement to achieve a 5-log reduction on pathogens for a promising food processing.

The reduction of *S. aureus* in milk was significantly greater than that in NB, which might be due to the differences in constituents of the two medium. Milk had a more complex composition (e.g. proteins, fats, sugars) while NB was mainly proteins. Normally, inactivation of microorganisms was more difficult in complex food materials than in simple buffer solutions [28]. A higher inactivation on bacteria in simple buffer solution was obtained than in food matrix was found by others [29]. However, our results were not in agreement with these findings, which might be caused by two reasons. Firstly, the presence of fats in milk might reflect or absorb sound waves, resulting in the improvement of cavitation intensity [30,31]. Secondly, NB was the optimum medium for *S. aureus*, which might have strong protective effects on the cells.

Inactivation of *S. aureus* by US, nisin and US + nisin followed two-stage kinetics or three-stage kinetics. Similarly, non-linear inactivation curves by US have been reported for the inactivation of *Listeria monocytogenes* [27,32], *Escherichia coli* [32–34], *Shigella* [35], and *Saccharomyces cerevisiae* [36]. Lee et al. [37] reported that the inactivation of *E. coli* K12 in phosphate buffer (0.01M, pH 7) by thermosonication (20 kHz, 61°C) exhibited a non-linear curve, including a fast initial inactivation followed by a slow reduction in microbial survival counts. The non-linear inactivation by sonication treatments could be explained by the vitalistic concept [38]. The vitalistic approach assumed that individual cells with different resistance to a lethal treatment existed in a population, which formed the foundation of log-logistic models to describe non-linear inactivation curves including tailing-off [39]. Cells that are sensitive to US treatment are

likely to be inactivated first, resulting in a rapid inactivation period, while cells with higher resistance to ultrasound are slowly killed and cause tailing-off [38]. Besides, the sterilization curve was also related to the number of experimental data and the choice of experimental conditions. When the data was small or the processing time was short, it would appear as two-stage kinetics, but when the experimental data was enough and the processing time was long enough, sterilization curve might be expressed as three-stage kinetics [40].

Under the most treatment conditions of this study (except for 968.16 W/cm^2 and 25°C for 10 min), the inactivation of US + nisin on *S. aureus* only showed the greater values between US and nisin treatments. The results indicated that US and nisin had no added or synergistic inactivation effect on *S. aureus* suspended in both NB and milk. It is, therefore, likely that the inactivation of *S. aureus* by US may be an "all or nothing" event: few or no injured but only alive or dead cells existed in the suspension medium treated by US [41]. Therefore, in the quest to achieve more effective pasteurization treatments, further studies on the combinations of US with other antimicrobials, or alternative technologies, will need to be conducted.

5. CONCLUSION

There was no significant difference in *S. aureus* reduction by nisin under different treatment temperatures. But the inactivation of *S. aureus* by US was increased with increasing the treatment power and temperature. A maximum reduction of 5.87 $\log_{10}$ cycles on *S. aureus* in NB was obtained by US at 968.16 W/cm^2 and 37°C for 15 min, which met the FDA's requirement of 5-log reduction on pathogen for a food processing technology. The reduction of *S. aureus* by US in milk was greater than in NB, which further confirmed that US was a promising technology in milk processing. However, the reduction of *S. aureus* by US + nisin was similar to that obtained by either nisin or US alone, indicating that US and nisin had no added or synergistic inactivation effect on *S. aureus* in both NB and milk under the tested conditions. Therefore, studies on the combination of US and other antimicrobials rather than nisin will need to be further conducted to improve inactivation effect.

CONFLICTS OF INTEREST

All authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflicts of interest.

AUTHORS' CONTRIBUTION

YS contributed to the data analysis, and critical revision of the manuscript. YL contributed to experimental design, samples and data collection, and the drafting of the manuscript. LC contributed to data analysis, figures plots, drafted the manuscript and revised it critically for important intellectual content. XB designed the study and contributed to the editing of the manuscript. LL and ZC contributed in supervision and project administration.

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