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Erythorbyl laurate as a potential food additive with multi-functionalities: Antibacterial activity and mode of action

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A B S T R A C T

The antibacterial activity and mechanism of erythorbyl laurate were investigated to provide information on practical applications as a multi-functional food additive. Antibacterial susceptibility screening demonstrated that erythorbyl laurate exerted both bacteriostatic and bactericidal effects on gram-positive foodborne pathogens (minimum inhibitory concentration: 0.48–0.88 mM, minimum bactericidal concentration: 0.65–1.00 mM). The growth curves of gram-positive strains showed increases in the λ (lag phase) accompanied by decreases in the μ_{max} (maximum specific growth rate) as the concentration of erythorbyl laurate increased. The results of the crystal violet uptake and LIVE/DEAD *Bac*Light assays suggested that the antibacterial mechanism of erythorbyl laurate might depend on alterations in the permeability and integrity of cell membranes. Furthermore, erythorbyl laurate showed strong synergistic effects when combined with a variety of antibacterial agents including nisin, kanamycin, and erythromycin (FIC index < 0.28), which might result from increases in bacterial membrane permeability induced by erythorbyl laurate.

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1. Introduction

An emulsion can be defined as a heterogeneous dispersion of two immiscible liquids (*e.g.* water and lipid) wherein droplets of one phase (dispersed or internal phase) are encapsulated within another phase (continuous or external phase) in the presence of surface-active agents (*i.e.* emulsifiers) (Friberg, Larsson, & Sjoblom, 2003). Lipid oxidation and microbial contamination of the emulsion-based products are major safety hazards for human consumption (Luther et al., 2007).

Under the strategy for simultaneously controlling the aforementioned hazards, our research group conducted lipase-catalyzed esterification between lauric acid (a lipophilic antimicrobial) and erythorbic acid (a hydrophilic antioxidant) (Park, Sung, Lee, & Chang, 2011). The erythorbyl laurate (6-*O*-lauroyl-erythorbic acid), which results from the enzymatic esterification between the C-6 hydroxyl group of erythorbic acid and the carboxyl group of lauric acid, was anticipated to be an amphiphilic material with multi-functionalities. Recently, we reported the interfacial characteristics and antioxidant activity of erythorbyl laurate (Park et al., 2017). From the previous report, it was revealed that erythorbyl laurate was surface-active and showed higher foaming stability than Tween 20 and Triton X-100. Furthermore, the surfactant property of erythorbyl laurate allowed the antioxidant molecules to be concentrated at the oil–water interface where oxidation is prevalent, which led to more effective retardation of lipid oxidation in emulsions.

Lauric acid, lipophilic moiety of the erythorbyl laurate, is a medium-chain fatty acid with a strong antimicrobial activities against a wide spectrum of foodborne pathogens such as *Staphylococcus aureus*, *Listeria monocytogenes*, and *Clostridium perfringens* (Altieri, Bevilacqua, Cardillo, & Sinigaglia, 2009; Lieberman, Enig, & Preuss, 2006). It has been reported that some derivatives of lauric acid inhibit the growth of a range of microorganisms, and

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adjustments to the non-fatty acid moiety on a molecule can significantly affect its antimicrobial activity (Lieberman et al., 2006; Nobmann, Bourke, Dunne, & Henehan, 2010; Sands, Landin, Auperin, & Reinhardt, 1979). Unlike antibiotics, fatty acids and their derivatives exhibit a variety of nonspecific modes of action, and there have been no reports of microbes developing resistance to fatty acids (Kabara, 1993). The precise mode of lauric acid's action on bacteria is not completely understood. However, the bacterial cell membrane is probably the primary target for antibacterial fatty acids and their esters (Desbois & Smith, 2010; Nakatsuji et al., 2009). The amphipathic structure and detergent properties of fatty acids and their derivatives may be responsible for their antibacterial effects, possibly by interacting with bacterial cell membranes and creating transient or permanent pores (Desbois & Smith, 2010).

The primary objective of this study was to evaluate the antimicrobial activity of erythorbyl laurate. The secondary aim was to understand its mode of action against food-borne pathogens. Antibacterial efficacy was assessed by comparing minimum inhibitory concentrations (MICs), and more detailed studies based on growth curves (*e.g.* changes in the lag phase [λ] and maximum specific growth rate [μ_{max}]) were also conducted. The investigation into erythorbyl laurate's antibacterial mode of action focused on alterations in the permeability and integrity of bacterial cell membranes using crystal violet uptake and LIVE/DEAD *Bac*Light kit assays, in addition to microscopic observations. The synergistic effects of erythorbyl laurate in combination with other antimicrobial agents were also assessed to generate information that might have a practical application.

2. Materials and methods

2.1. Materials

Novozym® 435 (*i.e.* lipase from *Candida antarctica* immobilized on a macroporous acrylic resin, approximate density 0.40 g/mL) with a catalytic activity of 10,000 PLU/g (one unit PLU was defined as the amount of enzyme that synthesizes 1 μmol/min propyl laurate at 60 °C) was kindly provided by Novozymes (Bagsværd, Denmark). Erythorbic acid (≥99.0%) and lauric acid (≥99.0%) were purchased from Fluka Co. (Buchs, Switzerland) and Sigma-Aldrich Co. (St. Louis, MO, USA), respectively. High-performance liquid chromatography (HPLC)-grade acetonitrile (J. T. Baker Co., Phillipsburg, NJ, USA) was dehydrated using 4 Å molecular sieves (8–12 mesh; Sigma-Aldrich Co.) and filtered through a membrane filter (0.45 μm) prior to use. All other chemicals were of analytical grade and were used without further purification.

2.2. Preparation of erythorbyl laurate

The enzyme-catalyzed synthesis, purification, and identification of erythorbyl laurate were performed according to our previously described method (Park et al., 2017). Erythorbic acid (0.12 mmol) and lauric acid (0.60 mmol) were mixed in a screw-capped glass vial with 20 mL acetonitrile and pre-incubated at 50 °C for 30 min in an orbital shaking water bath (200 rpm). The reaction was initiated by adding 200 mg immobilized lipase to the mixture. The temperature was maintained at 50 ± 1 °C during the entire reaction. After the synthesis reaction had been terminated, erythorbyl laurate was purified by solvent-separation. Quantitative analysis was performed using the LC-2002 HPLC apparatus (JASCO Inc., Tokyo, Japan) equipped with a Spherisorb-ODS column (5 μm, 100 Å, I.D. 4.6 × 250 mm; Waters Corp., Milford, MA, USA) and Auto Flex II mass spectrometer (Bruker Daltonics, Bremen, Germany) for matrix-assisted laser desorption ionization-time of flight mass spectrometry.

2.3. Bacterial strains and culture conditions

The *in-vitro* antibacterial activity of erythorbyl laurate against 13 foodborne pathogens was evaluated. The seven gram-positive strains assessed in this study were *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus*. The six gram-negative strains were *Escherichia coli*, *Salmonella* Typhimurium, and *Pseudomonas aeruginosa*. All microorganisms were cultured in tryptic soy broth (TSB) at 37 °C for 12–18 h to yield 10^8 colony-forming units per milliliter (CFU/mL).

2.4. Susceptibility screening (spot-on-the-lawn assays)

The susceptibility of different bacteria to erythorbyl laurate was assessed using spot-on-the-lawn assays (Lewus, Kaiser, & Montville, 1991). Tryptic soy agar plates were overlaid with 5 mL tryptic soy soft agar (0.4%, w/v) that had been inoculated with 10^8 CFU/mL of an exponentially growing bacterial suspension. After solidification, 5 μL of the sample solution containing the required concentration of erythorbyl laurate in 1% dimethyl sulfoxide were spotted onto the soft agar overlay. The plates were incubated at 37 °C for 24 h and then observed for clear zones caused by growth inhibition.

2.5. Bactericidal and bacteriostatic activities

The MIC and minimum bactericidal concentration (MBC) were determined using a slightly modified broth micro-dilution method (Wiegand, Hilpert, & Hancock, 2008). Serial dilutions to generate various concentrations of erythorbyl laurate were prepared in sterile TSB to final volumes of 100 μL in Costar 3595 96-well plates (Corning Inc., Corning, NY, USA). Each well was then inoculated with 100 μL of the test organism in TSB at a final concentration of 5×10^5 CFU/mL. The MICs were defined as the lowest concentration of erythorbyl laurate at which growth was inhibited after 12 h of incubation at 37 °C (Nobmann et al., 2010). Subsequently, the MBCs of erythorbyl laurate were also determined. The MBCs were defined as the lowest concentration leading to a 99.9% decrease in viable bacteria in the subcultured well contents relative to the initial inoculum. This was assessed immediately after inoculation by subculturing the positive control well onto tryptic soy agar plates and counting the colonies produced (Pridmore, Burch, & Lees, 2011).

Increases in the λ (lag phase) and μ_{max} (maximum specific growth rate) were calculated based on data from absorbance-based broth microdilution assays using SoftMax Pro software (ver. 5.3; Molecular Devices, Sunnyvale, CA, USA). The $\Delta\lambda$ was defined as the time taken to achieve an increase in the OD_{600} of 0.10 in a culture containing the test compound minus the time taken to achieve the same OD_{600} increase in a culture without the test compound (Nobmann et al., 2010).

2.6. Preparation of erythorbyl laurate-stabilized emulsion

Oil-in-water emulsions were prepared by mixing 5% (w/v) lipid phase (soybean oil) with 95% aqueous phase (0.2% [w/v] surfactant in distilled water). To prepare the surfactant solution, the surfactant was dissolved in 2% (v/v) ethanol solution, followed by stirring for 1 h at ambient temperature to remove any traces of ethanol. A

coarse emulsion premix was prepared by homogenizing the lipid and aqueous phases together in a T-18 basic high-speed blender (IKA-Werke GmbH & Co. KG, Staufen, Germany) for 60 s at 16,000 rpm and room temperature. The droplet size of the premixed emulsions was reduced by sonication for 2 min at 210 W using a duty cycle of 0.5 s at 4 °C.

2.7. Crystal violet assay

The bacterial strains were grown up overnight at 37 °C in TSB. Cells were then harvested and washed with phosphate-buffered saline (PBS, pH 7.4). The bacterial cells were treated with erythorbyl laurate dissolved in PBS at concentration of 0.4–3.2 mM (*i.e.* 0.5–4 × MIC) for 8 h at 37 °C (Bharali, Saikia, Ray, & Konwar, 2013; Devi, Nisha, Sakthivel, & Pandian, 2010). Nisin (a typical antibacterial agent that generates pores in bacterial cell membranes, 40 mg/mL) and ampicillin (an irreversible inhibitor of the transpeptidase, 50 μg/mL) and were used as positive and negative controls, respectively. Both erythorbyl laurate-treated and untreated samples were resuspended in crystal violet solution (30 μg/mL) prepared in PBS and incubated for 30 min at 37 °C. The percentage of crystal violet dye taken up by the samples was calculated using the following equation:

$$\text{Crystal violet uptake (\%)} = \frac{\text{OD}_{590}\text{ value of the sample}}{\text{OD}_{590}\text{ value of the crystal violet solution}} \times 100$$

2.8. LIVE/DEAD BacLight bacterial viability assay

A culture of *S. aureus* cells was grown to late log phase in TSB. The bacterial culture was concentrated by centrifugation at 10,000 × g for 10 min. The supernatant was removed and the pellet washed with PBS (pH 7.4). Aliquots of the bacterial suspension were added to sterile PBS to prepare a sample of live bacteria and to 70% isopropyl alcohol to prepare a suspension of dead bacteria. Both the live and dead cell suspensions were adjusted to 2×10^7 CFU/mL. Different proportions of the live and dead cells were mixed to obtain cell suspensions containing five different ratios (100:0, 75:25, 50:50, 25:75, and 0:100%) and to construct a standard curve. For the standard curve, the green/red (G/R) ratio was plotted versus the percentage of live cells. To assess the effect of erythorbyl laurate, *S. aureus* cells from an overnight culture were washed and resuspended in PBS. Bacterial suspensions were adjusted to 2×10^7 CFU/mL, treated with erythorbyl laurate, and incubated for 30 min at 37 °C. At the end of the incubation period, the suspensions were centrifuged at 10,000 × g for 10 min and washed with PBS. A volume of 100 μL of each bacterial suspension was added to separate wells of a black 96-well microplate with a translucent base (Nalge Nunc International, Rochester, NY, USA). Preparation of the staining solution and the fluorescent intensity measurements were performed according to the manufacturer's instructions. The staining solution was prepared by mixing 3.34 mM SYTO 9 (G) and 20 mM propidium iodide (R) in equal proportions. In total, 100 μL staining solution were added to each well, and the plates were incubated at room temperature for 15 min in the dark. After the incubation period, the measurements were performed using an excitation wavelength of 485 nm and emission wavelengths of 530 nm for G and 630 nm for R. The G:R intensity ratio was calculated for each bacterial suspension to estimate the relative percentages of live and dead cells as follows:

$$\text{Ratio}_{\,G/R} = \frac{\text{Green emission fluorescence intensity}}{\text{Red emission fluorescence intensity}}$$

2.9. Transmission electron microscopy (TEM)

To prepare specimens for TEM, overnight cultures of *S. aureus* were treated with erythorbyl laurate at the MIC and 4 × MIC for 12 h at 37 °C. Untreated (negative control) and erythorbyl laurate-treated cells were harvested by centrifugation at 10,000 × g for 10 min. The pellets were fixed using modified Karnovsky's fixative containing 2% paraformaldehyde and 2% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 4 h at 4 °C and then washed with 50 mM sodium cacodylate buffer. After primary fixation, the samples were post-fixed using 1% osmium tetroxide in 50 mM sodium cacodylate buffer for 2 h at 4 °C and then washed with distilled water at room temperature. After treatment with 100% propylene oxide, the samples were dehydrated using increasing concentrations of ethanol (30, 50, 70, 80, 90, and 100%) and then infiltrated with Spurr's resin. Subsequently, the samples were dried for 24 h. Specimens were visualized by TEM (LIBRA 120; Carl Zeiss, Oberkochen, Germany) under standard conditions (Y.-H. Kim & Chung, 2011).

2.10. Fluorescence microscopy

S. aureus cells grown overnight were washed and resuspended in 0.85% NaCl. The bacterial suspensions were adjusted to 1×10^5 CFU/mL and treated with erythorbyl laurate for 30 min at 37 °C. After incubation, 3 μL dye mixture were added for each 1 mL bacterial suspension and transferred to dark conditions at room temperature. A 5 μL aliquot of the stained bacterial suspension was applied to a microscope slide with a coverslip and examined using the DE/Axio Imager A1 fluorescence microscope (Carl Zeiss) equipped with fluorescent filters for SYTO 9 (filter set 38 HE; Carl Zeiss) and propidium iodide (filter set 43 HE; Carl Zeiss).

2.11. Synergistic effects in combination with antibiotics and food preservatives

The synergistic effects of erythorbyl laurate combined with a variety of antimicrobial agents were assessed using the checkerboard test (Gutierrez, Barry-Ryan, & Bourke, 2008; Magalhães & Nitschke, 2013). The checkerboard test was applied using 96-well microplates containing serial dilutions of erythorbyl laurate together with other antimicrobial agents. The diluted erythorbyl laurate samples were prepared vertically, and the dilutions of other antimicrobial agents were prepared in horizontal rows. Serial dilutions of the different test agents were mixed in TSB. After 12 h of incubation at 37 °C, the fractional inhibitory concentration (FIC) index was calculated using the MIC of each antimicrobial agent

alone and the MIC of the combined agents. Antimicrobial interactions were categorized according to the following parameters: FIC index ≤0.5 (synergistic interaction), 0.5 < FIC index ≤ 4 (no significant interaction), and FIC index > 4 (antagonistic interaction). The FIC index (Σ FIC) was determined using the following equation:

$$\sum \text{FIC} = \frac{\text{MIC of EL in combination}}{\text{MIC of EL}} + \frac{\text{MIC of antimicrobial agents in combination}}{\text{MIC of antimicrobial agent}}$$
$$= \text{FIC}_{\text{EL}} + \text{FIC}_{\text{Antimicrobial agent}}$$

2.12. Statistical analysis

All data are expressed as means ± standard deviations from triplicate experiments. Analysis of variance (ANOVA) was performed, and differences between means were detected using Duncan's multiple range test ($p < 0.05$). All statistical analyses were performed using SPSS software (ver. 13.0; SPSS Inc., Chicago, IL, USA).

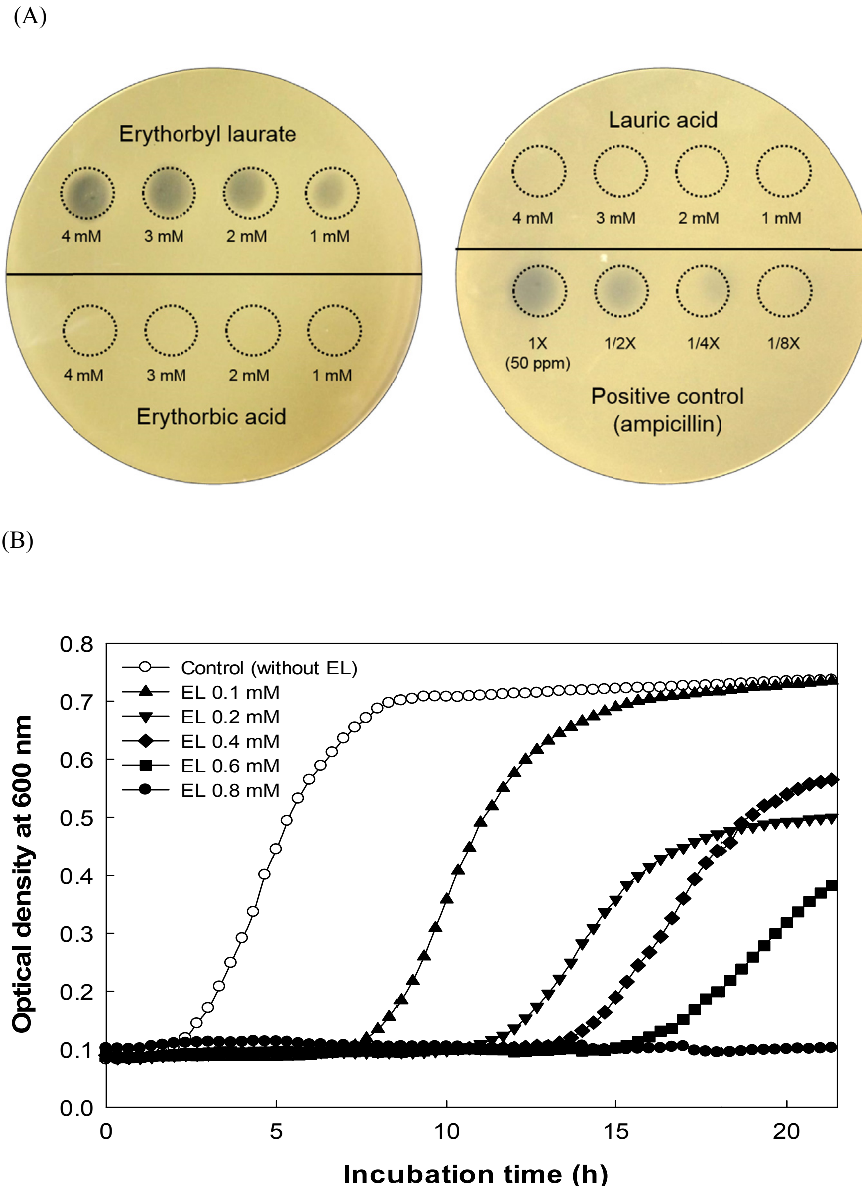

Fig. 1. Susceptibility screening using spot-on-the-lawn assays (A) and the bacteriostatic effect (B) of erythorbyl laurate on *S. aureus*.

Table 1
Effective concentrations (MICs and MBCs) of erythorbyl laurate against Gram-positive pathogens, and changes in lag time (λ) and maximum specific growth rate (μ_{max}) at the sub-MIC concentrations.

Bacterial strains	MIC (mM) [1)]	MBC (mM) [2)]	Concentration (mM) [3)]	λ (h)	μ_{max} (OD_{600}/h^{-1})
S. aureus ATCC 12692	0.88 ± 0.10	1.27 ± 0.12	0.6	4.50 ± 1.00^a	0.050 ± 0.002^a
			0.4	3.33 ± 0.58^a	0.083 ± 0.003^b
			Control [4)]	3.00 ± 0.58^a	0.123 ± 0.003^c
S. aureus ATCC 29213	0.88 ± 0.10	1.28 ± 0.27	0.6	2.83 ± 0.87^a	0.070 ± 0.001^a
			0.4	2.50 ± 0.58^a	0.092 ± 0.003^b
			Control	2.33 ± 0.50^a	0.149 ± 0.004^c
S. aureus ATCC 49444	0.48 ± 0.13	0.80 ± 0.17	0.4	9.33 ± 0.29^a	0.019 ± 0.008^a
			0.2	3.83 ± 0.58^b	0.055 ± 0.005^b
			Control	3.17 ± 2.02^b	0.090 ± 0.005^c
B. cereus ATCC 13061	0.73 ± 0.1	0.90 ± 0.00	0.4	5.33 ± 1.44^a	0.034 ± 0.012^a
			0.2	2.50 ± 1.32^{a,b}	0.078 ± 0.006^b
			Control	2.00 ± 0.58^b	0.190 ± 0.008^c
B. cereus ATCC 10876	0.65 ± 0.06	1.00 ± 0.00	0.4	4.50 ± 2.02^a	0.033 ± 0.003^a
			0.2	2.67 ± 0.00^a	0.077 ± 0.011^b
			Control	2.17 ± 0.29^a	0.137 ± 0.006^c
L. monocytogenes ATCC 7644	0.58 ± 0.05	0.65 ± 0.07	0.4	6.50 ± 0.00^a	0.011 ± 0.009^a
			0.2	4.50 ± 0.87^b	0.028 ± 0.009^a
			Control	3.00 ± 0.29^c	0.091 ± 0.012^b
L. monocytogenes ATCC 19115	0.53 ± 0.15	0.73 ± 0.12	0.4	7.50 ± 0.29^a	0.004 ± 0.003^a
			0.2	5.50 ± 0.29^b	0.026 ± 0.004^b
			Control	3.25 ± 0.71^c	0.094 ± 0.004^c

[1)] Minimum inhibitory concentration, [2)] Minimum bactericidal concentration, [3)] Sub-MIC concentrations, [4)] Without erythorbyl laurate.
[a–c] Means in the same column with different superscripts differ significantly ($P < 0.05$, Tukey HSD).

3. Results and discussion

3.1. Susceptibility screening and effective concentrations (MIC and MBC)

Susceptibility screening of erythorbyl laurate against a variety of major foodborne pathogens was performed using spot-on-the-lawn assays (Fig. 1A). Erythorbyl laurate showed antibacterial activity against gram-positive bacteria (*Staphylococcus*, *Listeria*, and *Bacillus* spp.) but no significant activity against gram-negative bacteria (*Escherichia*, *Salmonella*, and *Pseudomonas* spp.). This selective susceptibility is probably due to differences in membrane structure and composition between gram-positive and gram-negative bacteria and is consistent with the properties of lauric acid as the fatty acyl moiety of erythorbyl laurate (Bergsson, Arnfinnsson, Steingrimsson, & Thormar, 2001; Dayrit, 2015; Fischer et al., 2011). Results from the MIC and MBC assessments of gram-positive bacterial strains using the broth microdilution method are displayed in Table 1. The MICs of erythorbyl laurate against gram-positive bacteria ranged from 0.48 to 0.88 mM, and the MBCs were approximately 1.2–1.5 fold higher than the MICs, indicating that erythorbyl laurate has both bacteriostatic and bactericidal effects on gram-positive bacteria.

A detailed evaluation of the bacteriostatic effect of erythorbyl laurate was performed by analyzing the kinetics of the bacterial growth curves. Kinetic parameters for sigmoidal bacterial growth models include a period of physiological adjustment prior to exponential growth and allowing the bacterial culture to reach its maximum density during the stationary growth phase (Silva-Angulo, Zanini, Rodrigo, Rosenthal, & Martinez, 2014). The representative growth curve (Fig. 1B) shows how changes in the λ and μ_{max} were monitored at concentrations below the MIC. The growth curves for all gram-positive bacterial strains exhibited an increase in the λ accompanied by a decline in the μ_{max} as the concentration of erythorbyl laurate increased, indicating a bacteriostatic effect (Table 1). The corresponding increases in λ and decreases in μ_{max} were concentration-dependent and proportional to increases in the erythorbyl laurate concentration. Increases in λ are required to repair physicochemical damage and to synthesize the proteins and nucleic acids needed for multiplication of microorganisms (Shintani, 2006; Silva-Angulo et al., 2015).

Because one promising application of erythorbyl laurate is in emulsion-based products, the antibacterial activity of erythorbyl laurate against gram-positive pathogens was evaluated in emulsions. A 5% (w/v) soybean oil in water emulsion stabilized using 0.2% (w/v) erythorbyl laurate was prepared according to the method described in Section 2.6. A two-phase suspension with no emulsifier and a Tween 20-stabilized emulsion were used for the comparative analyses, because Tween 20 has the same hydrophobic moiety (*i.e.* lauric acid) as that of erythorbyl laurate. Within 6 h, the erythorbyl laurate-stabilized emulsion resulted in decreases of 5.72 and 5.42 log CFU/mL against *S. aureus* and *L. monocytogenes*, respectively, while no significant differences were observed for either the Tween 20-stabilized emulsion or the two-phase suspension ($p > 0.05$) (Supplementary Data S1). Therefore, erythorbyl laurate effectively inhibits food-borne pathogens in emulsions.

3.2. Effect of erythorbyl laurate on the permeability of cell membranes

The results of the susceptibility screening indicated that erythorbyl laurate exerts selective antibacterial activity against gram-positive but not gram-negative bacteria. This could be because of differences in membrane structure. Previous studies on the antibacterial mechanisms of fatty acid derivatives have indicated bacterial cell membranes as the primary target (Desbois & Smith, 2010; Nakatsuji et al., 2009). Therefore, the effect of erythorbyl laurate on cell membrane integrity was investigated. We selected *S. aureus* as a representative gram-positive bacteria and evaluated alterations in membrane permeability by measuring crystal violet uptake (Fig. 2A).

As expected, bacteria without erythorbyl laurate treatment accumulated little crystal violet (17.3%) and did not differ significantly from a negative control sample treated with ampicillin (an irreversible inhibitor of the transpeptidase) (T. S. Kim, Decker, & Lee, 2012). In contrast, crystal violet uptake (%) gradually increased with increasing concentrations of erythorbyl laurate from 0 to 1.6 mM, eventually reaching a level similar to that

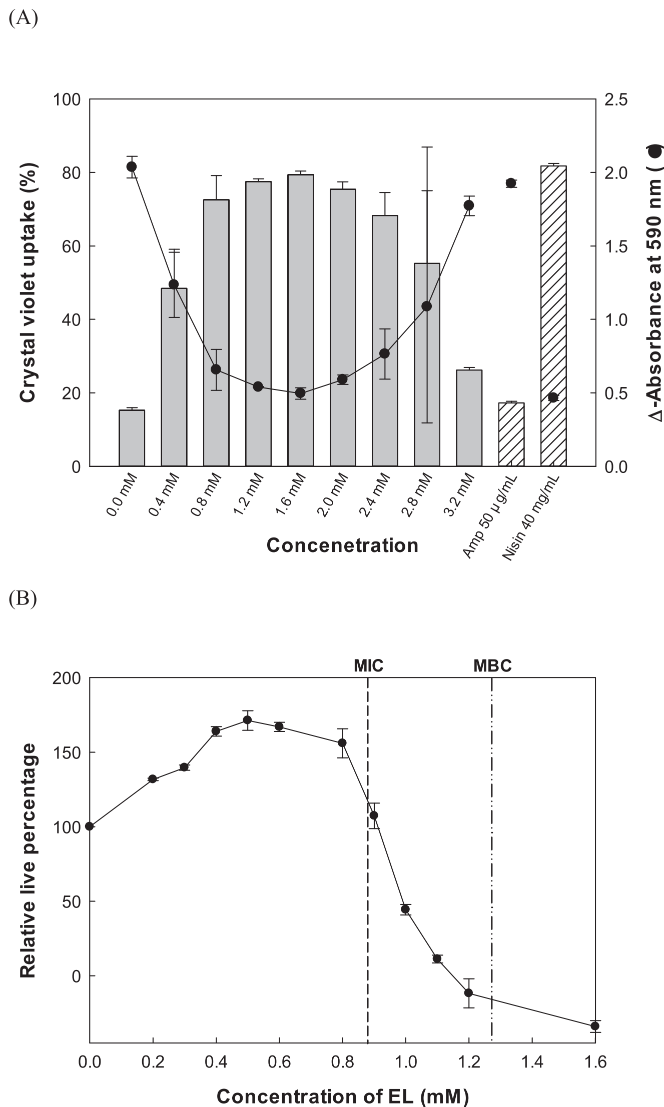

Fig. 2. Changes in *S. aureus* membrane permeability indicated by crystal violet uptake (A) and relative live cell percentages using the LIVE/DEAD *Bac*Light assay (B).

observed after treatment with 40 mg/mL nisin, an antibiotic that generates pores in bacterial cell membranes (Breukink et al., 1997). However, crystal violet uptake decreased as the concentration of erythorbyl laurate was further increased to greater than twofold the MIC. This may be due to disintegration of the cell membrane, which leads to a release of internal nucleic acids stained with crystal violet.

To monitor changes in plasma membrane integrity in real time, the commercial LIVE/DEAD *Bac*Light kit (Molecular Probes Inc., Eugene, OR, USA) was used to assess permeability of the plasma membrane using the nucleic acid-specific viability stains SYTO 9 and propidium iodide. These stains compete in the binding of bacterial nucleic acids. The green SYTO 9 stain is used to label cells with both damaged and intact membranes, whereas the red propidium iodide stain is used to label cells with damaged membranes only (Bischoff, Leathers, Price, & Manitchotpisit, 2015). Therefore, viable cells with intact plasma membranes are stained by SYTO 9, but if the permeability status changes because of damage to the plasma membrane, then SYTO 9 fluorescence is quenched and counterstained as propidium iodide enters the cell. Thus, the G/R intensity ratio is correlated with cell viability. As a result, it is easy to visualize the rapid decrease in *S. aureus* cell viability observed at the MIC and 0% cell viability at the MBC (Fig. 2B).

There was rapid decrease in the relative percentage of live cells at the approximate MIC for *S. aureus*, which decreased to 0% at the MBC. The apparent increases in the relative percentage of live cells at low concentrations of erythorbyl laurate may be related to increased cell permeability to the SYTO 9 dye (Stocks, 2004). The oscillating random Brownian motion of cell wall-attached molecules might cause stretching of the cell membrane or cell wall to produce larger pores, and the resulting increases in membrane permeability could lead to cell lysis and death (Sitohy, Mahgoub, & Osman, 2012).

3.3. *Erythorbyl laurate-induced alteration of cell membrane integrity*

To verify that erythorbyl laurate alters the permeability and integrity of cell membranes, fluorescence microscopy was performed on dual-stained (SYTO 9/propidium iodide) *S. aureus* cells. A fluorescence image of an erythorbyl laurate-free bacterial suspension (left column in Fig. 3) showed only a few red spots, indicating few stained cells with damaged membranes. However, in bacterial suspensions treated with erythorbyl laurate concentrations equivalent to 1 × and 2 × MIC (slightly greater than the MBC), both green and red fluorescence were present, resulting in yellow fluorescence (middle and right columns in Fig. 3). As the concentration of erythorbyl laurate increased, the green fluorescence intensity decreased with a concurrent increase in the red fluorescence intensity. At concentrations greater than the erythorbyl laurate MIC, the increased red and decreased green fluorescent intensities indicated that erythorbyl laurate inhibits cell growth.

In addition to the strong fluorescence microscopy evidence for erythorbyl laurate antibacterial activity, the effect of erythorbyl laurate on cell membrane integrity was evaluated by TEM. Energy-filtered (EF)-TEM was performed to reveal structural damage to *S. aureus* plasma membranes exposed to inhibitory (1 × MIC) and bactericidal (4 × MIC) concentrations of erythorbyl laurate for 12 h. A smooth continuous membrane structure was clearly observed in untreated *S. aureus* cells, highlighting the integrity of the plasma membrane (Fig. 4). In contrast, *S. aureus* cells exposed to inhibitory and bactericidal concentrations of erythorbyl laurate exhibited damaged membranes and loss of some intracellular contents. In addition, erythorbyl laurate-treated *S. aureus* cells had more disordered regions within the cytoplasm compared with untreated cells. At the MIC, membranes were uneven, and slight cytoplasmic convolutions were visible as indicated by the faint cytoplasmic membrane boundaries. Treatment with erythorbyl laurate at 4 × the MIC was enough to completely rupture *S. aureus* membranes and release cellular contents. EF-TEM images revealed that erythorbyl laurate caused dissolution of the cytoplasmic space, disruption of the smooth membrane surfaces, and cytoplasmic convolutions. The permeability of membranes and cell walls damaged by erythorbyl laurate was increased, and intracellular contents were released, consistent with the results of the crystal violet and LIVE/DEAD *Bac*Light assays. Therefore, disintegration of the cell wall and cell membrane is likely to be a major mechanism of erythorbyl laurate cytotoxicity.

3.4. *Synergistic effects of erythorbyl laurate in combination with other antibacterial agents*

The effect of combining erythorbyl laurate with other antibacterial agents was evaluated to determine the potential synergistic effects of erythorbyl laurate against *S. aureus*. When erythorbyl laurate was applied with commercial antimicrobial agents, several

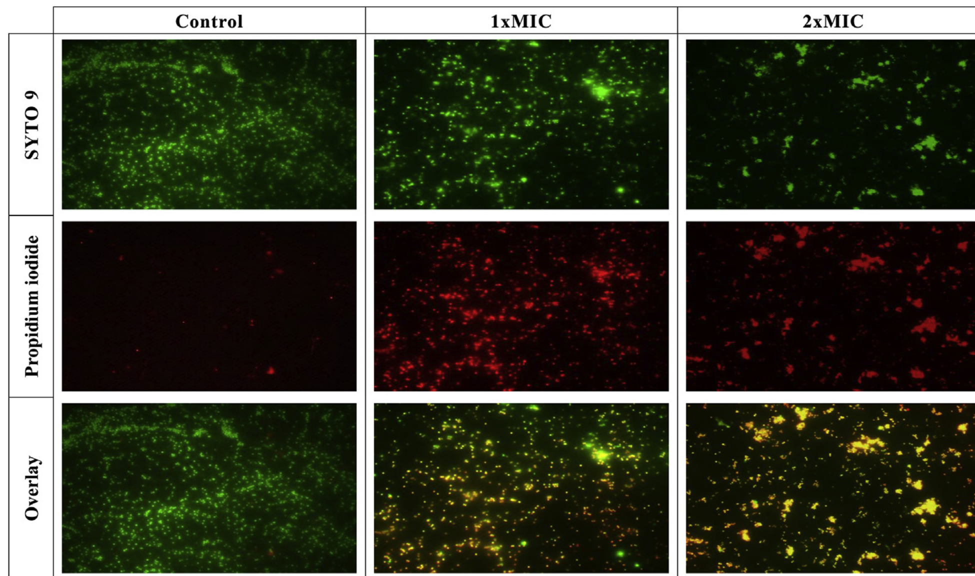

Fig. 3. Fluorescent images of *S. aureus* cells stained with SYTO 9 (green color; an indicator of whether cell membranes are damaged or intact) and propidium iodide (red color; only penetrates cells with damaged membranes). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

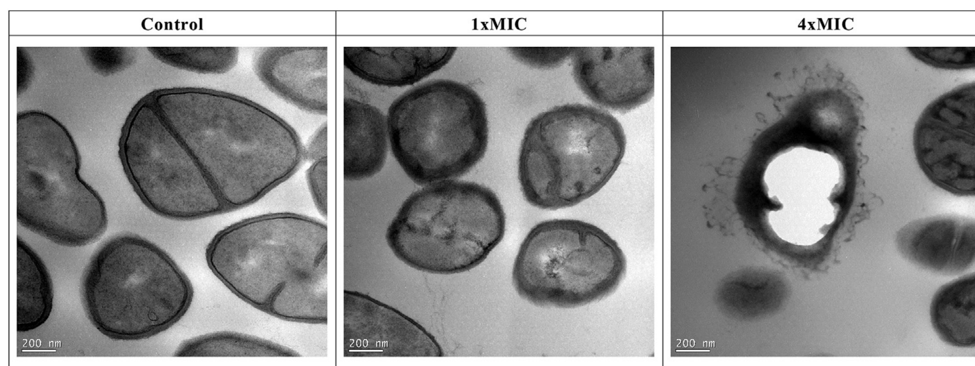

Fig. 4. Transmission electron micrographs showing alterations in membrane integrity and damage to *S. aureus* cells treated with erythorbyl laurate.

Table 2
Fractional inhibitory concentration (FIC) index of erythorbyl laurate with various antimicrobial agents against *S. aureus*.

Target mechanism	Antimicrobial agents	∑FIC	Effect
Electron transport system	Potassium sorbate	1.25	No significant interaction
Permeability of the cytoplasmic membrane	Nisin	<0.28	Synergistic interaction
Cell wall synthesis	Cephalexin	0.75	No significant interaction
	Ampicillin	0.50	Synergistic interaction
	Cloxacillin	1.25	No significant interaction
Protein synthesis	Sodium benzoate	0.75	No significant interaction
	Kanamycin	0.26	Synergistic interaction
	Erythromycin	0.27	Synergistic interaction
	Chloramphenicol	0.50	Synergistic interaction
	Streptomycin	0.50	Synergistic interaction
DNA/RNA synthesis	Nalidixic acid (DNA)	0.75	No significant interaction
	Rifampicin (RNA)	0.75	No significant interaction

treatment combinations enhanced the antibacterial activity against *S. aureus*. Synergistic studies *in-vitro* of the antibacterial agent combinations were quantified using the FIC index, and the results are displayed in Table 2. Erythorbyl laurate acted synergistically with nisin, ampicillin, kanamycin, erythromycin, chloramphenicol, and streptomycin. The synergistic effects of erythorbyl laurate with nisin, kanamycin, and erythromycin were particularly strong. Erythorbyl laurate and nicin both target the plasma membrane and strongly inhibited the growth of *S. aureus*. Synergistic effects against *S. aureus* were observed to a lesser extent between erythorbyl laurate and cephalexin, sodium benzoate, nalidixic acid, and rifampicin. The remaining antimicrobial agents evaluated showed no synergistic effects when combined with erythorbyl laurate.

One benefit of decreasing the quantities of antimicrobial agents

in widespread use is to reduce the risk of pathogens developing resistance. Because some of antibacterial agents tested in this study have been approved for clinical use, this combination strategy cannot be directly applied in food preservation. However, it is worthy of note that the erythorbyl laurate showed the synergistic activities in combination with diverse antibacterial agents that inhibit different pathways. Although not fully understood, it is reasonable to assume that the synergistic effects of erythorbyl laurate in combination is due to two possible mechanisms: [i] Because the antibacterial activity of erythorbyl laurate is primarily dependent on the formation of membrane pores and/or the disruption of cytoplasmic membranes, the resulting increase in membrane permeability might act synergistically with diverse antibacterial agents with different antibacterial mechanisms. [ii] The other is based on the hurdle technology. In general, each treatment can be used in combination with other disinfection strategies to enhance the inactivation effect (Chen & Jiang, 2014).

4. Conclusions

In this study, we investigated the antibacterial activity and mechanism of erythorbyl laurate, a promising food additive with multi-functionalities including antibacterial, antioxidant, and interfacial activities. Antibacterial susceptibility screening and MIC/MBC determination revealed that erythorbyl laurate exerted both bacteriostatic and bactericidal effects on gram-positive pathogens, including *S. aureus*, *L. monocytogenes*, and *B. cereus*. Furthermore, the results of the crystal violet uptake and LIVE/DEAD *Bac*Light assays indicated that the antibacterial mechanism of erythorbyl laurate might depend on alterations in the permeability and integrity of cell membranes. In addition, erythorbyl laurate showed synergistic effects when combined with diverse antibacterial agents, which could result from the increase in membrane permeability induced by erythorbyl laurate.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.foodcont.2017.11.008.

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