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Combinatorial treatment using citric acid, malic acid, and phytic acid for synergistical inactivation of foodborne pathogenic bacteria

Young-seok Seo*,‡, Gyenggeun Lee,‡, Sibum Song**, Kangmin Kim*, and Min Cho*,†**

*SELS center, Division of Biotechnology, Advanced Institute of Environment and Bioscience, College of Environmental and Bioresource Sciences, Jeonbuk National University, Iksan 54596, Korea
**R&D Center, Sanigen CO., Ltd., Iksan 54576, Korea
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Abstract−Inorganic germicides, such as chlorine and its derivatives, are widely used against surface pathogens in various food industries. Due to the potential toxicity of the disinfectants and their by-products, alternative and dosage-efficient methods should be developed to secure food safety and hygiene. Here, we present a natural organic acid-based combinatorial treatment that efficiently inactivated the selected foodborne pathogenic bacterial strains even at low concentration. The individual and/or combinatorial treatments of citric (CA), malic (MA), and phytic acid (PA) inactivated *Escherichia coli* and *Staphylococcus aureus* in concentration- and time-dependent fashion. At one selected concentration, the mixture of acids (CA+MA+PA) efficiently reduced *E. coli* and *S. aureus* viability by approximately 99.9% within 10 min. The combined application of three organic acids resulted in higher germicidal activity than the sum of the individual treatment inactivation levels, suggesting a synergistic effect among the acids. Our combined acid treatment disrupted bacterial membrane integrity and increased the intracellular reactive oxygen species. The inactivation efficiency of the presented organic acid mixture was also verified for *Salmonella* Typhimurium, *Pseudomonas aeruginosa*, and *Listeria monocytogenes*. In conclusion, we established a composition of natural acid-based mixture, allowing efficient surface disinfection against various Gram-positive and negative pathogenic bacteria through a synergistic effect mechanism.

Keywords: Combinatorial Treatment, Foodborne Pathogen, Inactivation Kinetics, Organic Acid, Synergistic Effect

INTRODUCTION

Foodborne pathogens are not only a threat to public health but cause various diseases worldwide every year [1-4]. The Centers for Disease Control and Prevention (CDC) recently estimated that more than 9 million cases of foodborne illnesses were reported annually in the United States, including more than 55,000 hospitalizations and 1,300 deaths [5]. Most foodborne disease cases are known to be caused by habitual pathogens such as *Salmonella* Typhimurium [6], *Listeria monocytogenes* [7], *Pseudomonas aeruginosa* [8], *Staphylococcus aureus* [9], and *Escherichia coli* [10]. To secure food safety or hygiene, several disinfectants have been developed to inactivate foodborne pathogens. To date, most formulae have been based on chlorination [11,12]. However, chlorination itself is toxic, odorous [13], and generates disinfection by-products (DBPs), such as carcinogenic trihalomethanes (THMs) [14,15].

To reduce or substitute the harmfulness of chlorination, chemicals with relatively moderate harshness have been evaluated for their disinfection efficiency against pathogens. Among them, organic acids (OAs), which are used as food additives and preservatives, have often been investigated for their germicidal activity alone or in combination with other disinfecting agents. The individual treatment of citric acid (CA) and lactic acid (LA) inactivated *Yersinia enterocolitica*, *L. monocytogenes*, and *E. coli* [16,17], propionic acid, acetic acid, lactic acid, malic acid, and citric acid also inactivated *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* [18]. Recently, disinfection efficiencies of OAs were examined in combination with physico-chemical disinfection techniques (e.g., mild heat and hydrogen peroxide, ultrasound, light-emitting diodes (LED), and sodium chloride etc.). Combinatorial treatment of various OAs (lactic acid, citric acid, malic acid, tartaric acid, and acetic acid) with hydrogen peroxide inactivated *E. coli* O157:H7 on baby spinach [19]. The simultaneous application of ultrasound and various OAs (malic acid, lactic acid, and citric acid) significantly reduced *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on iceberg lettuce [20]. Blue LED illumination was also reported to increase germicidal activity of citric acid, lactic acid, and malic acid [21]. The mixture of sodium chloride and various OAs (acetic acid, citric acid, lactic acid, malic acid, and phytic acid) disinfected both non-adapted and acid-adapted *E. coli* O157:H7 [22]. However, the germicidal activity of OAs is generally dependent on the acid composition and requires high concentration, leading to high cost, odor, and corrosion [23]. Therefore, it would be potentially interesting to explore the new combinations of OAs in order to achieve reasonable activity with lower amount and higher safety.

Among the OAs, CA and MA are commonly used as they exhibit confirmed antimicrobial properties [18,20] and are easy to secure because they are the predominant acids in most fruits [24]. Phytic acid (PA), a natural organic acid extracted from rice bran or legumes [25,26], also shows high antimicrobial activity compared to other OAs [22,27]. Based on these advantages, in this study, we investi-

†To whom correspondence should be addressed.
E-mail: cho317@jbnu.ac.kr
‡These authors contributed equally.

gated the bactericidal activity of citric acid (CA), malic acid (MA), and phytic acid (PA) against selected foodborne pathogenic bacteria, focusing on the function of composition, concentration, and treatment time. We determined the minimal concentration of each acid in the combined mixture, at which reasonable germicidal activity was achieved against both Gram-negative (*E. coli*) and Gram-positive bacteria (*S. aureus*). Finally, we extended the inactivation efficiency of a mixture of CA, MA, and PA to other pathogenic bacteria (i.e., *S.* Typhimurium, *P. aeruginosa*, and *L. monocytogenes*).

EXPERIMENTAL DETAILS

1. Chemical Reagents and Materials

All chemical reagents were used without further purification. Malic acid (MA, food-grade), citric acid (CA, foodgrade), phytic acid (PA, Sigma-Aldrich Co., USA), propidium iodide (PI, Sigma-Aldrich Co., USA), hydroethidine (HE, Life Technologies Co., USA), 3-(p-hydroxyphenyl) fluores-cein (HPF; Life Technologies Co., USA), Luria-Bertani (LB) broth (Difco Co., USA), Brain Heart Infusion (BHI) broth (Difco Co., USA), and tryptic soy (TS) broth (Difco Co., USA), as well as agar powder (Daejung Co., Korea), were commercially purchased. Stock solutions of OAs were prepared using tap water without free chlorine and were sterilized by filtering through a 0.22 μm syringe filters (PVDF, Millipore, USA). All glassware was washed with distilled water and sterilized by autoclaving at 121 °C for 15 min.

2. Preparation and Analysis of Microorganisms

Gram-negative *E. coli* (ATCC 8739) and Gram-positive *S. aureus* (ATCC 6538) bacteria were used in the inactivation experiment. They were cultured and counted following a previously described procedure [28]. Briefly, *E. coli* or *S. aureus* were inoculated into 500 ml baffled Erlenmeyer flasks containing 300 ml of TS broth and grown at 37 °C in a shaking incubator for 18 h. Cultured *E. coli* or *S. aureus* was harvested by centrifugation in 50 ml conical tubes at 4,000 rpm for 10 min with phosphate-buffered saline solution (PBS, pH 7.2, Sigma-Aldrich Co., USA). *S.* Typhimurium (pathogen SL1344, Gram-negative bacteria) [29], *P. aeruginosa* (opportunistic pathogen PAO1, Gram-negative bacteria) [30], and *L. monocytogenes* (pathogen BA00092, Gram-positive bacteria) [31] were cultivated in LB, TS, and BHI broths, respectively. The stock solutions (approximately 1×10^{10} colony forming units (CFU)/ml) of these five bacteria (*E. coli*, *S. aureus*, *S.* Typhimurium, *P. aeruginosa*, and *L. monocytogenes*) were prepared by re-suspending the washed pellets in 30 ml of PBS. The initial populations of these bacteria were adjusted to approximately 10^8 CFU/ml. Viable cell counts of these five bacteria were performed in triplicate using the spread plate method on their specific agar media, after incubation at 37 °C for 24-48 h.

3. Microbial Inactivation Experiments

Microbial inactivation experiments were conducted in 60 ml crystallizing dishes with 30 ml of OA (CA, MA, and PA) suspension and bacteria (approximately 10^8 CFU/ml) mixed intensely using a magnetic stirrer. Four sets of microbial inactivation experiments were performed under various conditions: (1) individual treatment (CA, MA, and PA), (2) combined treatment I (CA+MA), (3) combined treatment II (CA+MA+PA), and (4) Gram-negative *vs.* Gram-positive bacterial cells. First, individual treatments were performed to inactivate bacteria (*E. coli* and *S. aureus*) by applying different OA concentrations (CA and MA: 9-36 g/l, PA: 0.05-0.2 g/l). Second, for the combined CA and MA treatment, the concentration of CA and MA ranged between 4.5-36 g/l at a 1 : 1 ratio. Third, for the combined CA, MA, and PA treatment, the concentrations of CA and MA were fixed at 1.8 g/l, while different concentrations of PA, such as 0.05, 0.1, and 0.2 g/l, were used. Finally, the inactivation efficiency of various bacteria (Gram-negative: *E. coli*, *S.* Typhimurium, and *P. aeruginosa*, Gram-positive bacteria: *S. aureus*, and *L. monocytogenes*) were compared at suitable concentrations determined through the third experiment. For the viability assessments, 1 ml of each solution was serially diluted with PBS up to a 1/100,000 dilution ratio. Dilution aliquots (0.1 ml for each bacterial strain) were inoculated onto specific agar plates using the spread plate method. All inactivation studies were performed in triplicate, and the averaged values, with statistical deviations, were used for inactivation kinetics analysis.

4. Microbial Inactivation Mechanisms

To examine the possible cell membrane integrity changes, we performed propidium iodide (PI) staining [29]. Untreated or OA-treated *E. coli* (approximately 10^8 CFU/ml) were stained by adding 10 μl PI stock to it, followed by incubation in the dark for 30 min. The fluorescence intensity was measured using an absorbance/fluorescence microplate reader at excitation and emission wavelengths of 535 and 617 nm, respectively (Tecan Co., Austria).

The generation of reactive oxygen species (ROS) in *E. coli* cells was measured using cell-permeable fluorescence probes HE (for $O_2{\cdot}^-$) and HPF (for OH·) and according to a previously described method [32] with modifications. Briefly, untreated or OA-treated *E. coli* (approximately 10^8 CFU/ml) were mixed with HE or HPF (10 μM) and incubated for 1 h in the dark. Then, *E. coli* cells were washed, re-suspended in PBS, transferred into 96-well plates, and subjected to fluorescence intensity measurements using an absorbance/fluorescence microplate reader (Tecan Co., Austria) at 485 and 535 nm of excitation and emission, respectively, for HPF and 535 and 590 nm of excitation and emission, respectively, for HE. The fluorescence intensity ratio (FIR) was calculated as the ratio of the fluorescence of samples treated with various OAs over the fluorescence of the untreated control samples.

The changes in the intracellular enzymatic activities of the treated (CA, MA, and PA; 1 log inactivation) and untreated *E. coli* (about 10^8 CFU/ml) samples were investigated using the API-ZYM system (BioMerieux Co., France), according to the manufacturer's instructions [33].

RESULTS AND DISCUSSION

1. Bacterial Inactivation by Individual OA Treatments

To investigate the disinfection efficiency of different OAs against foodborne pathogenic bacteria, we primarily selected CA and MA and monitored their inactivation kinetics against *E. coli* and *S. aureus* (Fig. 1). The inactivation activity of CA against *E. coli* marginally increased in the concentration ranges of 9, 12, and 18 g/l within 5 min (<0.7 log scale). When applied at 36 g/l for 5 min, CA efficiently inactivated *E. coli* on a 1.22 log scale (Fig. 1(a)). CA exhib-

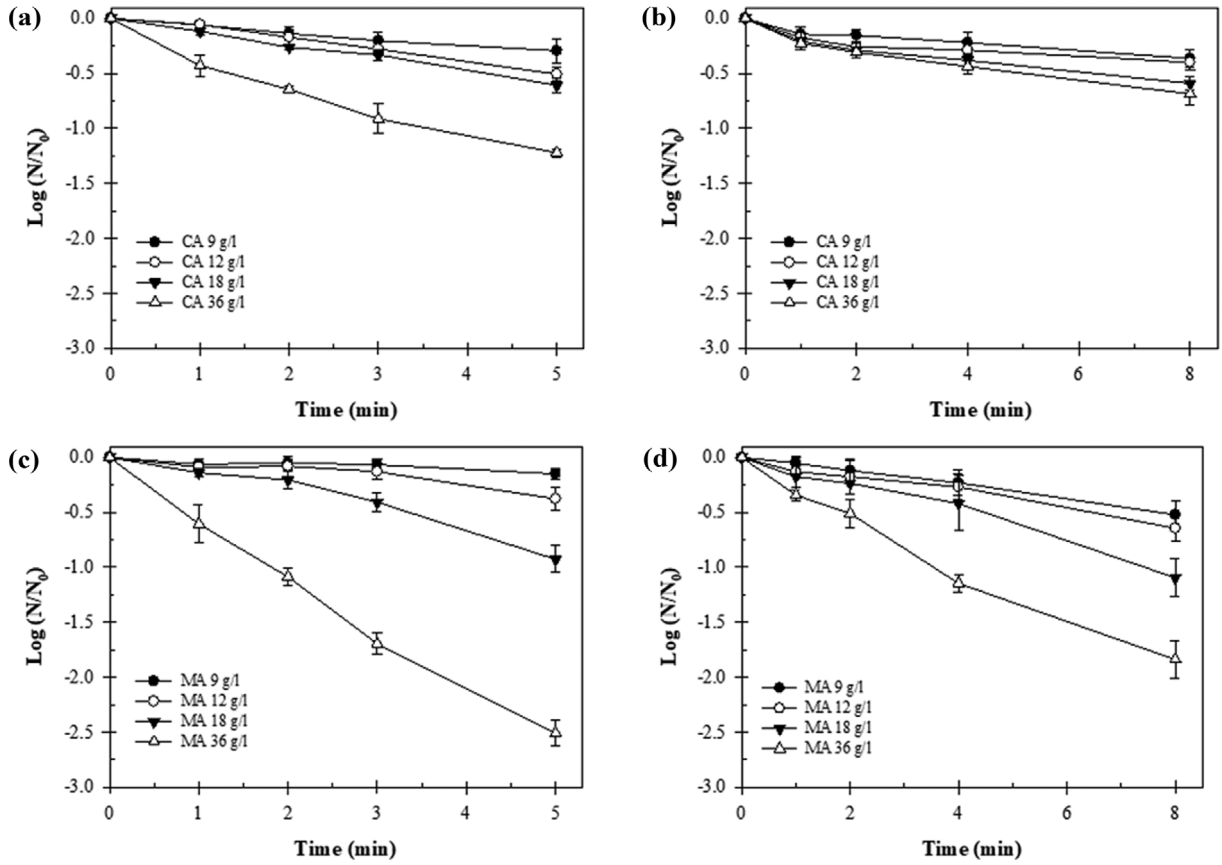

Fig. 1. The inactivation kinetics of the individual treatment of CA and MA against bacteria. A series of concentrations (9-36 g/l) of CA or MA were individually treated to *E. coli* (a), (c) and *S. aureus* (b), (d). The viable bacterial population (CFU/ml) was counted at the different time courses of organic acid treatment. N/N_0, the ratio of the numbers of viable bacteria at the designated time courses (N) to those (10^8 CFU/ml) at zero time (N_0).

ited no improvement in the inactivation kinetics against *S. aureus* within any tested concentration range (Fig. 1(b)). In the case of MA, inactivation activity against both pathogens increased in a dose-dependent manner (Fig. 1(c) and (d)). At 36 g/l, MA inactivated *E. coli* and *S. aureus* to 2.51 and 1.84 log scales for 5 and 8 min, respectively. Combined together, MA exhibited a higher inactivation rate than CA at the same concentration. *E. coli* was more sensitive to CA and MA than *S. aureus*. On the other hand, OA antimicrobial activity generally increases as the pH decreases. In our study, pH values in all CA and MA suspensions were 1.90-2.33 (Table 1). We observed no significant difference in pH values between the CA and MA solution, suggesting that the inactivation efficiency of MA was higher than that of CA, non-correlating with the pH. Alternatively, many other factors, such as chain length, degree of branching, and the ratio of undissociated forms, might affect OA antimicrobial activity [18]. In particular, OA size or molecular weight is known to be associated with diffusivity [17]. Based on this, MA (134.09 Da) penetration might be easier into the cell than that of CA (192.13 Da) [34]. In addition, Gram-negative bacteria are more sensitive to lower pH than Gram-positives [35] and are more susceptible to OA diffusion due to their thinner peptidoglycan layer [17].

Table 1. OA condition-dependent pH measurement

OA (g/l)	pH	OA (g/l)	pH
MA (9)	2.33	PA (0.4)	2.57
MA (12)	2.22	MA (4.5)+CA (4.5)	2.29
MA (18)	2.21	MA (9)+CA (9)	2.19
MA (36)	2.02	MA (18)+CA (18)	2.06
CA (9)	2.25	MA (36)+CA (36)	1.97
CA (12)	2.19	MA (1.8)+CA (1.8)	2.52
CA (18)	2.11	MA (1.8)+CA (1.8)+PA (0.05)	2.50
CA (36)	1.90	MA (1.8)+CA (1.8)+PA (0.1)	2.48
PA (0.1)	3.28	MA (1.8)+CA (1.8)+PA (0.15)	2.46
PA (0.2)	2.81	MA (1.8)+CA (1.8)+PA (0.2)	2.45

2. Bacterial Inactivation by Combined CA and MA Treatment

To further examine the germicidal activity of CA and MA, we monitored the inactivation kinetics of the CA+MA mixture against *E. coli* and *S. aureus* at various concentrations (Fig. 2). Overall, as the CA and MA concentrations increased, inactivation efficiency improved both against *E. coli* and *S. aureus*. Noticeably, similarly to individual treatments (Fig. 1), a mixture of CA and MA inactivated *E. coli* more efficiently compared to *S. aureus*. The mixture of CA (36 g/l) and MA (36 g/l) inactivated *E. coli* and *S. aureus* in

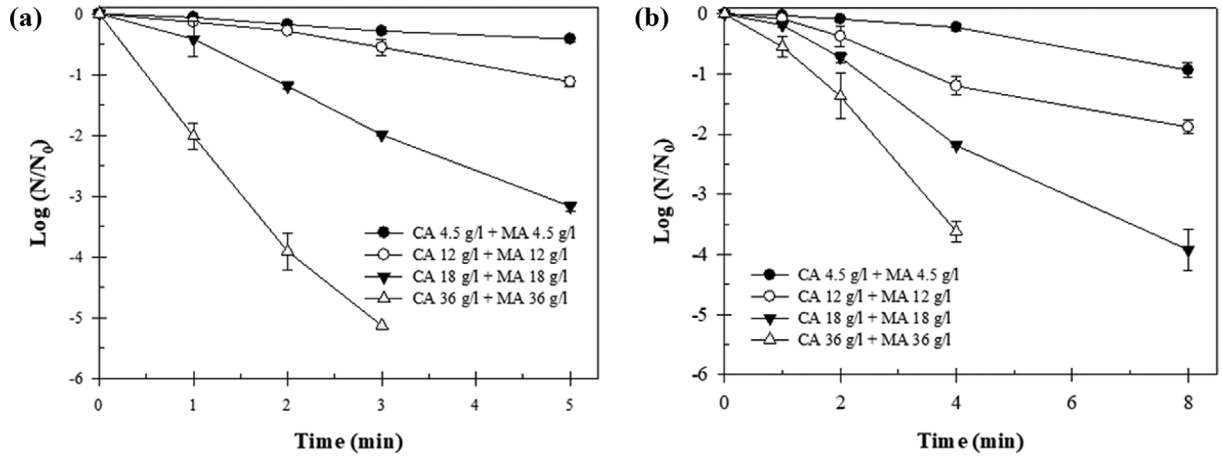

Fig. 2. The inactivation kinetics of the combinatorial treatment of CA and MA against bacteria. A series of concentrations (4.5-36 g/l) of CA and MA were treated to *E. coli* (a) and *S. aureus* (b) in combination. The viable bacterial population (CFU/ml) was counted at the different time courses of organic acid treatment. N/N_0, the ratio of the numbers of viable bacteria at the designated time courses (N) to those (10^8 CFU/ml) at zero time (N_0).

Table 2. Synergistic effect in combinatorial treatment with CA and MA on the inactivation of *E. coli* and *S. aureus* ($[N]_0$: 10^8 CFU/ml, pH not controlled)

Bacteria		*E. coli* (for 3 min)			*S. aureus* (for 4 min)		
Disinfection method	CA	12 g/l	18 g/l	3 g/l	12 g/l	18 g/l	36 g/l
	MA	12 g/l	18 g/l	36 g/l	12 g/l	18 g/l	36 g/l
Predicted inactivation[a]		0.41 log	0.74 log	2.61 log	0.56 log	0.80 log	1.58 log
Observed inactivation		0.55 log	1.99 log	5.13 log	1.20 log	2.19 log	3.62 log
Percent synergistic effect		34.1%	168.9%	96.6%	114.3%	173.8%	129.1%

[a]Arithmetic sums of inactivation levels (log N/N_0) through individual CA and MA treatments (e.g., 0.41 log was the sum of 0.28 log for CA at 12 g/l and 0.13 log for MA at 12 g/l).

3 log scale (99.9% inactivation) within 1.6 min and 3.5 min, respectively. The measured pH values were similar between the mixed and individual CA and MA treatments (Table 1). Interestingly, against both *E. coli* and *S. aureus*, the combined treatment (Fig. 2) of 18 g/l CA and 18 g/l MA (sum of total OAs=36 g/l) achieved higher inactivation than the individual treatments (Fig. 1) of 36 g/l MA or 36 g/l CA. Referred to as 'percent synergistic effect' in inactivation efficiency on log N/N_0 scale [36], the combination of CA and MA increased microbial inactivation by 34.1-168.9% for *E. coli* and 114.3-173.8% for *S. aureus*, when compared to the arithmetic sum of the individual treatments (Table 2). The combined CA+MA treatment inactivated bacteria through a synergistic rather than an additive effect.

3. Determination of Suitable Concentration in Combinatorial Treatment of OA for Bacterial Inactivation

CA+MA exhibited strong disinfecting activity under our experimental conditions (Fig. 2), but such high OA concentrations might not be suitable for practical usage. To overcome these limitations, OA concentrations must be reduced. Based on the synergistic effects between CA and MA (Fig. 2), we hypothesized that the incorporation of another OA would maintain or enhance the CA+MA inactivation activity even at low concentration. To test this, we assessed the inactivation kinetics of the CA+MA mixture at significantly lower concentration (1.8 g/l for each) in the presence of another OA, phytic acid (PA) (Fig. 3). The PA concentration was adjusted in the range of 0.05-0.2 g/l. When PA (0.1 g/l) or a CA+MA mixture were supplemented separately, no inactivation activity was observed even within 10 min (Supplementary Information Fig. S1 and Fig. 3). However, when treated in combination, the mixture of CA (1.8 g/l), MA (1.8 g/l), and PA (0.05-0.2 g/l) exhibited reasonable inactivation against both *E. coli* and *S. aureus*. As the PA concentration increased, inactivation activity of OA mixture increased (Fig. 3). Noticeably, the mixture of 1.8 g/l CA, 1.8 g/l MA, and 0.2 g/l PA exhibited inactivation in 3 log scale (99.9% inactivation) against *E. coli* and *S. aureus* within 6.9 and 8.6 min, respectively. In addition, as shown in Table 3, 'percent synergistic effects' in bacterial inactivation efficiencies by CA+MA+PA were 250.7-358.7% for *E. coli* and 1115.8-1571.4% for *S. aureus*, when compared to the arithmetic sums of the separate treatment of the CA+MA and PA. These data suggested that the combinatorial treatment of CA, MA, and PA efficiently inactivated *E. coli* and *S. aureus* in a synergistic fashion.

4. The Mechanism of OA Mixture (CA+MA+PA) on *E. coli* Inactivation

To explore the mechanisms underlying the synergistic effects of CA+MA+PA on the inactivation of pathogens, we first examined

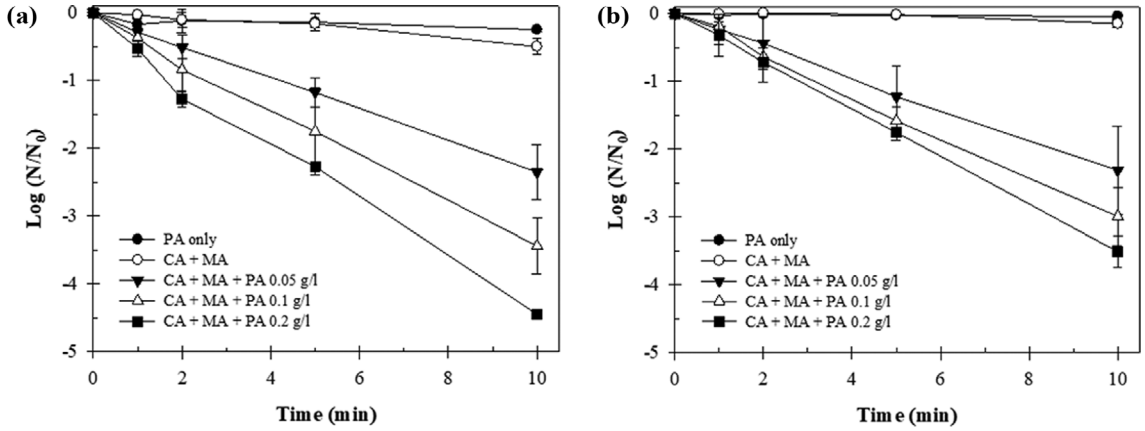

Fig. 3. The inactivation kinetics of the combinatorial treatment of CA, MA, and PA against bacteria. The effects of the combinatorial treatment of CA, MA, and PA on the inactivation kinetics of *E. coli* (a) and *S. aureus* (b). The viable bacterial population (CFU/ml) was counted at the different time courses of organic acid treatment. N/N_0, the ratio of the numbers of viable bacteria at the designated time courses (N) to those (10^8 CFU/ml) at zero time (N_0). [CA]=[MA]=1.8 g/l; [PA]=0.1 g/l.

Table 3. Synergistic effect in combinatorial treatment with CA+MA and PA on the inactivation of *E. coli* and *S. aureus* ($[N]_0$: 10^8 CFU/ml, pH not controlled)

Bacteria		*E. coli* (for 10 min)			*S. aureus* (for 10 min)		
Disinfection method	CA	1.8 g/l	1.8 g/l	1.8 g/l	1.8 g/l	1.8 g/l	1.8 g/l
	MA	1.8 g/l	1.8 g/l	1.8 g/l	1.8 g/l	1.8 g/l	1.8 g/l
	PA	0.05 g/l	0.1 g/l	0.2 g/l	0.05 g/l	0.1 g/l	0.2 g/l
Predicted inactivation[a]		0.67 log	0.75 log	1.12 log	0.19 log	0.19 log	0.21 log
Observed inactivation		2.35 log	3.44 log	4.44 log	2.31 log	2.99 log	3.51 log
Percent synergistic effect		250.7%	358.7%	296.4%	1,115.8%	1,473.7%	1,571.4%

[a]Arithmetic sums of inactivation levels (log N/N_0) through individual CA+MA and PA treatments (e.g., 0.67 log was the sum of 0.5 log for CA+MA at 1.8 g/l and 0.17 log for PA at 0.05 g/l).

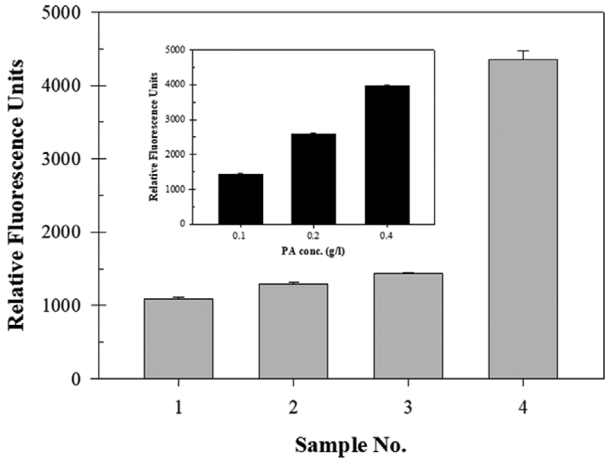

Fig. 4. The effects of the combinatorial treatments of CA, MA, and PA on the membrane integrity of *E. coli*. The cell-membrane integrity of *E. coli* (10^8 CFU/ml) was monitored by the measurement of the fluorescence of PI staining after the treatment with CA, MA, and/or PA for 3 min. 1) Untreated control; 2) CA+MA ([CA]=[MA]=1.8 g/l); 3) PA only (0.1 g/l); 4) CA+MA+PA ([CA]=[MA]=1.8 g/l, [PA]=0.1 g/l). Inset, the changes in the relative fluorescence unit of PI staining as the function of PA concentration.

E. coli membrane integrity after various OA combination treatments. In general, PI is widely used as a fluorescent dye that binds to DNA and stains the nucleus. PI staining is also often used to monitor membrane disintegration because it does not cross the intact lipid bilayer [37,38]. In our study, *E. coli* treated with CA+MA or PA showed higher relative fluorescence unit (RFU) values than untreated *E. coli* (Fig. 4). In particular, the combinatorial treatment with CA+MA+PA resulted in the highest fluorescence of PI. The relative fluorescence intensity gradually increased with the increasing PA concentration, indicating that the *E. coli* cell membrane was disintegrated by PA in a dose-dependent manner (Fig. 4 inset). Our data confirmed that PA facilitated the disintegration of membrane, which was similarly suggested previously [22,39,40]. PA is frequently called as myo-inositol hexakisphosphate (IP6). Six reactive phosphate groups carry negative charges and act as efficient cation chelators [41]. PA is known to bind to various divalent cations (e.g., Mg^{2+} and Ca^{2+}), which plays a role in the conservation of stable structure of the bacterial outer membrane [22,39]. Therefore, in the mixture of CA+MA+PA, PA might play a role to disrupt cell membrane of pathogens and made easier OAs to penetrate into the pathogenic bacteria by chelating cations in cell membrane.

On the other hand, OA dissociates into protons and acidic anions inside bacterial cells [42] and in turn causes the accumulation of

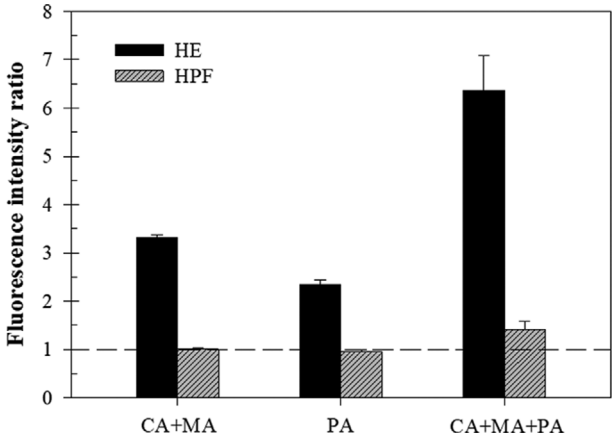

Fig. 5. The effect of the individual or combinatorial treatment of CA, MA, and PA on the accumulation of reactive oxygen species in *E. coli*. The level of reactive oxygen species was measured by monitoring the fluorescence of HE (for $O_2\cdot^-$) and HPF (for OH·) when *E. coli* (10^8 CFU/ml) were treated with the organic acid mixture for 10 min. CA, MA, 1.8 g/l for each: PA, 0.1 g/l.

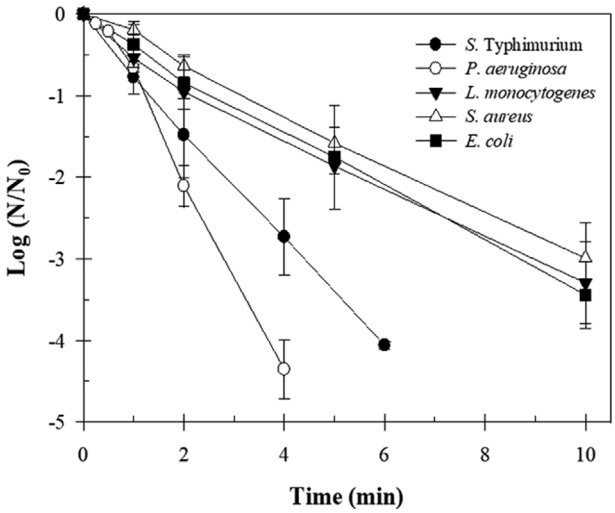

Fig. 6. The inactivation kinetics of bacteria by the combinatorial treatment of CA, MA, and PA. The combinatorial treatment of CA, MA, and PA was monitored for inactivation activity against the representative Gram-negative (*E. coli*, *S.* Typhimurium, and *P. aeruginosa*) and Gram-positive (*S. aureus* and *L. monocytogenes*). N/N_0, the ratio of the numbers of viable bacteria at the designated time courses (N) to those (10^8 CFU/ml) at zero time (N_0). CA, MA, 1.8 g/l for each; PA, 0.1 g/l.

reactive oxygen species (ROS) which disrupts the viability of bacteria [43,44]. Previously, $O_2\cdot^-$ and OH· were suggested to be powerful and non-selective oxidizing species in bacterial cells [45]. Based on this, we also measured the intracellular levels of $O_2\cdot^-$ and OH· in *E. coli* treated with CA, MA, and/or PA (Fig. 5). Compared with untreated condition (dashed line), it was found that the fluorescence intensity for HE inside *E. coli* increased in all conditions with each OAs treatment. This implies that an OA mixture produced $O_2\cdot^-$ and likely induced severe oxidative stress in the bacterial cells. When *E. coli* was treated with a mixture of CA+MA+PA, the fluorescence intensity ratio (FIR) was 6.4-times higher than that observed in the control sample. However, the FIR for HPF was approximately 1.4-times higher in the CA+MA+PA treatment than in the control sample, whereas that of individual treatment with CA+MA and PA was similar to that of the control sample. Taken together, the OA mixtures generated a large amount of $O_2\cdot^-$ and promoted the production of OH·.

The result of ROS production in the bacterial cell must be one of the factors contributing to the significant microorganism inactivation efficiency in a short time by a combination treatment of OAs of low concentration. The disruption of *E. coli* in presence of CA+MA+PA was also confirmed by decreased activity of alkaline phosphatase, leucine arylamidase, acid phosphatase, β-glucuronidase, and β-glucosidase in treated cells (Supplementary Information Fig. S2 and Table S1).

Combining all, combinatorial treatment of CA+MA+PA caused fatal damage to *E. coli* via the disintegration of the bacterial membrane, ROS accumulation, and concomitant inactivation of various intracellular enzymes.

5. Evaluation of Inactivation Activity of CA+MA+PA against Various Pathogenic Bacteria

Based on the inactivation efficiency of CA+MA+PA against *E. coli* and *S. aureus* (Fig. 3), we extended the evaluation of disinfecting activity of OA mixture against more various foodborne pathogenic bacteria (Fig. 6). Two species of Gram positive bacteria (*S. aureus* and *L. monocytogenes*) and three species of Gram negative bacteria (*P. aeruginosa*, *S.* Typhimurium, and *E. coli*) were selected for the evaluation. Overall, combinatorial treatment of CA (1.8 g/l)+MA (1.8 g/l)+PA (0.1 g/l) inactivated all of bacterial strains more than 99.9% within 10 min. Specifically, the elapsed time for CA+MA+PA mixture to achieve the inactivation in 3 log scale was 3, 4.5, and 8.7 min against *P. aeruginosa*, *S.* Typhimurium, and *E. coli*, respectively (Fig. 6). In case of Gram positive strains, CA+MA+PA inactivated *S. aureus* and *L. monocytogenes* to 3 log scale within 8.8 min and 10 min, respectively. In general, Gram negative bacteria were more sensitive to OA mixture than Gram positive bacteria [16,17,46]. The less complexity in peptidoglycan architecture in cell walls of Gram negative bacteria must be labile to the attacks of OAs. In the presence of CA+MA+PA, the inner membrane, the enzymes, transporters, and transmembrane antiporters of Gram-negative bacteria likely encountered fatal acid stress [47]. Therefore, the CA+MA+PA treatment efficiently inactivated both Gram-negative and Gram-positive bacteria with a higher inactivation efficiency for Gram-negative bacteria.

CONCLUSION

Natural OAs (CA, MA, and PA) were selected as alternatives to chlorine-based disinfectants. Through the combinatorial treatment of low concentration OAs, suitable concentrations (CA+MA+PA) that are economical, eco-friendly and highly efficient have been suggested. In spite of the low concentrations of CA and MA, the addition of PA induced high degree of synergism in microbial inactivation kinetics and mechanisms. We confirmed that this suitable

concentration (CA+MA+PA) effectively inactivated both Gram-negative and Gram-positive bacteria. The composition and concentration suggested in this study can manage to control various pathogenic bacteria that would normally cause food poisoning within a short time. Our study also provides an insight into the mechanism underlying the effect of suitable OA concentrations (CA+MA+PA) on the inactivation of bacteria. In practice, the simple mixing of natural OAs could be a promising alternative to conventional chemicals as cleaning products or disinfectants.

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SUPPORTING INFORMATION

Additional information as noted in the text. This information is available via the Internet at http://www.springer.com/chemistry/journal/11814.

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