LWT - Food Science and Technology 72 (2016) 9–18

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Antimicrobial activities of lauric arginate and cinnamon oil combination against foodborne pathogens: Improvement by ethylenediaminetetraacetate and possible mechanisms

Qiumin Ma, P. Michael Davidson, Faith Critzer, Qixin Zhong*

Department of Food Science and Technology, University of Tennessee, Knoxville, USA

ARTICLE INFO

Article history:
Received 29 November 2015
Received in revised form
23 March 2016
Accepted 15 April 2016
Available online 19 April 2016

Keywords:
Lauric arginate
Cinnamon oil
EDTA
Synergistic antimicrobial activity
Mechanism

ABSTRACT

The objective of this work was to study if ethylenediaminetetraacetate (EDTA) could enhance the antimicrobial activity of lauric arginate (LAE) and cinnamon oil (CO) combination, and the possible mechanisms. With 500 mg/L of EDTA, 5 mg/L of LAE and 200 mg/L of CO showed an increased log reduction of *Escherichia coli* O157: H7, *Salmonella* Enteritidis, and *Listeria monocytogenes* by ca. 4, 5, and 1 log CFU/mL, respectively. EDTA at 500 mg/L significantly increased the permeability of outer membrane of *E. coli* O157: H7 based on a crystal violet assay. Scanning electron microscopy (SEM) showed that 600 mg/L CO damaged the cell membrane of *S.* Enteritidis, while 40 mg/L LAE did not. Atomic force microscopy demonstrated that LAE caused the aggregation of DNA molecules. It was hypothesized that EDTA increased the permeability of the outer membrane of Gram-negative bacteria to facilitate the penetration of LAE and CO enabling enhanced antimicrobial activity. Compared to the treatment with LAE or CO alone, severe damage of *L. monocytogenes* membrane occurred with LAE and CO in combination based on SEM, increase in loss of intracellular nucleic acids, and increase of extracellular ATP level, suggesting LAE and CO acted synergistically on *L. monocytogenes* cell membranes.

1. Introduction

Lauric arginate (ethyl-N^{α}-lauroyl-L-arginine ethylester monohydrochloride; LAE) is a generally-recognized-as-safe preservative approved by the United States Food and Drug Administration in 2005, and the dosage limit in food products is 200 mg/L (USDA., 2005). It is a cationic surfactant derived from lauric acid, L-arginine and ethanol (Ruckman, Rocabayera, Borzelleca, & Sandusky, 2004). LAE has been shown to be non-toxic to human because it is metabolized rapidly *in vivo* to lauric acid and arginine, which are naturally occurring dietary components (Hawkins, Rocabayera, Ruckman, Segret, & Shaw, 2009). LAE has a broad antimicrobial spectrum and the minimum inhibitory concentration of LAE against *Listeria monocytogenes* Scott A is as low as 11.8 mg/L in tryptic soy broth (TSB) at 32 °C (Ma, Davidson, & Zhong, 2013). However, a much higher amount (>200 mg/L) of LAE is needed in complex food matrices due to its interaction with negatively charged food components (Bonnaud, Weiss, & McClements, 2010; Ma et al., 2013). Because a high concentration (>50 mg/L) of LAE can lead to a bitter taste (Zheng, 2014), strategies are needed to lower the LAE level used in food products.

Combinations of natural antimicrobials with synergistic antimicrobial effects are a possible way to lower the concentration of each antimicrobial needed in the food matrix (Ma et al., 2013; Noll, Prichard, Khaykin, Sinko, & Chikindas, 2012; Techathuvanan, Reyes, David, & Davidson, 2014). Essential oils (EOs) have gained a lot of attention for possible use as natural antimicrobial preservatives in recent years (Chen, Zhang, & Zhong, 2015; Ma et al., 2016; Pan, Chen, Davidson, & Zhong, 2014; Zhang, Ma, Critzer, Davidson, & Zhong, 2015). EOs have a broad spectrum of antimicrobial activity but, due to binding with hydrophobic components, are needed at high concentrations in complex food products to enable sufficient inhibition of foodborne pathogens. For example, 3000 mg/L eugenol in 2% reduced fat milk only reduced the amount of *L. monocytogenes* by less than 1 log CFU/mL at 32 °C after 24 h (Ma et al., 2013). Lowering the amount of EOs used in the food products is also desired since high concentrations of EOs affect sensory quality of food products. Previously, a synergistic effect against the

* Corresponding author. Department of Food Science and Technology, University of Tennessee, 2510 River Drive, Knoxville, TN, 37996-4539, USA.
E-mail address: qzhong@utk.edu (Q. Zhong).

http://dx.doi.org/10.1016/j.lwt.2016.04.021
0023-6438/

Gram-positive bacteria *L. monocytogenes* was found when combining LAE and cinnamon leaf oil or eugenol, while the combination was antagonistic against Gram-negative *Escherichia coli* O157:H7 and *Salmonella* Enteritidis (Ma et al., 2013).

The major difference between Gram-negative and Gram-positive bacteria is that the lipopolysaccharide (LPS) outer membrane of Gram-negative bacteria can protect against the penetration of antimicrobial compounds, especially hydrophobic compounds, while Gram-positive bacteria do not have an outer membrane (Bladen & Mergenhagen, 1964). Ethylenediaminetetraacetic acid (EDTA) can chelate divalent cations that are critical to the ordered structure of LPS outer membrane of Gram-negative bacteria (Nikaido, 2003; Ruiz, Kahne, & Silhavy, 2009), which increases the permeability of the outer membrane (Vaara, 1992). Studies have shown that EDTA can enhance activities of nisin, lysozyme, and monolaurin (Branen & Davidson, 2004). Therefore, we hypothesize that EDTA could overcome the antagonistic effect of LAE-EO combination against Gram-negative bacteria.

The objective of the present study was to test antimicrobial activities of the combinations of LAE, cinnamon oil (CO), and EDTA against both Gram-positive and Gram-negative bacteria and better understand the underlying mechanisms of these interactions. Possible mechanisms for the enhancement of activity by EDTA and/or interactions among antimicrobials investigated included disruption of the cell membrane, loss of intracellular nucleic acids, and loss of ATP, as well as observation of cell morphology. Interaction of antimicrobials and bacterial DNA was also investigated.

2. Materials and methods

2.1. Materials

LAE with a brand name of Mirenat®-TT was provided by Vedeqsa Inc. (New York, NY). The commercial product contained 15.5 g/100 g LAE. CO and EDTA were purchased from Sigma–Aldrich Corp. (St. Louis, MO).

2.2. Bacterial culture

L. monocytogenes Scott A, *E. coli* O157:H7 ATCC 43895, and *S.* Enteritidis were used in the present study. All strains were from Department of Food Science and Technology at University of Tennessee in Knoxville. Strains were stored in sterile 20 g/100 g glycerol at −20 °C and transferred at least 2 times in TSB for *E. coli* O157:H7 and *S.* Enteritidis or in TSB supplemented with yeast extract (TSBYE) for *L. monocytogenes* before testing. Unless stated otherwise, *L. monocytogenes* Scott A was incubated at 32 °C, while *E. coli* O157:H7 ATCC43895 and *S.* Enteritidis were incubated at 37 °C.

2.3. Microbial growth kinetics in tryptic soy broth

Growth curves of bacteria were determined in 96-well microtiter plates using a spectrophotometric plate reader (Synergy HT MultiMode Microplate Reader, BioTek, Winooski, VT). Culture with ca. 10^7 CFU/mL bacteria and an antimicrobial solution were added at 120 μL each into each well. The optical density (OD) at 600 nm was automatically recorded at an interval of 30 min during incubation at 37 °C (for *S.* Enteritis and *E. coli* O157:H7) or 35 °C (for *L. monocytogenes*) for up to 12 h. Stock solutions with 500 mg/L LAE or 4 g/100 mL EDTA were prepared in water and adjusted to pH 6.8 using 1.0 M NaOH or HCl. The stock solution of CO was prepared by dissolving 5 g/100 mL CO in 90 mL/100 mL aqueous ethanol. The same ethanol concentration as in CO sample was used as an ethanol control, while wells without antimicrobial were treated as positive controls. Concentrations of antimicrobials used in inhibiting *S.* Enteritidis and *E. coli* O157:H7 were 5 mg/L LAE, 500 mg/L EDTA, and 200 mg/L CO, while those used against *L. monocytogenes* were 2.5 mg/L LAE, 100 mg/L EDTA, and 100 mg/L CO. Experiments were performed in triplicate.

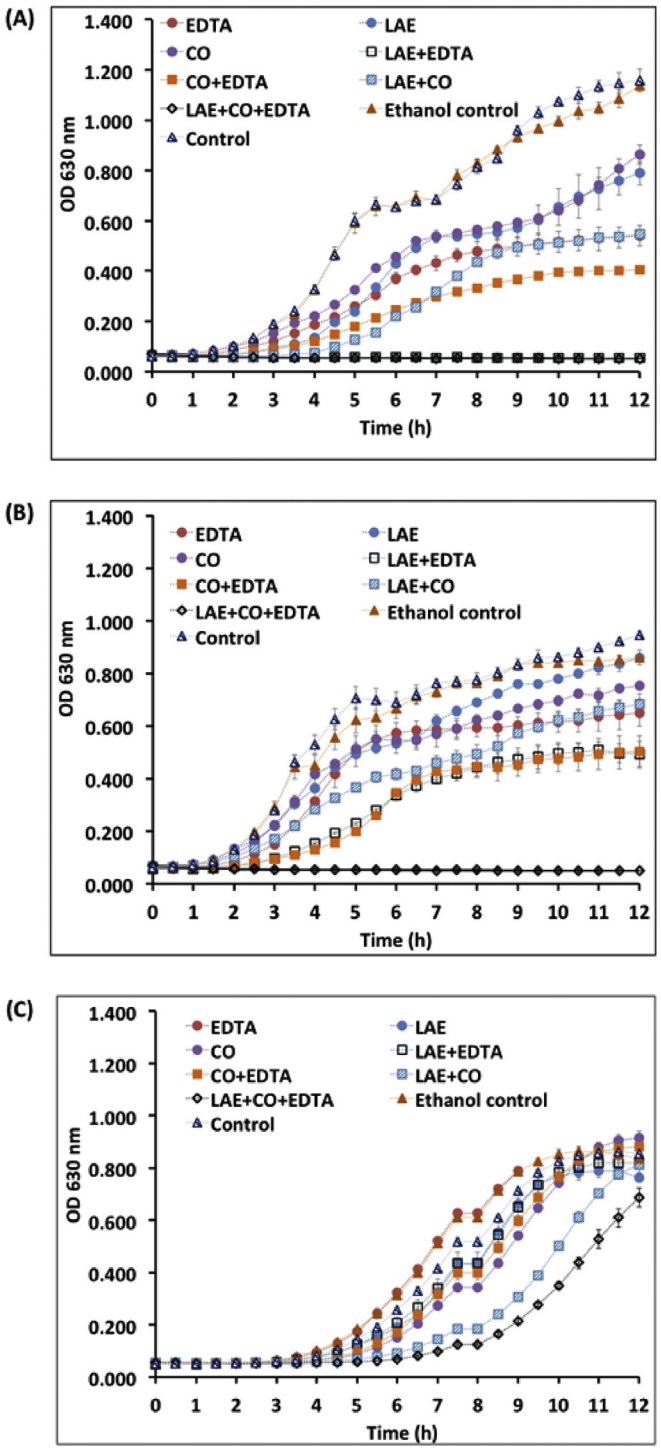

Fig. 1. Growth curves of (A) *Escherichia coli* O157:H7 ATCC 43895, and (B) *Salmonella* Enteritidis at 37 °C and (C) *Listeria monocytogenes* Scott A at 35 °C in tryptic soy broth. Treatments for *E. coli* O157:H7 and *S.* Enteritidis contained 5 mg/L lauric arginate (LAE), 500 mg/L EDTA, and 200 mg/L cinnamon oil (CO) alone or in combinations. Treatments for *L. monocytogenes* contained 2.5 mg/L lauric arginate (LAE), 100 mg/L EDTA, and 100 mg/L CO alone or in combination. Errors are standard deviations (n = 3).

2.4. Microbial survivability end-point analysis in tryptic soy broth

To confirm the antimicrobial effect of the antimicrobials alone or in combination, viable bacterial cells were enumerated after treatments. Five mg/L of LAE, 200 mg/L CO, and 500 mg/L EDTA were added alone or in combination in TSB. One mL culture with ca. 10^7 CFU/mL *L. monocytogenes*, *S.* Enteritidis or *E. coli* O157:H7 was added to 9 mL TSB containing antimicrobials at the above concentrations to obtain a bacterial population of ca. 10^6 CFU/mL. After incubating the mixtures for 2 h, viable bacteria were enumerated using surface plating on tryptic soy agar (TSA) for *S.* Enteritidis and *E. coli* O157:H7 or TSA supplemented with yeast extract (TSAYE) for *L. monocytogenes*. The detection limit was 1 log CFU/mL. The experiments were done in triplicate.

2.5. Crystal violet assay

Alteration in outer membrane permeability was detected by the crystal violet assay, where a higher uptake% of the dye indicates greater membrane permeability (Devi, Nisha, Sakthivel, & Pandian, 2010). Suspensions of bacteria in TSB were harvested by centrifugation at 6700×*g* for 5 min at 21 °C (Sorvall Legend 23R, Thermo Scientific, Waltham, MA). The pellets were washed twice with and resuspended in 10 mM phosphate-buffered saline (PBS, pH 7.4). Antimicrobials (5 mg/L LAE, 500 mg/L EDTA, and 200 mg/L CO alone or in combinations) were added into 1 mL suspension and incubated for 2 h. Cells without treatment were used as control. After treatment, the cells were harvested at 6700×*g* for 5 min and resuspended in PBS (10 mM, pH 7.4) containing 10 μg/mL of crystal violet. After incubating for another 15 min, the suspensions were centrifuged at 13,000×*g* for 15 min and the absorbance of the cell free supernatant was measured at 590 nm using the above microplate reader. The uptake% of crystal violet was calculated using Eq. (1):

$$\text{Uptake}(\%) = \left(1 - \frac{\text{Abs value of supernant}}{\text{Abs value of the crystal violet solution}}\right) \times 100 \quad (1)$$

2.6. Nucleic acids released from bacteria cells

The release of cellular nucleic acids was detected by measuring A_{260} (Diao, Hu, Zhang, & Xu, 2014) of cells treated with antimicrobials. Bacteria were incubated overnight and washed twice in Tris–HCl buffer (10 mM, pH 7.2). After centrifugation at 10,000 ×*g* for 5 min at 20 °C, 1 mL suspension with ca. 10^{10} CFU/mL bacteria cells was incubated with 10 mg/L LAE, 1000 mg/L EDTA, and 400 mg/L CO alone or in combination for 3 h. Cells were then centrifuged at 13,000×*g* for 15 min at 20 °C, and the A_{260} of the supernatant was measured using a UV–Vis spectrophotometer (model Evolution 201, Thermo Scientific, Waltham, MA). The net increase of A_{260} due to antimicrobial treatments was obtained after subtracting the A_{260} of supernatants collected from suspensions with same concentrations of bacteria and antimicrobials without incubation. Measurement was done in triplicate.

2.7. Extracellular adenosine triphosphate (ATP) level

Bacteria were concentrated by centrifugation at 7500×*g* for 3 min at 25 °C, washed twice with and then resuspended in PBS (10 mM, pH 7.4) and 1 mL (ca. ~ 10^9 CFU/mL) treated with 5 mg/L LAE, 500 mg/L EDTA and 200 mg/L CO alone or in combination and incubated for 30 min. Then, bacterial suspensions were centrifuged at 7500×*g* for 4 min and the supernatants were collected and immediately placed to an ice bath to prevent ATP loss. The Enliten™ ATP assay system with bioluminescence detection kit (Promega Corp., Madison, WI) was used for ATP assay. The rL/L reagent was rehydrated in the reconstitution buffer and incubated at room temperature (21 °C) for 1 h before use. Ten μL of a sample and 100 μL of a reagent solution were added into wells of a 96-well microtiter plate, and the luminescence values were determined with a luminescence plate reader (BioTek). A standard curve was made to quantify the ATP concentration in bacterial suspensions. Each treatment was measured in duplicate.

2.8. Cell morphology studied with scanning electron microscopy

SEM was used to study the morphology of bacterial cells after treatment with antimicrobials (Yahyazadeh, Omidbaigi, Zare, & Taheri, 2008). Bacterial cells were collected at 7500×*g* for 4 min at 20 °C after 24 h incubation. After washing twice with and resuspension in PBS (10 mM, pH 7.4), 40 mg/L LAE, 1000 mg/L EDTA, and 600 mg/L CO alone or in combinations were added to a 1 mL suspension with 10^{10} CFU/mL of cells and incubated for 2 h. Cells were re-washed, and pre- and post-fixed using 3 mL/100 mL glutaraldehyde and 2 g/100 mL osmium tetroxide, respectively, for 1 h at room temperature (21 °C). Subsequently, cells were gradually dehydrated using first 25 mL/100 mL, then 50 mL/100 mL, 75 mL/100 mL, 95 mL/100 mL and 100 mL/100 mL ethanol for 20 min at each concentration. The dehydrated cells were placed on a silicon wafer, coated with gold, and imaged using a LEO 1525 surface SEM

Table 1
Log reduction of *Escherichia coli* O157:H7 ATCC 43895 (initial count of 6.17 log CFU/mL) and *Salmonella* Enteritidis (initial count of 6.23 log CFU/mL) at 37 °C and *Listeria monocytogenes* Scott A (initial count of 6.41 log CFU/mL) at 32 °C after treatment by lauric arginate (LAE), cinnamon oil (CO), and/or EDTA alone or in combination in tryptic soy broth for 2 h.

Treatment (conc. in mg/L)	Log reduction[a]		
	E. coli O157:H7	*S.* Enteritidis	*L. monocytogenes*
LAE (5)	-0.16 ± 0.05^{c}	-0.60 ± 0.18^{d}	-0.31 ± 0.16^{c}
EDTA (500)	-0.18 ± 0.08^{c}	-0.51 ± 0.15^{d}	-0.07 ± 0.44^{bc}
CO (200)	0.03 ± 0.05^{c}	-0.49 ± 0.10^{d}	-0.42 ± 0.51^{c}
LAE (5) + EDTA (500)	0.91 ± 0.27^{b}	0.34 ± 0.18^{b}	0.10 ± 0.30^{bc}
CO (200) + EDTA (500)	0.44 ± 0.11^{bc}	0.07 ± 0.17^{bc}	-0.06 ± 0.16^{bc}
LAE (5) + CO (200)	0.42 ± 0.12^{bc}	-0.24 ± 0.16^{cd}	0.76 ± 0.20^{b}
LAE (5) + CO (200) + EDTA (500)	4.70 ± 0.53^{a}	5.01 ± 0.26^{a}	1.71 ± 0.08^{a}

Negative values indicate an increase in bacteria population after treatments. Different superscript letters in each column indicate significant differences ($p < 0.05$).
[a] Numbers are mean ± standard deviation ($n = 3$).

(LEO Electron Microscopy, Oberkochen, Germany). Viable bacteria cells after treatment with the antimicrobials for 2 h before fixation were also enumerated using the same method described in the microbial survivability end-point analysis.

2.9. Interaction of antimicrobials with bacteria DNA

2.9.1. Extraction of DNA

The DNA of bacteria cells was extracted using a genomic DNA purification kit from Thermo Scientific (Waltham, WA). The purity of the extracted DNA was evaluated based on the ratio of absorbance at 260 and 280 nm, which was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA) at room temperature (21 °C). In our study, the absorbance ratio was about 1.82.

2.9.2. Interaction between DNA and LAE studied using atomic force microscopy (AFM)

LAE was added in to Tris–HCl (10 mM, pH 7.2) buffer containing about 30 μg/mL DNA to reach a final LAE concentration of 10 or 50 mg/L. The DNA-LAE mixture was incubated at room temperature (21 °C) for 15 min and diluted 10 times with 10 mM Tris–HCl buffer (pH 7.2). Ten μL of the diluted mixture was spread onto a freshly cleaved mica sheet mounted on a sample holder. After drying at room temperature for about 6 h, samples were imaged using a Multimode VIII nanoscope AFM (Bruker Corp., Santa Barbara, CA) operating in the tapping mode. All images were captured at a scanning speed of 1.78 Hz.

2.9.3. Absorption spectra of antimicrobial-DNA mixture

To specify the possible interactions of the antimicrobials with bacterial DNA, the extracted DNA was diluted using 10 mM Tris–HCl buffer (pH 7.2) to about 10 μg/mL and the 1 g/100 mL LAE or 4 g/100 mL CO stock solution was titrated into DNA solution gradually to reach LAE concentration of 0, 25, 50 and 100 mg/L and CO concentration of 0, 50, and 100 mg/L. The absorption spectra of the mixtures were determined in a quartz cuvette with a light path length of 1 cm from 200 to 400 nm using a UV–Vis spectrophotometer (Evolution 201, Thermo Scientific, Waltham, MA).

2.10. Statistical analysis

Experiment data were analyzed using ANOVA with Turkey's test (SPSS 22, IBM, Armonk, NY) to discriminate among means.

3. Results

3.1. Microbial growth kinetics in tryptic soy broth

Growth curves of bacteria measured at an OD of 600 nm are shown in Fig. 1. For *E. coli* O157:H7 ATCC 43895 (Fig. 1A), the treatment with a combination of LAE, EDTA and CO showed no growth over 12 h and had a significantly lower OD than other treatments except the one with a combination of LAE and EDTA (Fig. 1A). For *S.* Enteritidis (Fig. 1B), the combination of LAE, EDTA and CO also prevented growth followed by the combinations of LAE and EDTA, and CO and EDTA. Similarly, the triple antimicrobial combination showed the highest efficiency inhibiting the growth of *L. monocytogenes* Scott A (Fig. 1C) although the bacterium did demonstrate growth with this treatment after 7–8 h. Overall, antimicrobial activities of the combination of LAE, EDTA and CO were significantly better than treatments with double or single antimicrobials, which indicated potential of synergistic effect among LAE, EDTA and CO.

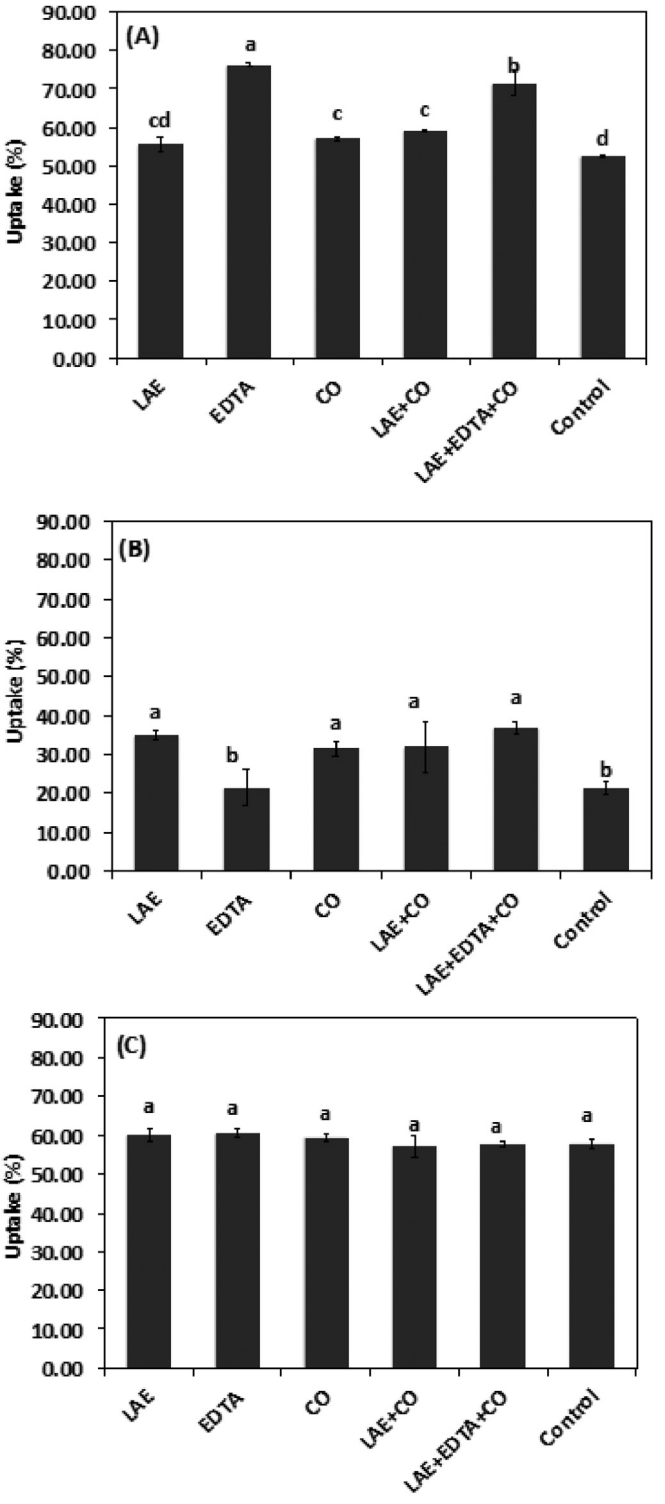

Fig. 2. Uptake% of crystal violet by (A) *Escherichia coli* O157:H7 ATCC 43895, (B) *Salmonella* Enteritidis, and (C) *Listeria monocytogenes* Scott A in 0.1 M PBS (pH 7.4) at 37 °C (for *E. coli* O157:H7 and *S.* Enteritidis) or 32 °C (for *L. monocytogenes*) after 2-h treatment with 5 mg/L lauric arginate (LAE), 500 mg/L EDTA, and 200 mg/L cinnamon oil (CO) alone or in combinations. Errors are standard deviations (n = 3). Different letters above bars indicate significant difference of treatments in the same plot.

3.2. Microbial survivability end-point analysis in tryptic soy broth

To further evaluate the potential for enhanced antimicrobial activity by the triple combination, a test was done to determine the

lethality of the treatments in TSB after 2 h exposure (Table 1). Log-reductions of Gram-negative bacteria in treatments with the combination of 5 mg/L LAE, 500 mg/L EDTA and 200 mg/L CO were significantly greater ($p < 0.05$) than treatments with one or two compounds. After 2 h at 37 °C, the triple combination resulted in a reduction of 4.70 and 5.01 log CFU/mL for *E. coli* O157: H7 and *S.* Enteritidis, respectively, which contrasted with no more than 0.91 log CFU/mL reduction for other treatments. For the Gram-positive *L. monocytogenes*, a reduction of about 1.7 log CFU/mL was observed for the triple combination after 2 h incubation at 32 °C, which was also significantly greater ($p < 0.05$) than other treatments with reductions of no more than 0.76 log CFU/mL. Additionally, a much higher ($p < 0.05$) reduction of *L. monocytogenes* was observed in the treatment of LAE and CO combination (0.76 log CFU/mL) compared to treatments of LAE (−0.31 log CFU/mL) or CO (−0.42 log CFU/mL) alone.

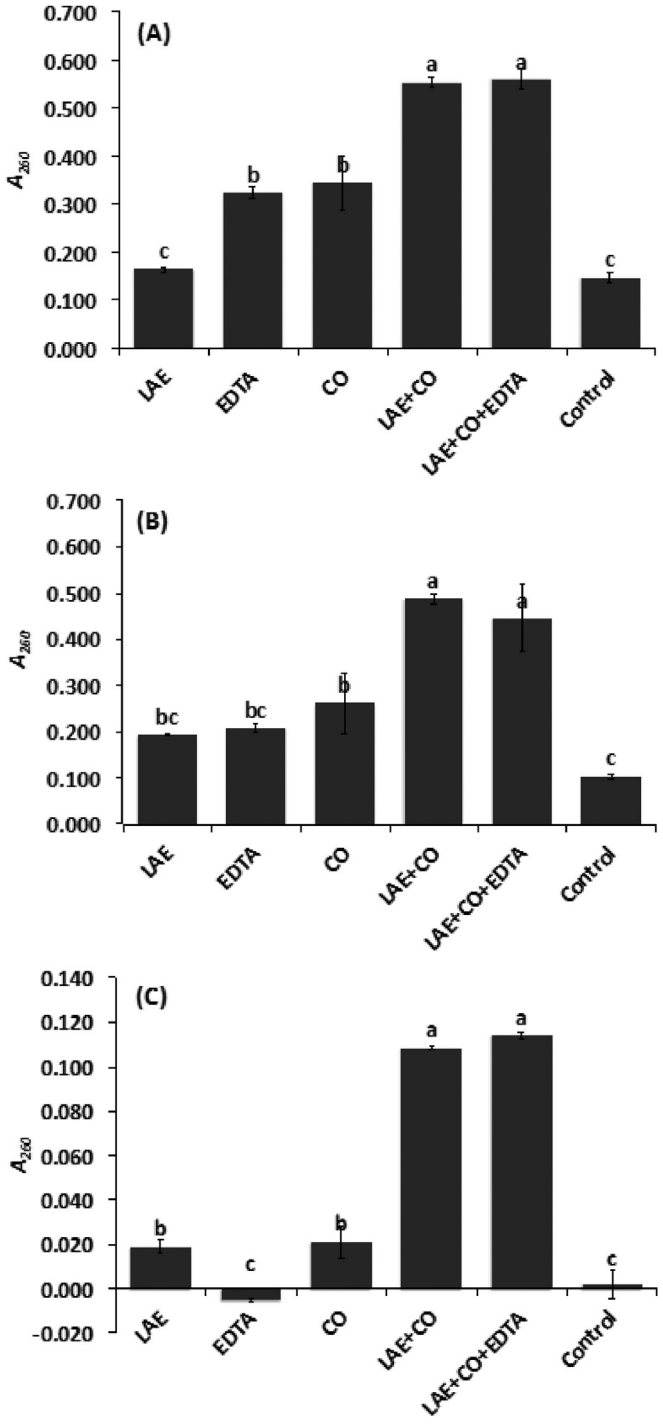

Fig. 3. Absorbance at 260 nm (A_{260}) of extracellular contents after treating (A) *Escherichia coli* O157:H7 ATCC 43895 and (B) *Salmonella* Enteritidis at 37 °C, and (C) *Listeria monocytogenes* Scott A at 32 °C in 10 mM Tris−HCl (pH 7.2) for 2 h with 10 mg/L lauric arginate (LAE), 1000 mg/L EDTA, and 400 mg/L cinnamon oil (CO) alone or in combinations. Errors are standard deviations (n = 3). Different letters above bars indicate significant difference of treatments in the same plot.

3.3. Membrane permeability

To determine the potential mechanisms of the antimicrobials against the test bacterium, their influence on membrane permeability was determine using the crystal violet assay, loss of nucleic acids, and loss of ATP. Fig. 2A shows the uptake% of crystal violet by *E. coli* O157:H7 after different treatments. The highest uptake% was observed in the treatment with EDTA (>70%). A significantly higher uptake% ($p < 0.05$) was observed for LAE + CO + EDTA (>70%) than treatments with LAE + CO, CO, or LAE. There were no consistent differences detected among treatments for *S.* Enteritidis (Fig. 2B) and for *L. monocytogenes*, no significant ($p > 0.05$) differences of crystal violet uptake% were detected (Fig. 2C).

To further detect the integrity of cell membranes after antimicrobial treatments, A_{260} (nucleic acids) released from bacterial cells was determined in the supernatant after centrifugation of treated cell suspensions. As shown in Fig. 3, the treatments of LAE + CO and

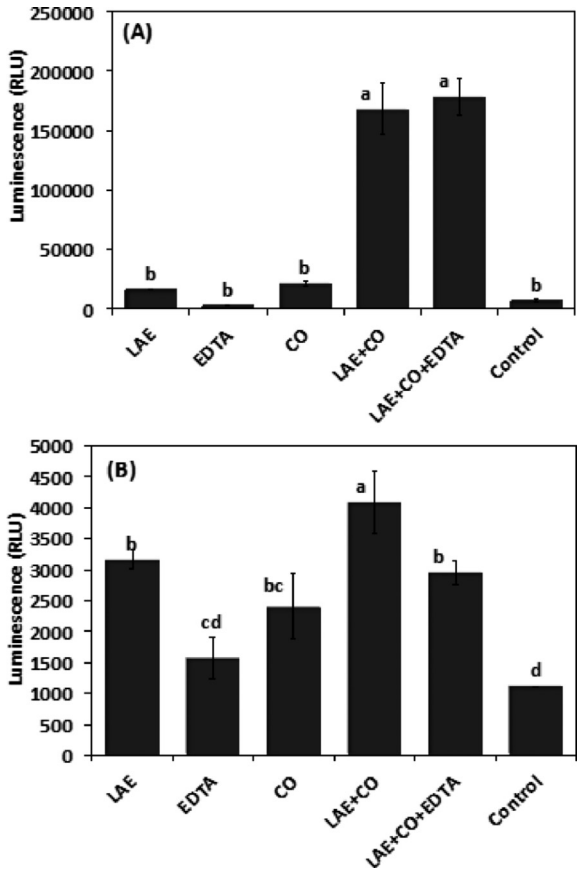

Fig. 4. Luminescence showing extracellular ATP content of (A) *Listeria monocytogenes* Scott A at 37 °C and (B) *Salmonella* Enteritidis at 32 °C after 30 min incubation in tryptic soy broth with 5 mg/L lauric arginate (LAE), 500 mg/L EDTA, and 200 mg/L cinnamon oil (CO) alone or in combinations. Errors are standard deviations (n = 2).

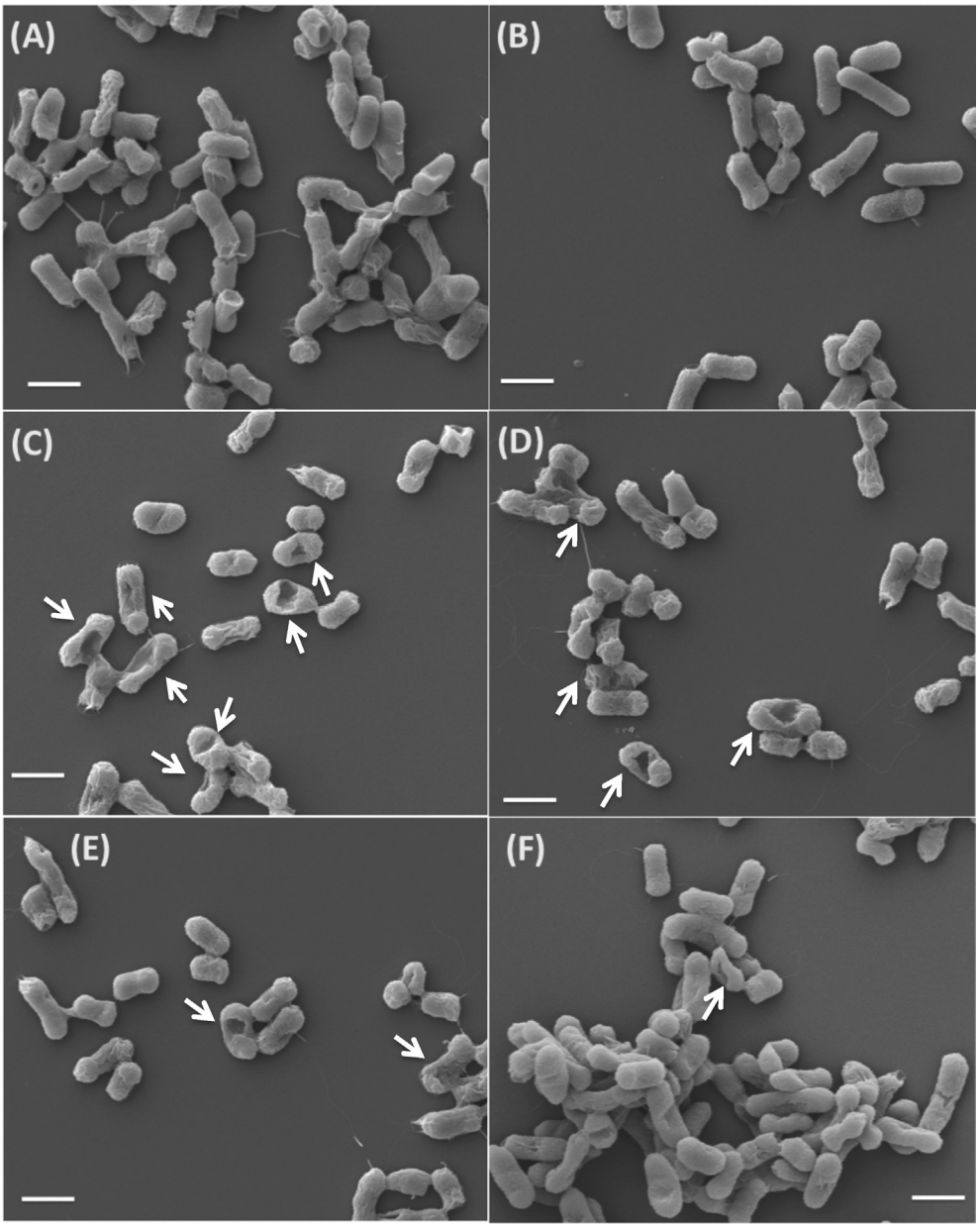

Fig. 5. SEM images of *Salmonella* Enteritidis after treatment by (A) 40 mg/L lauric arginate (LAE), (B) 1000 mg/L EDTA, (C) 600 mg/L cinnamon oil (CO), (D) 40 mg/L LAE+600 mg/L CO, or (E) 40 mg/L LAE+600 mg/L CO+1000 mg/L EDTA at 37 °C for 2 h, with comparison to the untreated sample (F). Arrows indicated disrupted cells. Bar = 1 μm.

LAE + CO + EDTA ($A_{260} > 0.500$) demonstrated significantly higher ($p < 0.05$) release of cellular constituents from *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* than other treatments.

As shown in Fig. 4A and similar to the results of A_{260}, extracellular ATP from *L. monocytogenes* in treatments LAE + CO and LAE + CO + EDTA was significantly higher ($p < 0.05$) than that in other treatments, while the addition of EDTA had no significant impact on the extracellular ATP level ($p > 0.05$). For *S.* Enteritidis (Fig. 4B), extracellular ATP was the highest in the treatment of LAE + CO ($p < 0.05$), and similarly to *L. monocytogenes*, addition of EDTA did not increase the extracellular ATP ($p > 0.05$).

3.4. Cell morphology after antimicrobial treatments

Fig. 5 shows SEM images of *S.* Enteritidis cells after various treatments. Compared to the treatment with LAE or EDTA (Fig. 5A and B), a greater extent of disruption of bacteria cell membranes was observed for the treatment with 600 mg/L CO (Fig. 5C). Based on inspection of about 50 cells on SEM images, ca. 10–20% cells were disrupted in the control group or treatments with LAE or EDTA alone, while more than 40% cells were disrupted in treatments with CO alone, the combination of LAE and CO, and the combination of LAE, CO and EDTA. For *L. monocytogenes*, < 10% of cells were damaged in untreated control (Fig. 6F) or in treatment with LAE, EDTA or CO alone (Fig. 6A–C), while about 20–30% of cells were damaged in treatments with combinations of LAE and CO or LAE, EDTA and CO (Fig. 6D and E).

Log reduction results after antimicrobial treatments at conditions corresponding to SEM experiments are presented in Table 2. Treatment with LAE, CO or EDTA alone did not cause large reduction of viable bacteria cells. Only 0.10, 0.01 and 0.08 log CFU/mL reduction of *S.* Enteritidis were detected in the treatments with 40 mg/L LAE, 1000 mg/L EDTA and 600 mg/L CO, respectively. Treatment by combinations of LAE and CO or LAE, CO and EDTA

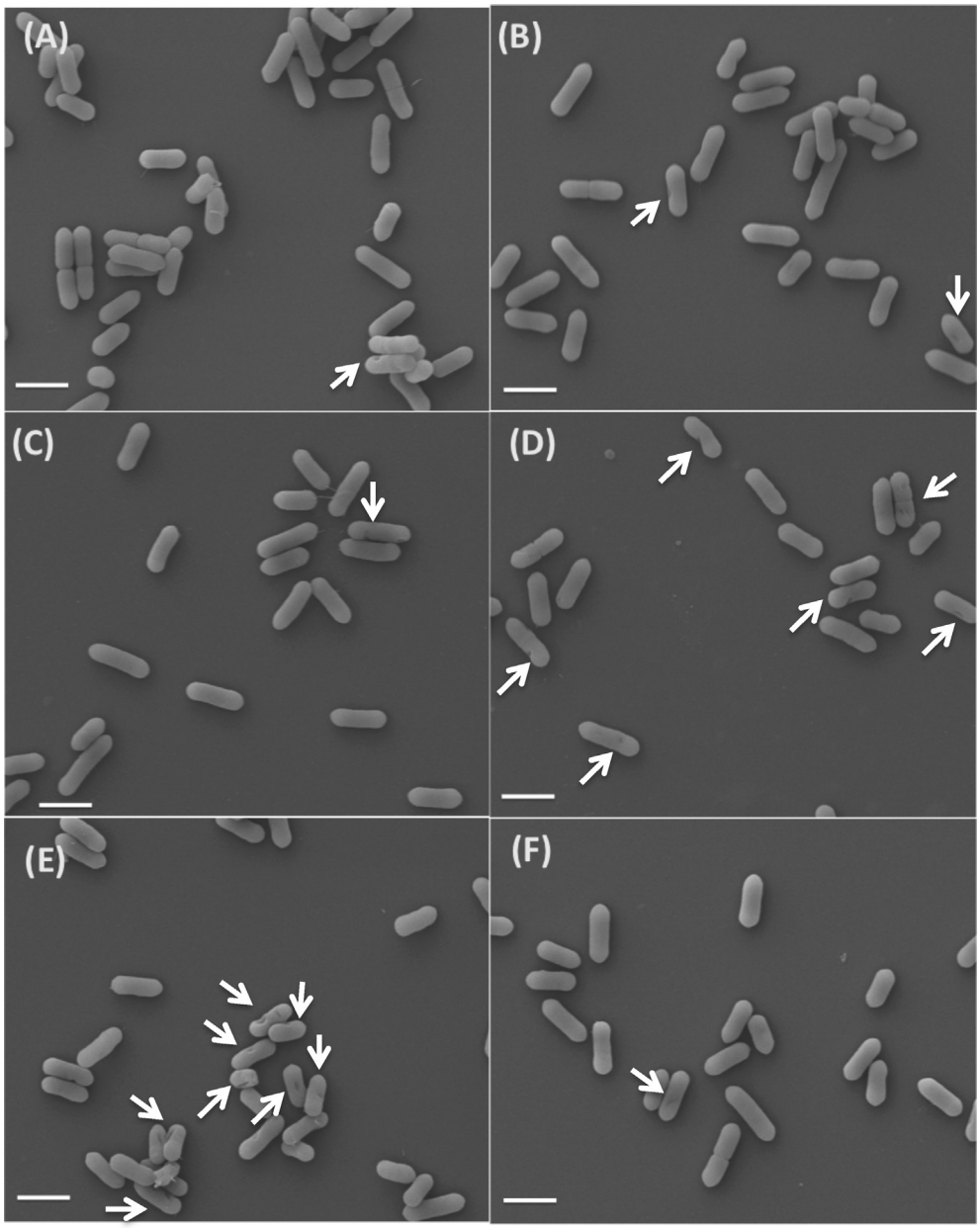

Fig. 6. SEM images of *Listeria monocytogenes* Scott A after treatment by (A) 40 mg/L lauric arginate (LAE), (B) 1000 mg/L EDTA, (C) 600 mg/L cinnamon oil (CO), (D) 40 mg/L LAE+600 mg/L CO, or (E) 40 mg/L LAE+600 mg/L CO+1000 mg/L EDTA at 32 °C for 2 h, with comparison to the untreated sample (F). Arrows indicated disrupted cells. Bar = 1 μm.

resulted in significantly higher ($p < 0.05$) log reductions for *S.* Enteritidis of 1.43 or 1.71 log CFU/mL, respectively. Similarly, much higher ($p < 0.05$) log reductions (>6 log) of *L. monocytogenes* were observed in treatments with combinations of LAE and CO or LAE, CO and EDTA, while only an 0.84 log CFU/mL reduction was found with 40 mg/L LAE, and no log reduction was observed in the treatments with the other two antimicrobials alone.

3.5. Interaction between DNA and LAE

To test whether there was any binding between LAE and bacterial DNA, which may lead to the morphology change of DNA molecules, bacterial DNA morphology was observed using AFM before and after antimicrobial treatments. As shown in Fig. 7, DNA of *L. monocytogenes* and *S.* Enteritidis was regularly distributed. However, with the addition of 10 or 50 mg/L LAE, DNA assembled to aggregated structures (Fig. 7B, C, E and F).

To further confirm the interaction between antimicrobials and DNA, the absorption spectra of solutions with about 10 μg/mL of *S.* Enteritidis DNA were measured before and after the addition of antimicrobials. As shown in Fig. 8A, the absorbance of DNA centered on 260 nm increased as the addition of 50 mg/L LAE; but negligible absorbance of LAE was detected at 260 nm. The absorbance peak of 100 mg/L CO was at 280 nm and the absorbance at 280 nm was decreased after mixing CO with the DNA solution.

4. Discussion

4.1. Effects of antimicrobials on bacteria membrane structures

The bacterial cell envelope is the first barrier for antimicrobial action and differs significantly between Gram-positive and Gram-

Table 2
Log reduction of *Salmonella* Enteritidis (initial count of 10.06 log CFU/mL) at 37 °C and *Listeria monocytogenes* Scott A (initial count of 9.79 log CFU/mL) at 32 °C after treatment by 40 mg/L lauric arginate (LAE), 600 mg/L cinnamon oil (CO), and 1000 mg/L EDTA alone or their combination in PBS (10 mM, pH 7.4) for 2 h.

Treatment	Log reduction[a]	
	S. Enteritidis	*L. monocytogenes*
LAE	0.10 ± 0.04[b]	0.84 ± 0.65[b]
EDTA	0.01 ± 0.04[b]	−0.01 ± 0.19[b]
CO	0.08 ± 0.08[b]	−0.03 ± 0.11[b]
LAE + CO	1.43 ± 0.44[a]	6.19 ± 1.41[a]
LAE + CO + EDTA	1.71 ± 0.33[a]	6.37 ± 1.19[a]

Negative values indicate an increase in bacteria population after treatments. Different superscript letters in each column indicate significant differences ($p < 0.05$).
[a] Numbers are mean ± standard deviation ($n = 6$).

negative bacteria. The envelope of Gram-positive bacteria consists of an outer peptidoglycan cell wall and a cytoplasmic membrane, while that of Gram-negative bacteria is comprised of an outer LPS-containing membrane, a thin peptidoglycan layer, and the inner cytoplasmic membrane (Bladen & Mergenhagen, 1964, Kong, Chen, Xing, & Park, 2010). Gram-positive bacteria possess relatively porous hydrophilic cell wall that allows most antimicrobials such as phenols, alcohols, aldehydes to freely cross the cell wall (Lambert, 2002). Thus, Gram-positive bacteria are generally more sensitive to small molecular weight antimicrobials. For Gram-negative bacteria, the existence of an extra LPS-containing outer membrane slows or prevents diffusion of antimicrobials. Hydrophobic interactions between LPS molecules together with hydrogen bonds and ionic bridging by multivalent cations (Ca^{2+} and Mg^{2+}) enable the low fluidity and impermeability of the outer membrane to large hydrophilic and small hydrophobic antimicrobials (Nikaido, 2003; Ruiz et al., 2009).

EDTA is known to improve the permeability of LPS outer membrane because it chelates divalent Ca^{2+} and Mg^{2+} ions that are essential to the membrane stability (Hancock, 1984; Nikaido, 2003; Vaara, 1992). In the present study, increased membrane permeability, as measured by uptake% of crystal violet, was observed for *E. coli* O157:H7 cells to be the highest for EDTA alone followed by the LAE + CO treatment with EDTA (Fig. 2A). However, no significant difference ($p > 0.05$) was observed for same treatments with *S.* Enteritidis (Fig. 2B). The EDTA concentration (500 mg/L) may not be sufficient to affect the membrane structure of *S.* Enteritidis compared to *E. coli* O157:H7 under the conditions studied (Table 1 and Fig. 1). Results in other study also showed that *S.* Enteritidis was more resistant to EDTA than *E. coli* O157:H7 (Branen & Davidson, 2004). As for *L. monocytogenes*, because of missing LPS outer membrane, EDTA with or without antimicrobials did not significantly impact crystal violet uptake% (Fig. 2C).

The amount of nucleic acids released from bacteria was measured for A_{260} as an indicator of the integrity of bacteria cytoplasmic membranes (Fig. 3). The A_{260} of *E. coli* O157:H7 after the 10 mg/L LAE treatment was similar to the control without treatment, which indicated that 10 mg/L LAE may not cause cell leakage. This could be due to the LAE concentration being lower than the 11.5 mg/L minimum inhibitory concentration and minimum bactericidal concentration of *E. coli* O157:H7 (Ma et al., 2013). This may also result if the antimicrobial mechanism of LAE does not involve cell membrane disruption and leakage which was evident in the SEM images of *S.* Enteritidis or *L. monocytogenes* treated with 40 mg/L LAE. Forty mg/L LAE did have lethal effect on *S. Enteritidis* and *L. monocytogenes* cells (Table 2). The results in the present study agreed with some previous studies. Disruption of cell membranes but no cell lysis of the Gram-negative *S.* Typhimurium and Gram-positive *Staphylococcus aureus* treated with 32 and 8 mg/L LAE, respectively, was observed based on transmission electron microscopy (TEM) images (Rodriguez, Seguer, Rocabayera, &

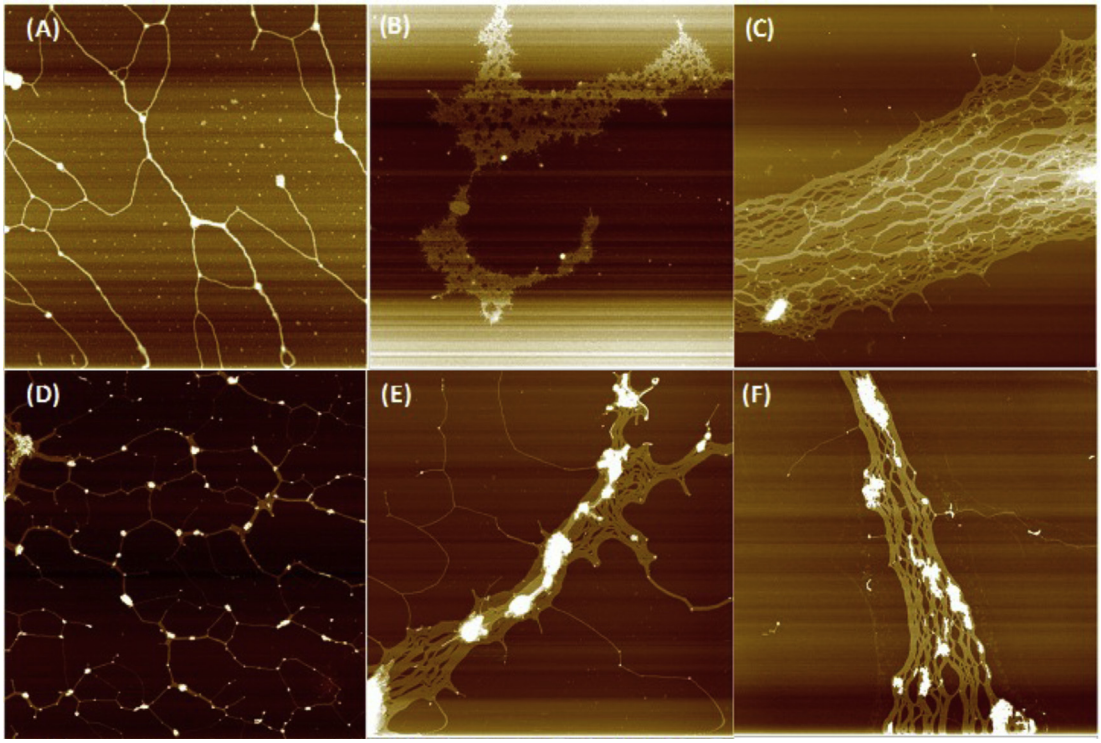

Fig. 7. AFM images of *Listeria monocytogenes* Scott A (A–C) and *Salmonella* Enteritidis DNA (D–F) before (A and D) and after treatment by 10 mg/L (B and E) or 50 mg/L (C and F) LAE). Image dimensions are 10 μm × 10 μm.

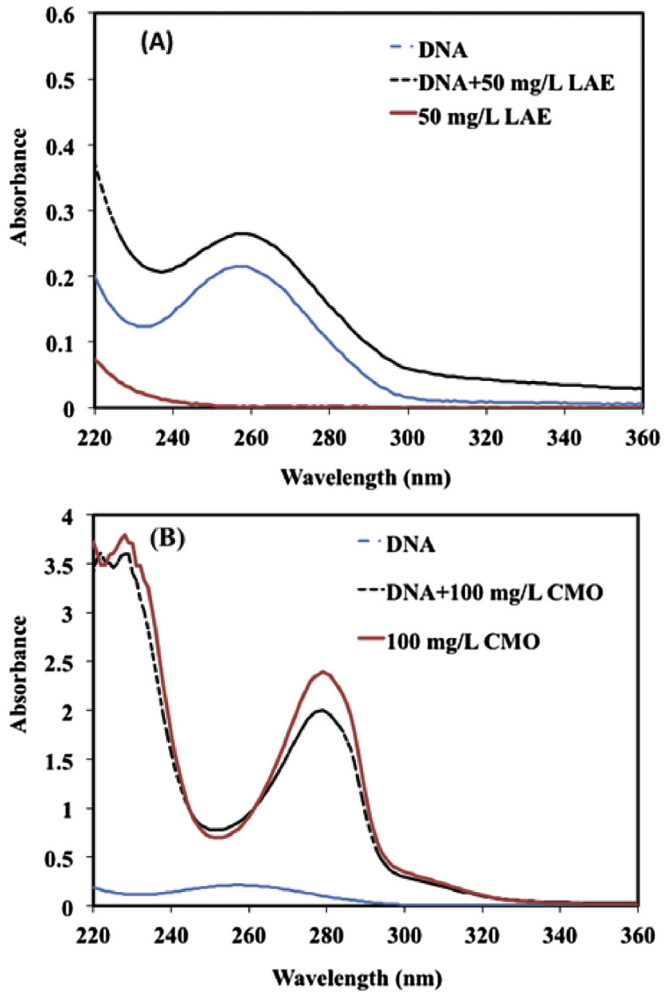

Fig. 8. Absorption spectra of *Salmonella* Enteritidis DNA with the addition of (A) 50 mg/L lauric arginate (LAE) or (B) 100 mg/L cinnamon oil (CO).

Manresa, 2004). Similarly, no cell lysis of *E. coli* O157:H7 or *L. monocytogenes* treated by 200 mg/L LAE was detected based on SEM and TEM images (Pattanayaiying, Aran, & Cutter, 2014).

Extracellular ATP of *S.* Enteritidis was the highest in the treatment of LAE + CO. Interestingly, lower extracellular ATP was detected in the treatment of LAE + CO + EDTA (Fig. 4B). One possible explanation for this may be found in a study by Gill and Holley (2004). They showed that EOs inhibit ATP generation in bacteria cells. Addition of EDTA may increase permeability of the outer membrane of *S.* Enteritidis thus facilitating penetration of LAE + CO. With greater penetration of LAE and CO, the generation of cellular ATP may be inhibited which would lead to lower extracellular ATP detected.

When treated by 600 mg/L CO, severe damage of *S.* Enteritidis cells was observed (Fig. 5), although only 0.08 log CFU/mL reduction of *S.* Enteritidis could be detected (Table 2), which indicates the main target of CO is the cell membrane. Similar results have been reported for the bactericidal action of EOs against Gram-negative and Gram-positive bacteria analyzed using the crystal violet assay, SEM, and AFM (Bajpai, Sharma, & Baek, 2013; Devi et al., 2010; Lv, Liang, Yuan, & Li, 2011; Oussalah, Caillet, & Lacroix, 2006; Rhayour, Bouchikhi, Tantaoui-Elaraki, Sendide, & Remmal, 2003). However, at this level of CO, severe damage of cell morphology was not observed for *L. monocytogenes* (Fig. 5). This may have resulted from the greater resistance of *L. monocytogenes* to CO, as reported previously (Ma et al., 2013).

Compared to *E. coli* O157:H7 or *S.* Enteritidis treated with LAE or CO alone, A_{260} of the treatment of LAE + CO was significantly higher ($p < 0.05$) and was nearly equivalent to the sum of treatments with LAE and CO alone. Since the total antimicrobial concentration was higher in the combination of LAE + CO, A_{260} values suggest the additive effects of the two antimicrobials in releasing intra-cellular materials from *E. coli* O157:H7 and *S.* Enteritidis. For *L. monocytogenes*, A_{260} of the LAE + CO treatment was significantly higher ($p < 0.05$) than the sum of LAE and CO alone treatments, which agreed with the potential synergistic antilisterial effect of LAE and CO, as reported in a previous study (Ma et al., 2013). This also suggests that the disruption of cytoplasmic membrane by the combination of LAE and CO may be the major mechanism for lethality of *L. monocytogenes*, which was further confirmed by SEM (Fig. 7) and extracellular ATP results (Fig. 4).

4.2. Binding between DNA and antimicrobials

DNA has a negatively charged phosphate backbone and cationic surfactants are known to interact with DNA molecules through electrostatic attraction and hydrophobic interaction, causing precipitation of DNA (Bathaie, Moosavi-Movahedi, & Saboury, 1999; Bhattacharya & Mandal, 1997; Ishaq, Wolf, & Ritter, 1990). Strong interactions between positively charged LAE and bacterial DNA were also observed in our study (Figs. 7 and 8), and the assembly of DNA was observed at the lowest concentration (10 mg/L) of LAE tested (Fig. 7), which suggested that DNA may be an intracellular target for LAE bactericidal action. Since this was an *in vitro* test, further *in vivo* studies are needed to confirm the interaction. A recent study did suggest that LAE may have multiple targets excluding the bacterial cell membrane (Coronel-León et al., 2016), because a large number of dead bacterial cells had undamaged membranes after being treated with LAE.

Increased absorption of DNA at 260 nm after addition of 50 mg/L LAE (Fig. 8A) suggested the distortion of stacking interactions between nucleic acid base pairs and a significant change of DNA secondary structure after binding with LAE (Morrissey, Kudryashov, Dawson, & Buckin, 1999). Decreased absorbance of CO at 280 nm (Fig. 8B) indicated CO can intercalate into the double helix of DNA and change the native structure of DNA, as previously demonstrated for hydrophobic binding between lipid and DNA molecules (Matulis, Rouzina, & Bloomfield, 2002).

5. Conclusions

In the present study, enhanced antimicrobial activity of LAE and CO against Gram-negative bacteria was observed in the presence of EDTA, which was in contrast with a normally antagonistic interaction for the pair. EDTA is suggested to improve the permeability of the LPS outer membrane and enable greater penetration by LAE and CO to the cytoplasmic membrane. The main target of CO is thought to be the bacterial cell membrane, and the hydrophobic binding with DNA can be another possible mechanism. LAE did not cause lysis of cells but affected DNA structures by causing them to aggregate through ionic bridging. Thus, it is hypothesized that EDTA improved the permeability of outer membrane of Gram-negative bacteria to facilitate the penetration of LAE and CO which targeted the cytoplasmic membrane and intracellular structures to enable the enhanced antimicrobial activity. For the Gram-positive bacteria *L. monocytogenes*, LAE and CO had synergistically antimicrobial activity and caused severe damage of the cytoplasmic membrane, which may be a major mechanism for lethality of the bacterium. Findings in present study could lower the amount of each antimicrobial used in food matrixes thus reduce the

possible negative effect of the antimicrobials on the sensory qualities of food products.

Acknowledgments

This work was supported by the Center for Produce Safety, under grant number SCB12062, and the University of Tennessee, the USDA National Institute of Food and Agriculture Hatch Project 223984.

References

Bajpai, V. K., Sharma, A., & Baek, K.-H. (2013). Antibacterial mode of action of *Cudrania tricuspidata* fruit essential oil, affecting membrane permeability and surface characteristics of food-borne pathogens. *Food Control, 32*(2), 582–590.

Bathaie, S., Moosavi-Movahedi, A., & Saboury, A. (1999). Energetic and binding properties of DNA upon interaction with dodecyl trimethylammonium bromide. *Nucleic Acids Research, 27*(4), 1001–1005.

Bhattacharya, S., & Mandal, S. S. (1997). Interaction of surfactants with DNA. Role of hydrophobicity and surface charge on intercalation and DNA melting. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 1323*(1), 29–44.

Bladen, H. A., & Mergenhagen, S. E. (1964). Ultrastructure of Veillonella and morphological correlation of an outer membrane with particles associated with endotoxic activity. *Journal of Bacteriology, 88*(5), 1482–1492.

Bonnaud, M., Weiss, J., & McClements, D. J. (2010). Interaction of a food-grade cationic surfactant (lauric arginate) with food-grade biopolymers (pectin, carrageenan, xanthan, alginate, dextran, and chitosan). *Journal of Agricultural and Food Chemistry, 58*(17), 9770–9777.

Branen, J. K., & Davidson, P. M. (2004). Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin. *International Journal of Food Microbiology, 90*(1), 63–74.

Chen, H., Zhang, Y., & Zhong, Q. (2015). Physical and antimicrobial properties of spray-dried zein–casein nanocapsules with co-encapsulated eugenol and thymol. *Journal of Food Engineering, 144*, 93–102.

Coronel-León, J., López, A., Espuny, M., Beltran, M., Molinos-Gómez, A., Rocabayera, X., et al. (2016). Assessment of antimicrobial activity of N^{α} -lauroyl arginate ethylester (LAE®) against *Yersinia enterocolitica* and *Lactobacillus plantarum* by flow cytometry and transmission electron microscopy. *Food Control, 63*, 1–10.

Devi, K. P., Nisha, S. A., Sakthivel, R., & Pandian, S. K. (2010). Eugenol (an essential oil of clove) acts as an antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane. *Journal of Ethnopharmacology, 130*(1), 107–115.

Diao, W.-R., Hu, Q.-P., Zhang, H., & Xu, J.-G. (2014). Chemical composition, antibacterial activity and mechanism of action of essential oil from seeds of fennel (*Foeniculum vulgare Mill.*). *Food Control, 35*(1), 109–116.

Gill, A. O., & Holley, R. A. (2004). Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*. *Applied and Environmental Microbiology, 70*(10), 5750–5755.

Hancock, R. E. (1984). Alterations in outer membrane permeability. *Annual Reviews in Microbiology, 38*(1), 237–264.

Hawkins, D., Rocabayera, X., Ruckman, S., Segret, R., & Shaw, D. (2009). Metabolism and pharmacokinetics of ethyl N^{α}-lauroyl-L-arginate hydrochloride in human volunteers. *Food and Chemical Toxicology, 47*(11), 2711–2715.

Ishaq, M., Wolf, B., & Ritter, C. (1990). Large-scale isolation of plasmid DNA using cetyltrimethylammonium bromide. *BioTechniques, 9*(1), 19–20, 22, 24.

Kong, M., Chen, X. G., Xing, K., & Park, H. J. (2010). Antimicrobial properties of chitosan and mode of action: a state of the art review. *International Journal of Food Microbiology, 144*(1), 51–63.

Lambert, P. (2002). Cellular impermeability and uptake of biocides and antibiotics in gram-positive bacteria and mycobacteria. *Journal of Applied Microbiology, 92*(s1), 46S–54S.

Lv, F., Liang, H., Yuan, Q., & Li, C. (2011). In vitro antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms. *Food Research International, 44*(9), 3057–3064.

Ma, Q., Davidson, P. M., & Zhong, Q. (2013). Antimicrobial properties of lauric arginate alone or in combination with essential oils in tryptic soy broth and 2% reduced fat milk. *International Journal of Food Microbiology, 166*(1), 77–84.

Matulis, D., Rouzina, I., & Bloomfield, V. A. (2002). Thermodynamics of cationic lipid binding to DNA and DNA condensation: roles of electrostatics and hydrophobicity. *Journal of the American Chemical Society, 124*(25), 7331–7342.

Ma, Q., Zhang, Y., Critzer, F., Davidson, P. M., Zivanovic, S., & Zhong, Q. (2016). Physical, mechanical, and antimicrobial properties of chitosan films with microemulsions of cinnamon bark oil and soybean oil. *Food Hydrocolloids, 52*, 533–542.

Morrissey, S., Kudryashov, E., Dawson, K., & Buckin, V. (1999). Surfactant-DNA complexes in low ionic strength dilute solutions. In *Trends in Colloid and Interface Science* (vol. XIII, pp. 71–75). Springer.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews, 67*(4), 593–656.

Noll, K. S., Prichard, M. N., Khaykin, A., Sinko, P. J., & Chikindas, M. L. (2012). The natural antimicrobial peptide subtilosin acts synergistically with glycerol monolaurate, lauric arginate, and ε-poly-L-lysine against bacterial vaginosis-associated pathogens but not human lactobacilli. *Antimicrobial Agents and Chemotherapy, 56*(4), 1756–1761.

Oussalah, M., Caillet, S., & Lacroix, M. (2006). Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of *Escherichia coli* O157: H7 and *Listeria monocytogenes*. *Journal of Food Protection, 69*(5), 1046–1055.

Pan, K., Chen, H., Davidson, P. M., & Zhong, Q. (2014). Thymol nanoencapsulated by sodium caseinate: physical and antilisterial properties. *Journal of Agricultural and Food Chemistry, 62*(7), 1649–1657.

Pattanayaiying, R., Aran, H., & Cutter, C. N. (2014). Effect of lauric arginate, nisin Z, and a combination against several food-related bacteria. *International Journal of Food Microbiology, 188*, 135–146.

Rhayour, K., Bouchikhi, T., Tantaoui-Elaraki, A., Sendide, K., & Remmal, A. (2003). The mechanism of bactericidal action of oregano and clove essential oils and of their phenolic major components on *Escherichia coli* and *Bacillus subtilis*. *Journal of Essential Oil Research, 15*(4), 286–292.

Rodriguez, E., Seguer, J., Rocabayera, X., & Manresa, A. (2004). Cellular effects of monohydrochloride of l-arginine, N^{α}-lauroyl ethylester (LAE) on exposure to *Salmonella typhimurium* and *Staphylococcus aureus*. *Journal of Applied Microbiology, 96*(5), 903–912.

Ruckman, S. A., Rocabayera, X., Borzelleca, J. F., & Sandusky, C. B. (2004). Toxicological and metabolic investigations of the safety of N-α-Lauroyl-l-arginine ethyl ester monohydrochloride (LAE). *Food and Chemical Toxicology, 42*(2), 245–259.

Ruiz, N., Kahne, D., & Silhavy, T. J. (2009). Transport of lipopolysaccharide across the cell envelope: the long road of discovery. *Nature Reviews Microbiology, 7*(9), 677–683.

Techathuvanan, C., Reyes, F., David, J. R., & Davidson, P. M. (2014). Efficacy of commercial natural antimicrobials alone and in combinations against pathogenic and spoilage microorganisms. *Journal of Food Protection, 77*(2), 269–275.

USDA. (2005). *Agency response letter GRAS notice no. GRN 000164*. Retrieved 03/17, 2015, from http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn000164.pdf.

Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiological Reviews, 56*(3), 395–411.

Yahyazadeh, M., Omidbaigi, R., Zare, R., & Taheri, H. (2008). Effect of some essential oils on mycelial growth of *Penicillium digitatum Sacc*. *World Journal of Microbiology and Biotechnology, 24*(8), 1445–1450.

Zhang, Y., Ma, Q., Critzer, F., Davidson, P. M., & Zhong, Q. (2015). Physical and antibacterial properties of alginate films containing cinnamon bark oil and soybean oil. *LWT-Food Science and Technology, 64*(1), 423–430.

Zheng, Z. (2014). Ingredient technology for food preservation. *Industrial Biotechnology, 10*(1), 28–33.