Effective control of antibiotic resistance using a sonication-based combinational treatment and its application to fresh food

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ABSTRACT

Antibiotics have been widely used to treat several infectious diseases. However, the overuse of antibiotics has promoted the emergence and spread of antibiotic resistant bacteria (ARB) in various fields, including the food industry. In this study, the antimicrobial efficacies of two conventional sterilization methods, mild heat, and sonication, were evaluated and optimized to develop a new strategy against ARB. Simultaneous mild heat and sonication (HS) treatment led to a significant reduction in viable cell counts, achieving a 5.58-log reduction in 4 min. However, no remarkable decrease in viable cell counts was observed in individually treated groups. Interestingly, the release of antibiotic resistance genes (ARGs) increased in a time-dependent manner in the heat-treated and HS-treated groups. The inactivation levels of ARGs increased as the HS treatment time increased from 2 to 8 min, and most ARGs were degraded after 8 min. In contrast, no significant inactivation of ARGs was observed in the heat-treated and sonication-treated groups after 8 min. These results reveal the synergistic effect of the combination treatment in controlling not only ARB but also ARGs. Finally, on applying this newly developed combination treatment to fresh food (cherry tomato and carrot juice), 3.97- and 4.28-log microbial inactivation was achieved, respectively. In addition, combination treatment did not affect food quality during storage for 5 days. Moreover, HS treatment effectively inactivated ARGs in fresh food systems.

1. Introduction

Antimicrobial resistance (AMR) causes serious medical problems worldwide and is one of the greatest threats to public health [1,2]. Globally, pathogens with AMR caused 700,000 deaths per year as of 2019, with 10 million deaths per year being expected by 2050; this number is even higher than the 8.2 million deaths per year caused by cancer at present [3,4]. Antibiotics have been widely used in the medical industry and animal husbandry since the invention of penicillin in 1927; however, the excessive use of antibiotics leads to bacterial resistance and stimulates the emergence of multidrug-resistant (MDR) bacteria. In particular, food production industries, such as livestock and fisheries, are considered the main reservoirs of MDR bacteria [5,6]. Antibiotic resistant bacteria (ARB) can be easily transferred to the food chain through the contamination of soil and water resources by animal feces [7]. In addition, due to rapid globalization, ARB can spread across countries by food transportation. Therefore, it is necessary to develop an efficient technology that can control antibiotic resistance in the food industry.

At present, various physical treatments (e.g., ultraviolet radiation, heat, and cold plasma) have been proposed as solutions to control various foodborne pathogens, including ARB, in the food system [8,9]. For example, recent studies have reported that a combination of physical treatments may lead to the synergistic inactivation of various pathogenic bacteria, including *Escherichia coli*, *Listeria* spp., and *Staphylococcus aureus* [9–19]. However, during bacterial inactivation, nucleic acids can be released from the dead bacteria into the food environment. Some of these genes can be antibiotic resistance genes (ARGs) and can remain undestroyed. Finally, these ARGs can be horizontally transferred to other bacteria, resulting in the emergence of new ARB [20,21]. However, the development of technologies to control various antibiotic resistance factors related to ARB is often neglected. Although some studies have been conducted to monitor ARB distribution and reduce the horizontal gene transfer (HGT) of released ARGs, strategies to inactivate both ARB and ARGs have not been widely studied so far [6,17,18,22–25].

Therefore, in the present study, we assessed the efficacy of conventional sterilization methods in controlling ARB. In addition, we aimed to

develop a new combination treatment for effectively controlling both ARB and ARGs. Usually, excessive physical treatments can affect food quality [26]. Therefore, we tried to minimize the physical treatments by applying them in combination [10]. Finally, we applied our novel method to fresh food (cherry tomato and carrot juice) to evaluate its efficacy as a potential sterilization method.

2. Materials and methods

2.1. Reagents

Plasmids were extracted using the FavorPrep™ Plasmid DNA Extraction Mini Kit (FAVORGEN Biotech Corp., Taiwan). DNA-Staining Bandi Load (6 ×) was purchased from Smartgene (SJ Bioscience, Daejeon, Korea). The ExcelBand™ 1 KB DNA ladder (DM3100; SMOBIO Technology) was used as the DNA size marker. Moreover, 50 × Tris-acetate-EDTA (TAE) buffer and 20 × phosphate-buffered saline (PBS) were purchased from Dongin Biotech (Seoul, Korea). Ampicillin sodium and kanamycin sulfate monohydrate were purchased from Duchefa (Duchefa Biochemie, Netherlands). Luria–Bertani agar (LB agar) and LB broth were purchased from Difco (Miller, BD, Franklin Lakes, NJ, USA).

2.2. Bacterial strains, plasmids, culture methods, and bacterial enumeration

E. coli DH5α cells containing the ampicillin resistance plasmid (Amp^r plasmid) pYD1 (ampicillin-resistant *E. coli*: ARE) and those containing the kanamycin resistance plasmid (Kan^r plasmid) pET29b (kanamycin-resistant *E. coli*: KRE) were used as the model bacterial strains in the present study. The use of ARE and KRE cells enabled selective bacterial culture in ampicillin- and kanamycin-containing growth media, respectively. Each bacterial strain was routinely grown aerobically in LB broth in the presence of the appropriate antibiotics (50 μg/mL of ampicillin or 50 μg/mL of kanamycin, final concentration) at 37 °C for 12 h. After cell harvesting, the bacterial cultures were diluted to 10^6–10^7 CFU/mL and subjected to antibacterial treatments. After 10-fold serial dilution, residual bacterial counts were determined using the standard plate counting method by incubation at 37 °C for 24 h.

2.3. Assessment of antimicrobial efficacy and nucleic acid release

2.3.1. Antimicrobial efficacy test

Two conventional physical treatments, mild heat (55 °C) and sonication, were used either alone or in combination (HS) for the microbial inactivation test. Treatment conditions (temperature and time) were determined based on the previous results (data not shown). A laboratory-scale water bath (WB-11GDN, Samheng Scientific, Korea) was used for the mild heat treatment, and a low-frequency (40 kHz) bath sonicator (power density range, 300–400 W; Powersonic 410, Hwashin Technology, Korea) was used for the sonication treatment [27,28]. The water temperature was monitored and maintained at room temperature (25 °C) by continuously adding cold water during the treatments. For the combination treatment (HS), the water tank temperature of the bath sonicator was set at 55 °C and ultrasound was performed simultaneously. Overnight grown ARE cells were harvested by centrifugation at 12,300 × g for 2 min, and the cell pellet was washed with 1 × PBS. A bacterial sample (1 mL) was prepared by dilution with 1 × PBS to reach an optical density (OD) of 1. Each physical treatment was performed in 3 mL glass vials for 0, 0.5, 1, 2, and 4 min. Samples were collected at the indicated time points and plated on LB agar plates containing the appropriate antibiotics. The counts of viable bacterial cells were determined after incubation at 37 °C for 24 h. All experiments were performed in triplicates.

2.3.2. Nucleic acid release assay

A nucleic acid release assay was performed to determine the number of ARGs released from damaged cells after each physical treatment [10,11]. In brief, ARE cells were treated with mild heat, sonication, and HS under the same conditions as those in the antimicrobial efficacy test. All the treated samples were centrifuged at 12,300 × g for 2 min to remove residual cells and to obtain nucleic acid-containing supernatants. Nucleic acid concentrations were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) at 260 nm. The supernatant without any treatment was used as a negative control. All experiments were performed in triplicates. Finally, the results of the nucleic acid release test were compared with those of the antimicrobial efficacy test.

2.4. Inactivation of ARGs

2.4.1. Assessment of nucleic acid cleavage by agarose gel electrophoresis

Agarose gel electrophoresis was performed to assess the cleavage of ARGs following physical treatments (mild heat, sonication, and HS). In brief, 50 μL PBS containing pYD1 and pET29b plasmids (10 μg/mL, final concentration) was subjected to each physical treatment for 0, 2, 4, 6, and 8 min. This treatment time was determined based on the results of section 3.1. Nucleic acid damage was assessed by loading 50 ng of each DNA sample on 1 % agarose gel and visualized using a Blue Light LED illuminator gel documentation system (Fast Gene FAS-Digi PRO, Bionics, Korea).

2.4.2. Assessment of nucleic acid integrity by heat shock transformation

To assess plasmid integrity after the physical treatments, heat shock transformation was performed with some modifications [29]. In brief, physically treated pYD1 and pET29b plasmids (50 ng, final concentration) were mixed with 200 μL of competent *E. coli* DH5α cells and incubated on ice for 20 min. After incubation, the cells were subjected to heat shock for 80 s at 42 °C, chilled on ice for 1 min, and recovered by adding 1 × LB broth. The cells were plated on LB agar containing the appropriate antibiotics, and the numbers of transformant cells were counted after incubation at 37 °C for 24 h.

2.5. Food application

2.5.1. Inactivation of ARB in fresh food

Cherry tomato (*Lycopersicon esculentum* var. *cerasiforme*) and sterilized carrot juice were purchased from a local market. ARE cells (cell inoculum, 2.0×10^8 CFU/mL) were used to artificially contaminate the food samples. For preparing the tomato sample, approximately 20 g and 3.27 ± 0.25 cm in diameter of tomatoes were rinsed with 70 % ethanol and air dried under UV light at room temperature (25 ± 1 °C) for 15 min to remove indigenous bacteria. The tomato samples were spiked with the cell inocula, followed by air drying for 40 min on a clean bench [30]. After drying, combination treatment (HS) was performed for 0, 4, 6, 8, and 10 min. This treatment time was determined based on the results of section 3.3 and 3.4. For preparing artificially contaminated carrot juice, 10 mL of carrot juice was aseptically transferred to a glass vial and 100 μL of cell inoculum was added to it. Following this, HS treatment was performed for 0, 2, 4, 6, and 8 min. This treatment time was determined based on the results of section 3.3 and 3.4. Viable cell counts were determined using the plate counting method, as described in section 2.3.1. All experiments were performed in triplicates.

2.5.2. Inactivation of ARGs in fresh food

Agarose gel electrophoresis was performed to assess the cleavage of ARGs following combination treatment (HS) in fresh food systems (cherry tomato and carrot juice). In brief, approximately 2.5 g of tomato samples were placed in 10 mL PBS containing pYD1 plasmid (5 μg/mL, final concentration) followed by HS treatment for 0, 10, and 14 min. This treatment time was determined based on the results of section 3.5. For carrot juice, 5 mL of carrot juice was aseptically transferred to a glass vial and 0.025 mg of pYD1 plasmid (5 μg/mL, final concentration) was
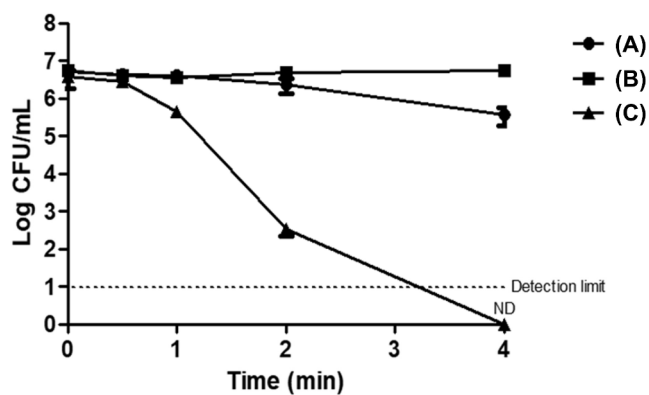
Fig. 1. Microbial inactivation efficacy of physical treatments. Antimicrobial efficacy of individual physical treatments and combinational treatment (0, 0.5, 1, 2, and 4 min) against antibiotic resistant *Escherichia coli* (pYD1/*E. coli*). (A) Mild heat treatment at 55℃, (B) sonication treatment, and (C) simultaneous treatment of mild heat and sonication (HS), respectively. ND: Not detected.

added to it and performed by HS treatment for 0, 8, and 12 min. This treatment time was determined based on the results of section 3.5. Determination of nucleic acid damage was assessed as described in section 2.4.1. In addition, to assess plasmid integrity after the physical treatment (HS), heat shock transformation and microbial enumeration was performed as described in section 2.4.2.

2.6. Food quality analysis

Food color was measured using a colorimeter (CR-400, Minolta, Co., Tokyo, Japan) at room temperature (25 ± 1 °C) as described previously, with slight modifications [12,31]. The colorimeter was calibrated using white standard tiles, and the results were expressed as $L*$ (whiteness/darkness), $a*$ (redness/greenness), and $b*$ (yellowness/blueness). Color values of cherry tomatoes were determined by measuring three locations on each sample surface. Color values of carrot juice were measured by filling a Petri dish (35 × 10 mm) with 10 mL of carrot juice. Nine readings were obtained from three cherry tomato samples and one carrot juice samples after HS treatment. Changes in the firmness of cherry tomatoes were assessed using a TA-XT2 texture analyzer (Stable Micro System, Godalming, UK) equipped with a cylinder probe of 2 mm and set at 1 mm/s and 10 mm, as described previously [32]. The maximum peak force was recorded as firmness, and the results were expressed as Newton (N). Color and texture analysis for all food samples were assessed at 0, 1, and 5 days after the treatments, respectively. All experiments were performed in triplicates.

3. Results and discussion

3.1. Microbial inactivation efficacy

The physical treatments used in the present study were mild heat and ultrasonic waves. Of the various conventional sterilization treatments, these two treatments have been mainly used during food processing [33–36]. Previous studies have revealed that sonication with high heat (61 °C, thermosonication) provided enhanced antimicrobial effects [13,23,37]. However, the synergistic antimicrobial activity of mild heat (55 °C) and sonication (40 kHz) has not been investigated so far. Fig. 1 shows the antimicrobial efficacy of mild heat, sonication, and their combination (HS) against ARE. HS treatment resulted in significant bacterial inactivation to nondetection levels, achieving a reduction of 5.58 log in viable ARE in 4 min ($P < 0.05$). However, individual treatments (mild heat or sonication) had no significant ($P > 0.05$) effect and resulted in a reduction of 1.14 log CFU/mL and 0.01 log CFU/mL, respectively. Previously, ultrasound-based combination treatments,
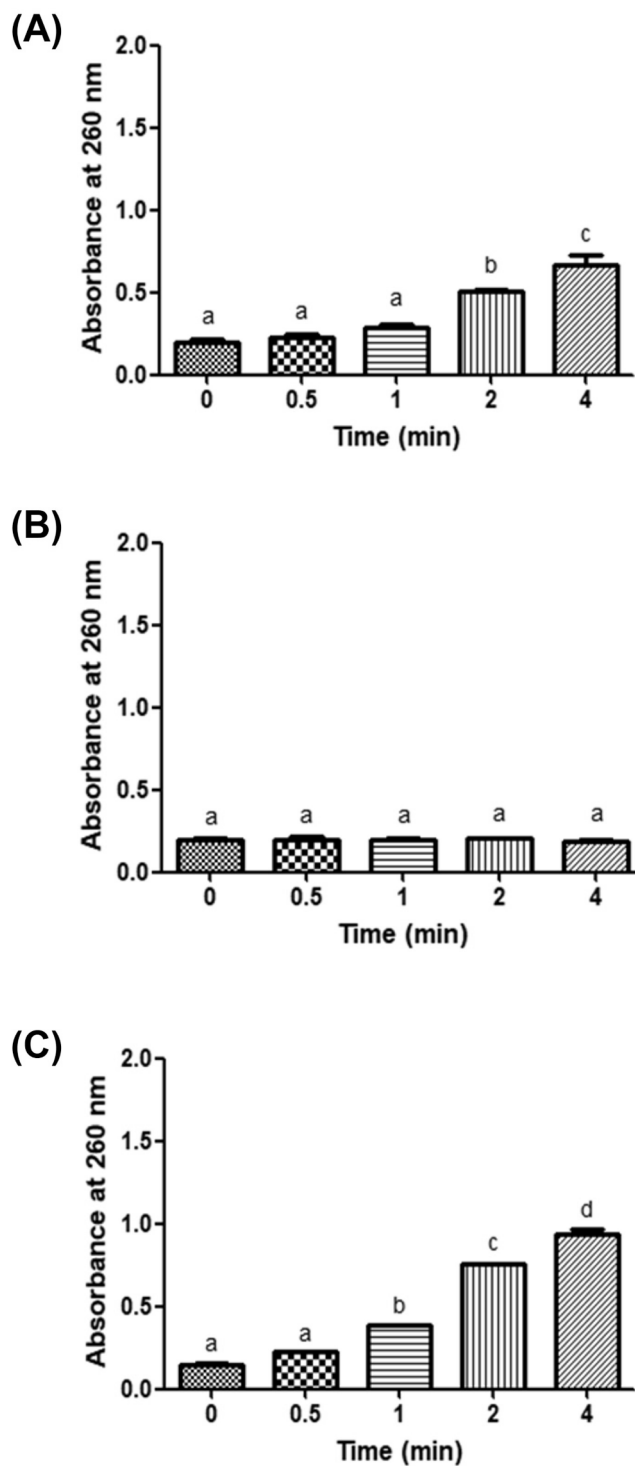

Fig. 2. Nucleic acid release assay. Confirmation of release of antibiotic resistance genes (ARGs) from antibiotic resistant *Escherichia coli* after physical treatments (0, 0.5, 1, 2, and 4 min). (A) Mild heat treatment at 55℃, (B) sonication treatment, and (C) simultaneous treatment of mild heat and sonication (HS), respectively. Different letters above the bars represent significant differences ($P < 0.05$) among the treated groups, as assessed using Duncan's new multiple range test.

particularly those in combination with chemical reagents, have been widely used to control microbial contamination. For example, a combination of low-frequency ultrasound (LFU) and a food-grade antioxidant, propyl gallate (PG), significantly (>4 log CFU/mL) inactivated *E. coli* O157:H7 [14]. In addition, ultrasound combined with ozone
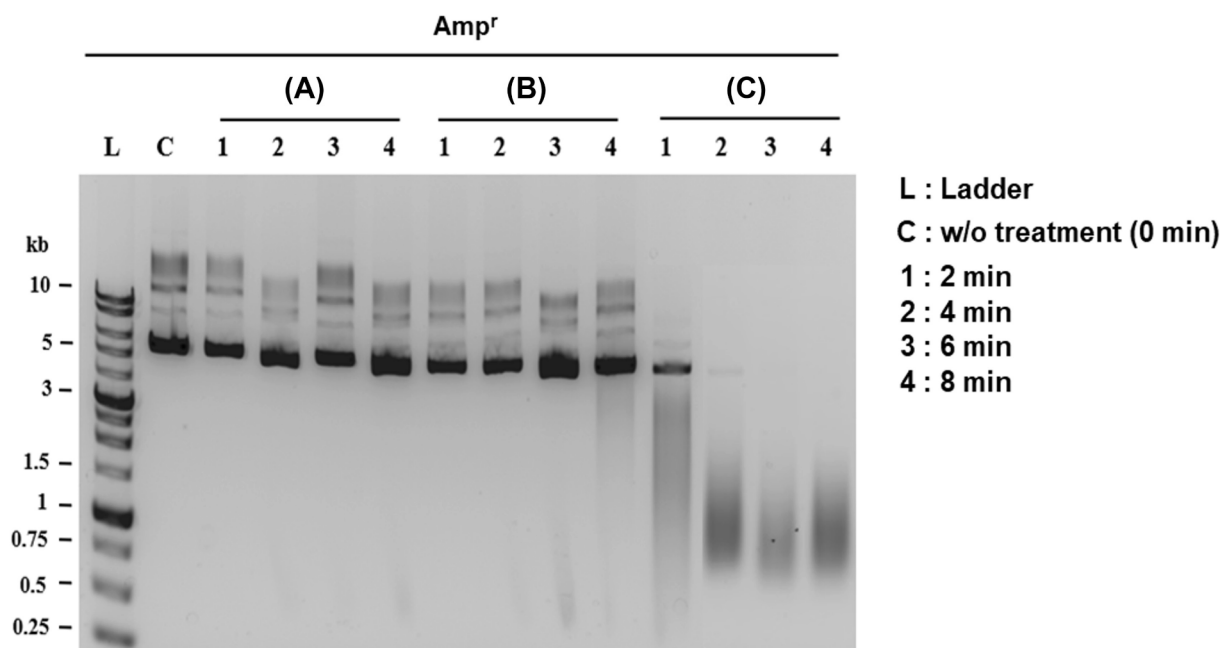
Fig. 3. Agarose gel electrophoresis. Gel electrophoresis image indicates the degree of inactivation of antibiotic resistance genes (ARGs) by physical treatments. (A) Mild heat treatment at 55℃, (B) sonication treatment, and (C) simultaneous treatment of mild heat and sonication (HS), respectively. Amp^r: ampicillin resistant plasmid pYD1.

pretreatment effectively inactivated enteric indicator bacteria [24]. Our result clearly revealed effective antimicrobial activity of the novel combination of the two physical treatments without using chemical compounds. In general, ultrasound treatment causes cavitation bubbles in the liquid phase. The violent collapse of these bubbles can generate high shearing forces and free radicals, leading to membrane disruption, pore formation, and eventual breakage or fragmentation [38–44]. Cavitation-induced reactive oxygen species generation is one of the most frequently described bactericidal mechanisms of ultrasound treatment [14]. We speculated that the high antimicrobial efficacy of HS treatment against *E. coli* could be because gram-negative bacteria have a thinner cell wall than gram-positive bacteria, providing less protection to the cell membrane against ultrasonic effects [45,46]. Moreover, membrane damage caused by mild heat may enhance the cavitation effect of sonication on the *E. coli* cell membrane [47–50]. Thus, our results indicate that HS has the potential to be developed as a low-energy (mild heat) and ecofriendly (without chemical treatment) sterilization strategy in the food industry.

3.2. Nucleic acid release

In general, physical sterilization methods induce pore formation in the bacterial membrane, which can result in the leakage of cytoplasmic components, including nucleic acids [38,42,51–53]. Therefore, the level of nucleic acids released after individual treatments (mild heat or sonication) and combined treatment (HS) was assessed (Fig. 2). Compared with the control (blank) sample, nucleic acids were not released in the bacterial cell samples treated with sonication only ($P < 0.05$). In contrast, significantly increased nucleic acid release was observed over time in the bacterial cell samples treated with mild heat alone or HS ($P < 0.05$). Interestingly, statistical comparison revealed that the combination treatment (HS) significantly increased the leakage of nucleic acids in comparison with mild heat treatment alone ($P < 0.05$). These results are in accordance with those of the synergistic antimicrobial activity of HS. According to previous studies, mild heat treatment increases bacterial cell membrane permeabilization by removing exopolysaccharides, a major component of extracellular polymeric substances [10,16]. In addition, ultrasound treatment generates highly reactive

radicals that can destroy bacterial cell walls and cause the release of intracellular ATP and nucleic acids [11,17]. Thus, the significant increase in the release of nucleic acids in the HS-treated group correlated with the synergistic antimicrobial activity of the combined HS treatment (Fig. S1).

3.3. Agarose gel electrophoresis

Under stressful conditions, ARB lose their membrane integrity. Consequently, cytoplasmic contents are released and DNA is leaked out of the cell [18]. The released ARGs can be absorbed by other bacteria, resulting in further spread of ARGs in the environment [54]. Therefore, we assessed whether the physical treatments (individual and in combination) could inactivate the released ARGs. The degree of inactivation of two antibiotics (ampicillin and kanamycin) resistance factors by individual treatment (mild heat or sonication) and combination treatment (HS) was assessed through agarose gel electrophoresis. HS treatment for >6 min inactivated most resistance factors under *in vitro* conditions; the inactivation mechanism involved DNA cleavage (Fig. 3 and S2). We also confirmed that some double-stranded DNA was denatured into single-stranded DNA when treated with mild heat alone (Fig. 3 and S2). In a previous study, treatment of wastewater with ultrasound alone (180 min) did not significantly reduce ARGs (*sul*1, *sul*2, *tet*W, *tet*M, and *amp*) and the integrase-encoding gene *intI*1 under the following experimental conditions: 150 W, 53 kHz. However, combination treatment with ultrasound and O_3 showed higher inactivation efficacy than ultrasound treatment alone [17]. In addition, combination treatment with UV and H_2O_2 decreased the abundance of *bla*$_{TEM}$ after the inactivation of *E. coli* [25]. Moreover, the photo-Fenton process (2.8 mg/L of 0.5 mM Fe^{2+}, 340.2 mg/L of 10 mM of H_2O_2, and 19.2 mW/cm^2 of visible LED light irradiation) increased the degradation of extracellular ARGs (*tet*A and *bla*$_{TEM-1}$) after 10 min of treatment, with higher doses eventually degrading the DNA into tiny pieces [18]. Collectively, when ultrasound is used to control ARG, it should be combined with other appropriate treatment methods. In particular, our data revealed that a longer HS treatment time (over 6 min) is required to completely inactivate ARGs than to completely inactivate ARB (4 min). We speculated that the underlying mechanism of synergistic effect may be due to DNA
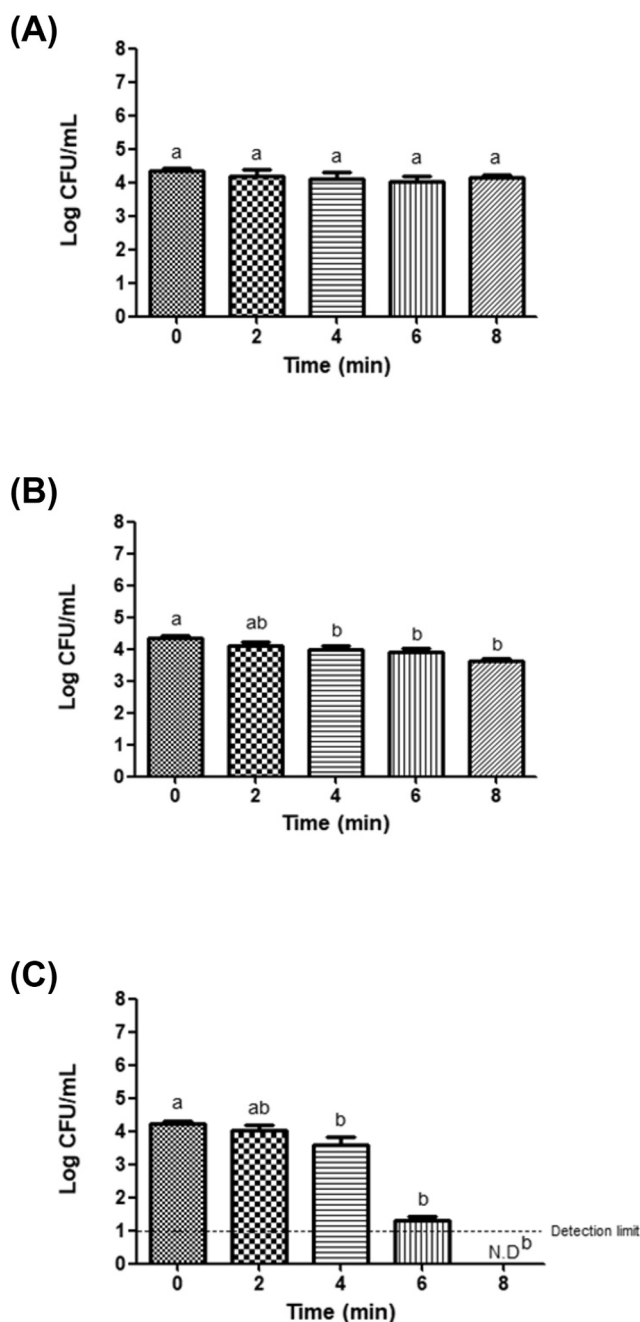
(A)

(B)

(C)

Fig. 4. Horizontal gene transfer efficacy test. The horizontal gene transfer efficiency of physically treated antibiotic resistance genes (pYD1: ampicillin resistant) into normal *Escherichia coli* cells (DH5α). (A) Mild heat treatment at 55℃, (B) sonication treatment, and (C) simultaneous treatment of mild heat and sonication (HS), respectively. Different letters above the bars represent significant differences ($P < 0.05$) among the treated groups, as assessed using Duncan's new multiple range test.

denaturation by heat and physical cleavage by sonication [55,56].

3.4. HGT efficacy

Antibiotic resistant *E. coli* strains are widely present in various foods, indicating the prevalence of HGT in the food system [57]. We assessed and compared the impact of individual treatments (mild heat or sonication) and combinational treatment (HS) on the transfer of ARGs (Amp[r] plasmid and Kan[r] plasmid) to normal *E. coli* cells, which did not contain the resistance genes (Fig. 4 and S3). Overall, the transfer efficacy of the

Amp[r] plasmid was higher than that of the Kan[r] plasmid. The rate of HGT significantly ($P < 0.05$) decreased after 8 min of HS treatment for both ARGs. A previous study reported that plasmids (containing *tet*A, *sul*2, and *bla*CTX-M or *bla*CMY) could be transferred by conjugation from antibiotic resistant *E. coli* cells when heated at 60 °C (high temperature) for > 10 min [57]. However, treatment with sonication alone did not protect the transfer of ARGs (*tet*G, *tet*O, *tet*W, *sul*I, and *sul*II) and the integrase-encoding gene *int*I1 (an indicator of HGT) [24]. We found that our newly developed combination treatment (HS) required lesser time (8 min) and lower temperature (55 °C) than the individual treatments for the complete inactivation of ARGs and thus inhibited the transfer of ARGs. Therefore, HS has the potential to be used as a new sterilization method to control both ARB and ARGs.

3.5. Inactivation of ARB in fresh food

The combinational treatment (HS) resulted a 3.97-log reduction in viable ARE counts in 8 min and 4.28-log reduction in viable ARE counts in 6 min in the cherry tomato (Fig. 5A) and carrot juice (Fig. 5B) samples, respectively. Previously, the antimicrobial activity of sonication-based combination treatments has been assessed by many research groups. For instance, treatment with sonication (40 kHz, 30 min) in combination with antimicrobial compounds (3 % lactic acid and 0.1 % nisin) effectively reduced both *L. monocytogenes* and *E. coli* O157:H7 counts below the detection limits in *enoki* mushrooms [19]. In addition, treatment with 1 % ascorbic acid in combination with thermosonication at 50 °C for 30 min effectively inhibited both *E. coli* and *L. monocytogenes* in soft persimmon juice [58]. Interestingly, sonication combined with heat treatment usually showed enhanced antimicrobial efficacy in a short time. For example, increased microbial inactivation was observed in thermosonication (heat and sonication)-treated juices of hog plum, carrot, grapefruit, and orange. Most thermosonication treatments at 50 °C and 60 °C resulted in no detectable increase in the bacterial count in juices within 5 to 30 min [38,59–61]. This result is similar to our result that combined HS treatment could result in complete microbial inactivation in fresh food within 6–8 min. Ultrasonic cavitation can easily affect the heat-shocked cell structure and result in increased damage to bacteria and bacterial enzymes [48,50,62]. However, excessive heat treatment over 55 °C can limit bubble formation by ultrasonic cavitation, which is one of the hurdles that needs to be considered during the development of ultrasound-based technology [50,63,64]. We speculated that the synergistic antimicrobial activity of HS treatment is associated with the cavitation of cellular structures that can further destabilize and disrupt the *E. coli* membrane, in combination with heat-induced cell membrane damage [38,50].

3.6. Inactivation of ARGs in fresh food

ARGs inactivation of mild heat (55 °C) and sonication (40 kHz) in fresh food systems has not been investigated yet. We assessed whether the combination treatment (HS) could inactivate the ARGs in fresh food systems. The degree of inactivation of ARG (Amp[r] plasmid) by HS treatment was determined. HS treatment for > 14 and 12 min efficiently cleaved ARG in cherry tomato and carrot juice samples, respectively (Fig. 6A and B). Following transformation test clearly showed that the HGT rates were significantly ($P < 0.05$) decreased after 14 and 12 min of HS treatment for each fresh food sample (Fig. 6C and 6D). Consequently, the synergistic inactivation of ARG was also observed in foods as in the buffer system (Figs. 3 and 6), again supported the potential use of HS as a new sterilization method to control of ARGs in food system.

3.7. Evaluation of color and firmness

Ultrasound is an emerging food processing technology that has been developed to minimize processing, maximize quality, and ensure the safety of food products [53]. Recently, studies have been conducted to
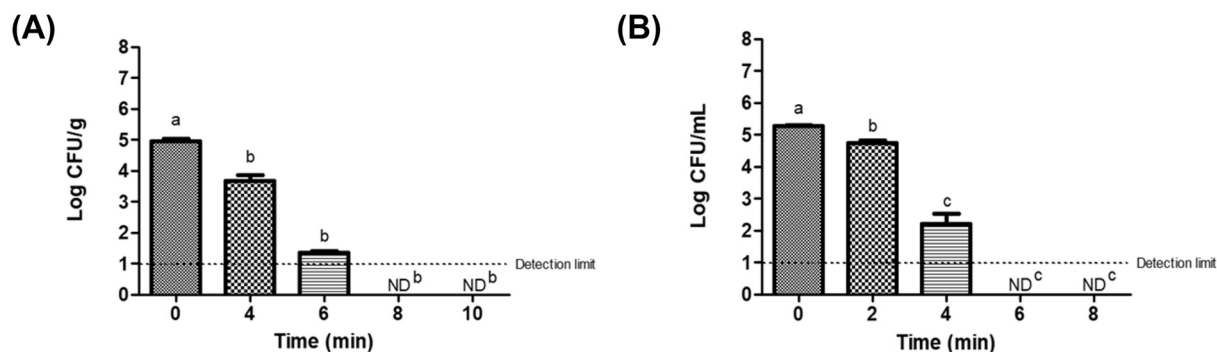
Fig. 5. Microbial inactivation efficacy of HS treatment in foods. Antimicrobial efficacy of combinational treatment [mild heat and sonication (HS)] (0, 2, 4, 6, 8, and 10 min) on *Escherichia coli*-contaminated foods. (A) Cherry tomato and (B) carrot juice, respectively. Different letters above the bars represent significant differences ($P < 0.05$) among the treated groups. Statistical significance of the means was assessed by Duncan's new multiple range test using SPSS software. ND: Not detected.
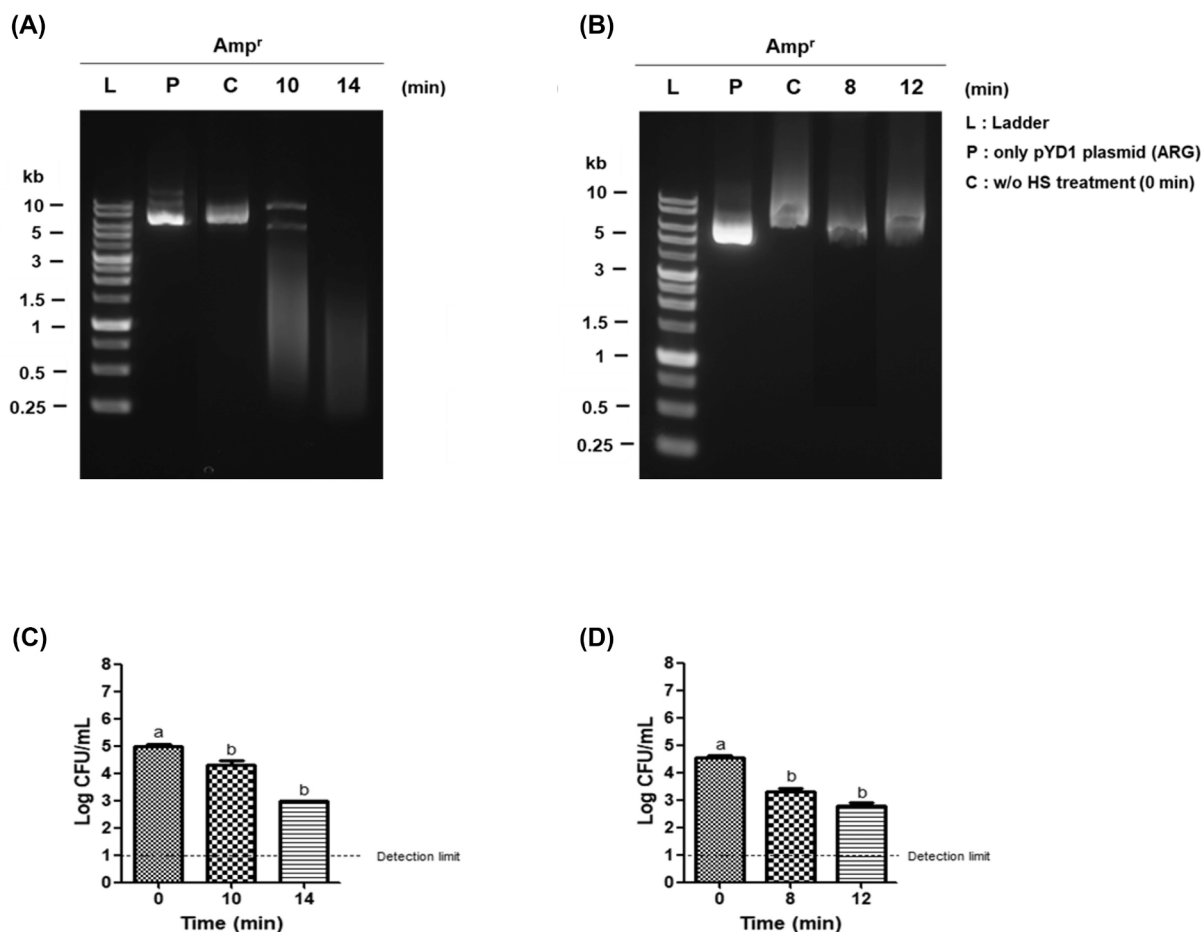

Fig. 6. Inactivation of antibiotic resistance genes (ARGs) in foods. Gel electrophoresis image indicates the degree of inactivation of antibiotic resistance genes (ARGs) by combination treatment [mild heat and sonication (HS)] (0 to 14 min) in (A) cherry tomato and (B) carrot juice, respectively. Horizontal gene transfer efficacy after the HS (0 to 14 min) in the presence of (C) cherry tomato and (D) carrot juice, respectively. Amp^r: ampicillin resistance plasmid pYD1. Different letters above the bars represent significant differences ($P < 0.05$) among the treated groups, as assessed using Duncan's new multiple range test.

improve the quality of fresh food while controlling pathogens using various combination treatments involving ultrasound [12,32,38,50,60]. Color is a vital parameter for assessing the microbial safety and sensory quality of juice [38,50]. The texture (firmness) of fruits, such as cherry tomatoes, is one of the major parameters for assessing the quality of the fruits, especially for consumers [65]. Therefore, in the present study, changes in the quality parameters (color and texture) of fresh food (cherry tomato and carrot juice) were assessed during the storage period after the newly developed HS treatment (Table 1 and S1).

Changes in color values were analyzed using the Hunter color system. In both cherry tomato and carrot juice samples, no significant ($P > 0.05$) differences in L^*, a^*, and b^* values were observed during the storage period after HS treatment (Table 1). Previously, high heat treatment (98 °C, 10 min) of fruits and vegetables (e.g., cherry, nectarine, apricot, peach, plum, carrot, and red bell pepper) was found to result in the release of anthocyanins or carotenoids, which may be responsible for the higher a^* or b^* values [66]. In addition, carrot slices treated with thermosonication at different frequency modes exhibited a

Table 1

Evaluation of color for 5 days storage at 4℃ after HS treatment. Effect of combination treatment [mild heat and sonication (HS)] (0, 6, 8, and 10 min) on (A) color parameters (L^*, a^*, and b^*) of cherry tomato and (B) color of carrot juice. All values are the means ± standard deviations (n = 12) of three replicates.

(A)

Storage time (days)	Control			HS (8 min)			HS (10 min)		
	L^*	a^*	b^*	L^*	a^*	b^*	L^*	a^*	b^*
0	37.32 ± 0.47[a]	24.80 ± 0.44[a]	18.64 ± 0.47[a]	37.29 ± 0.26[a]	24.76 ± 0.57[a]	18.57 ± 0.25[a]	37.21 ± 0.21[a]	24.76 ± 0.41[a]	18.54 ± 0.56[a]
1	37.04 ± 0.28[a]	24.73 ± 1.01[a]	19.26 ± 0.47[a]	36.98 ± 0.13[a]	24.79 ± 0.66[a]	19.22 ± 0.81[a]	36.97 ± 0.21[a]	24.94 ± 0.65[a]	19.22 ± 0.82[a]
5	37.04 ± 0.35[a]	24.48 ± 1.07[a]	19.28 ± 0.24[a]	36.95 ± 0.23[a]	24.84 ± 0.36[a]	19.23 ± 0.38[a]	36.92 ± 0.27[a]	24.96 ± 0.67[a]	19.23 ± 0.62[a]

(B)

Storage time (days)	Control			HS (6 min)			HS (8 min)		
	L^*	a^*	b^*	L^*	a^*	b^*	L^*	a^*	b^*
0	44.52 ± 0.42[a]	14.91 ± 0.09[a]	17.87 ± 0.37[a]	44.52 ± 0.42[a]	14.91 ± 0.09[a]	17.87 ± 0.37[a]	44.51 ± 0.16[a]	14.94 ± 0.12[a]	18.10 ± 0.39[a]
1	43.81 ± 0.28[a]	14.56 ± 0.08[a]	17.79 ± 0.10[a]	43.83 ± 0.07[a]	14.57 ± 0.19[a]	17.85 ± 0.32[a]	43.83 ± 0.32[a]	14.62 ± 0.07[a]	17.94 ± 0.56[a]
5	43.73 ± 0.11[a]	14.55 ± 0.17[a]	17.76 ± 0.21[a]	43.78 ± 0.22[a]	14.57 ± 0.08[a]	17.82 ± 0.33[a]	43.79 ± 0.21[a]	14.59 ± 0.08[a]	17.89 ± 0.06[a]

[a] Within the column, values with same letter are not significantly different ($p < 0.05$).

yellower color than raw and sonicated carrot slices, with some exceptions (40 kHz and 22/33 kHz). The changes in a^* and b^* values of the carrot samples were closely related to the changes in total carotenoids [67]. In addition, thermosonication treatment increased L^* and b^* values at 40 °C and 50 °C. This increase could be associated with the partial precipitation of unstable suspended particles during cavitation, resulting in the induction of more colored compounds [38,60]. We found that HS treatment did not affect food color even after 8–10 min, which is the complete inactivation time for ARE (Fig. 6). Generally, Thermal and non-thermal technologies have been applied to inhibit enzyme function of which activity can affect food quality [68]. A previous study reported that thermal processing (55 °C for 10 min) inactivates 50 % of polyphenol oxidase (PPO) in strawberry [69]. In addition, ultrasound (20 kHz for 30 min) inactivated 63.7 % of PPO in satsuma mandarin extract [70]. Interestingly, thermo-sonication (25 kHz, 55 °C for 10 min) effectively inactivated 96 % of PPO in mushroom extract [71]. However, our results showed that no food quality changes were observed for 5 days storage in HS treated samples. Therefore, we cannot ensure whether HS treatment inactivated those enzyme activities in tested fresh food systems. Further analyses are required to elucidate the relationship between HS treatment and enzymatic activity. As shown in Table S1, no significant ($P > 0.05$) differences were observed between the control and treated samples, indicating that our HS treatment did not result in remarkable structural changes in cherry tomatoes during the storage period. In a previous study, ultrasound treatment at a density of 66.64 W/L had no effects on cherry tomatoes. The same result was observed after ultrasound treatment at an increased time and power level (167 W/L, 3 min) [65]. However, after multiple-frequency (e.g., dual-frequency or tri-frequency) ultrasound, a decrease in the firmness of cherry tomatoes was observed, which could negatively impact their quality [72]. Unlike the effect of excessive (e.g., high power or long duration) ultrasound treatment on cell wall stability, the HS treatment performed in the present study did not cause a significant loss of firmness because of the low frequency of sonication. Therefore, the HS treatment developed in the present study is beneficial for maintaining the firmness of cherry tomatoes while effectively controlling microbial contamination.

4. Conclusion

In the present study, an HS sterilization method was newly developed and successfully utilized for bacterial decontamination in fresh food (cherry tomato and carrot juice). Compared with individual treatments (mild heat or sonication), the combined HS treatment synergistically enhanced bacterial inactivation, resulting in a 5.58-log reduction in *E. coli* within 4 min. The release of ARGs significantly

increased with an increase in inactivation levels of ARB. In addition, an increase in treatment time from 4 to 8 min resulted in complete ARGs degradation and inhibited the HGT of ARGs. Thus, the present study revealed the synergistic effect of HS against both ARB and ARGs. Moreover, synergistic antimicrobial activity and ARGs inactivation by HS treatment were also observed in fresh food systems (cherry tomato and carrot juice) without any impact on the food quality, representing the potential application of HS in the food industry, especially for preparing minimally processed foods. Further optimization for scale-up and research on the effects of HS on nutritional values will be performed in the future.

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.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2022.106198.

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