Master's Thesis of Science
in Agricultural Biotechnology

Synergistic bactericidal effect of hot water and citric acid against pathogens in biofilm formed on food contact surface

- Identifying mechanism of synergistic bactericidal effect -

식품 접촉면에 형성된 바이오필름 속 식중독균에 대한 온수와
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ABSTRACT

This study investigated the antimicrobial effect of hot water with citric acid against *Escherichia coli* O157:H7 in biofilm formed on stainless steel (SS). The hot water with citric acid of 50, 60, or 70°C exhibited synergistic bactericidal effect on the pathogen biofilm. At this time, it was shown that sub-lethally injured cells should be considered when applying this technology because it generated sub-lethally injured cells. Meanwhile, in this study, the 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 polysaccharide, a major component of the extracellular polymeric substances (EPS), to increase the contact of the 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 inactivity of superoxide dismutase (SOD) in *E. coli* O157:H7, resulting in a synergistic generation of reactive oxygen species (ROS), and thus these ROS lead synergistic cell death by activating synergistic incidence of lipid peroxidation, which induces synergistic destruction of cell membrane. Therefore, it is interpreted that when hot water with citric acid is applied to the *E. coli* O157:H7 biofilm on SS, the synergy effects on the biofilm matrix and cell itself have a complex

interaction with each other, thus causing a dramatic synergistic bactericidal effect. Based on these results, I conducted the experiments on pipe to evaluate applicability of this combined treatment in the food industry.

Keywords: **biofilm, foodborne pathogens, hot water, citric acid, food contact surfaces, synergistic effect**
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I. INTRODUCTION

Contamination by foodborne pathogens is a continuing concern for producers and consumers (1). Food contamination is usually caused by contact with food contact surfaces such as knives, containers, and conveyor belts, etc (2). Thus, inappropriate cleaning or disinfection in food processing plants can lead to contamination of pathogens in food products, resulting in serious health risks to consumers (3). In particular, the microorganisms attached to the wet surface have a natural tendency to multiply and become trapped in a slimy matrix composed of the extracellular polymeric substances (EPS) they produce, forming biofilm (4). Pathogens in biofilms exhibit enhanced resistance to mechanical actions or commonly used sanitizers, and about 80% of all bacterial infections are associated with biofilm; thus eliminating them from food processing plants is a huge challenge (5-7). Therefore, the process of effectively removing pathogens in biofilm formed on food contact surfaces is critical to control the outbreaks of foodborne illness.

Escherichia coli O157:H7 is typical Gram-negative foodborne pathogenic bacteria and causes diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome in humans (8). *E. coli* O157:H7 surviving on the food contact surface is capable of producing biofilm and thus can increase the probability

of food contamination (9-11). It is known that *E. coli* O157:H7 can form biofilm on various type of food contact surfaces such as glass, plastic, and stainless steel (SS) (12-15).

Hot water sanitation is commonly applied in the food industry to ensure the microbial safety of fruits, vegetables, meat, and food contact surfaces. (16). It is believed that the hot water treatment is familiar and easy to use in food processing plant. Indeed, the United States Food and Drug Administration (FDA) has approved hot water for surface decontamination because of its effectiveness (17). However, it requires a high level of thermal energy, which is costly, even though cleaning surfaces through hot water is very effective (18). Thus, it is considered that there is a need to apply hurdle technology, which is known as combined two more treatments and induces synergistic bactericidal effect. Through this combination representing the synergistic effect, it is possible to reduce the input energy by decreasing the treatment time or temperature required for reducing the pathogen to a desired level, and also to ensure safety against pathogens by the increased inactivation effect (19, 20).

Organic acids are usually selected in the food processing industry as well as hot water and known to effective not only in pathogens but also in biofilms (21-23). They pass through cell membrane as un-dissociated form and then

lowers the intracellular pH, resulting in depletion of cellular energy to regulate internal pH, which means inhibits metabolic activities, and eventually lead to cell death (24). They are approved as GRAS for use in the manufacture of fresh and processed meats and poultry products (25) and are a representative substitute for chlorinated water which has the drawbacks of rapidly decomposing and forming carcinogenic halogenated by-products by reacting with organic matter (26). They have the characteristics of rapidly killing a wide range of bacteria, being effective within a broad range of temperature and not affected by water hardness (27).

In fact, Ban et al.'s study (28) reported that inactivation effect of combining heat (100^{o}C steam) and organic acid (lactic acid) was higher than the sum of inactivation effect by individual treatment of each treatment against *E. coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes* in biofilms on food contact surface (SS and polyvinyl chloride coupons). Since heat and organic acid combination treatment have shown synergistic bactericidal effect on biofilm pathogens from that previous study, I expected that the combination of hot water and organic acid may have synergistic bactericidal effect.

Therefore, this study confirmed the inactivation effect of combination treatment of hot water and citric acid on *E. coli* O157:H7 biofilms formed on

food-contact surface. At this time, 2% of citric acid was selected among several organic acids for use in this study because it is known to exhibit powerful antimicrobial effect due to its low pH (29). Also, among various types of food-contact surface, biofilm was formed on SS because it is the most commonly used material for application to food contact surfaces in the food industry due to its properties of heat transfer efficiency, hygienic, corrosion resistance and rigidity (30, 31). Furthermore, identification of mechanism of synergistic bactericidal effect on biofilm pathogens exhibited by treatment of that combination was performed.

II. MATERIALS AND METHODS

2.1. Bacterial strains and culture preparation

Three strains of *E. coli* O157:H7 (8624, 2026, 2029), *S. aureus* (ATCC 25923 ATCC 27213 ATCC 29213) were obtained from the bacterial culture collection at Seoul National University (Seoul, Korea) and used in this study. Stock cultures were grown in tryptic soy broth (TSB; Difco, Becton, Dickinson and Company, Sparks, MD, USA) and stored at -80^{o}C in 0.7 ml TSB and 0.3 ml of 50% glycerol. Working cultures were obtained by streaking bacteria onto tryptic soy agar (TSA; Difco), incubated at 37^{o}C for 24 h, and stored at 4^{o}C. Each strain of *E. coli* O157:H7 was cultivated in 15 ml of TSB at 37^{o}C for 24 h of shaking incubation. The cell pellets were collected by centrifugation (4000 x g at 4^{o}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 approximately 10^8 – 10^9 colony-forming-units (CFU)/ml. This inoculum was used for biofilm formation or treatment of planktonic cell in this study.

2.2. Biofilm formation

Prepared inoculum (10 ml) was inoculated into 500 ml of PBS to approximately 10^7 - 10^8 CFU/ml. SS coupons (type 304, no.4 finish) were

prepared into size of 5 × 2 cm^2 and Stainless steel (SUS), Polyethylene-Raised Temperature (PE-RT) pipes were cut into size of 5 cm in length, 1 cm in diameter. Prepared surface samples were soaked in 70% ethanol for 24 h and washed with sterile distilled water. The washed surface samples were sterilized by autoclaving. Each prepared sterile surface sample was transferred to a sterile 50 ml conical centrifuge tube containing 30 ml of cell suspension of *E. coli* O157:H7 and *S. aureus* in PBS. Conical centrifuge tubes with surface samples were incubated at 4°C for 24 h to facilitate cell attachment. After attachment, surface samples were gently stirred for 5 s in sterile distilled water to remove unattached cells. The rinsed surface samples were put into sterile 50 ml conical centrifuge tubes containing 30 ml of TSB and incubated at 25°C for 5 days for biofilm formation. This method was adapted from reference (32).

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

Treatment solutions of citric acid (CA; 99.5%, Samchun Pure Chemical Co. LTD., Korea) were prepared with sterile distilled water at a concentration of 2% (pH 1.3). For hot water and hot water with citric acid treatments, sterile distilled water and CA solution were adjusted to 50, 60, 70, and 75°C. The temperature was controlled and maintained with a water bath, and monitored

using a K-type Teflon-coated thermocouple. For citric acid treatment alone, CA solution was held at room temperature (22 ± 2°C).

2.3.1. Coupon biofilm

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

For planktonic cells treatment, 1 ml of prepared inoculum was inoculated into the 15 ml glass tube containing 9 ml of DW and CA solution adjusted to target temperature (22, 50, 60, or 70°C) for 5, 10, 15, or 20 s. Immediately after treatment, the glass tube was put into ice-water to lower temperature of treated solution.

2.3.2. Pipe biofilm

Biofilm formed pipes were rinsed as described previously to remove unattached cells. The surface of the rinsed pipes were wiped with 70%

alcohol to observe the reduction of biofilm cells inside the pipe. DW or CA solution adjusted to target temperature (22, 60, 70, or 75ºC) were flowed at a flow rate of 0.3, 0.5, 0.7 LPM using a peristaltic pump to be treated inside surface of each pipe (FIGURE 2). *E. coli* O157:H7 and *S. aureus* biofilm cells were exposed to each solution for 3 s and 10 s, respectively. Immediately after treatment, pipe was transferred into 50 ml conical centrifuge tube containing 30 ml of PBS with 3 g of sterile glass beads (425–600 μm; Sigma-Aldrich, St. Louis, MO, USA).

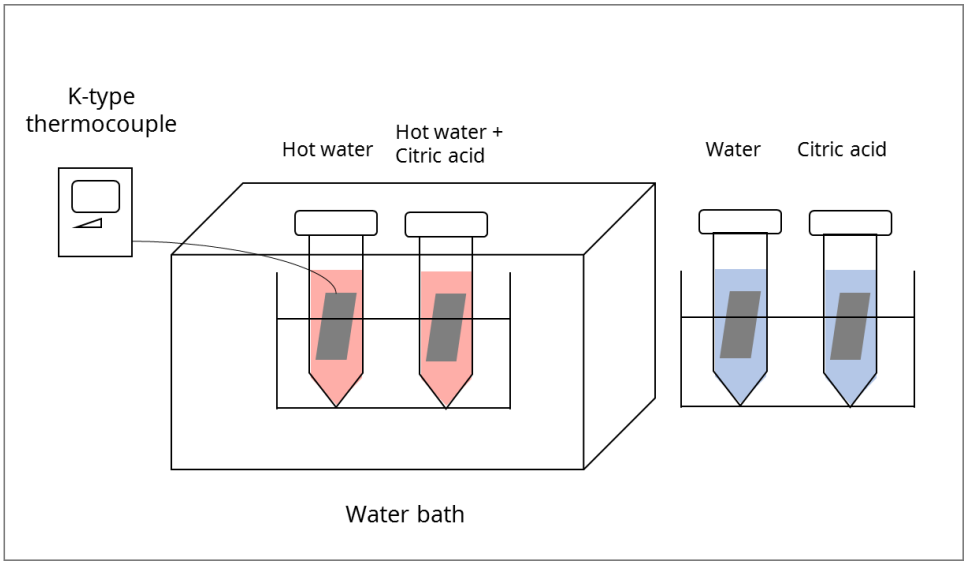

FIGURE 1. Schematic diagram of the hot water and citric acid treatment on coupon.

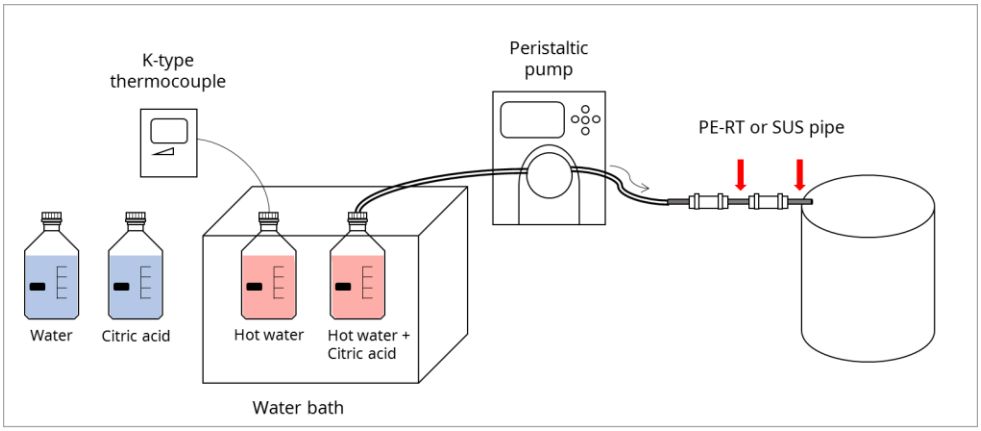

FIGURE 2. Schematic diagram of the hot water and citric acid treatment on pipe.

2.4. Bacterial enumeration

For biofilm cells enumeration, treated and untreated (control) surface samples placed in 50 ml conical centrifuge tubes containing 30 ml of PBS with 3 g of sterile glass beads were vortexed at maximum speed for 1 min with benchtop vortex mixer for detaching the pathogenic biofilm cells from surface samples. After detachment, 1 ml of undiluted cell suspensions were 10-fold serially diluted in 9 ml of PW and spread-plated onto selective media. For planktonic cells enumeration, 1 ml of treated and untreated (control) cell suspensions were 10-fold serially diluted in 9 ml of Dey/Engley (DE) neutralizing broth (Difco) and 0.1 ml of undiluted cell suspensions or diluents were spread-plated onto selective media. At this time, sorbitol MacConkey agar (SMAC; Difco), Baird Parker Agar (BPA; MB cell) were used as a selective media to enumerate the number of *E. coli* O157:H7 and *S. aureus*. All plates were incubated at 37^{o}C for 24 h and after incubation, the number of typical colonies were counted.

2.5. Enumeration of injured cells

Enumeration of injured *E. coli* O157:H7 planktonic or biofilm cells was accomplished with SPRAB (33). One-tenth-milliliter aliquots of undiluted cell suspensions and diluents were spread-plated onto SPRAB plates. The

plates were incubated at 37°C for 24 h and white colonies characteristic of *E. coli* O157:H7 were enumerated, and simultaneously, serological confirmation (RIM; *E. coli* O157:H7 latex agglutination test; Remel, Lenexa, KS, USA) was performed on randomly selected presumptive *E. coli* O157:H7 colonies.

2.6. Identification of mechanism of synergistic bactericidal effect

I conducted the experiments to identify the cause of synergistic inactivation effect when combined with hot water and citric acid. Cell suspension was prepared as in the above method and optical density at 600 nm (OD_{600}) was adjusted to 0.3. In the case of measurement of EPS, biofilms formed SS coupons were used. The treatment was carried out under the same conditions as for the inactivation experiment, and the treatment time was fixed to 10 s.

2.6.1. Amount of biofilm EPS

The amount of EPS of *E. coli* O157: H7 biofilm formed on the SS coupon was measured as follows with reference to two studies (34, 35). To measure the change in EPS amount by treatment, the amount of polysaccharides which is the main constituent of EPS was assessed using calcofluor white staining. Treated SS coupons were transferred to sterile 50 ml conical

centrifuge tubes containing 30 ml of calcofluor white solution (1 mg of fluorescent brightener 28 in 1 ml of dH_2O) and incubated for 15 min in the dark. After incubation, stained SS coupons were placed in 50 ml conical centrifuge tubes containing 30 ml of PBS with 3 g of sterile glass beads and vortexed at maximum speed for 1 min with vortex mixer for detaching polysaccharides bound calcofluor white. The PBS in which the polysaccharides were dispersed was centrifuged at 10,000 × g for 10 min and then fluorescence of the supernatant was measured with a spectrophotometer (Spectramax M2e; Molecular Devices, CA, USA) at excitation and emission wavelength of 360 and 460 nm, respectively.

2.6.2. Measurement of cell membrane damage

I conducted two assays to quantitatively determine 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 assess the change of cell membrane permeability and incidence of lipid peroxidation on cell membrane, respectively. Treated cell suspensions were incubated with PI or DPPP at final concentration of 2.9 or 50 μM for 10 or 20 min, respectively, at 37°C. After incubation, cells were collected by centrifugation (10,000 × g for 10

min) followed by washing twice with PBS to remove residual dye. The cell pellets were re-suspended in PBS and fluorescence was measured with a spectrophotometer at excitation/emission wavelength of 493/630 nm or 351/380 nm for PI uptake or DPPP assay, respectively.

2.6.3. Measurement of intracellular total reactive oxygen species (ROS) and superoxide (O_2^-) generation

To detect the degree of intracellular ROS generation, I evaluated total ROS and superoxide generation with cellular probe, 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 with CM-H2DCFDA or HDE at a final concentration of 5 μM for 15 or 30 min, respectively, at 37ºC. After incubation, cells were centrifuged at 10,000 × g for 10 min followed by washing twice with PBS to remove residual dye. The cell pellets were re-suspended in PBS and fluorescence was measured with a spectrophotometer (Spectramax M2e; Molecular Devices, CA, USA) at excitation/emission wavelength of 495/520 nm or 518/605 nm for total ROS assay or superoxide assay, respectively.

2.6.4. Measurement of superoxide dismutase (SOD) activity

I assess the activity of superoxide dismutase (SOD) which is representative of ROS scavenging enzyme using a SOD assay kit -WST (Sigma-Aldrich). Treated cells were collected by centrifugation at 10,000 × g and 4^{o}C for 10 min and cell pellets were re-suspended in PBS. Re-suspended cells in the PBS were disrupted by sonicator (10s on and 10s off, six times) in an ice bath and then centrifuged at 10,000 × g for 10 min at 4^{o}C to obtain a supernatant containing SOD. Then, SOD activity measurement was followed manufacturer's instructions.

2.7. Confocal laser scanning microscopy

A Live/Dead BacLight Bacterial Viability Kit (L7012, Molecular probes, USA) was used to evaluate membrane integrity of biofilm cells inside the pipes. Because of experimental limitations biofilm was formed on the surface of coupons instead of the pipe. The staining solution was prepared by diluting 3 μl of SYTO9 and PI into 1 ml of distilled water. Stainless steel coupons with *S. aureus* biofilm were treated with HW and CA and 10 ul of the staining solution was applied to the coupons. The coupons were stained for 30 min at room temperature in the dark. Biofilm samples were imaged with an upright confocal laser scanning microscope (CLSM, Eclipse 90i,

Nikon, Japan) using a 60X water immersion objective lens with a numerical aperture of 0.9. Image stacks at various foci collected through the CLSM were reconstructed in three-dimension using IMARIS software. (Bitplane, Zurich, Switzerland) (36)

2.8. Statistical analysis

All experiments were repeated three times with duplicate samples. 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.

III. RESULTS

3.1. Inactivation of foodborne pathogens in biofilm on stainless steel coupons

3.1.1. Synergistic bactericidal effect of hot water with citric acid against biofilm E. coli O157:H7 or planktonic cell of the pathogen

TABLE 1 shows the viable-count reductions of *E. coli* O157:H7 in biofilm formed on stainless steel (SS) during citric acid, hot water, or hot water with citric acid treatment. The initial populations of *E. coli* O157:H7 biofilm on SS enumerated on SMAC and SPRAB were 5.90 and 5.91 log CFU/cm^2, respectively.

In case treated cells were enumerated on SMAC, the 20 s treatment of citric acid, 50°C hot water and 60°C hot water resulted in reductions of 0.62, 0.44 and 0.54 log CFU/cm^2, respectively. These reduction levels were not significantly ($P > 0.05$) difference from the reduction level obtained after 20 s treatment of DW. On the other hand, 70°C hot water reduced the *E. coli* O157: H7 biofilm to below the detection limit (< 0.18 log CFU/cm^2) after 20 s treatment. Meanwhile, when citric acid and hot water were combined, 50°C hot water with citric acid achieved a 2.49 log reduction of *E. coli* O157:H7 biofilm after 20 s treatment, and hot water with citric acid of 60°C and 70°C

reduced *E. coli* O157:H7 biofilm to below the detection limit (< 0.18 log CFU/cm^2) after 15 and 5 s treatments, respectively. This combination treatment of hot water and citric acid exhibited synergistic bactericidal effect: combination treatment resulted in significantly (P < 0.05) larger reduction level than the sum of the reduction level produced by the individual treatments of hot water and citric acid. Specifically, the 50°C hot water with citric acid showed a synergistic effect from 10 s treatment, and the hot water with citric acid of 60°C and 70°C showed synergistic effect from 5 seconds.

In case treated cells were enumerated on SPRAB, similar to the results from the case enumerated on SMAC, citric acid and 50°C or 60°C hot water led to slight log reduction of *E. coli* O157:H7 biofilm after 20 s treatment (0.54, 0.42, and 0.44 log CFU/cm^2, respectively), and these reductions were not significantly (P > 0.05) different from the 0.44 log reduction obtained from 20 s treatment of DW. 70°C hot water treatment resulted in 5.54 log reduction of *E. coli* O157:H7 biofilm after 20 s treatment. 50°C or 60°C hot water with citric acid resulted in 1.45 or 2.90 log reduction 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 to below the detection limit (< 0.18 log CFU/cm^2) after 10 s treatment. This combination of hot water and citric acid also induced synergistic bactericidal effect on the cells enumerated

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 its combination were also investigated for planktonic cells of *E. coli* O157:H7 in sterile DW (TABLE 2). At this time, treated cells were enumerated on both SMAC and SPRAB media and initial populations of planktonic cell of *E. coli* O157:H7 enumerated on SMAC and SPRAB were 7.88 and 8.13 log CFU/ml, respectively. Overall, the reduction tendency of planktonic cells of *E. coli* O157:H7 following treatments were similar to that of the pathogen biofilm. For both cells enumerated on SMAC and SPRAB after treatment, citric acid, 50°C and 60°C hot water induced slight reduction (< 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 synergistic bactericidal effect for planktonic cells of *E. coli* O157:H7. For the cells enumerated on SMAC after treatment, 50, 60 and 70°C hot water with citric acid showed synergistic effect after 5 s treatment, and for the cells enumerated on SPRAB after treatment, those showed synergistic effect after 15, 15 and 5 s treatments, respectively.

3.1.2. Comparative reduction levels of cells enumerated on SMAC and SPRAB to citric acid, hot water or hot water with citric acid

For both *E. coli* O157:H7 biofilm and planktonic cells of the pathogen, after treatments, survival cells were enumerated not only on selective medium, SMAC, but also on recovery medium, SPRAB (TABLE 1 and 2). In case biofilm *E. coli* O157:H7 was treated with citric acid or all temperature hot water (50, 60 or 70°C), the reduction levels of cell enumerated on SMAC were not significantly ($P > 0.05$) different from those of cell enumerated on SPRAB for all treatment time (5- 20 s). However, hot water with citric acid of 50, 60 or 70°C resulted in significant ($P < 0.05$) less reduction levels in cell enumerated on SPRAB than those in cells enumerated on SMAC occurred after 20, 5 or 5 s treatment, respectively. In case of planktonic cells of *E. coli* O157:H7, also, citric acid and hot water treatment (50, 60 or 70°C) did not produce significant ($P > 0.05$) differences between the reduction levels of treated cells enumerated on SMAC and SPRAB for all treatment (5 – 20 s), whereas hot water with citric acid of 50 or 60°C resulted in significant ($P < 0.05$) less reduction levels in cells enumerated on SPRAB than those in cells enumerated on SMAC for all treatment (5 – 20 s). Meanwhile, there were no significant ($P > 0.05$) differences between the

reduction levels of treated cells enumerated on SMAC and SPRAB after hot water with citric acid of 70°C.

3.1.3. Different resistance to citric acid, hot water, or hot water with citric acid treatment between biofilm E. coli O157:H7 and planktonic cells of the pathogen

To compare the differences in resistance between *E. coli* O157:H7 biofilm and planktonic cells of the pathogen for treatments, the reduction results of *E. coli* O157:H7 biofilm (TABLE 1) and planktonic cells of the pathogen (TABLE 2) were compared. In both cells enumerated on SMAC and SPRAB, there were no significantly ($P > 0.05$) differences of reduction level between *E. coli* O157:H7 biofilm and planktonic cells of the pathogen during citric acid or hot water of 50 or 60°C treatment (5 – 20 s), whereas reduction levels in planktonic cells of *E. coli* O157:H7 were significantly ($P < 0.05$) greater than those in the pathogen biofilm during 70°C hot water or 60°C hot water with citric acid treatment (5 – 20 s). Meanwhile, 50°C hot water with citric acid treatment induced significant ($P < 0.05$) greater reductions in planktonic cells of *E. coli* O157:H7 than those in biofilm the pathogen for cells enumerated on SMAC, but this treatment produced no significant ($P > 0.05$) differences of reduction level between planktonic cells of *E. coli* O157:H7

and the pathogen biofilm for the cells enumerated on SPRAB. On the other hand, 70°C hot water with citric acid treatment produced no significant ($P > 0.05$) differences in reduction level between planktonic cells of *E. coli* O157:H7 and the pathogen biofilm in case of the cells enumerated on SMAC, whereas this treatment resulted in significant ($P < 0.05$) greater reduction levels in planktonic cells of *E. coli* O157:H7 than those in the pathogen biofilm in case of the cells enumerated on SPRAB.

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

Treatment type	Cell enumeration medium	Log reduction [Log_{10} (N_0/N)][a] by treatment type and medium			
		Treatment time (s)			
		5	10	15	20
Untreated control	SMAC	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	0.55 ± 0.35 Aa	0.67 ± 0.21 Aa	0.62 ± 0.23 Ab
50°C HW		0.12 ± 0.11 Aab	0.35 ± 0.17 Aa	0.33 ± 0.16 Aa	0.44 ± 0.19 Aab
50°C HW-CA		1.00 ± 0.29 Ac	1.56 ± 0.26 ABb	1.69 ± 0.30 Bb	2.49 ± 0.54 Cc
60°C HW		0.37 ± 0.25 Aab	0.39 ± 0.09 Aa	0.43 ± 0.14 Aa	0.54 ± 0.21 Ab
60°C HW-CA		2.15 ± 0.03 Ad	4.23 ± 0.20 Bc	> 5.73 Cd	-
70°C HW		0.46 ± 0.25 Ab	1.91 ± 0.86 Bb	4.17 ± 1.06 Cc	> 5.73 Dd
70°C HW-CA		> 5.73 e	-	-	-
Untreated control	SPRAB	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 Aab	0.40 ± 0.29 Aab	0.54 ± 0.15 Ab	0.54 ± 0.13 Aa

50°C HW	0.13 ± 0.06 Aa	0.34 ± 0.06 Aa	0.32 ± 0.06 Aab	0.42 ± 0.23 Aa
50°C HW-CA	0.61 ± 0.08 Abc	1.05 ± 0.50 Bc	1.06 ± 0.29 BCc	1.45 ± 0.20 Cb*
60°C HW	0.23 ± 0.13 Aab	0.29 ± 0.24 Aa	0.35 ± 0.17 Aab	0.44 ± 0.20 Aa
60°C HW-CA	0.87 ± 0.41 Ac*	1.06 ± 0.50 Ac*	1.72 ± 0.55 ABd*	2.90 ± 0.92 Bc*
70°C HW	0.25 ± 0.33 Aab	0.83 ± 0.35 Abc	3.94 ± 0.52 Be	5.54 ± 0.66 Cd
70°C HW-CA	3.62 ± 0.54 Ad*	> 5.74 Bd	-	-

[a]Values are means ± standard deviations from three replications. Means with same uppercase letters within the same row are not significantly different ($P > 0.05$). Means with different lowercase letters within same column at same media are significantly different ($P < 0.05$). Significant differences ($P < 0.05$) in the means of SPRAB medium from the means of SMAC medium at the same treatment time and type are marked with asterisks.

DW, distilled water treatment; SMAC, sorbitol MacConkey agar; SPRAB, phenol red agar base with 1% sorbitol; N_0, initial population; N, population after treatment.

TABLE 2. Log reductions of 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	Cell enumeration medium	Log reduction [Log_{10} (N_0/N)][a] by treatment type and medium			
		Treatment time (s)			
		5	10	15	20
Untreated control	SMAC	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa
CA		0.11 ± 0.08 Aa	0.21 ± 0.09 Aab	0.14 ± 0.11 Aab	0.25 ± 0.04 Aab
50°C HW		0.09 ± 0.10 Aa	0.19 ± 0.22 Aab	0.23 ± 0.05 Aab	0.24 ± 0.09 Aab
50°C HW-CA		1.79 ± 0.53 Ab	2.20 ± 0.22 ABc	2.76 ± 0.71 ABc	3.01 ± 0.61 Bc
60°C HW		0.28 ± 0.30 Aa	0.48 ± 0.34 Ab	0.57 ± 0.48 Ab	0.72 ± 0.50 Ab
60°C HW-CA		4.81 ± 0.15 Ad	5.20 ± 0.32 Ae	> 6.88 Be	-
70°C HW		3.35 ± 0.63 Ac	4.23 ± 0.13 Bd	5.06 ± 0.11 Cd	> 6.88 Dd
70°C HW-CA		5.19 ± 0.22 Ad	> 6.88 Bf	-	-
Untreated control	SPRAB	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa
CA		0.20 ± 0.05 Aab	0.15 ± 0.07 Aa	0.12 ± 0.06 Aa	0.28 ± 0.02 Aa
50°C HW		0.20 ± 0.07 Aab	0.10 ± 0.14 Aa	0.16 ± 0.12 Aa	0.12 ± 0.11 Aa
50°C HW-CA		0.67 ± 0.42 Ab*	0.95 ± 0.45 ABb*	1.55 ± 0.33 ABb*	1.74 ± 0.70 Bb*
60°C HW		0.19 ± 0.17 Aab	0.22 ± 0.17 Aa	0.33 ± 0.15 Aa	0.29 ± 0.22 Aa

60°C HW-CA	2.96 ± 0.41 Ac*	3.56 ± 0.34 ABc*	4.46 ± 0.53 BCc*	5.25 ± 0.88 Cc
70°C HW	2.73 ± 0.52 Ac	4.07 ± 0.35 Bd	5.05 ± 0.22 Cd	6.73 ± 0.71 Dd
70°C HW-CA	5.30 ± 0.58 Ad	> 7.13 Be	-	-

[a]Values are means ± standard deviations from three replications. Means with same uppercase letters within the same row are not significantly different ($P > 0.05$). Means with different lowercase letters within same column at same media are significantly different ($P < 0.05$). Significant differences ($P < 0.05$) in the means of SPRAB medium from the means of SMAC at the same treatment time and type are marked with asterisks.

DW, distilled water treatment; SMAC, sorbitol MacConkey agar; SPRAB, phenol red agar base with 1% sorbitol; N_0, initial population; N, population after treatment.

3.2. Identification of mechanism of synergistic bactericidal effect

3.2.1. Removal of EPS from SS surface

TABLE 3 represent residual exopolysaccharide associated with *E. coli* O157:H7 biofilm after citric acid, hot water or hot water with citric acid treatment. Citric acid treatment slightly reduced exopolysaccharide on SS surface (by 3.82%), but there was no significant ($P > 0.05$) difference when compared to control. However, treatment of hot water or hot water with citric acid of all temperature (50, 60 or 70°C) significantly ($P < 0.05$) removed exopolysaccharide from SS surface. In both hot water and hot water with citric acid, the degree of exopolysaccharide removal was significantly ($P < 0.05$) increased with increasing treatment temperature from 50 to 70°C. Specifically, 50, 60, or 70°C of hot water treatment reduced exopolysaccharide on SS surface by 40.68, 71.39 or 87.39%, respectively, and 50, 60 or 70°C hot water with citric acid reduced exopolysaccharide on SS surface by 45.93, 76.38 or 88.03%, respectively. For all treatment temperature, however, the removal of exopolysaccharide between hot water and hot water with citric acid was not significantly ($P > 0.05$) different at the same treatment temperature.

TABLE 3. The amount 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 uppercase letters within the same column are significantly different ($P < 0.05$). The amount of exopolysaccharide was expressed as a percentage value relative to that of the control. Sterile distilled water treated biofilm SS was used as a control.

3.2.2 The extent of cell membrane damage following citric acid, hot water or hot water with citric acid

To quantitatively assess the damage of the cell membrane, the treated cells were stained with fluorescent dye, propidium iodine (PI) or diphenyl-1-pyrenylphosphine (DPPP), which can indicate the degree of destruction or occurrence of lipid peroxidation of cell membrane, respectively. The values obtained from these assays were expressed as PI uptake and DPPP=O values, respectively and are presented in TABLE 4. All treatments except for 50°C hot water treatment resulted in significantly ($P < 0.05$) increased values of PI or DPPP=O compared to that for untreated control. Furthermore, combination treatment of hot water and citric acid of all temperature (50, 60 or 70°C) induced synergistic increase in DPPP=O as well as PI value, which is significantly ($P < 0.05$) greater than the sum of the values obtained by the individual treatments of hot water and citric acid.

TABLE 4. Levels of the 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 uppercase letters within the same column are significantly different ($P < 0.05$). The data were normalized by subtracting fluorescence (OD_{600}) values obtained from untreated cells as follows: (fluorescence value after treatment − fluorescence value of untreated control)/OD_{600}.

3.2.3. Generation of intracellular reactive oxygen species (ROS) and superoxide (O_2^-)

TABLE 5 shows the levels of generation of total ROS and O_2^- in *E. coli* O157:H7 following with citric acid, hot water, or hot water with citric acid. All treatments except 50°C hot water treatment resulted in significantly ($P < 0.05$) increased values of generation of total ROS or O_2^- compared to that for untreated control. In particular, for the both values of total ROS and O_2^-, hot water treatment of 50 or 60°C combined with citric acid produced significantly ($P < 0.05$) greater value than the sum of values obtained from the individual treatments. However, for the total ROS as well as O_2^- value, the combination treatment of citric acid and hot water with 70°C did not induce synergistic increased of the value and even the value obtained by hot water treatment of 70°C with citric acid were significantly ($P < 0.05$) less than the value obtained by hot water treatment of 70°C.

TABLE 5. Levels of the generation 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 uppercase letters within the same column are significantly different ($P < 0.05$). The data were normalized by subtracting fluorescence (OD_{600}) values obtained from untreated cells as follows: (fluorescence value after treatment − fluorescence value of untreated control)/OD_{600}.

3.2.4. Superoxide dismutase (SOD) activity

TABLE 6 shows SOD activities in *E. coli* O157:H7 after treatment with citric acid, hot water or hot water with citric acid. Treatment of hot water with 50, 60 or 70°C did not result in significant ($P > 0.05$) reduction of the SOD activity in *E. coli* O157:H7 compared with that of untreated control. However, the activity of SOD in *E. coli* O157:H7 decreased by 15.02% after citric acid treatment, and furthermore, the activity of SOD in *E. coli* O157:H7 decreased by 29.23, 78.91, or 98.13% after combination treatment of citric acid and hot water of 50, 60, or 70°C, respectively.

TABLE 6. 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 uppercase 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.

3.3. Evaluation the antimicrobial effect of combination treatment of hot water and citric acid against food pathogens in biofilm on pipes

3.3.1. Reduction of E. coli O157:H7 in biofilm on SUS and PE-RT pipes

TABLE 7 shows the viable-count reduction of *E. coli* O157:H7 in biofilm formed on PE-RT and SUS pipes during distilled water, hot water, citric acid and hot water with citric acid treatment. The initial populations of *E. coli* O157:H7 in biofilm on PE-RT and SUS pipe were 5.59, 5.59 log CFU/cm^2, respectively. There were no significant ($P > 0.05$) differences in reduction level between two materials of pipe. Despite the only distilled water was treated without any other treatment, a 0.43 – 1.05 log reduction and 0.21 – 0.93 log reduction were shown in the PE-RT and SUS pipe, respectively at the flow range of 0.3 – 0.7 LPM, which were significantly ($P < 0.05$) different from the untreated control. Treatment of citric acid induced significant ($P < 0.05$) greater reductions than the reductions obtained from the treatment of distilled water. The log reduction values of all temperature (60, 70, or 75°C) of hot water treatment, except for 60°C of hot water treatment on PE-RT pipes, were significantly ($P < 0.05$) different according to the flow rates. Specifically, when the temperature was 60, 70°C, there were no significant ($P > 0.05$) difference in reduction level of *E. coli* O157:H7 biofilm by temperature at the same flow rate. Therefore, it was

shown that the flow rate is more important factor than the temperature during below 70°C of hot water treatment on *E. coli* O157:H7 biofilm. On the other hand, when temperature of hot water was over 75°C, the temperature and the flow rate were influenced simultaneously on decontamination of *E. coli* O157:H7 biofilm formed on both PE-RT and SUS pipes. Meanwhile, when citric acid and hot water were combined, 60°C, 70°C, and 75°C hot water with citric acid reduced *E. coli* O157:H7 biofilm to below the detection limit (< 0.28 log CFU/cm^2) after 0.3, 0.5 and 0.5 LPM treatments, respectively.

TABLE 7. Log reductions of *E. coli* O157:H7 in biofilms developed on pipes subjected to citric acid (CA), hot water (HW), or hot water with citric acid (HW-CA) treatment

Treatment condition	PE-RT			SUS		
	Flow rate (LPM)			Flow rate (LPM)		
	0.3	0.5	0.7	0.3	0.5	0.7
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
DW	0.43 ± 0.09 Ab	0.84 ± 0.35 ABb	1.05 ± 0.38 Bb	0.21 ± 0.13 Aa	0.56 ± 0.23 ABb	0.93 ± 0.39 Bb
CA	0.98 ± 0.23 Ac	1.35 ± 0.20 ABbc	1.63 ± 0.40 Bc	0.99 ± 0.28 Ab	1.19 ± 0.25 Ac	1.46 ± 0.27 Ac
60℃ HW	1.43 ± 0.28 Ad	2.17 ± 0.05 Ac	3.14 ± 0.23 Bd	1.50 ± 0.05 Ac	2.02 ± 0.35 Bd	3.07 ± 0.13 Cd
70℃ HW	1.55 ± 0.30 Ad	2.55 ± 0.08 Bd	3.24 ± 0.11 Cd	1.53 ± 0.20 Ac	2.22 ± 0.26 Bd	3.36 ± 0.22 Cd
75℃ HW	1.82 ± 0.51 Ad	3.15 ± 0.27 Be	4.75 ± 0.15 Ce	1.60 ± 0.07 Ac	2.99 ± 0.26 Be	4.90 ± 0.25 Ce
60℃ HW-CA	2.96 ± 0.09 Ae	> 5.31 Bf	> 5.31 Bf	2.65 ± 0.46 Ad	> 5.31 Bf	> 5.31 Bf
70℃ HW-CA	> 5.31 Af	> 5.31 Af	> 5.31 Af	> 5.31 Ae	> 5.31 Af	> 5.31 Af
75℃ HW-CA	> 5.31 Af	> 5.31 Af	> 5.31 Af	> 5.31 Ae	> 5.31 Af	> 5.31 Af

[a]Values are means ± standard deviations from three replications. Means with same uppercase letters within the same row are not significantly different ($P > 0.05$). Means with different lowercase letters within same column at same media are significantly different ($P < 0.05$). DW, distilled water treatment.

3.3.2. Reduction of Staphylococcus aureus in biofilm on SUS and PE-RT pipes

The decontamination effects of hot water, citric acid, and its combination were also investigated for *S. aureus* in biofilm formed on PE-RT and SUS pipes (TABLE 8). The initial populations of *S. aureus* in biofilm on PE-RT and SUS pipe were 5.85, 6.12 log CFU/cm^2, respectively. There were no significant ($P > 0.05$) differences in reduction level between the two materials of pipe as in the results of *E. coli* O157:H7. In case of *S. aureus* biofilm, different to the results from the case of *E. coli* O157:H7, distilled water induced slight log reduction of *S. aureus* biofilm on PE-RT and SUS pipes at three flow rates (0.03–0.52 log reduction and 0.15-0.35 log reduction, respectively), and these reductions were not significantly ($P > 0.05$) different from untreated control. However, the reduction levels of citric acid were significantly ($P < 0.05$) greater than those of untreated control. Unlike the results of *E. coli* O157:H7, the decontamination of *S. aureus* biofilm from the pipe was affected by both temperature and flow rate when hot water was treated on *S. aureus* biofilm. The log reductions of 60°C hot water with citric acid treatment were significantly greater than those of hot water treatment at the same temperature, whereas those were statistically same with 70°C hot water treatment. Meanwhile, when 70°C hot water and

citric acid were combined, it achieved a 3.09, 3.61 log reduction of *S. aureus* biofilm at 0.3 LPM of flow rate on PE-RT and SUS pipes, respectively, and it reduced *S. aureus* biofilm to below the detection limit (< 0.28 log CFU/cm^2) at 0.5 and 0.7 LPM. For 75°C hot water with citric acid treatment, *S. aureus* in biofilm was reduced below the detection limit (< 0.28 log CFU/cm^2) at all flow rates (0.3-0.7 LPM).

TABLE 8. Log reductions of *S. aureus* in biofilms developed on pipes subjected to citric acid (CA), hot water (HW), or hot water with citric acid (HW-CA) treatment

Treatment condition	PE-RT			SUS		
	Flow rate (LPM)			Flow rate (LPM)		
	0.3	0.5	0.7	0.3	0.5	0.7
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
DW	0.03 ± 0.05 Aa	0.24 ± 0.24 ABab	0.52 ± 0.11 Bab	0.15 ± 0.19 Aab	0.14 ± 0.28 Aa	0.35 ± 0.11 Ab
CA	0.40 ± 0.03 Aab	0.80 ± 0.35 ABbc	1.10 ± 0.29 Bbc	0.51 ± 0.22 Ac	0.84 ± 0.06 ABb	1.16 ± 0.23 Ac
60℃ HW	0.66 ± 0.17 Ab	0.99 ± 0.15 Bc	1.71 ± 0.14 Cc	0.46 ± 0.08 Abc	0.98 ± 0.16 Bb	1.50 ± 0.11 Cc
70℃ HW	1.67 ± 0.45 Ac	2.88 ± 0.21 Bd	3.36 ± 0.26 Bd	1.87 ± 0.12 Ae	2.36 ± 0.23 Bc	3.66 ± 0.16 Ce
75℃ HW	2.84 ± 0.08 Ad	3.49 ± 0.38 Be	4.51 ± 0.36 Ce	2.53 ± 0.33 Af	3.81 ± 0.70 Bd	4.86 ± 0.13 Cf
60℃ HW-CA	1.42 ± 0.27 Ac	2.90 ± 0.61 Bd	3.26 ± 0.77 Bd	1.19 ± 0.17 Ad	2.78 ± 0.36 Bc	3.29 ± 0.44 Bd
70℃ HW-CA	3.09 ± 0.38 Ad	> 5.57 Bf	> 5.57 Bf	3.61 ± 0.21 Ag	> 5.84 Be	> 5.84 Bg
75℃ HW-CA	> 5.57 Ae	> 5.57 Af	> 5.57 Af	> 5.84 Ah	> 5.84 Ae	> 5.84 Ag

[a]Values are means ± standard deviations from three replications. Means with same uppercase letters within the same row are not significantly different ($P > 0.05$). Means with different lowercase letters within same column at same media are significantly different ($P < 0.05$). DW, distilled water treatment.

3.3.3. Effect of combination treatment of hot water and citric acid on membrane integrity of foodborne pathogens in biofilm

Confocal laser scanning microscope (CLSM) images were obtained to analyze the degree of membrane integrity and detachment of *S. aureus* cells in biofilm when treated with distilled water, hot water, citric acid, and hot water with citric acid (FIGURE 3). CLSM images of *S. aureus* were captured after treatment with each solutions at a flow rate of 0.5 LPM and 70°C for 10 s. FIGURE 3 (b), (c) treated with distilled water and citric acid, respectively, on *S. aureus* biofilm showed that the amount of cells was slightly reduced compared to the untreated control of FIGURE 3 (a). In particular, *S. aureus* biofilm cells after citric acid treatment were stained with yellow, damaged cells, as shown in FIGURE 3 (c). When coupons were treated with hot water and hot water with citric acid, biofilm cells were stained mostly red, as shown in FIGURE 3 (d), (e). Moreover most of the cells with the combination of hot water and citric acid treatment disappeared.

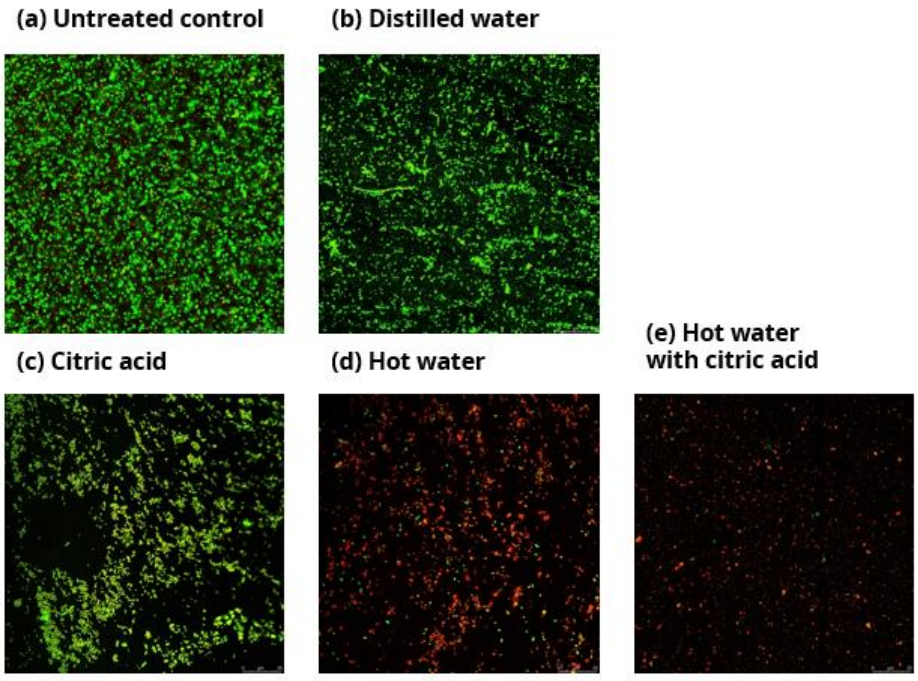

FIGURE 3. Membrane integrity of *S. aureus* biofilm on stainless steel observed by CLSM. The biofilm was treated with (a) untreated control, (b) Distilled water (DW), (c) citric acid (CA), (d) 70℃ hot water (70℃ HW) , (e) 70℃ hot water with citric acid (70℃ HW-CA) (Green: viable; red: dead).

IV. DISCUSSION

This study investigated the antimicrobial effect of hot water combined with citric acid for the purpose of enhancing the antimicrobial effect of hot water sanitation on foodborne pathogens biofilm on food contact surfaces. First, I confirmed the antimicrobial effect of *E. coli* O157:H7 biofilm on coupons. At this time, antimicrobial effect on planktonic cells of *E. coli* O157:H7 was examined to confirm the difference in the resistance to treatment between biofilm type- and suspended cells. Also, it is important to inactivate pathogens suspended in treatment solution because there is a risk that suspended cells in treatment solution can developed into biofilm cells on the surface again if they survive. Microorganisms can be killed completely in response to various antimicrobial treatments, becoming dead cells, but they also can be killed incompletely for insufficient treatment, which is commonly referred to as injured cells (37-39). These injured cells are believed to be potentially as dangerous as their normal counterparts because they can be resuscitated from favorable environment and regain their normal pathogenicity (39). Because 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 in a non-selective medium, where they can be resuscitated (37, 40). Therefore, in this

study, treated cells of *E. coli* O157:H7 were enumerated not only on selective media Sorbitoal MacConkey Agar (SMAC) but also on phenol red agar with 1% sorbitol media (SPRAB), which can recover injured cells, to confirm the occurrence of injured cells following treatments (41).

In this study, citric acid as well as 50 or 60°C hot water treatment did not significantly ($P > 0.05$) reduce *E. coli* O157:H7 in the biofilm formed on the SS surface for 20 sec. However, 70°C hot water reduced the biofilm *E. coli* O157:H7 to below the detection limit (> 5.73 log reduction) after 20 sec, and this treatment did not produce injured cells because there were no significant ($P > 0.05$) differences between cells enumerated on SMAC and SPRAB after treatments. Similar to these results, Oh and Marshall's study (42) showed that 55°C hot water did not significantly ($P > 0.05$) reduce *L. monocytogenes* in the biofilm formed on the SS during the 5 min treatment, whereas the 65°C hot water treatment reduced the biofilm the pathogen by 1.4 log after 5 min treatment. However, unlike this study in which a coupon was immersed in a treatment solution set at a treatment temperature, the Oh and Marshall's study was conducted by immersing a tube containing a solution in which a coupon was immersed in a water bath set at a treatment temperature, thus the scale of the treatment time in their study differs from that of the treatment time of this study. Meanwhile, Wahlen et al.'s study (16) reported that when

waterborne pathogens *Sphingomonas parapaucimobilis*, which is resistant to heat treatment, were present in the form of biofilms on the surface of the SS, 70, 75, or 80°C of hot water treatment required 44:46, 1:32, 1:27 (min:sec) to reduce the biofilm pathogen by 5-log. Therefore, it is important to establish the appropriate temperature and treatment time for hot water sanitation according to the type of the pathogenic bacteria and the type of treatment, and additionally, my results suggest that hot water treatment of 70°C or higher is required to reduce the biofilm *E. coli* O157:H7 on SS by 5-log or more within 20 sec.

When the hot water was combined with citric acid, this treatment showed a dramatic enhancement in the antimicrobial effect compared with that of hot water on biofilm *E. coli* O157:H7 because the combination of the two led to a synergistic bactericidal effect. However, when the cells treated with this combination were enumerated on SMAC and SPRAB, respectively, the level of reduction in cells enumerated on SPRAB was significantly ($P < 0.05$) less than that in cells enumerated on SMAC, that is, it means that hot water with citric acid generates injured cells. Therefore, when applying hot water with citric acid to biofilm *E. coli* O157:H7 on SS surface, it is important to consider the occurrence of injured cells to avoid over-estimating its antimicrobial effect. However, considering the injured cells, hot water with

citric acid showed a synergistic bactericidal effect, thus combining citric acid with hot water seems to be an effective strategy to control *E. coli* O157:H7 biofilm on SS.

When planktonic cells of *E. coli* O157:H7 and the pathogen biofilm were enumerated on SPRAB after treatment to compare the difference in resistance, for the treatment of 70°C hot water and 60 or 70°C hot water with citric acid, planktonic cells showed significantly ($P < 0.05$) more reduction levels than the pathogen biofilm. That is, this means that *E. coli* O157:H7 attaches on the SS surface and to form a biofilm, which enhances resistance to some treatments. Indeed, many studies have reported that pathogens in biofilm exhibit greater resistance to hot water (16, 42, 43) or sanitizer (44-46) treatment than their planktonic counterparts. Since biofilm matrix composed of EPS act as a barrier to block or reduces a contact with antimicrobial agents, biofilm pathogens are more resistance to antimicrobial treatment than planktonic counterparts (47). Therefore, it can be deduced that *E. coli* O157:H7 biofilm showed more resistance than planktonic cells of the pathogen to 70°C hot water and 60 or 70°C hot water with citric acid due to a protective effect of biofilm matrix. On the other hand, for citric acid, 50 or 60°C hot water, and 50°C hot water with citric acid treatments, no significant ($P > 0.05$) differences in reduction level were observed between *E. coli*

O157:H7 biofilm and planktonic cells of the pathogen. With regard of this, it is considered that the difference in resistance to the treatments between *E. coli* O157:H7 biofilm and planktonic cells of the pathogen was not revealed because the reduction levels by each treatment were considerably low to make a difference.

It was confirmed in this study that the combination of hot water and citric acid, an organic acid, exhibits a synergistic bactericidal effect. As with this result, Ban et al.'s study (28) also showed that the combination of heat (steam) and lactic acid showed a synergistic bactericidal effect on *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* in biofilm formed on polyvinyl chloride (PVC) or SS surface. However, the mechanism of this synergistic bactericidal effect has not been elucidated. A deeper understanding of the inactivation mechanism of the developed technology is important in identifying the rate-limiting step during the inactivation process, as well as creating a more effective disinfection strategy (48). Therefore, I considered to necessary to more in detail elucidate the mechanism of synergistic bactericidal effect of the combination of hot water and citric acid, and thus tried to identify the mechanism through several approaches. At this time, I divided the mechanism into two parts: the cell itself and the biofilm matrix.

Firstly, since extracellular polysaccharides are known to be important for the structure and stability of the biofilm matrix (49), residual polysaccharides on the surface of the SS coupon after treatment were measured (TABLE 3). In this case, the effect of washing was neglected because the treatment with DW was used as a control. As a result, citric acid did not achieve significant ($P > 0.05$) removal of polysaccharides, but hot water significantly ($P < 0.05$) removed polysaccharides and the degree of removal increased with increasing temperature. However, hot water with citric acid treatment showed removal of polysaccharides not significantly ($P > 0.05$) different from hot water treatment at the same temperature. This means that only heat treatment has the ability to remove polysaccharides on the SS surface. Therefore, in aspect of biofilm matrix, it can be interpreted that the additional contact of citric acid with cells on surface due to the removal of EPS by hot water result in an additional bactericidal effect leading to a synergistic bactericidal effect. In other words, the combined treatment of hot water and citric acid results in synergistic bactericidal effect by increasing penetration ability of this antimicrobial solution due to the elimination of the EPS induced by hot water treatment.

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 bactericidal effect 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 a synergistic damage site in the cell. The results of the PI uptake value of TABLE 4 showed that hot water with citric acid of 50, 60, or 70°C led to a synergistic increase in the PI uptake value. PI does not penetrate the intact cell membrane, but when the destruction of the cell membrane occurs, such as a pore-forming, it can penetrate into the cell, forms a complex with nucleic acids, and exhibits fluorescence (50). That is, an increase in the PI uptake value means that cell membrane destruction is increased. Meanwhile, this form of damage, which increases the permeability of the cell membrane, makes it difficult for the cell to maintain homeostasis and ultimately leads to cell death (51, 52). Therefore, it can be interpreted that the synergistic bactericidal effect by treatment with hot water with citric acid is caused by the synergistic damage on the cell membrane in form of destruction. Furthermore, the results of the DPPP=O value of TABLE 3 indicates that these values were also increased synergistically by hot water with citric acid. Non-fluorescent DPPP reacts with lipid hydroperoxide in the cell membrane to turn into fluorescent diphenyl-1-pyrenylphosphine oxide

(DPPP=O) (53). Therefore, this DPPP=O value serves as an indicator of the incidence of lipid peroxidation in the cell membrane. Lipid peroxidation in the cell membrane induces an increase in permeability as well as a decrease in potential and fluidity of the cell membrane (54). Therefore, since lipid peroxidation in the cell membrane is synergistically induced by the combined treatment of hot water and citric acid, it can be interpreted that lipid peroxidation in the cell membrane is the cause of synergistic destruction of the cell membrane by this combination treatment.

In the next step, it was necessary to determine why synergistic lipid peroxidation occurred. ROS is known to be one of the leading causes of cell death by activating lipid peroxidation in the cell membrane leading to destruction (55). Based on this fact, I confirmed the occurrence of intracellular ROS following each treatment. At this time, since superoxide (O_2^-) among various forms of ROS is known to play an important role in the inducing of other oxygen radicals with potential to cause biological oxidative damage (56, 57), 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 oxidized by ROS and hydrolyzed into fluorescent 2′,7′-dichlorofluorescein (DCF), was used to measure total ROS (58). Intracellular superoxide was identified with

hydroethidine (HDE), which is converted to ethidium bromide as it react with superoxide within the cell, which emit fluorescence as it intercalate into DNA (59). Interestingly, as shown in TABLE 5, hot water with citric acid at 50 or 60°C 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, the 70°C hot water with citric acid showed that the less occurrence of ROS than 70°C hot water. This result is in contrast to the result of induction of lipid peroxidation in the cell membrane by hot water with citric acid at 70°C. In this regard, it is interpreted that 70°C hot water with citric acid treatment induced too much destruction of cell membrane, resulting in the leakage of ROS within the cell, and consequently led to reduced intracellular ROS. That is, 70°C hot water citric acid also caused synergistic ROS, but the large damage of the cell membrane led to ROS leakage, which is considered to be a result of reduced intracellular ROS generation.

In order to complete the mechanism identification, it was necessary to find the cause of the synergistic occurrence of ROS. In this study, it was confirmed that citric acid treatment produces intracellular ROS. Although some studies have reported that organic acid induces damage to the outer

membrane through intercalation, chelation, or protonation (60, 61), the result of this study identifying that citric acid generates the intracellular ROS inducing lipid peroxidation in the cell membrane is interesting because it is a new discovery of another inactivation mechanism of organic acids. Organic acids have many other complexed inactivation mechanisms besides the cell membrane damage. Possible inactivation mechanisms of organic acids are listed in the literature reviewed by Mani-Lopez et al. (62). This literature referred that when the organic acid diffuses through the cell membrane, it causes the cell to maintain its internal pH normally by increasing the consumption of adenosine triphosphate (ATP), resulting in depletion of the energy, and the internal pH lowered by the organic acid damages the enzyme, DNA, or structural protein, and thus changes their function. Meanwhile, since organisms with aerobic metabolism produce ROS such as superoxide (O_2^-), hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2) as a by-products in the normal metabolic process and thus 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 (63). The most well-known non-enzymatic ROS defense system is glutathione (GSH) and the enzymatic ROS defense system includes superoxide dismutase, catalase (CAT), and glutathione peroxidase (GPx) (64,

65). From the inactivation mechanisms of organic acids and the characteristic of the aerobic organisms, I assumed that the reduction of ROS scavenging ability of enzymatic ROS defense systems by citric acid induced generation of intracellular ROS. Furthermore, based on these properties, the principle of the synergistic generation of ROS by combined treatment of hot water and citric acid can be deduced as follows: when combined with hot water and citric acid treatments, destruction of the cell membrane caused by the hot water treatment induces penetration of more citric acid into the cell, leading to more functional loss of the enzymatic ROS defense systems resulting in synergistic generation of ROS. To demonstrate this hypothesis, the activity of the ROS defense enzyme in *E. coli* O157:H7 was measured after each treatment. At this time, among the various antioxidant enzymes, SOD activity that decomposes superoxide into hydrogen peroxide, which has a lower oxidative damage potential, was confirmed because SOD is generally assumed to play a major role in defending against oxidative damage due to the characteristic of SOD present in all aerobic organisms and most subcellular compartments where active oxygen is produced (66, 67). As shown in TABLE 6, treatment of hot water with citric acid at all temperatures (50 - 70°C) led to a synergistic reduction of SOD activity in *E. coli* O157:H7. Above hypothesis can be proved through this result, and to

sum up comprehensively, it is concluded when hot water and citric acid are combined, increased penetration of citric acid into the cell due to destruction of the cell membrane by heat treatment leads to a synergistic reduction of SOD activity, and thus the synergistic generation of ROS lead to synergistic lipid peroxidation and destruction of the cell membrane sequentially, eventually resulting in the synergistic inactivation of the cells.

Based on the above results, I selected method to flow the treatment solution into the biofilm formed pipes at a constant flow rate to verify the applicability in real food industry by setting the experimental apparatus as shown in the following FIGURE 2 to be as close as possible to the actual environment. Therefore, the results of this study included not only the reduction of foodborne pathogenic biofilm by hot water and citric acid treatments but also the cell detachment which is physically washed down by the flow of the solution.

In this study, there was no significant ($P > 0.05$) differences in reduction level according to the type of pipe materials. However, the previous study was found that the inactivation effect by ClO_2 treatment was various due to hydrophobicity, roughness, crevice of surface difference for each material (68, 69). Also, Ban et al. (70) implied that the thermal conductivity of stainless steel (16 W/m·K) is higher than that of polyvinyl chloride (0.19

W/m·K), resulting in a greater antibacterial effect by heat. It seems that this study is due to different processing methods such as flow rate and shorter processing time, which are less affected by surface properties and thermal conductivity.

Meanwhile, when *E. coli* O157:H7 biofilms were treated with distilled water, the log reduction level was significantly ($P < 0.05$) different from that of untreated control at most flow rates, but *S. aureus* did not show any significant ($P > 0.05$) difference. From this result, it can be deduced that the ability to attach to the surface of *E. coli* O157:H7 differs from *S. aureus*. In fact, various factors are involved in the initial attachment to abiotic surfaces (71, 72). These factors include bacterial hydrophobicity, cellular surface charge, cellular surface structures, and surface free energy (73). Bacterial hydrophobicity is measured by the MATH test (microbial adherence to hydrocarbons), HIC (hydrophobic interaction chromatography), and water contact angle measurements, and according to Mark et al. (74), hydrophobic cells have a greater tendency to adhere than hydrophilic cells. The other factor, the cell surface charge, is determined by measuring the zeta potential. It is known that plastic and stainless steel have a negative charge (75, 76), and because bacterial cells also have a negative charge, the electric repulsive force affects the adherence (73). Gilbert et al.'s (77) study reported that *S.*

epidermidis is more hydrophobic and less electronegative than *E. coli*, so *S. aureus* can be deduced to be better adhered to surface, but more research is needed about *S. aureus*. However, there are several studies that showed the correlation between cell hydrophobicity and electrostatic charge and cell attachment is not clear, thus other factors should be considered together. An experiment comparing the surface properties of *E. coli* O157:H7 and *S. aureus* are also needed (78, 79).

Overall, *S. aureus* biofilm showed more resistance than *E. coli* O157:H7 biofilm to all treatments, which can only be explained in part by the cell attachment mentioned above. Instead, the difference in resistance between *S. aureus* and *E. coli* O157:H7 can be attributed to a cause. *S. aureus* is known to be more resistant to heat than other pathogens because it produces heat-stable enterotoxins (SEs) in contaminated food (80). Because of the high heat resistance of *S. aureus*, the reduction level was lower than that of *E. coli* O157:H7 despite the longer treatment time.

On the other hand, when combined treatment of hot water and citric acid, the reduction was remarkably increased above a certain flow rate, which is consistent with the tendency of the synergistic inactivation effect described in the previous study with SS coupons, and it was confirmed that it decreased below the detection limit more rapidly. Furthermore, it was also supported

by the CLSM images that biofilm control was more effective when combined with hot water and citric acid.

V. CONCLUSION

The results of this study reveal that hot water with 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 bactericidal effect of this combination in this study is worthy of utilization as a baseline for further research or industry applications related to this. Also, a study on the control ability of this technology on the pathogen in biofilm formed in pipe was conducted to broaden the applicability in the food industry where biofilm is a problem. Finally, this study has demonstrated that hot water, citric acid, and hot water with citric acid treatments for foodborne pathogens in biofilm are effective methods for practical food processing facilities.

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VI. 국문초록

본 연구에서는 식품 접촉면에 형성된 바이오필름 속 식중독균에 대한 온수와 구연산의 조합처리의 저감화 효과를 살펴보았다. 우선, 스테인리스 스틸 쿠폰에 *Escherichia coli* O157:H7 바이오필름을 형성시켜서 확인하였다. 50, 60, 70°C 의 온수와 구연산을 바이오필름에 함께 처리하였을 때, 저감화에 있어서 시너지 효과가 나타났다. 이때, 나중에 회복될 수 있는 손상된 균들이 많이 발생하였기 때문에 이 기술을 실제로 적용할 시 손상된 균들을 고려해주어야 한다. 한편, 본 연구에서 온수와 구연산을 조합처리하였을 때 저감화에 있어서 시너지 효과가 나타나는 기작을 두 가지 측면에서 규명하였다. 첫번째로, 바이오필름 매트릭스 측면에서 보면 온수에 의해 Extracellular polymeric substances (EPS)의 대부분을 차지하는 다당류가 제거되어 구연산과 균과의 접촉이 증가하여 시너지 효과를 나타냈다. 두번째로, 균의 측면에서는 온수에 의해 세포막이 파괴되어 세포 내에 구연산이 더 많이 유입되고 그로 인해 superoxide dismutase (SOD)의 활성이 감소하면 reactive oxygen species (ROS)가

시너지스틱하게 발생하여 이가 지질 과산화에 의한 세포막 파괴를 더 일으키게 되어 시너지 효과를 나타냈다. 그러므로 온수와 구연산의 조합처리를 *E. coli* O157:H7 바이오필름에 처리하면, 바이오필름 매트릭스와 균 자체에서 시너지 효과가 복합적으로 일어나 저감화에 있어서 극대화된 시너지 효과를 발생시킨다. 이러한 결과를 바탕으로 온수와 구연산의 조합처리를 식품 산업에서 적용할 수 있는지 검증하기 위하여 관 내부에 *E. coli* O157:H7 과 *Staphylococcus aureus* 바이오필름을 형성시켜 실험을 해본 결과 이 조합처리가 관 내의 바이오필름도 효과적으로 제어하였다.

주요어: 바이오필름, 식중독균, 온수, 구연산, 식품 접촉면, 시너지효과
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