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Ultrasound treatment combined with fumaric acid for inactivating foodborne pathogens in apple juice and its mechanisms

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ABSTRACT

The purpose of this study was to evaluate the synergistic bactericidal efficacy of combining ultrasound (US) and fumaric acid (FA) treatment against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* in apple juice and to identify the synergistic bactericidal mechanisms. Additionally, the effect of combination treatment on juice quality was determined by measuring the changes in color, pH, non-enzymatic browning index, and total phenolic content. A mixed cocktail of the three pathogens was inoculated into apple juice, followed by treatment with US (40 kHz) alone, FA (0.05, 0.1, and 0.15%) alone, and a combination of US and FA for 1, 2, 3, 4, and 5 min. Combined US and 0.15% FA treatment for 5 min achieved 5.67, 6.35, and 3.47 log reductions in *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively, with the 1.55, 2.37, and 0.57 log CFU reductions attributed to the synergistic effect. Although the pH value slightly decreased as FA increased, there were no significant ($P > 0.05$) differences in color values, browning indices, and phenolic content between untreated and treated samples. To identify the mechanism of this synergistic bactericidal action, membrane integrity, malfunctions in the membrane efflux pump, and intracellular enzyme activity were measured. The analyses confirmed that damage to the cell envelope (membrane integrity and efflux pump) was strongly related to the synergistic microbial inactivation. These results suggest that simultaneous application of US treatment and FA is a novel method for ensuring the microbial safety of apple juice.

1. Introduction

There has been a steady increase in the consumption of apple juice in the last three decades (USDA, 2012). The acidic pH of apple juice can inhibit contamination by and survival of foodborne pathogens. However, there have been several disease outbreaks caused due to the contamination of apple juice with pathogens, such as *Escherichia coli* O157:H7 and *Salmonella* spp. (Sung et al., 2014). Between 1995 and 2005, ten disease outbreaks were reported to the Centers for Disease Control and Prevention (CDC) in the United States caused due to the pathogen-contaminated apple juice (Vojdani et al., 2008). *E. coli* O157:H7 was first recognized as a pathogen in 1982 and is the causative agent of serious illnesses, such as hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Moreira et al., 2005). HUS is a life-threatening disease that is characterized by hemolytic anemia, thrombocytopenia, and renal insufficiency (Gould et al., 2009). Salmonellosis results from ingesting food contaminated with the members of the bacterial genus *Salmonella*. The symptoms of salmonellosis, which persist for an average of 9 days, include diarrhea, stomachache, fever, blood-tinged feces, and vomiting (Kim et al., 2007). The United States Food and Drug Administration (FDA) has issued a series of regulatory guidelines for fruit processing that include a minimum 5-log reduction of pathogen count in the processed fruit juice (U.S. FDA, 2001). There have been no reports of disease outbreaks that are associated with the contamination of fruit juice by *Listeria monocytogenes*. However, the FDA recommends that *L. monocytogenes* contamination should also be considered as a criterion for evaluating the safety of fruit juice (U.S. FDA, 2001).

Traditional thermal processing techniques are commonly used to extend the shelf-life of fruit juice (Tiwari et al., 2009). However, the emergence of non-thermal food processing technologies has ensured not only the microbial safety of food products but also the retention of desired nutritional value and quality of food products. Recently, ultraviolet (UV) light has been used as a non-thermal method for decontaminating apple cider. However, this method is mainly used by small producers because of its lower transmittance (Ugarte-Romero et al., 2006). The other alternative to thermal sterilization is the ultrasonic energy. The FDA recommends that the ultrasound (US) treatment can be an alternative to thermal pasteurization (U.S. FDA, 2000). Moreover, there has been an increase in the number of studies on the

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application of US processing in the food industry (Park et al., 2014; Sagong et al., 2013).

US is a compressional wave with a frequency of over 20 kHz. The frequency of US used in the food industry for microbial inactivation ranges from 20 kHz to 10 MHz (Piyasena et al., 2003). The bactericidal action of US is mainly due to the cavitation process, in which microbubbles are produced and collapsed within a liquid medium. During the cavitation process, the temperature can increase to as high as 5500 °C and the pressure can increase up to 100 MPa, resulting in localized microbial sterilization (Gabriel, 2012). However, US treatment alone does not achieve a 5-log reduction in the microbial count (Barba et al., 2017). Thus, for industrial applications, US must be used in combination with other preservation processes to enhance the microbial inactivation (García et al., 1989; Ordoñez et al., 1987; U.S. FDA, 2000). Fumaric acid (FA) is a food grade acidulant used in the food industry. Organic acids such as FA are generally recognized as safe (GRAS) and are known to exhibit bactericidal activity (Kondo et al., 2006). However, the use of high concentration of organic acids can result in the development of sour taste and odor in the food product and can cause corrosion of the processing equipment. Therefore, it is necessary to reduce the concentration of organic acids without compromising their ability to inactivate the foodborne pathogens (Sagong et al., 2013).

Recently, Salleh-Mack and Roberts (2007) reported that treating simulated juice using a combination of US and organic acid (citric and malic acids) markedly reduced the cell number of *E. coli* ATCC 25922. However, there are no reports on the combined application of US and FA for the pathogen inactivation in apple juice. Both US and FA are safe, non-toxic, and environment-friendly for pathogen inactivation in food products. In this study, we determined the microbicidal effect of US and FA combination against *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* in apple juice. We also evaluated the effect of this combined treatment on the food quality factors, and sought to elucidate the mechanism of bacterial inactivation.

2. Materials and methods

2.1. Bacterial strains

Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, and ATCC 43890), *S.* Typhimurium (ATCC, 19585, ATCC 43971, and DT 104), and *L. monocytogenes* (ATCC 15313, ATCC, 19111, and ATCC, 19115), were obtained from the bacterial culture collection of Hankyong National University (Anseong, South Korea) and used in the experiments. All strains were kept frozen at −80 °C in tryptic soy broth (TSB; MB Cell, Los Angeles, CA, USA) containing 20% glycerol. Working cultures were streaked onto tryptic soy agar (TSA; MB Cell), incubated at 37 °C for 24 h, and stored at 4 °C.

2.2. Preparation of pathogen inocula

Each strain of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was cultured individually in 5 ml of TSB with 0.6% yeast extract (YE; Difco, Sparks, MD, USA) at 37 °C for 24 h, followed by centrifugation (4000 rpm for 20 min at 4 °C), and the supernatant was discarded. The final pellets were resuspended in 9 ml of 0.2% sterile peptone water (PW; Difco), corresponding to approximately 10^8–10^9 colony forming units (CFU)/ml. The suspended pellets of each strain of the three pathogenic species were combined to produce mixed culture cocktails (nine strains total). These cocktails, consisting of a final concentration of approximately 10^8 CFU/ml, were used for the inactivation study. To analyze the mechanism of inactivation, each final three strain pellets of *E. coli* O157:H7, *S.* Typhimurium, or *L. monocytogenes* was resuspended in 15 ml of phosphate-buffered saline (PBS; 0.1 M), respectively.

2.3. Sample preparation and inoculation

Apple juice concentrate (72 °Brix and pH 3.3) was purchased from SERIM FOOD (Bucheon, South Korea). Apple juice samples were diluted with sterile distilled water (DW) to 12 °Brix to reflect that in commercial apple juice products. For inoculation, 1 ml of the pathogen cocktail was inoculated into 100 ml of each sample and mixed using a sterile magnetic stirrer. The final cell concentration in apple juice was approximately 10^6–10^7 CFU/ml.

2.4. Fumaric acid and ultrasound treatment

For treatment with fumaric acid (FA; 99.0%; Sigma-Aldrich, St. Louis, MO, USA), 0.5% (w/v) FA solution was prepared in sterile distilled water. The apple juice or PBS inoculated with the pathogens was treated with FA solution in a glass test tube to a final concentration of 0.05, 0.1, and 0.15% in a 10 ml volume. The mixture was mixed using a vortex. For ultrasound (US) treatment, we used an US treatment chamber (Power sonic 420; Hwashin Co., Seoul, South Korea) filled with 15 L of DW with the following settings: 40 kHz operating frequency, 25 mm wavelength, and 700 W maximum power. A glass test tube containing the inoculated apple juice or PBS sample was placed in the middle of the US tank and mixed with FA solution to final concentrations of 0 (US treatment alone), 0.05, 0.1, and 0.15%. All single FA or US and US-FA treatments were conducted at room temperature for a maximum of 5 min in apple juice and maximum of 9 min in PBS.

For mechanism evaluation, cell suspensions in PBS in a glass test tube were treated with 0.15% FA, US, and US-FA for 7 min. The FA concentration (0.15%) and treatment time (7 min) were selected from the results of preliminary experiments.

2.5. Bacterial enumeration

After treatment, 1 ml aliquots of each sample were serially diluted by 10-fold in 9 ml of sterile 0.2% PW, and 0.1 ml of sample or diluent was spread-plated onto each selective medium. Sorbitol MacConkey agar (SMAC; MB Cell), xylose lysine desoxycholate agar (XLD; MB Cell), and Oxford agar base (OAB; MB Cell) with Bacto Oxford antimicrobial supplement (MB Cell) were used as the selective media for the enumeration of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. All selective media were incubated at 37 °C for 24–48 h and typical colonies were counted. To confirm the identity of the pathogens, colonies were randomly selected from the enumeration plates and subjected to biochemical and serological tests. These tests consisted of the *E. coli* O157:H7 latex agglutination assay (RIM; Remel, Lenexa, KS, USA), the *Salmonella* latex agglutination assay (Oxoid; Ogdensberg, NY, USA), and the API *Listeria* test (bioMérieux, Inc., Marcy-lÉtoile, France) for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively.

2.6. Enumeration of injured cells

The liquid repair method was used to enumerate injured cells of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*. One milliliter aliquots of treated samples were serially diluted by 10-fold in 9 ml of TSB with 0.6% YE, and incubated at 25 °C for 2 h to allow injured cells to resuscitate. Following incubation, 0.1 ml of diluent was spread-plated onto each selective medium. All agar plates were incubated for 22–46 h at 37 °C and typical colonies were counted.

The Geeraerd and Van Impe Inactivation model Fitting Tool (GInaFiT) and the Weibull model were used in order to fit survival curves obtained after a resuscitation step (Geeraerd et al., 2005; Mafart et al., 2002). The Weibull model is given by

$$\log \frac{N}{N_0} = -\left(\frac{t}{\alpha}\right)^{\beta}$$

where α = the scale parameters (min), β = the shape parameters, t = treatment time (min), N = the number of survivors at treatment time, and N_0 = initial population of pathogen.

2.7. Cell membrane damage measurement

The fluorescent dye propidium iodine (PI; sigma-Aldrich) was used to quantitatively assess membrane damage to pathogen cells induced by each treatment. For PI uptake values, treated *E. coli* O157:H7, *S.* Typhimurium, or *L. monocytogenes* cells adjusted to an optical density of 680 nm (OD_{680}) of approximately 0.2 in PBS and then centrifuged (8000 rpm for 15 min). The cell pellets were resuspended in PBS and then PI solution was added to a final concentration of 2.9 μM, followed by 10 min incubation in the dark at 37 °C. Samples were centrifuged at 8000 rpm for 15 min and washed twice with PBS to remove excess dye. Pelleted cells were resuspended in PBS, and fluorescence was measured with a spectrofluorophotometer (Spectra Max Gemini XS; Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 495 nm and emission wavelength of 615 nm. PI uptake values calculated by subtracting the obtained fluorescence data from untreated and treated cells, and the data were normalized against the OD_{680} of the cell suspensions.

2.8. Efflux activity assay

In order to determine efflux pump damage to pathogen cells, ethidium bromide (EB; Sigma-Aldrich) was used. For measurement of EB values, *E. coli* O157:H7, *S.* Typhimurium, or *L. monocytogenes* cells were adjusted to an OD_{680} of approximately 0.2 in PBS. *E. coli* and *S.* Typhimurium suspensions were incubated in pure PBS containing 10 mg/ml EB for 15 min at 37 °C in the dark. For L. *monocytogenes* suspensions, incubated in PBS (pH 7.4) containing 1% glucose (99.5%; Sigma-Aldrich) and 5 mg/ml EB at 37 °C for 5 min in the dark. After incubation, the cells were collected by centrifugation at 8000 rpm for 15 min and washed twice with PBS to remove excessive EB. The cell pellet was resuspended in PBS, and fluorescence was measured using a spectrofluorometer at excitation and emission wavelengths of 360 and 590 nm, respectively. Fluorescence values of untreated cells were subtracted from those of treated cells, and the data were normalized to the OD_{680} of the cell suspensions.

2.9. Enzymatic activity assay

To evaluate intracellular enzyme damage to the cells, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Sigma-Aldrich) was used as a fluorescent dye. *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* cells were resuspended in PBS to adjust the OD_{680} to approximately 0.2. Each suspension of bacteria cells was incubated in PBS with 1% glucose and 5 mM CTC for 30 min at 37 °C while stirring at 250 rpm. After incubation, the cells were collected by centrifugation at 8000 rpm for 15 min and washed twice with PBS. Fluorescence was measured with a spectrofluorometer at excitation and emission wavelengths of 450 and 630 nm, respectively. The CTC fluorescence values for each bacteria cell suspension were normalized to the OD_{680} of the cell suspensions, and data obtained for untreated cells were subtracted from those for treated cells.

2.10. Measurement of product quality

A model CR400 colorimeter (Minolta Co., Osaka, Japan) was used to measure the color changes of treated samples. Two ml of sample was poured into the bottom half of the measurement device. The color attributes were quantified by the values L^*, a^*, and b^*, which indicate color lightness, redness, and yellowness of the sample, respectively.

The pH changes were measured with an Orion Star™ A211 pH meter (Thermo Fisher Scientific, Waltham, MA, USA). The pH meter was calibrated using pH 4.0, 7.0, and 10.0 standard solutions (Thermo Scientific).

The non-enzymatic browning index was determined by mixing 5 ml of sample with 5 ml of 95% ethanol, centrifugation for 15 min at 4000 rpm, and absorbance measurement of the supernatant with a spectrophotometer (X-ma 1000; Human Corp., Seoul, South Korea) at 420 nm. The obtained value was considered as the non-enzymatic browning index (Caminiti et al., 2012).

Total phenolic content was determined colorimetrically by the Folin-Ciocalteu method. Briefly, 0.1 ml of a 10-fold diluted apple juice in sterile DW was mixed with 5 ml of 0.2 N Folin–Ciocalteu reagent (Sigma-Aldrich). After maintaining the mixture at room temperature for 3 min, 4 ml of 7.5% sodium carbonate (Junsei Chemicals Co., Ltd., Tokyo, Ja-pan) solution was added to the mixture and the mixture was shaken. After storage for 2 h at room temperature in the dark, absorbance was measured at 765 nm against a blank sample using a spectrophotometer. Phenolic contents were expressed as gallic acid equivalents.

2.11. Statistical analysis

All experiments were repeated three times with duplicate samples. Data were analyzed by Duncan's multiple-range test using a statistical analysis system (SAS Institute, Cary, NC, USA). A P-value of < 0.05 indicated a significant difference.

3. Results

3.1. Inactivation of pathogenic bacteria by US-FA simultaneous treatment in apple juice

When the apple juice, contaminated with *E. coli* O157:H7, *S.* Typhimurium, or *L. monocytogenes*, was subjected to US treatment for 5 min, it resulted in the reduction of pathogen load by 0.80, 0.87, or 1.05 log CFU/ml, respectively. The bactericidal effect of FA against the three pathogens in the apple juice was dose-dependent. Reductions of 1.23, 2.42, and 3.32 log CFU/ml of *E. coli* O157:H7 cells were observed after 5 min of FA treatment alone at 0.05, 0.1, and 0.15%, respectively. Similarly, treating with 0.05, 0.1, and 0.15% FA resulted in the reduction of *S.* Typhimurium load by 1.14, 2.54, and 3.11 log CFU/ml, respectively. After treatment with 0.05, 0.1, and 0.15% FA, 1.10, 1.57, and 1.85 log CFU/ml reductions, respectively, were observed for *L. monocytogenes* (Tables 1–3).

When the contaminated apple juice was treated using a combination of US and 0.1% FA for 5 min, the mean load of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was reduced by 3.59, 5.88, and 2.22 log CFU/ml, respectively. The additional log reduction in the pathogen number resulting from the synergistic effect was calculated by subtracting the sum of pathogen load reduction observed after individual (US or 0.1% FA) treatment from that observed after the combination treatment (US and 0.1% FA). Treatment with the combination of US and 0.1% FA for 5 min resulted in an additional 2.47-log reduction in the cell number of *S.* Typhimurium (Table 2). Further, treatment with the combination of US and 0.15% FA for 5 min achieved 5.67-, 6.35-, and 3.47-log reduction in the cell number of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. The combination of US and 0.15% treatment resulted in a synergistic log reduction in the cell number of *E. coli* O157:H7 and *S.* Typhimurium by 1.55 and 2.37, respectively (Table 3). Thus, some combinations of US and FA were synergistic while others were additive or antagonistic, depending on the FA concentration. Among the combinations evaluated in this study, the combination of US and 0.15% FA was the most effective for reducing the pathogen count.

Table 1
Reductions of viable *E.coli* O157:H7, *S*. Typhimurium, and *L. monocytogenes* cells in apple juice treated with single ultrasound (US) or 0.05% fumaric acid (FA) and US treatment combined with 0.05% FA [a].

Organism	Treatment time (min)	Log reduction [$\log_{10}$ (*N0/N*)] by treatment type and selection medium							
		US		FA		US-FA			
		SA		SA		SA		SAR	
E. coli O157:H7	0	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	Aa	0.00 ± 0.00	Aa
	1	0.11 ± 0.03	A	0.12 ± 0.07	A	0.57 ± 0.03	Ba	0.17 ± 0.13	ABb
	2	0.24 ± 0.14	AB	0.65 ± 0.11	B	1.11 ± 0.26	Ca	0.38 ± 0.19	Bb
	3	0.53 ± 0.23	BC	0.91 ± 0.34	BC	2.04 ± 0.23	Da	0.72 ± 0.14	Cb
	4	0.67 ± 0.32	C	1.00 ± 0.26	BC	2.27 ± 0.18	Da	0.93 ± 0.15	CDb
	5	0.80 ± 0.18	C	1.23 ± 0.16	C	2.75 ± 0.37	Ea	1.10 ± 0.06	Db
		SA		SA		SA		SAR	
S. Typhimurium	0	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	Aa	0.00 ± 0.00	Aa
	1	0.34 ± 0.15	B	0.30 ± 0.07	B	0.62 ± 0.04	Ba	0.35 ± 0.05	Bb
	2	0.62 ± 0.08	C	0.40 ± 0.19	B	0.90 ± 0.13	BCa	0.61 ± 0.22	Ca
	3	0.67 ± 0.07	CD	0.54 ± 0.16	BC	1.14 ± 0.14	Ca	1.04 ± 0.18	Da
	4	0.81 ± 0.04	DE	0.71 ± 0.12	C	1.69 ± 0.31	Da	1.17 ± 0.07	DEb
	5	0.87 ± 0.02	E	1.14 ± 0.16	D	1.98 ± 0.35	Da	1.40 ± 0.18	Ea
		SA		SA		SA		SAR	
L. monocytogenes	0	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	Aa	0.00 ± 0.00	Aa
	1	0.51 ± 0.27	B	0.08 ± 0.04	A	0.21 ± 0.16	ABa	0.50 ± 0.12	Ba
	2	0.58 ± 0.25	B	0.51 ± 0.25	B	0.38 ± 0.21	Ba	0.68 ± 0.22	BCa
	3	0.66 ± 0.21	B	0.64 ± 0.32	B	0.82 ± 0.23	Ca	0.89 ± 0.08	CDa
	4	0.86 ± 0.14	BC	1.01 ± 0.17	C	0.90 ± 0.28	Ca	0.98 ± 0.06	Da
	5	1.05 ± 0.23	C	1.10 ± 0.22	C	1.18 ± 0.18	Ca	1.09 ± 0.11	Da

[a] Mean values ± standard deviations from three replications. Means with the same uppercase letter in the same column are not significantly different ($P > 0.05$). Within the US-FA columns, values in the same row followed by the same lowercase letter are not significantly different ($P > 0.05$). SA, plating directly on selective agar; SAR, plating on selective agar preceded by a resuscitation step.

3.2. Inactivation of pathogenic bacteria by US-FA simultaneous treatment in PBS

The reductions in the numbers of the three pathogens in PBS during US, 0.15% FA, and combined US-FA treatment are presented in Table 4. For all three pathogens, the sum of US and FA inactivation was lower than that observed by simultaneous application of both treatments, and the existence of a synergistic effect could be deduced. Furthermore, the mean synergistic effect value of 7 min treatment was higher than those of other treatment times. Log reductions in *E. coli* O157:H7, *S.*

Table 2
Reductions of viable *E.coli* O157:H7, *S*. Typhimurium, and *L. monocytogenes* cells in apple juice treated with single ultrasound (US) or 0.1% fumaric acid (FA) and US treatment combined with 0.1% FA [a].

Organism	Treatment time (min)	Log reduction [$\log_{10}$ (*N0/N*)] by treatment type and selection medium							
		US		FA		US-FA			
		SA		SA		SA		SAR	
E. coli O157:H7	0	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	Aa	0.00 ± 0.00	Aa
	1	0.11 ± 0.03	A	0.28 ± 0.13	A	1.79 ± 0.23	BCa	0.99 ± 0.09	BCb
	2	0.24 ± 0.14	AB	1.38 ± 0.26	B	1.24 ± 0.57	Ba	0.62 ± 0.29	Ba
	3	0.53 ± 0.23	BC	1.78 ± 0.48	BC	1.68 ± 0.11	BCa	0.71 ± 0.11	Bb
	4	0.67 ± 0.32	C	2.01 ± 0.37	CD	2.47 ± 0.69	Ca	1.28 ± 0.14	CDb
	5	0.80 ± 0.18	C	2.42 ± 0.37	D	3.59 ± 0.69	Da	1.64 ± 0.46	Db
		SA		SA		SA		SAR	
M	0	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	Aa	0.00 ± 0.00	Aa
	1	0.34 ± 0.15	B	0.77 ± 0.23	B	0.74 ± 0.20	Ba	0.96 ± 0.15	Ba
	2	0.62 ± 0.08	C	1.06 ± 0.15	B	1.00 ± 0.17	BCa	0.96 ± 0.13	Ba
	3	0.67 ± 0.07	CD	1.37 ± 0.33	BC	1.56 ± 0.39	CDa	2.18 ± 0.18	Ca
	4	0.81 ± 0.04	DE	1.83 ± 0.57	C	2.19 ± 0.80	Da	2.51 ± 0.44	Ca
	5	0.87 ± 0.02	E	2.54 ± 0.42	D	5.88 ± 0.39	Ea	3.34 ± 0.92	Db
		SA		SA		SA		SAR	
L. monocytogenes	0	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	Aa	0.00 ± 0.00	Aa
	1	0.51 ± 0.27	B	0.45 ± 0.05	B	0.69 ± 0.05	Ba	0.59 ± 0.07	Ba
	2	0.58 ± 0.25	B	0.76 ± 0.08	BC	0.73 ± 0.19	Ba	0.91 ± 0.10	Ca
	3	0.66 ± 0.21	B	1.04 ± 0.16	CD	1.04 ± 0.30	Ba	1.20 ± 0.27	Da
	4	0.86 ± 0.14	BC	1.21 ± 0.19	DE	1.66 ± 0.27	Ca	1.54 ± 0.01	Ea
	5	1.05 ± 0.23	C	1.57 ± 0.48	E	2.22 ± 0.45	Da	1.80 ± 0.12	Fa

[a] Mean values ± standard deviations from three replications. Means with the same uppercase letter in the same column are not significantly different ($P > 0.05$). Within the US-FA columns, values in the same row followed by the same lowercase letter are not significantly different ($P > 0.05$). SA, plating directly on selective agar; SAR, plating on selective agar preceded by a resuscitation step.

Table 3
Reductions of viable *E.coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* cells in apple juice treated with single ultrasound (US) or 0.15% fumaric acid (FA) and US treatment combined with 0.15% FA [a].

Organism	M	Log reduction [$\log_{10}$ (*N0/N*)] by treatment type and selection medium							
	Treatment time (min)	US		M		US-FA			
		SA		SA		SA		SAR	
E. coli O157:H7	0	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	Aa	0.00 ± 0.00	Aa
	1	0.11 ± 0.03	A	1.89 ± 0.16	B	1.87 ± 0.13	Ba	0.73 ± 0.25	Bb
	2	0.24 ± 0.14	AB	2.21 ± 0.17	C	2.31 ± 0.03	Ca	1.21 ± 0.35	Cb
	3	0.53 ± 0.23	BC	2.43 ± 0.16	C	3.18 ± 0.07	Da	1.35 ± 0.15	Cb
	4	0.67 ± 0.32	C	2.74 ± 0.09	D	3.68 ± 0.06	Ea	1.44 ± 0.15	Cb
	5	0.80 ± 0.18	C	3.32 ± 0.09	E	5.67 ± 0.38	Fa	1.59 ± 0.27	Cb
		SA		SA		SA		SAR	
S. Typhimurium	0	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	Aa	0.00 ± 0.00	Aa
	1	0.34 ± 0.15	B	1.32 ± 0.10	B	1.61 ± 0.18	Ba	0.79 ± 0.10	Bb
	2	0.62 ± 0.08	C	1.75 ± 0.04	C	2.68 ± 0.20	Ca	1.43 ± 0.25	Cb
	3	0.67 ± 0.07	CD	2.06 ± 0.07	D	3.34 ± 0.07	Da	2.12 ± 0.14	Db
	4	0.81 ± 0.04	DE	2.98 ± 0.10	E	4.13 ± 0.18	Ea	3.23 ± 0.60	Ea
	5	0.87 ± 0.02	E	3.11 ± 0.07	E	6.35 ± 0.34	Fa	6.42 ± 0.16	Fa
		SA		SA		SA		SAR	
L. monocytogenes	0	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	Aa	0.00 ± 0.00	Aa
	1	0.51 ± 0.27	B	0.74 ± 0.02	B	0.97 ± 0.19	Ba	0.83 ± 0.16	Ba
	2	0.58 ± 0.25	B	0.94 ± 0.12	B	1.56 ± 0.11	Ca	1.33 ± 0.07	Cb
	3	0.66 ± 0.21	B	1.59 ± 0.08	C	1.84 ± 0.04	Da	1.96 ± 0.21	Da
	4	0.86 ± 0.14	BC	1.79 ± 0.13	CD	2.28 ± 0.23	Ea	2.41 ± 0.18	Ea
	5	1.05 ± 0.23	C	1.85 ± 0.26	D	3.47 ± 0.17	Fa	3.42 ± 0.09	Fa

[a] Mean values ± standard deviations from three replications. Means with the same uppercase letter in the same column are not significantly different ($P > 0.05$). Within the US-FA columns, values in the same row followed by the same lowercase letter are not significantly different ($P > 0.05$). SA, plating directly on selective agar; SAR, plating on selective agar preceded by a resuscitation step.

Typhimurium, and *L. monocytogenes* resulting from the synergistic effects after 7 min of treatment were 3.68, 1.84, and 1.43 log units, respectively.

3.3. Resuscitation of injured cells

Tables 1–3 show levels of sublethally injured *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* cells in apple juice following simultaneous US-FA treatment. Evaluation of the difference between inactivation of samples subjected to injured cell recovery methods and those plated directly on selective media revealed 0.58, 2.54, and 0.00 log units of injured *S.* Typhimurium cells after 0.05, 0.1, and 0.15% FA combined treatments for a maximum of 5 min, respectively. For L. *monocytogenes*, 0.09, 0.42, and 0.05 log CFU/ml of injured cells was observed after 0.05, 0.1, and 0.15% FA combined treatments for 5 min, respectively. No statistically significant ($P > 0.05$) differences between the levels of surviving cells, including sublethally injured *S.* Typhimurium and L. *monocytogenes* cells, were observed at 5 min for

Table 4
Reductions of viable *E.coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* cells in PBS treated with single ultrasound (US) or 0.15% fumaric acid (FA) and US treatment combined with 0.15% FA [a].

Organism	Treatment time (min)	Log reduction [$\log_{10}$ (*N0/N*)] by treatment type and selection medium					
		US		FA		US-FA	
		SA		SA		SA	
E. coli O157:H7	0	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	A
	1	0.92 ± 0.19	B	0.75 ± 0.11	B	1.48 ± 0.46	B
	3	0.97 ± 0.14	B	1.20 ± 0.24	BC	2.07 ± 0.49	C
	5	1.02 ± 0.20	B	1.51 ± 0.23	CD	3.54 ± 0.15	D
	7	1.11 ± 0.35	B	1.80 ± 0.41	DE	6.59 ± 0.11	E
	9	1.34 ± 0.31	B	2.24 ± 0.48	E	6.59 ± 0.11	E
		SA		SA		SA	
S. Typhimurium	0	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	A
	1	0.50 ± 0.04	B	1.23 ± 0.10	B	2.19 ± 0.07	B
	3	0.61 ± 0.09	M	3.04 ± 0.07	C	3.48 ± 0.40	C
	5	0.66 ± 0.09	B	3.80 ± 0.20	D	6.20 ± 0.35	D
	7	0.55 ± 0.23	B	3.81 ± 0.27	D	6.20 ± 0.35	D
	9	0.66 ± 0.15	B	4.40 ± 0.30	E	6.20 ± 0.35	D
		SA		SA		SA	
L. monocytogenes	0	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	A
	1	0.04 ± 0.04	AB	0.07 ± 0.04	A	0.43 ± 0.09	B
	3	0.21 ± 0.11	AB	0.39 ± 0.09	B	0.52 ± 0.04	B
	5	0.26 ± 0.08	B	0.54 ± 0.03	BC	1.17 ± 0.20	C
	7	0.53 ± 0.16	C	0.83 ± 0.16	C	2.79 ± 0.20	D
	9	0.59 ± 0.22	C	1.19 ± 0.37	D	4.38 ± 0.20	E

[a] Mean values ± standard deviations from three replications. Means with the same uppercase letter in the same column are not significantly different ($P > 0.05$).

Table 5
Levels of membrane damage of ultrasound treatment (US), fumaric acid (FA), and US-FA simultaneously treated cells obtained from the propidium iodine (PI) uptake test [a].

Treatment type	PI uptake value[b]					
	E. coli O157:H7		*S.* Typhimurium		*L. monocytogenes*	
Untreated control	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	A
US	1.40 ± 0.09	B	0.79 ± 0.61	A	0.51 ± 0.22	A
FA	1.85 ± 0.09	B	2.69 ± 0.53	B	3.82 ± 1.01	B
US-FA	3.08 ± 0.67	C	4.37 ± 0.82	C	5.32 ± 1.01	C

[a] Data represent mean values ± standard deviations from three replications. Different letters within the same pathogen column indicate significant difference ($P < 0.05$).
[b] The data were normalized by subtracting fluorescence values obtained from untreated cells and against OD_{680} as follows: PI uptake value = (fluorescence value after treatment – fluorescence value of non-treated)/OD_{680}.

combinations of 0.15% FA. In the case of *E. coli* O157:H7, 1.65, 1.95 and 4.08 log CFU/ml of injured cells were observed after 0.05, 0.1, and 0.15% FA concentration combinations for 5 min, respectively. Significant ($P < 0.05$) recovery of injured *E. coli* O157:H7 cells was observed in all FA concentration combined treatments.

3.4. Determination of membrane damage by PI uptake

As a quantitative test of membrane disruption, US-, FA-, and US-FA-treated cells were stained with the fluorescent dye PI, which only passes through damaged cell membranes. The PI uptake values of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* after each treatment are shown in Table 5. The overall result pattern of *S.* Typhimurium was similar to that of *L. monocytogenes*. Based on PI uptake values, there was no significant ($P > 0.05$) damage to the cellular membranes of both pathogens following US treatment. The degree of PI uptake by FA- and US-FA-treated cells was much higher than that by US-treated cells. Among them, the US-FA simultaneous treatments showed significantly ($P < 0.05$) higher PI uptake values compared to the other treatments. In *E. coli* O157:H7 cells, the PI uptake values of US treatment and FA treatment were similar. Furthermore, cells subjected to US-FA simultaneous treatment showed significantly ($P < 0.05$) higher PI uptake values than cells subjected to the other treatments.

3.5. Inhibition of efflux pumps

To test if the efflux pump malfunctions were involved in the US, FA, and US-FA inactivation mechanisms, treated bacteria were stained with the fluorescent dye EB. Data on the accumulation of EB in *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* after US, FA, and US-FA treatments are shown in Table 6. In US treatment, the EB accumulation values of *E. coli* O157:H7 and *L. monocytogenes* were not significantly ($P > 0.05$) different compared to those of untreated cells. However, for *S.* Typhimurium, the EB accumulation value was significantly different following US treatment ($P < 0.05$). All bacterial cells subjected to FA and US-FA treatments showed significantly ($P < 0.05$) higher EB accumulation values than cells treated with US alone. Among them, US-FA treatment showed the highest EB accumulation values.

Table 6
Levels of inactivated efflux pump in bacterial cells treated with ultrasound treatment (US), fumaric acid (FA), and US-FA inferred from EB accumulation values [a].

Treatment type	EB accumulation value[b]					
	E. coli O157:H7		*S.* Typhimurium		*L. monocytogenes*	
Untreated control	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	A
US	1.40 ± 0.05	A	5.41 ± 0.64	B	3.72 ± 0.28	A
FA	57.89 ± 3.84	B	38.97 ± 0.68	C	25.89 ± 3.90	B
US-FA	84.16 ± 9.77	C	51.10 ± 0.18	D	36.64 ± 7.91	C

[a] Mean values of three replications ± standard deviations. Different letters within the same pathogen column indicate significant difference ($P < 0.05$).
[b] The data were normalized by subtracting fluorescence values obtained from untreated cells and against OD_{680} as follows: EB accumulation value = (fluorescence value after treatment – fluorescence value of non-treated)/OD_{680}.

Table 7
Levels of intracellular enzyme inactivation in bacterial cells treated with ultrasound treatment (US), fumaric acid (FA), and US-FA obtained from the CTC conversion tests [a].

Treatment type	CTC conversion value[b]					
	E. coli O157:H7		*S.* Typhimurium		*L. monocytogenes*	
Untreated control	0.00 ± 0.00	A	0.00 ± 0.00	A	0.00 ± 0.00	A
US	2.63 ± 0.65	B	2.43 ± 0.46	B	1.12 ± 0.36	A
FA	2.79 ± 0.51	B	4.53 ± 0.47	C	34.02 ± 0.79	B
US-FA	3.01 ± 0.44	B	5.32 ± 0.93	C	34.86 ± 0.96	B

[a] Mean values of three replications ± standard deviation. Values followed by the same letters within the column per parameter are not significantly different ($P > 0.05$).
[b] The data were normalized by subtracting fluorescence values obtained from untreated cells and against OD_{680} as follows: CTC conversion value = |(fluorescence value after treatment − fluorescence value of non-treated)/OD_{680}|.

3.6. Loss of respiratory chain dehydrogenase

In order to measure respiratory activity, treated bacteria were stained with the fluorescent dye CTC. The CTC conversion values following each treatment of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* are shown in Table 7. In *E. coli* O157:H7, although the value of US-FA treatment was higher than those of the single treatments, there were no significant ($P > 0.05$) differences between treatment types. In both *S.* Typhimurium and L. *monocytogenes* cells, the degrees of CTC conversion in FA- and US-FA-treated cells were much higher than that in US-treated cells. However, the CTC conversion level of US-FA treatment was not significantly ($P > 0.05$) different from that of FA treatment.

3.7. Effect of US-FA simultaneous treatment on product quality

The color, pH, browning indices, and phenolic content changes of apple juice after US-FA treatment are presented in Table 8. The L*, *a**, and *b** color values were not significantly different ($P > 0.05$) from those of untreated samples. There were no significant ($P > 0.05$) differences in browning indices and phenolic contents between untreated and treated samples. The pH value of apple juice decreased, regardless of treatment time.

4. Discussion

For application in the apple juice industry, novel non-thermal processing must not only achieve inactivation of foodborne pathogens, but also preserve the nutritional value and quality of the apple juice. Ultrasound treatment is one of the most promising non-thermal processing systems for effectively inactivating the foodborne pathogens and for the decontamination of processing conditions (U.S. FDA, 2000). However, long-term treatment with ultrasound may not be suitable for application in the food industry as the quality of the food product deteriorates and the sterilization maybe inefficient (Sagong et al., 2013). In this study, we confirmed that ultrasound treatment alone resulted in

Table 8
Color values, pH, browning indices and total phenolic content of US-FA simultaneously treated apple juice [a].

Treatment time (min)	Color value for parameter											
	L^*		a^*		b^*		pH		ΔNEBI		Total phenols	
0	20.40 ± 1.00	A	1.18 ± 0.26	A	10.40 ± 1.32	A	3.22 ± 0.03	A	0.444 ± 0.001	A	109.7 ± 0.4	A
1	20.05 ± 0.18	A	1.19 ± 0.07	A	10.98 ± 0.36	A	2.98 ± 0.03	B	0.433 ± 0.014	A	108.9 ± 4.4	A
2	20.15 ± 0.90	A	1.19 ± 0.10	A	10.83 ± 0.99	A	2.98 ± 0.01	B	0.433 ± 0.016	A	108.4 ± 1.9	A
3	20.16 ± 0.57	A	1.17 ± 0.15	A	10.59 ± 0.98	A	2.98 ± 0.02	B	0.426 ± 0.006	A	106.6 ± 7.5	A
4	20.43 ± 0.09	A	1.16 ± 0.65	A	11.78 ± 0.90	A	3.00 ± 0.03	B	0.434 ± 0.006	A	107.2 ± 1.5	A
5	20.44 ± 0.27	A	1.22 ± 0.21	A	10.17 ± 0.84	A	2.99 ± 0.02	B	0.445 ± 0.006	A	108.4 ± 4.9	A

[a] Mean values ± standard deviations from three replications. Values in the same column followed by the same letter are not significantly different ($P > 0.05$). L^*, lightness; a^*, redness; b^*, yellowness. ΔNEBI: non-enzymatic browning index.

the low inactivation of foodborne pathogens (Tables 1–4). Several studies have reported that combining ultrasound treatment with heat or pulsed light treatment can improve the efficacy of microbial inactivation (Munoz et al., 2012; Ugarte-Romero et al., 2006). It was previously reported that ultrasound treatment in combination with heat (40 °C) treatment achieved a 5.3-log reduction in the load of *E. coli* K12 in apple cider. However, this treatment was also associated with minor changes in the color and turbidity of the apple cider (Ugarte-Romero et al., 2006). Similarly, ultrasound treatment in combination with pulsed light treatment achieved a cell count reduction of *E. coli* K12 by approximately 6 log CFU/ml, but this was associated with the change in color of apple juice (Munoz et al., 2012). In this study, we used a combination of US and FA to treat apple juice for the inactivation of pathogens. The synergistic effect of US and FA (0.15%) treatment achieved 5.67-log reduction in the cell count of *E. coli* O157:H7 (Table 3) without affecting the product quality (Table 8). Although the pH value decreased slightly with the addition of FA, the pH remained within the reported range (2.9–4.5) for fresh apple juice (Kiskó and Roller, 2005).

The concentration of organic acids used for pathogen inactivation must be low. Previously, several studies have unsuccessfully employed various physical techniques to lower the use of high concentration of organic acids. Shin et al. (2006) reported that treating pickled asparagus puree using a combination of mild heating (> 50 °C) and acetic acid (> 1%) achieved a 5-log reduction in the cell count of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*. Fernández-Molina et al. (2005) used a combination of pulsed electric field and 0.3% organic acid (acetic or propionic acid) to treat skim milk, which resulted in the reduction of *Pseudomonas fluorescens* cell count by only 1.8 log CFU/ml. Sagong et al. (2013) used a combination of US and a high concentration of organic acids (2% malic, lactic, or citric acid) to treat lettuce, which resulted in 2.75-, 3.18-, and 2.87-log reduction in the cell count of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. However, we used a combination of US and low concentration of FA (0.15%) to treat apple juice that decreased the cell count of foodborne pathogens by 3.47–5.67 log CFU/ml (Table 3). Thus, the combination of US and low concentration of FA can be an effective decontamination procedure in the food industry.

As foodborne pathogens that are subjected to sublethal injury can regain pathogenicity under suitable conditions, it is important to confirm the presence of injured cells after bactericidal treatment (Wu, 2008). In this study, the extent to which sublethally injured pathogens survived after treatment with the combination of US and FA was evaluated by plating the treated juice on selective media with and without a resuscitation step. We did not observe any significant ($P > 0.05$) recovery of *L. monocytogenes* injured cells throughout the treatment at all treatment combinations (Tables 1–3). However, the injured cells of *S.* Typhimurium significantly ($P < 0.05$) recovered after treatment with a combination of US and 0.1% FA. Further, treatment with the combination of US and 0.15% FA effectively inactivated this pathogen in apple juice without causing apparent sublethal injury to the bacterial cells (Tables 1–3). Contrastingly, the injured cells of *E. coli* O157:H7 significantly recovered ($P < 0.05$) at all treatment combinations (Tables 1–3). The sensitivity of these pathogens to the combined treatment with US and 0.15% FA was evaluated based on the cell count reduction after the resuscitation step (SAR column in Table 3). The t3D value, which is the treatment time necessary to reach 3 decimal reduction, for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was 64.00 ($R^2 = 0.99$), 6.31 ($R^2 = 0.97$), and 8.21 min ($R^2 = 0.98$) in apple juice, respectively. Among the three pathogens, *E. coli* O157:H7 was significantly more resistant to the combined treatment with US and FA than the other two pathogens in apple juice. These results concur with those of previous reports. Miller and Kasper (1994) reported that *E. coli* O157:H7 strains were tolerant to low pH. Foster (2004) reported that the *E. coli* is nearly as acid-tolerant as *Helicobacter pylori* and can survive at pH 2 for several hours.

As mentioned before, mechanism experiments of the bacterial inactivation were performed in PBS. The PBS has been used in place of food to minimize interference from food particles in many mechanism studies (Kim et al., 2017; Ha et al., 2017). As shown in Table 4, the reduction levels of the three pathogens were lower in PBS than in apple juice after the same treatment time. This is because components such as malic acid in apple juice increase the sensitivity of bacteria cells (Splittstoesser et al., 1996). Park and Kang (2013) reported that the levels of surviving cells subjected to both ohmic and conventional heating in apple juice were decreased more than in buffered peptone water. Our results indicate that the combined treatment for 7 min in PBS had similar effects as inactivation of pathogens in apple juice by US-FA treatment for 5 min. Therefore, mechanism experiments were conducted in PBS with a treatment time of 7 min. In addition, it is well known that ultrasound application increases the temperature of the samples. However, the temperature of PBS increased by only 4.03 °C (from 12.73 ± 0.06 °C to 16.77 ± 0.15 °C) when treated with US for 7 min in the present study. Therefore, it was considered that the temperature rise of PBS by US treatment did not affect the inactivation mechanism.

The main bactericidal mechanisms of ultrasound are breakage of cell walls, disruption and thinning of cell membranes, and free radical activity due to the collapse of cavitation bubbles (Birmpa et al., 2013; Piyasena et al., 2003; Wu et al., 2012). Organic acids such as fumaric acid diffuse across the membrane, reduce intracellular pH, and acid pH in the internal cell, damage or modify the functions of enzymes, structural proteins, and DNA (Mani-López et al., 2012). In the present study, we focused on the cell membrane and intracellular enzyme damage to identify the mechanism of the synergistic lethal effect of US-FA treatment. The inactivation mechanisms of single US or FA and US-FA treatment were investigated based the cell membrane integrity, efflux pump activity on the cell membrane, and intracellular enzyme activity (dehydrogenase).

PI emits red fluorescence when bound to nucleic acids (DNA and RNA) and only enters cells with damaged membranes (Ha et al., 2017). For this reason, the degree of cell membrane damage can be measured

as the fluorescence value of the dye that accumulates inside cells. The PI uptake values following US-FA application was greater than that with separate US and FA treatments for *S.* Typhimurium and L. *monocytogenes* (Table 5). As a result, we confirmed that damage to the cell membrane was the main factor affecting the synergistic lethal effect of US and FA combined treatment. However, the PI uptake values of simultaneous US-FA treatment for *E. coli* O157:H7 were lower than total values of individual US and FA treatments (Table 5). Accordingly, for *E. coli* O157: H7, the synergistic lethal effect of US-FA combination treatment was not associated with cell membrane damage.

EB can freely diffuse across cell membranes but is actively pumped out of the cell via a non-specific proton antiport transport system in healthy cells. When the efflux pump malfunctions, EB binds to nucleic acid in cells (Sousa and Souza, 2017). Therefore, accumulation of EB can be indicator of efflux pump activity. In the present study, EB accumulation values of US-FA combined treatments were higher than the total values for US or FA treatments in all three pathogens. In other words, significant efflux pump malfunction following US-FA treatment was evident in all three pathogens ($P < 0.05$; Table 6). Therefore, compromised efflux pump activity may be one of the mechanisms related to the synergistic lethal effect of simultaneous US-FA treatment.

We also measured the loss of enzymatic activity inside pathogenic bacteria. CTC is reduced from a colorless complex to red colored CTC formazan (CTF) by electron transfer through intracellular dehydrogenase enzymes (Sousa and Souza, 2017; Joux and Lebaron, 2000). Since electron transport is directly related to cellular energy metabolism in respiring cells, the ability of cells to reduce CTC dye can be considered an indicator of respiratory activity. As shown in Table 7, overall, the sums of CTC conversion values for US or FA treatment were higher than those reached by simultaneous application of both technologies. Thus, the inactivation of dehydrogenase is not related to the synergistic bactericidal effect of US-FA combination treatment. According to these results, we confirmed that damage to the cell envelope (membrane integrity and efflux pump) was the main factors in the synergistic microbicidal effect of US-FA treatment.

In conclusion, the combined treatment of FA and US can effectively inactivate the major pathogenic bacteria in the apple juice owing to their synergistic action without affecting the product quality. Although the inactivation of *L. monocytogenes* did not meet the FDA criteria for fruit juice processing, treatment with the combination of US and FA significantly reduced the population of *E. coli* O157:H7 and *S.* Typhimurium, which are associated with disease outbreaks. Therefore, further studies are needed to evaluate various high efficacy conditions for the industrial application of this combined treatment system.

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