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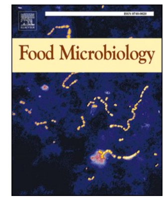

Synergistic bactericidal effect of hot water with citric acid against *Escherichia coli* O157:H7 biofilm formed on stainless steel

Jun-Won Kang [a,b,1], Hae-Yeon Lee [a,b,1], Dong-Hyun Kang [a,b,*]

[a] Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, Center for Food and Bioconvergence, Research Institute for Agricultural and Life Sciences, Seoul National University, Seoul, 08826, Republic of Korea

[b] Institutes of Green Bio Science & Technology, Seoul National University, Pyeongchang-gun, Gangwon-do, 25354, Republic of Korea

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ABSTRACT

This study investigated the antimicrobial effect of hot water with citric acid against *Escherichia coli* O157:H7 biofilm on stainless steel (SS). Hot water (50, 60, or 70 °C) with 2% citric acid exhibited a synergistic bactericidal effect on the pathogen biofilm. It was revealed that hot water and citric acid combination induced sub-lethally injured cells. Additionally, mechanisms of the synergistic bactericidal effects of hot water with citric acid were identified through several approaches. In terms of biofilm matrix, hot water removes exopolysaccharides, a major component of extracellular polymeric substances (EPS), thereby increasing contact between surface cells and citric acid, resulting in a synergistic bactericidal effect. In terms of the cell itself, increased permeability of citric acid through cell membranes destructed by hot water promotes the inactivation of superoxide dismutase (SOD) in *E. coli* O157:H7, which induce synergistic generation of reactive oxygen species (ROS) which promote inactivation of cell by activating lipid peroxidation, resulting in destruction of the cell membrane. Therefore, it is interpreted that when hot water with citric acid is applied to *E. coli* O157:H7 biofilm, synergy effects on the biofilm matrix and cell itself have a complex interaction with each other, thus causing a dramatic synergistic bactericidal effect.

1. Introduction

Contamination with foodborne pathogens is a continuing concern for producers and consumers. (Niemira et al., 2014). Cross-contamination from surface is known as one of the main cause of outbreaks (Miranda and Schaffner, 2016). Thus, inadequate cleaning or disinfection of food processing equipment can induce to cross-contamination of pathogens to food products, resulting in potential risks to consumers (Gutiérrez et al., 2012). In particular, microorganisms attached to wet surfaces have a natural tendency to multiply and can produce sticky structure consisting of the extracellular polymeric substances (EPS) that can trap themselves, resulting in the formation of a biofilm (Simoes et al., 2010). Pathogens in biofilms exhibit enhanced resistance to mechanical action or commonly used sanitizers, and most bacterial infections (about 80%) are related with biofilms; thus removing them from food processing plants is a crucial issue (Carpentier and Cerf, 1993; Janssens et al., 2008; Simões et al., 2006). Therefore, a process for effectively removing pathogen biofilms on the surfaces where food is in contact is pivotal to reducing foodborne illness outbreaks.

Escherichia coli O157:H7 surviving on the surface where food is in contact contain the possibility of producing biofilm and thus can increase the probability of food contamination (Dourou et al., 2011; Ryu and Beuchat, 2005; Uhlich et al., 2006). It is known that *E. coli* O157:H7 can create biofilm on multiple type of surfaces such as glass, plastic, and stainless steel (SS) (Dewanti and Wong, 1995; Dourou et al., 2011; Oh et al., 2007; Ryu et al., 2004).

Hot water sanitation is a technique that can be effectively utilized for the microbial safety of fruits, vegetables, meat, and the surfaces in contact with foods (Wahlen et al., 2016). Indeed, the United States Food and Drug Administration (FDA) approves hot water to be applied for surface decontamination because of its effectiveness (FDA, 2017). Furthermore, if hurdle technology, in which two or more mild treatments in combination induce a synergistic inactivation effect, is applied to hot water sanitation, it can be more effectively utilized by reducing the processing time or temperature required to inactive the pathogens to an appropriate level and ensuring more microbial safety of food

* Corresponding author. Department of Agricultural Biotechnology, Seoul National University, Seoul, 08826, Republic of Korea.
E-mail address: kang7820@snu.ac.kr (D.-H. Kang).

[1] These authors contributed equally to this work.

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products (Khan et al., 2017; Leistner, 2000).

Organic acids, which are approved as GRAS for use in the food manufactures (US Department of Agriculture and Service, 2018), can pass through the cell membrane in their un-dissociated form and then lower the intracellular pH, resulting in depletion of cellular energy needed to regulate internal pH, which in turn inhibits metabolic activities, eventually leading to cell death (Davidson et al., 2005). Especially, they have the characteristics of a wide range of bactericidal effect, being effective within a broad range of temperature and stable regardless of water hardness (Marriott et al., 2018). Furthermore, Ban et al.'s study (Ban et al., 2012) reported that combining heat (100 °C steam) and organic acid (lactic acid) showed synergistic bactericidal effect on biofilm. Therefore, it can be considered that the organic acid is a suitable candidate for combining with hot water sanitation.

This study confirmed the inactivation effect of hot water (50, 60, or 70 °C) and citric acid combination against *E. coli* O157:H7 biofilms on food-contact surfaces. Two percent citric acid was selected among several organic acids for use in this study because it is known that its low pH induce a strong antimicrobial effect (Park et al., 2011). Also, among various types of food-contact surfaces, we selected SS as a substrate for biofilm formation, which has a variety of benefits such as corrosion resistance, heat transfer efficiency, hygiene, and rigidity and is thus the most commonly utilized for fabricating food contact surfaces in the food industry (Brooks and Flint, 2008; Huang et al., 2008). Moreover, the identification of the mechanism of the synergistic bactericidal effect on pathogen biofilms exhibited by this treatment combination was performed.

2. Materials and methods

2.1. Preparation of bacterial strains and culture

E. coli O157:H7 (ATCC 8624, 2026, and 2029) were provided from Seoul National University (Seoul, Korea). Working cultures were prepared by streaking stock cultures stored at −80 °C in tryptic soy broth (TSB; Difco, Becton, Dickinson and Company, Sparks, MD, USA) with sterile 50% glycerol (TSB:50% glycerol = 7:3) onto tryptic soy agar (TSA; Difco), incubated at 37 °C for 24 h, and stored at 4 °C. Each strain of *E. coli* O157:H7 was cultivated in 15 ml of TSB at 37 °C for 24 h with shaking incubation. After incubation, TSBs in which each strain was grown were combined to make a mixed cultural cocktail, and then cell pellets were collected by centrifugation (4000×*g* at 4 °C for 20 min) and washed three times with sterile phosphate-buffered saline (PBS; 0.1 M). Subsequently, final pelleted cells were re-suspended in 10 ml of PBS for a final concentration of approximately 10^8–10^9 colony-forming-units (CFU)/ml confirmed by plating onto TSA. This inoculum was used for biofilm formation or treatment of planktonic cells in this study.

2.2. Biofilm formation

Prepared inoculum (10 ml) was transferred into 500 ml of PBS (approximately 10^7–10^8 CFU/ml). SS coupons (type 304, no.4 finish) were cut into 5 × 2 cm pieces, soaked in 70% ethanol for 24 h and washed with sterile distilled water (DW). Washed SS samples were sterilized by autoclave (121 °C, 15 min). Sterile SS coupon was transferred to a sterile 50 ml conical tube containing 30 ml of *E. coli* O157:H7 cell suspension in PBS. Conical tubes with SS sample were maintained at 4 °C for 24 h to perform initial cell attachment. After attachment, the coupon was gently stirred for 5 s in sterile DW to remove unattached cells. The rinsed sample was put into 30 ml of TSB in conical tube and incubated at 25 °C for 5 days for biofilm formation. This method was adapted from Kim et al. (Kim et al., 2006).

2.3. Citric acid, hot water, and hot water with citric acid treatment

Treatment solution of citric acid (CA; 99.5%, Samchun Pure Chemical Co. LTD., Korea) was prepared with sterile DW to a concentration of 2% (pH 1.3). For hot water and hot water with citric acid treatments, sterile distilled water (DW) and CA solution were tempered to 50, 60, and 70 °C. The temperature was controlled and maintained with a water bath, and monitored using a K-type Teflon-coated thermocouple. For CA treatment alone, CA solution was held at room temperature (22 ± 2 °C).

To perform inactivation experiments on biofilm cells, the biofilm-colonized SS coupon was rinsed as described previously to remove unattached cells and then immersed into 30 ml of DW or CA solution in conical tube adjusted to target temperature (22, 50, 60, or 70 °C) for 5, 10, 15, or 20 s. Immediately after treatment, the coupon was transferred into 30 ml of PBS in conical tube with 3 g of sterile glass beads (425–600 μm; Sigma-Aldrich, St. Louis, MO, USA) and agitated for 1 min with a benchtop vortex mixer to detach biofilm cells from coupons.

For planktonic cell treatment, one ml of prepared inoculum was inoculated into 15 ml glass tubes containing 9 ml of DW or CA solution adjusted to target temperature (22, 50, 60, or 70 °C) and maintained for 5, 10, 15, or 20 s. Immediately after treatment, one ml of treated solutions was transferred into 9 ml of Dey/Engley (DE) neutralizing broth (Difco) in the glass tubes into crushed ice-water to quickly lower temperature or/and terminate reaction of citric acid.

2.4. Bacterial enumeration

For biofilm or planktonic cell enumeration, one ml from the 30 ml suspension in PBS of cells detached from biofilm or from the 9 ml suspension in DE neutralizing broth of neutralized or/and cooled planktonic cells, respectively, was 10-fold serially diluted in 9 ml of PW and spread-plated onto selective and recovery media. Since injured cells are not able to be cultured in selective media due to their high sensitivity to some selective components, they can be distinguished from dead cells by culturing them on non-selective medium, where they can undergo repair and be resuscitated (Wesche et al., 2009; Yuste et al., 2004). Therefore, sorbitol MacConkey agar (SMAC; Difco) and phenol red agar with 1% sorbitol medium (SPRAB) were used as a selective and a non-selective recovery medium to enumerate surviving cells and confirm the injured cells of *E. coli* O157:H7, respectively (Rhee et al., 2003). All plates were incubated at 37 °C for 24 h and after incubation, the number of typical colonies were counted and serological confirmation was performed on randomly selected presumptive *E. coli* O157:H7 colonies (RIM; *E. coli* O157:H7 latex agglutination test; Remel, Lenexa, KS, USA).

2.5. Identification of mechanism of synergistic bactericidal effect

We conducted experiments to investigate the cause of the synergistic inactivation effect of combined hot water and citric acid.

For this experiment (i), biofilm-colonized SS coupons were treated with the same treatment protocol carried out as for the inactivation experiment with the treatment time fixed at 10 s.

For the experiments (ii), (iii), and (iv), 1 ml of cell inoculum prepared as described previously was inoculated into 9 ml of DW or CA solution adjusted to target temperature (22, 50, 60, or 70 °C) and maintained for 10 s. In this case, since the suspended particles in the DE neutralization broth can interfere with further analysis, the reaction was terminated by immediate centrifugation and elimination of supernatant instead of using the DE neutralization broth after treatment. Therefore, immediately after treatment, cell pellets were collected by centrifugation (10,000×*g* at 4 °C for 1 min) and re-suspended in PBS.

(i) **Measurement of biofilm EPS** The amount of EPS in *E. coli* O157:H7 biofilms on SS coupons was measured following a protocol based on two studies (Jung et al., 2013; Stiefel et al., 2016). To measure the change in EPS amount by treatment, the amount of polysaccharides, which are the main constituent of EPS, was assessed using calcofluor white staining. Calcofluor white can be

used as a useful dye to measure extracellular polysaccharides in *E. coli* O157:H7 biofilm because it selectively binds to polysaccharides with β-1,3 and β-1,4 linkages and does not stain gram-negative bacteria cells (Biotium; Vogeleer et al., 2016). Treated SS coupons were transferred to 30 ml of calcofluor white solution (1 mg of fluorescent brightener 28 in 1 ml of dH_2O) in conical tube and maintained for 15 min in the dark. After incubation, stained SS coupons were placed in 30 ml of PBS in conical tube with 3 g of sterile glass beads and vortexed at maximum speed for 1 min with a vortex mixer for detaching polysaccharides-bound calcofluor white. The PBS in which the polysaccharides were dispersed was measured with a spectrophotometer (Spectramax M2e; Molecular Devices, CA, USA) at excitation/emission wavelengths of 360/460 nm, respectively. The fluorescent signal obtained from SS without biofilm was used as negative control, and the amount of polysaccharides on SS was derived by the following equation.

$$Polysaccharides(\%) = \frac{F_T - F_{NC}}{F_0 - F_{NC}} \times 100$$

(F_T, Fluorescence after treatment; F_{NC}, Negative control; F_0, Fluorescence of untreated control).

(ii) Measurement of cell membrane damage. We conducted two assays to quantify the degree of cell membrane destruction by each treatment. The fluorescent dye propidium iodide (PI; Sigma-Aldrich, USA) and diphenyl-1-pyrenylphosphine (DPPP; Sigma-Aldrich, USA) were used to evaluate the incidence of structural damage and lipid peroxidation of the cell membrane, respectively. Treated cell suspensions were incubated at 37 °C with PI or DPPP at a concentration of 2.9 or 50 μM for 10 or 20 min, respectively and were centrifuged (10,000×*g* for 2 min) to collect cells followed by washing with PBS. The collected cell pellets were re-suspended in PBS and fluorescence was measured with a spectrophotometer at excitation/emission wavelengths of 493/630 nm for PI uptake or 351/380 nm for DPPP assay, respectively. Florescent signals were normalized by dividing the cell suspension OD_{600} value, and then data was obtained by subtracting the fluorescent value of untreated control from that of treated cells.

(iii) Measurement of reactive oxygen species (ROS) and superoxide (O_2^-) generation within cells. To detect the degree of intracellular ROS generation, we evaluated total ROS and superoxide generation with the cellular dye, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Molecular Probes, ThermoFisher Scientific) and hydroethidine (HDE; Molecular Probes, ThermoFisher Scientific), respectively. Treated cell suspensions were incubated at 37 °C with CM-H2DCFDA or HDE at a concentration of 5 μM for 15 or 30 min, respectively. were centrifuged at 10,000×*g* for 2 min followed by washing twice with PBS. The cell pellets were re-suspended in PBS and fluorescence was measured with a spectrophotometer at excitation/emission wavelengths of 495/520 nm for ROS assay or 518/605 for nm superoxide assay, respectively. Florescent signals were normalized by dividing the cell suspension OD_{600} value, and then data was obtained by subtracting the fluorescent value of untreated control from that of treated cells.

(iv) Measurement of superoxide dismutase (SOD) activity. We assessed the activity of superoxide dismutase (SOD) which is representative of ROS scavenging enzymes using a SOD assay kit -WST (Sigma-Aldrich). Treated cell suspensions were disrupted with a sonicator (10s on and 10s off, six times) in an ice bath and then centrifuged at 10,000 × g for 2 min at 4 °C to obtain SOD remaining supernatant. Then, SOD activity (%) measurement was performed following manufacturer's instructions.

2.6. Statistical analysis

All data were obtained from three independent replicates. Data was analyzed by the analysis of variance (ANOVA) and LSD *t*-test of Statistical Analysis System (SAS Institute, Cary, NC, USA). A probability level of $P < 0.05$ was used to determine significant differences.

3. Results

3.1. Synergistic bactericidal effect of hot water with citric acid against E. coli O157:H7 biofilm or planktonic cells

Viable-count reductions of *E. coli* O157:H7 biofilm cells on SS during treatment of citric acid, hot water, or hot water with citric acid were investigated (Table 1). The initial populations of *E. coli* O157:H7 biofilm on SS counted on SMAC and SPRAB were 5.90 and 5.91 CFU/cm^2, respectively.

In the case of treated cells enumerated on SMAC, 20 s treatment with citric acid, 50 °C hot water or 60 °C hot water resulted in reductions of 0.62, 0.44 or 0.54 log CFU/cm^2, respectively. These reduction levels were not significantly ($P > 0.05$) different from the reduction level obtained after 20 s treatment with DW of room temperature (22 ± 2 °C). On the other hand, 70 °C hot water reduced *E. coli* O157:H7 biofilm cells to below the detectable level (<0.18 CFU/cm^2) after 20 s treatment. Meanwhile, when citric acid and hot water were combined, 50 °C hot water with citric acid represented a 2.49 log reduction of *E. coli* O157:H7 biofilm cells after 20 s treatment, and 60 and 70 °C hot water with citric acid reduced *E. coli* O157:H7 biofilm cells to below the detectable level (<0.18 CFU/cm^2) after 15 and 5 s treatments, respectively. This combined treatment of hot water and citric acid exhibited the synergistic bactericidal effect: combined treatment led a significantly ($P < 0.05$) larger reduction level than that of the sum of individual hot water and citric acid treatments. Specifically, 50 °C hot water with citric acid showed a synergistic effect after 10 s treatment, and 60 °C and 70 °C hot water with citric acid showed synergism after 5 s.

The treated cells were enumerated on SPRAB giving counts similar to counts given by SMAC. The 20 s treatment with 50 °C hot water, 60 °C hot water, or citric acid reduced E. coli O157:H7 biofilm cells counts by $\log_{10}$ 0.54, 0.42, or 0.44 CFU/cm2, respectively, and these reductions were not significantly ($P > 0.05$) different from the 0.44 log reduction obtained from 20 s treatment of DW of room temperature (22 ± 2 °C). 70 °C hot water treatment resulted in 5.54 log reduction of *E. coli* O157:H7 biofilm cells after 20 s treatment. 50 or 60 °C hot water with citric acid resulted in 1.45 or 2.90 log reductions of *E. coli* O157:H7 biofilm after 20 s treatment, respectively, and 70 °C hot water with citric acid reduced *E. coli* O157:H7 biofilm cells to below the detectable level (<0.18 CFU/cm^2) after 10 s treatment. This combination of hot water and citric acid also induced a synergistic bactericidal effect on cells enumerated on SPRAB medium after treatment. The synergistic effect of 50, 60 or 70 °C hot water with citric acid appeared after 20, 15 or 5 s treatment, respectively.

The inactivation effects of hot water, citric acid, and their combination were also investigated for planktonic cells of *E. coli* O157:H7 in sterile DW (Table 1). Treated cells were enumerated on both SMAC and SPRAB media and initial levels of *E. coli* O157:H7 planktonic cells were 7.88 and 8.13 CFU/ml, respectively. Overall, the reduction tendency of planktonic cells of *E. coli* O157:H7 following treatments was similar to that of biofilm cells. For both cell types enumerated on SMAC and SPRAB, individual citric acid or hot water of 50 or 60 °C treatment induced slight reductions (<1.0 log CFU/ml) after 20 s treatment, whereas 70 °C hot water achieved log reductions of 6 or more after 20 s treatment. Furthermore, combination treatment of citric acid and hot water generated the synergistic bactericidal effect for planktonic cells of *E. coli* O157:H7. For cells enumerated on SMAC after treatment, 50, 60 and 70 °C hot water with citric acid showed the synergistic effect after 5 s treatment, and for the cells enumerated on SPRAB after treatment,

Table 1
Log reductions of biofilms developed on stainless steel or planktonic cells of *E. coli* O157:H7 subjected to citric acid (CA), hot water (HW), or hot water with citric acid (HW-CA) treatment.

Treatment type	Log reduction [Log_{10} (N_0/N)][a] by treatment type and medium							
	Treatment time (s)							
	5		10		15		20	
	Biofilm	Planktonic	Biofilm	Planktonic	Biofilm	Planktonic	Biofilm	Planktonic
Enumeration medium, SMAC								
Untreated control	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa
DW	0.26 ± 0.04 Aab	–	0.34 ± 0.07 Aa	–	0.37 ± 0.07 Aa	–	0.49 ± 0.13 Aab	
CA	0.48 ± 0.40 Ab	0.11 ± 0.08 Aa	0.55 ± 0.35 ABa	0.21 ± 0.09 Aab	0.67 ± 0.21 Ba	0.14 ± 0.11 Aab	0.62 ± 0.23 ABb	0.25 ± 0.04 ABab
50 °C HW	0.12 ± 0.11 ABab	0.09 ± 0.10 Aa	0.35 ± 0.17 BCa	0.19 ± 0.22 ABab	0.33 ± 0.16 ABCa	0.23 ± 0.05 ABCab	0.44 ± 0.19 Cab	0.24 ± 0.09 ABCab
50 °C HW-CA	1.00 ± 0.29 Ac	1.79 ± 0.53 ABCb	1.56 ± 0.26 ABb	2.20 ± 0.22 BCDc	1.69 ± 0.30 ABb	2.76 ± 0.71 DEc	2.49 ± 0.54 CDc	3.01 ± 0.61 Ec
60 °C HW	0.37 ± 0.25 Aab	0.28 ± 0.30 Aa	0.39 ± 0.09 Aa	0.48 ± 0.34 Ab	0.43 ± 0.14 Aa	0.57 ± 0.48 Ab	0.54 ± 0.21 Ab	0.72 ± 0.50 Ab
60 °C HW-CA	2.15 ± 0.03 Ad	4.81 ± 0.15 Cd	4.23 ± 0.20 Bc	5.20 ± 0.32 Ce	>5.73 Dd	>6.88 Ee	–	–
70 °C HW	0.46 ± 0.25 Ab	3.35 ± 0.63 Cc	1.91 ± 0.86 Bb	4.23 ± 0.13 CDd	4.17 ± 1.06 CDc	5.06 ± 0.11 DEd	>5.73 Ed	>6.88 Fd
70 °C HW-CA	>5.73 Ae	5.19 ± 0.22 Ad	–	>6.88 Bf	–	–	–	–
Enumeration medium, SPRAB								
Untreated control	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa
DW	0.20 ± 0.04 Aab	–	0.26 ± 0.14 Aa	–	0.32 ± 0.05 Aab	–	0.44 ± 0.06 Aa	
CA	0.38 ± 0.28 ABab	0.20 ± 0.05 Aab	0.40 ± 0.29 ABab	0.15 ± 0.07 Aa	0.54 ± 0.15 Bb	0.12 ± 0.06 Aa	0.54 ± 0.13 Ba	0.28 ± 0.02 ABa
50 °C HW	0.13 ± 0.06 ABCa	0.20 ± 0.07 ABCab	0.34 ± 0.06 CDa	0.10 ± 0.14 Aa	0.32 ± 0.06 BCDab	0.16 ± 0.12 ABCa	0.42 ± 0.23 Da	0.12 ± 0.11 ABa
50 °C HW-CA	0.61 ± 0.08 Abc	0.67 ± 0.42 Ab*	1.05 ± 0.50 ABc	0.95 ± 0.45 ABb*	1.06 ± 0.29 ABc	1.55 ± 0.33 BCb*	1.45 ± 0.20 BCb*	1.74 ± 0.70 Cb*
60 °C HW	0.23 ± 0.13 Aab	0.19 ± 0.17 Aab	0.29 ± 0.24 Aa	0.22 ± 0.17 Aa	0.35 ± 0.17 Aab	0.33 ± 0.15 Aa	0.44 ± 0.20 Aa	0.29 ± 0.22 Aa
60 °C HW-CA	0.87 ± 0.41 Ac*	2.96 ± 0.41 Bc*	1.06 ± 0.50 Ac*	3.56 ± 0.34 BCc*	1.72 ± 0.55 Ad*	4.46 ± 0.53 CDc*	2.90 ± 0.92 Bc*	5.25 ± 0.88 Dc
70 °C HW	0.25 ± 0.33 Aab	2.73 ± 0.52 Bc	0.83 ± 0.35 Abc	4.07 ± 0.35 Cd	3.94 ± 0.52 Ce	5.05 ± 0.22 Dd	5.54 ± 0.66 Dd	6.73 ± 0.71 Ed
70 °C HW-CA	3.62 ± 0.54 Ad*	5.30 ± 0.58 Bd	>5.74 Bd	>7.13 Ce	–	–	–	–

DW, distilled water treatment; SMAC, sorbitol MacConkey agar; SPRAB, phenol red agar base with 1% sorbitol; N_0, initial population; N, population after treatment.
[a] Values are means ± standard deviations from three replications. Means with the same uppercase letters within the same row are not significantly different ($P > 0.05$). Means with different lowercase letters within the same column for the same medium are significantly different ($P < 0.05$). Significant differences ($P < 0.05$) between the means of SPRAB medium and those of SMAC medium at the same treatment time and type are marked with asterisks.

those showed the synergistic effect after 15, 15 and 5 s treatment, respectively.

3.2. Comparative reduction of cells enumerated on SMAC and SPRAB after treatment with citric acid, hot water or hot water with citric acid

For both *E. coli* O157:H7 biofilm and planktonic cells following treatments, surviving cells were enumerated not only on selective medium, SMAC, but also on recovery medium, SPRAB (Table 1). In the case of *E. coli* O157:H7 biofilm treated with citric acid or all temperatures of hot water (50, 60 or 70 °C), reduction levels of cells enumerated on SMAC were not significantly ($P > 0.05$) different from those enumerated on SPRAB for all treatment times (5–20 s). However, 50, 60 or 70 °C hot water with citric acid combinations resulted in significantly ($P < 0.05$) less reduction among biofilm cells enumerated on SPRAB than in those enumerated on SMAC after 20, 5 or 5 s treatment, respectively. Also, in the case of planktonic cells of *E. coli* O157:H7, citric acid or hot water (50, 60 or 70 °C) single treatment did not induce significant ($P > 0.05$) differences in reduction among survivors enumerated on SMAC and SPRAB for all treatment times (5–20 s), whereas 50 or 60 °C hot water plus citric acid resulted in significantly ($P < 0.05$) less reduction in cells enumerated on SPRAB than in those enumerated on SMAC for all treatment times (5–20 s). Meanwhile, there were no significant ($P > 0.05$) differences between reduction levels of treated cells enumerated on SMAC and SPRAB after 70 °C hot water plus citric acid.

3.3. Differential resistance to citric acid, hot water, or hot water with citric acid treatment between E. coli O157:H7 biofilm and planktonic cells

To compare resistances between biofilm and planktonic cells, reduction results of biofilm and planktonic cells were compared (Table 1). Among both types of cells enumerated on SMAC and SPRAB, there were no significant ($P > 0.05$) differences of reduction between biofilm and planktonic cells during citric acid or 50 or 60 °C hot water treatment (5–20 s), whereas reduction of planktonic cells was significantly ($P < 0.05$) greater than that of biofilm cells during 70 °C hot water or 60 °C hot water plus citric acid treatment (5–20 s). Meanwhile, 50 °C hot water plus citric acid induced significantly ($P < 0.05$) greater reductions of planktonic cells compared to biofilm cells enumerated on SMAC, but this treatment did not produce significant ($P > 0.05$) differences in reduction between planktonic and biofilm cells when enumerated on SPRAB. On the other hand, 70 °C hot water with citric acid treatment did not produce significant ($P > 0.05$) differences in reduction between planktonic and biofilm cells in case of the cells enumerated on SMAC, whereas this treatment resulted in significantly ($P < 0.05$) greater reduction in planktonic cells than in biofilm cells

enumerated on SPRAB.

3.4. Removal of EPS from SS surface

Table 2 depicts residual exopolysaccharides associated with *E. coli* O157:H7 biofilm after citric acid, hot water or hot water with citric acid treatment. Citric acid treatment slightly reduced (by 3.82%) exopolysaccharides on SS surfaces, but there was no significant ($P > 0.05$) difference compared to the control. However, treatment with hot water or hot water with citric acid at all temperatures (50, 60 or 70 °C) significantly ($P < 0.05$) removed exopolysaccharides from SS. For both hot water and hot water with citric acid treatments, the degree of exopolysaccharide removal was significantly ($P < 0.05$) increased with increasing treatment temperature from 50 to 70 °C. Specifically, hot water treatment of 50, 60, or 70 °C reduced exopolysaccharides on SS by 40.68, 71.39 or 87.39%, respectively, and hot water with citric acid treatment of 50, 60 or 70 °C reduced exopolysaccharides on SS surfaces by 45.93, 76.38 or 88.03%, respectively. For each temperature, however, removal of exopolysaccharides by hot water and hot water with citric acid treatments was not significantly ($P > 0.05$) different.

3.5. The extent of cell membrane damage following citric acid, hot water or hot water with citric acid treatment

To quantitatively assess damage to the cell membrane, treated cells were reacted with the PI or DPPP, respectively. Values obtained from these assays were expressed as PI uptake and DPPP = O values, respectively, and are presented in Table 3. All treatments except for hot water treatment of 50 °C resulted in significantly ($P < 0.05$) increased PI or DPPP = O values compared to those of controls (Untreated). Furthermore, combination treatment with hot water and citric acid at all temperatures (50, 60 or 70 °C) induced synergistic increase in DPPP = O as well as PI value, which was significantly ($P < 0.05$) higher than the sum of values obtained from individual treatments of hot water and citric acid.

3.6. Generation of intracellular ROS and O_2^-

Table 4 shows generation levels of total ROS and O_2^- in *E. coli* O157:H7 following citric acid, hot water, or hot water with citric acid treatments. All treatments except for hot water treatment of 50 °C resulted in significantly ($P < 0.05$) increased generation of total ROS or O_2^- compared to untreated controls. In particular, for both total ROS and O_2^- values, 50 or 60 °C hot water with citric acid combined treatments produced significantly ($P < 0.05$) greater values than the sum of values obtained from the individual treatments. However, for total ROS as well as O_2^- value, the combination treatment of 70 °C citric acid and hot water did not induce a synergistic increase and even this value was significantly ($P < 0.05$) lower than that of 70 °C hot water treatment.

Table 3
Levels of destruction and lipid peroxidation of cell membrane of *E. coli* O157:H7 subjected to citric acid (CA), hot water (HW) or hot water with citric acid (HW-CA) inferred from PI and DPPP probes.

Treatment type	Value	
	PI uptake	DPPP = O
Untreated control	0.0 ± 0.0 A	0.0 ± 0.0 A
CA	154.4 ± 19.5 CD	2687.8 ± 276.0 B
50 °C HW	16.6 ± 5.8 AB	333.6 ± 165.3 A
50 °C HW-CA	205.5 ± 1.9 DE	4291.1 ± 546.3 D
60 °C HW	97.8 ± 15.8 BC	2296.7 ± 324.5 B
60 °C HW-CA	1066.7 ± 99.6 F	6358.9 ± 442.7 E
70 °C HW	275.6 ± 8.4 E	3418.9 ± 621.6 C
70 °C HW-CA	1407.1 ± 109.4 G	7934.4 ± 219.1 F

[a]Values are means ± standard deviations from three replications. Means with different letters within the same column are significantly different ($P < 0.05$). Fluorescent signals were normalized by dividing the cell suspension OD_{600} value, and then value was obtained by subtracting the fluorescent value of untreated control from that of treat cells as follows: (fluorescence value after treatment)/OD_{600} – (fluorescence value of untreated control)/OD_{600}.

Table 4
Generation levels of intracellular total reactive oxygen species (ROS) and superoxide (O_2^-) of *E. coli* O157:H7 subjected to citric acid (CA), hot water (HW) or hot water with citric acid (HW-CA) obtained using CM-H_2DCFDA or HDE probe.

Treatment type	Value	
	Total ROS	O_2^-
Untreated control	0.00 ± 0.00 A	0.00 ± 0.00 A
CA	72.22 ± 22.19 B	35.56 ± 8.39 B
50 °C HW	21.11 ± 16.44 A	3.33 ± 3.33 A
50 °C HW-CA	244.44 ± 30.25 C	77.78 ± 27.96 C
60 °C HW	107.78 ± 26.74 B	45.56 ± 8.39 B
60 °C HW-CA	397.78 ± 12.62 E	152.22 ± 18.36 D
70 °C HW	513.33 ± 38.44 F	180.00 ± 26.46 D
70 °C HW-CA	318.41 ± 41.14 D	105.56 ± 15.75 C

[a]Values are means ± standard deviations from three replications. Means with different letters within the same column are significantly different ($P < 0.05$). Fluorescent signals were normalized by dividing the cell suspension OD_{600} value, and then value was obtained by subtracting the fluorescent value of untreated control from that of treat cells as follows: (fluorescence value after treatment)/OD_{600} – (fluorescence value of untreated control)/OD_{600}.

3.7. Superoxide dismutase (SOD) activity

Table 5 shows SOD activities of *E. coli* O157:H7 after treatment with

Table 2
Removal of polysaccharide associated with *E. coli* O157:H7 biofilm on stainless steel (SS) surface following citric acid (CA), hot water (HW) or hot water with citric acid (HW-CA) treatment.

Treatment type	Amount of polysaccharide (%)[a]
Control	100.00 ± 0.00 A
CA	96.18 ± 2.06 A
50 °C HW	59.32 ± 10.52 B
50 °C HW-CA	54.07 ± 10.17 B
60 °C HW	28.61 ± 6.59 C
60 °C HW-CA	23.62 ± 3.02 C
70 °C HW	12.61 ± 4.23 D
70 °C HW-CA	11.97 ± 1.11 D

[a] Values are means ± standard deviations from three replications. Means with different letters within the same column are significantly different ($P < 0.05$). The amount of exopolysaccharides was expressed as a percentage value relative to that of the control. Sterile distilled water treated biofilm SS was used as a control.

Table 5
Superoxide dismutase (SOD) activity in *E. coli* O157:H7 after citric acid (CA), hot water (HW) or hot water with citric acid (HW-CA) treatment.

Treatment type	SOD activity (%)[a]
Untreated control	100.00 ± 0.00 A
CA	84.98 ± 4.85 B
50 °C HW	99.85 ± 0.30 A
50 °C HW-CA	70.77 ± 6.60 C
60 °C HW	99.96 ± 0.42 A
60 °C HW-CA	21.09 ± 7.08 D
70 °C HW	99.45 ± 1.05 A
70 °C HW-CA	1.87 ± 1.68 E

[a] Values are means ± standard deviations from three replications. Means with different letters within the same column are significantly different ($P < 0.05$). The activity of SOD was expressed as a percentage value relative to the activity of the untreated control.

citric acid, hot water or hot water with citric acid. Hot water treatment at 50, 60 or 70 °C did not result in significant ($P > 0.05$) reduction of SOD activity in *E. coli* O157:H7 compared with that of untreated controls. However, SOD activity decreased by 15.02% after citric acid treatment, and it furthermore decreased by 29.23, 78.91, or 98.13% after 50, 60, or 70 °C citric acid and hot water combination treatment, respectively.

4. Discussion

Our result shows that citric acid as well as 50 or 60 °C hot water treated up to 20 s had no ability to inactivate *E. coli* O157:H7 biofilm on SS, but 70 °C hot water can affect biofilm of *E. coli* O157:H7 from 5 s treatment. Similar to these results, Oh and Marshall's study (Oh and Marshall, 1995) showed that 55 °C hot water cannot reduce *L. monocytogenes* biofilm formed on SS during 5 min treatment, whereas 65 °C hot water treatment reduced the pathogen biofilm by 1.4 log after 5 min treatment. However, unlike this study in which a coupon was immersed in a treatment solution preheated to a set treatment temperature, Oh & Marshall's study (1995) was conducted by immersing a tube containing a solution in which a coupon was immersed into a water bath set at a treatment temperature, and thus the scale of the treatment time in their study differs from that of our study. Meanwhile, Wahlen et al.'s study (Wahlen et al., 2016) reported that when the waterborne pathogen *Sphingomonas parapaucimobilis*, which is resistant to heat treatment, was present in biofilm formed on the SS surface, 70, 75, or 80 °C hot water treatment required 44:46, 1:32, or 1:27 (min:sec), respectively, to reduce biofilm cells by 5-log. Therefore, since the type of pathogen and the application form of the hot water sanitation varies according to the actual industry environment, it is important to develop an effective application form of hot water with organic acid sanitation suitable for the environment to be applied while targeting the most resistant pathogen.

Hot water combined with citric acid resulted in a dramatic enhancement of the antimicrobial effect on biofilm of *E. coli* O157:H7 compared with that of hot water because the combination of the two produced a synergistic bactericidal effect. However, this hot water with citric acid treatment generates injured cells. Microorganisms can be killed completely in response to various antimicrobial treatments, becoming dead cells, but they also can be killed incompletely with insufficient treatment, producing what are commonly referred to as injured cells (Ray, 1979; Wesche et al., 2009; Wu, 2008). These injured cells can be recovered under proper condition and regain their normal pathogenicity, and thus it make them as dangerous as their normal counterparts (Wu, 2008). Thus, when applying hot water with citric acid to biofilm of *E. coli* O157:H7 on SS surfaces, considering the occurrence of injured cells is important to avoid over-estimating its antimicrobial effect. Even considering the occurrence of these injured cells, combining citric acid with hot water seems to be an effective strategy to control biofilm of *E. coli* O157:H7 on SS because hot water with citric acid showed a superior control ability due to its synergistic bactericidal effect.

When comparing differences in resistance of *E. coli* O157:H7 biofilm cells, planktonic cells were more vulnerable to 70 °C hot water and 60 or 70 °C hot water with citric acid treatments than biofilm cells. Many studies have also reported that pathogens in biofilm exhibit greater resistance to hot water (Kiskó and Szabó-Szabó, 2011; Oh and Marshall, 1995; Wahlen et al., 2016) or sanitizer (Joseph et al., 2001; Scher et al., 2005; Steed and Falkinham, 2006) treatment than their planktonic counterparts. Since biofilm matrix is composed of EPS which acts as a barrier to block or reduce contact with antimicrobial agents, pathogen shows increased resistant to antimicrobial treatment when in biofilm compared to when they are in the planktonic state (O'Toole et al., 2000). In addition, it is known that not only the phenotypic adaptation of bacteria by harsh environments within the biofilm such as insufficient oxygen/nutrient or high metabolic waste products, but also gene transfer or mutation due to high cell density of biofilm can increase resistance of biofilm cells (Bridier et al., 2011). Therefore, it can be deduced that *E. coli* O157:H7 biofilm showed increased resistance compared to planktonic cells to the treatments of 70 °C hot water and 60 or 70 °C hot water with citric acid due to the complex action of the biofilm matrix.

This study showed that the combined treatment of hot water and citric acid, an organic acid, exhibits a synergistic bactericidal effect. Even if Ban et al.'s study (Ban et al., 2012) also showed that the combined treatment of heat (steam) and lactic acid showed a synergistic bactericidal effect on *Listeria monocytogenes, Salmonella* Typhimurium, and *E. coli* O157:H7 biofilms on polyvinyl chloride (PVC) or SS surfaces, this synergistic inactivation mechanism has not been elucidated. Understanding the inactivation principle of the developed technology is important in creating a more effective application strategy (Cho et al., 2010). Therefore, we tried to identify this mechanism through several approaches. In this study, we divided the mechanism into two parts: the cell itself and the biofilm matrix.

Firstly, residual polysaccharides which is major component to form the structure and impart stability of the biofilm matrix (Branda et al., 2005) on the surface of SS coupons after treatment were measured (Table 2). Our results indicate that citric acid has no ability to remove polysaccharides, but hot water can remove polysaccharides and the removal rate increased with increasing temperature. However, hot water with citric acid treatment removed the same amount of polysaccharides as hot water at the same temperature. Since polysaccharides are representative materials of EPS composed of various substances such as proteins, S-layer glycoproteins, glycolipids extra-cellular DNA, metal ions, and other surface-active components (Branda et al., 2005; Jahid and Ha, 2012), it can be considered that only heat treatment has the ability to remove EPS from SS surfaces. Therefore, with respect to the biofilm matrix, it can be interpreted that improved contact of citric acid with cells on the surface due to the removal of EPS by hot water resulted in an enhanced, and thus synergistic, bactericidal effect.

Meanwhile, combined treatment of hot water and citric acid showed a synergistic bactericidal effect on planktonic cells of *E. coli* O157:H7. This means that, in addition to the biofilm matrix aspect, this combination treatment generates synergistic bactericidal effect on the cell itself, leading to a synergistic inactivation result on *E. coli* O157:H7 biofilm on SS. In order to identify the synergistic bactericidal effect on the cell itself, it was first necessary to find damage site in the cell. Table 3 shows that 50, 60, or 70 °C hot water with citric acid led to a synergistic increase in the PI uptake value. PI does not penetrate the intact cell membrane, but when destruction of the cell membrane occurs, such as with pore-formation, it can penetrate into the cell, form a complex with nucleic acids, and exhibit fluorescence (Breeuwer and Abee, 2000). That is, the increased PI uptake value indicates that cell membrane destruction has increased. Meanwhile, this form of damage increasing the cell membrane permeability makes the cell difficult to retain homeostasis and ultimately leads to cell inactivation (Pagán and Mackey, 2000; Park and Kang, 2013). Therefore, it can be interpreted that the synergistic inactivation effect by combined treatment of hot water plus citric acid is attributed to destructive synergistic damage to the cell envelope. Furthermore, tabulated DPPP = O values as shown in Table 3 indicate that these values also increased synergistically by with hot water with citric acid treatment. Non-fluorescent DPPP is converted into fluorescent DPPP = O when reacting with hydroperoxide in the cell membrane (Okimoto et al., 2000). Therefore, DPPP = O value can represent the incidence of lipid peroxidation in the cell membrane, which induces an increase in permeability as well as a decrease in potential and fluidity of the cell membrane (Gutteridge, 1995). Consequently, since lipid peroxidation in the cell membrane is synergistically induced from combined hot water and citric acid treatment, it can be interpreted that lipid peroxidation is the cause of this synergistic destruction of the cell membrane resulting from this combination treatment.

Next, it was necessary to determine why synergistic lipid

peroxidation occurred. It is known that ROS is one of the leading causes activating lipid peroxidation in the cell membrane inducing to destruction (von Moos and Slaveykova, 2014). Based on this fact, we confirmed the occurrence of intracellular ROS following each treatment. Since among various forms of ROS O_2^- play an key role in the induction of other oxygen radicals with potential to cause biological oxidative damage (Gülçin, 2006; Pietta, 2000), the occurrence of superoxide as well as total ROS was also confirmed. CM-H_2DCFDA, which is a cellular probe that is converted to dichlorofluorescein (DCFH) within the cell, which is in turn oxidized by ROS and hydrolyzed into fluorescent 2′, 7′-dichlorofluorescein (DCF), was used to measure total ROS (Wojtala et al., 2014). Intracellular superoxide was identified with hydroethidine (HDE), which is converted to ethidium bromide as it reacts with superoxide within the cell, which in turn emits fluorescence as it intercalates into DNA (Gomes et al., 2005). Interestingly, as shown in Table 4, 50 or 60 °C hot water with citric acid induced synergistic generation of ROS or superoxide. Therefore, it can be interpreted that the synergistic incidence of lipid peroxidation in the cell membrane was attributed to the occurrence of synergistic ROS generation. However, treatment with 70 °C hot water with citric acid showed less occurrence of ROS than with 70 °C hot water. This result is in contrast to the result of induction of lipid peroxidation in the cell membrane by 70 °C hot water with citric acid. In this regard, it can be interpreted that 70 °C hot water with citric acid treatment induced excessive destruction of the cell membrane, inducing the ROS leakage within the cell, which consequently led to reduced intracellular ROS. That is, 70 °C hot water plus citric acid also caused synergistic ROS generation, but extensive damage to the cell membrane led to ROS leakage, which is considered to be the cause of reduced intracellular ROS generation.

In order to identify the mechanism in more detail, it was necessary to find the cause of the synergistic occurrence of ROS. Although some studies have already reported that organic acids induce outer membrane damage through intercalation, chelation, or protonation (Alakomi et al., 2000, 2007), the result of this study identifying that citric acid generates intracellular ROS-inducing lipid peroxidation in the cell membrane is interesting because it is a new discovery of another inactivation mechanism of organic acids. Furthermore, organic acids have many other complex inactivation mechanisms besides cell membrane damage and possible inactivation mechanisms of organic acids are listed in the literature review of Mani-Lopez et al. (2012). Their article states that when an organic acid enters into the cell, it stimulates the cell to keep its internal pH normally by increasing the consumption of adenosine triphosphate (ATP), resulting in depletion of energy, and the lowering of internal pH by the organic acid damages enzymes, DNA, and structural proteins, and thus changes their function. Meanwhile, since organisms with aerobic metabolism produce ROS such as the O_2^-, hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH) as by-products in the normal metabolic process and thus are always confronted with the risk of oxidative damage caused by ROS, aerobic organisms have several defense systems that scavenge ROS to protect themselves from these stresses (Yao et al., 2006). The most well-known non-enzymatic ROS defense system is glutathione (GSH) and the enzymatic ROS defense system includes SOD, glutathione peroxidase (GPx), and catalase (CAT) (Li et al., 2007; Sies, 1999). From the inactivation mechanisms of organic acids and the characteristics of aerobic organisms, we assumed that reduction of the ROS scavenging ability of enzymatic ROS defense systems by citric acid induced generation of intracellular ROS. Furthermore, based on these properties, the principle of synergistic generation of ROS by combined treatment of hot water and citric acid can be deduced as follows: with combined hot water and citric acid treatment, destruction of the cell membrane caused by hot water induces penetration of more citric acid into the cell, leading to more functional loss of enzymatic ROS defense systems resulting in synergistic generation of ROS. To demonstrate this hypothesis, activity of a particular ROS defense enzyme in *E. coli* O157:H7 was measured following each treatment. Among the various antioxidant enzymes, SOD activity that decomposes superoxide into hydrogen peroxide, which has a lower oxidative damage potential, was investigated in our study because SOD is generally assumed to play a major role in defending against oxidative damage due to the characteristic of SOD being present in all aerobic organisms and most subcellular compartments where active oxygen is produced (Nakonieczna et al., 2010; Scandalios, 1993). As shown in Table 5, treatment with hot water with citric acid at all temperatures (50–70 °C) led to a synergistic decease in SOD activity in *E. coli* O157:H7. T above hypothesis can be proved by this result, and to sum up comprehensively, we conclude that when hot water and citric acid are combined, increased penetration of citric acid into cells due to increased cell membrane permeability by heat treatment leads to a synergistic reduction of SOD activity, and concurrent synergistic ROS generation induces synergistic cell membrane damage, eventually leading to synergistic inactivation of the cells.

In conclusion, our results reveal that combination of hot water and citric acid effectively controls *E. coli* O157:H7 biofilm on SS surface through synergy effect. However, since the combined treatment of hot water and citric acid generates injured cells, it is important to consider injured cells to avoid over-estimating the inactivation effect when applying this technique. Meanwhile, data identifying the mechanism for the synergistic inactivation effect of this combination revealed in this study is worthy of utilization as a baseline for further research or industry applications related to this. Also, we believe it is necessary to perform research to investigate the control ability of this technology on pathogen biofilms formed inside pipes in processing plants to broaden its applicability in the food industry where biofilms are a problem.

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