Nanoemulsions of thymol and eugenol co-emulsified by lauric arginate and lecithin

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Abstract

Lauric arginate (LAE) is a cationic surfactant with excellent antimicrobial activities. To incorporate essential oil components (EOCs) in aqueous systems, properties of EOC nanoemulsions prepared with a LAE and lecithin mixture were studied. The LAE-lecithin mixture resulted in stable translucent nanoemulsions of thymol and eugenol with spherical droplets smaller than 100 nm, contrasting with the turbid emulsions prepared with individual emulsifiers. Zeta-potential data suggested the formation of LAE-lecithin complexes probably through hydrophobic interaction. Negligible difference was observed for antimicrobial activities of nanoemulsions and LAE in tryptic soy broth. In 2% reduced fat milk, nanoemulsions showed similar antilisterial activities compared to free LAE in inhibiting *Listeria monocytogenes*, but was less effective against *Escherichia coli* O157:H7 than free LAE, which was correlated with the availability of LAE as observed in release kinetics. Therefore, mixing LAE with lecithin improved the physical properties of EOC nanoemulsions but did not improve antimicrobial activities, especially against Gram-negative bacteria.

Keywords: Lauric arginate, lecithin, nanoemulsion, essential oil, antimicrobial properties.

1. Introduction

Lauric arginate (LAE; ethyl-N^α-lauroyl-L-arginine ethyl ester monohydrochloride) is a cationic antimicrobial derived from lauric acid, arginine and ethanol (Ruckman, Rocabayera, Borzelleca, & Sandusky, 2004). LAE has been approved as a generally recognized as safe (GRAS) preservative by the United States Food and Drug Administration (USDA, 2005). LAE has very low toxicity because it is rapidly metabolized *in vivo* to lauric acid and arginine, both of which are naturally occurring dietary components (Hawkins, Rocabayera, Ruckman, Segret, & Shaw, 2009). These features make LAE a promising antimicrobial preservative to control foodborne pathogens in food systems. It inhibits a broad spectrum of foodborne pathogens (Ma, Davidson, & Zhong, 2013; Ma, Zhang, & Zhong, 2016; Porto-Fett et al., 2010) and, to date, LAE has been reported in many studies to be a highly efficient antimicrobial agent (Higueras, López-Carballo, Hernández-Muñoz, Gavara, & Rollini, 2013; Noll, Prichard, Khaykin, Sinko, & Chikindas, 2012; Saini, Barrios, Marsden, Getty, & Fung, 2013). In a recent study in our laboratories, the minimum inhibitory concentration (MIC) of LAE for inhibiting *Listeria monocytogenes* Scott A was found to be 11.8 ppm in tryptic soy broth (TSB), while the MIC for *Escherichia coli* O157:H7 ATCC 43895 or *Salmonella* Enteritidis was 23.5 ppm (Ma et al., 2013).

One problem with LAE is that, as a cationic antimicrobial, its antimicrobial activity is reduced considerably when applied in complex food matrices (Ma et al., 2013) due to binding with food components, such as anionic biopolymers (Asker, Weiss, & McClements, 2008; Bonnaud, Weiss, & McClements, 2010). For example, even at 750 ppm, LAE did not completely inhibit 6 log CFU/mL of *E. coli* O157:H7 ATCC 43895 or *S. Enteritidis* in 2% reduced fat milk after incubation at 21°C for 48h (Ma et al., 2013). Additionally, the cationic nature of LAE

causes a bitter taste at high concentrations (Zheng, 2014), which affects the acceptability of food products. Thus, strategies are needed to improve the functionality of LAE.

Some spice essential oils (EOs) or essential oil components (EOCs) have strong antimicrobial activity (Burt, 2004; Ma, Zhang, Critzer, et al., 2016; Zhang, Ma, Critzer, Davidson, & Zhong, 2015) and are promising natural antimicrobial preservatives. Like LAE, binding by proteins and lipids requires high concentrations of EOs/EOCs to obtain sufficient inhibition of foodborne pathogens in complex food matrices such as milk (Chen, Davidson, & Zhong, 2014; Ma et al., 2013). EOs/EOCs can also affect the sensory aspects and acceptability of food products (Busatta et al., 2008; Nielsen & Rios, 2000). Therefore, approaches for lowering the usage level of EOs/EOCs in foods are needed.

Preservation using antimicrobial combinations is an effective way to lower the concentration of each antimicrobial if synergistic antimicrobial effectiveness can be obtained. In our recent study, combining LAE and EO/EOC (eugenol, thymol, and cinnamon leaf oil) pre-dissolved in ethanol showed a synergistic antimicrobial effect against *L. monocytogenes* Scott A (Ma et al., 2013). Since EOs/EOCs are hydrophobic and have limited solubility in water (Chen et al., 2014), colloidal systems, such as oil-in-water nanoemulsions, are needed to incorporate EOs/EOCs in aqueous systems (Chang, McLandsborough, & McClements, 2015; Guan, Wu, & Zhong, 2016; Moghimi, Ghaderi, Rafati, Aliahmadi, & McClements, 2016). Because LAE is also an emulsifier, it can be used to prepare EO/EOC nanoemulsions (Ziani, Chang, McLandsborough, & McClements, 2011). To reduce the level of LAE as an emulsifier, another GRAS emulsifier may be used to co-emulsify EOs/EOCs. In recent studies, we have observed synergistic surface activity when hydrophobic lecithin was used in combination with water-soluble sodium caseinate, gelatin, or Tween 20 to prepare nanoemulsions or microemulsions of EOs/EOCs (Chen, Guan, &

Zhong, 2015; Xue & Zhong, 2014a, 2014b). Therefore, the objective of the present study was to prepare and characterize emulsions of eugenol or thymol using a combination of LAE and lecithin. Physical properties were studied for dimension, storage stability, zeta-potential, and morphology of emulsion droplets, as well as release kinetics of LAE. Antimicrobial activities of emulsions were characterized in TSB and 2% reduced fat milk using a Gram-positive bacterium, *L. monocytogenes* Scott A, and two Gram-negative bacteria, *E. coli* O157:H7 ATCC43895 and *S. Enteritidis*.

2. Materials and methods

2.1. Materials

LAE was provided by Vedeqsa Inc. (New York, NY). The commercial product Mirenat-TT contained 15.5%w/w LAE, with other components being propylene glycol and polysorbate. Eugenol (98% purity) and thymol ($\geq 99\%$ purity) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Soy lecithin (major component being phosphatidylcholine) was from Thermo Fisher Scientific Inc. (Waltham, MA). Chemicals were used without further purification. Simple Truth[®] 2% ultra-pasteurized reduced fat milk with a fat content of 2.08% w/v and a protein content of 3.33% w/v (Kroger Co., Cincinnati, OH) was bought from a local store.

2.2. Bacterial culture

L. monocytogenes Scott A, *E. coli* O157:H7 ATCC43895, and *S. Enteritidis* were from the culture collection of Department of Food Science and Technology at the University of Tennessee in Knoxville. All strains were stored in sterile 20% glycerol at -20°C and transferred at least 2 times in TSB with an interval of 24h before use. *L. monocytogenes* was incubated at 32°C, while *E. coli* O157:H7 and *S. Enteritidis* were incubated at 37°C.

2.3.Preparation of nanoemulsions

Lecithin was mixed at 1% w/w in deionized (DI) water, followed by adding 3-7%w/w Mirenat-TT (corresponding to 0.47-1.09% w/w LAE) and 1%w/w eugenol. The mixture was then homogenized at 15,000rpm for 6min using a T25 digital ULTRA TURRAX[®] homogenizer (IKA[®] Works, Inc., Wilmington, NC). Absorbance at 600nm of emulsions was measured using a UV-Vis spectrophotometer (Evolution 201, Thermo Scientific, Waltham, MA). The optimized conditions identified for eugenol were then used to prepare the nanoemulsion of thymol. **Treatments with both LAE and lecithin were prepared in triplicate, while those with LAE only were prepared in duplicate.**

2.4.Dimension and stability of emulsion droplets

The hydrodynamic diameter of nanoemulsions was measured **usinga model Delsa™ Nano C particle size/zeta-potential analyzer (Beckman Coulter, Atlanta, GA)**during 30-daystorage at room temperature (21°C). Samples were diluted in deionized(DI) water before measurement. Three nanoemulsion replicates were studied.

2.5. Atomic force microscopy (AFM)

The morphology of nanoemulsion droplets was studied usingAFM. Nanoemulsions were diluted 1,000 times in DI water. Ten microliter of the diluted sample was spread on a freshly cleaved mica sheet and mounted on a sample holder (Bruker Corp., Santa Barbara, CA). After about 2-h drying, samples were scanned in the tapping mode with a Multimode VIII microscope(Bruker AXS, Billerica, MA, USA). Topography images scanned at a dimension of 1.0×1.0 μm were collected.

2.6. Zeta-potential measurement

The zeta-potential of LAE, lecithin, LAE and lecithin mixture, and eugenol nanoemulsions prepared with LAE and lecithin were measured at 25°C (model Nano-ZS Zetasizer, Malvern Instruments Ltd, Worcestershire, UK). Nanoemulsions were diluted in DI water and adjusted to pH 4.0-7.0 using 1.0 M HCl or NaOH before measurement. Three measurements with 3 runs each were done for each sample.

2.7. Release kinetics of LAE

Release kinetics of LAE from nanoemulsions was studied by dialysis against DI water at room temperature (21°C). Regenerated cellulose dialysis tubing with a molecular weight cut-off of 3,500 Da (Thermo Fisher Scientific Inc., Waltham, MA) was loaded with 5 mL nanoemulsions or a 6000 ppm LAE solution that was identical to the LAE concentration of the nanoemulsion. The sealed tubes were placed in beakers containing 200 mL DI water that was mixed on a stir plate at 300 rpm. 20 mL of solution outside the dialysis tubing was withdrawn after 0, 1, 2, 4, 8, 24, 48, 72, 96 h, and 20 mL of fresh DI water was added to the beakers to maintain the volume at each sampling. LAE concentration in the sample withdrawn was quantified with HPLC (Higueras et al., 2013). Briefly, the reversed-phase HPLC system (Agilent 1200 series; Agilent Technologies, Waldbronn, Germany) was equipped with a UV detector (204.16 nm). A Zorbax Eclipse Plus C18 HPLC column (4.6 × 150 mm, 5 µm; Agilent, Palo Alto, CA) protected by a Zorbax Eclipse Plus C18 guard column (4.6 × 12.5 mm, 5 µm) was used. The sample injection volume was 10 µL and the mobile phase with equal volumes of acetonitrile and water acidified with 0.1% trifluoroacetic acid was run at 1.0 mL/min. The cumulatively released LAE was calculated using the following equation (Xiao & Zhong, 2011):

$$R_{t_i}(\%) = \frac{\sum_{n=1}^{i-1} a_n \times 20 + a_i \times 200}{A \times 5} \times 100\%$$

where R_{ti} is the cumulatively released LAE at time t_i , a_i is the concentration of LAE outside the dialysis tube at time t_i , and A is the original concentration of LAE in the dialysis tube. All experiments were repeated in triplicate.

2.8. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) in tryptic soy broth

MICs and MBCs of antimicrobials were determined by a microbroth dilution method (Barry, 1976). In addition to nanoemulsions, samples with LAE only, lecithin only, or both at concentrations identical to nanoemulsions were tested as controls. Nanoemulsions and the controls were diluted to various concentrations in TSB as working solutions. The culture was diluted to ca. 10^6 CFU/mL bacteria using TSB. Then 120 μ L of a bacteria culture and 120 μ L of an antimicrobial working solution were added into wells of sterile microtiter plates. After 24 h incubation at 32°C for *L. monocytogenes* or 37°C for *E. coli* O157:H7 and *S. Enteritidis*, plates were observed for growth of bacteria by visual inspection. MICs were defined as the lowest antimicrobial concentration inhibiting bacteria growth. MBCs were defined as the lowest antimicrobial concentration corresponding to at least 3 log reduction of viable cells by spreading the negative cells in MIC tests on tryptic soy agar (TSA) plates and incubating for 48 h at 32°C (for *L. monocytogenes*) or 37°C (for *E. coli* O157:H7 and *S. Enteritidis*) (Barry, 1976). For nanoemulsions, the MICs and MBCs were reported in LAE concentrations only to compare with free LAE treatments. Experiments were repeated once with 3 replications each time.

2.9. Microbial growth kinetics in 2% reduced fat milk

Free LAE, a mixture of LAE and lecithin, or nanoemulsions were added at an LAE overall concentration of 750 ppm in the 2% reduced fat milk. One milliliter of cultures containing 10^7 CFU/mL *L. monocytogenes* Scott A or *E. coli* O157:H7 ATCC 43895 were added to 9 mL of

milk to achieve a final population of ca. 10^6 CFU/mL. The mixtures were incubated at room temperature (21°C) for up to 120h. Viable cells were enumerated after incubation for 0, 4, 8, 24, 48, 72 and 120h by surface plating on TSA plates and incubating at the appropriate optimum temperature for each bacterium for 24 h. Experiments were done in triplicate.

2.10. Statistical analysis

Data were analyzed with ANOVA Tukey's test using SPSS 20 (IBM, Armonk, NY) at a significance level of 5%.

3. Results

3.1. Conditions of preparing emulsions

The appearance and absorbance at 600 nm of emulsions with 1%w/w eugenol emulsified by 0.47-1.09% w/w LAE with and without 1%w/w lecithin are shown in Fig. 1. Emulsions prepared with combinations of LAE and lecithin had much lower absorbance values than those emulsified by LAE alone. The emulsion prepared by 1% w/w lecithin alone was the most turbid.

Translucent emulsions were obtained using 1%w/w lecithin and 0.78-1.09% w/w LAE, and increasing the concentration of LAE to 1.09% w/w did not further reduce the absorbance of the emulsion. Thus, the combination of 0.93% w/w LAE and 1% w/w lecithin was chosen to prepare emulsions for further study.

3.2. Droplet structure and zeta-potential of nanoemulsions

The hydrodynamic diameters of nanoemulsions during 30-day storage are shown in Fig. 2A. The hydrodynamic diameter was around 55 and 75 nm for eugenol and thymol nanoemulsions, respectively, and remained stable for 30 days at room temperature (21°C). Particle size

distributions (Fig. 2B) of the nanoemulsions before or after 30-day storage showed only one sharp peak, which suggested the nanoemulsions were stable after one month. The AFM morphology of nanoemulsion droplets is shown in Fig. 2C and D. Both eugenol and thymol nanoemulsions had mostly spherical particles. The average diameter estimated over 50 particles of eugenol and thymol nanoemulsions was about 90 and 100nm, respectively, which was about 30nm larger than the hydrodynamic diameter (Fig. 2A). This can result from the drying process during sample preparation that caused flattening of the droplets with volatile EOCs.

The zeta-potentials of LAE, lecithin, LAE-lecithin mixture, and eugenol nanoemulsion emulsified with LAE-lecithin at pH 4.0-7.0 are shown in Fig. 3. Lecithin had a highly negative zeta-potential at pH 4.0-7.0, and the decrease from pH 4.0 to 7.0 was significant ($p < 0.05$). No significant difference ($p > 0.05$) in positive zeta potential of LAE, the mixture of LAE and lecithin, and eugenol nanoemulsion was found at pH 4.0-7.0.

3.3. Release kinetics of LAE

Release kinetics of LAE from nanoemulsions is shown in Fig. 4. A rapid release of LAE from the dialysis tube to bulk water was observed in the first 8h for nanoemulsions and free LAE. The cumulative release of LAE at 8 h reached 72%, 58%, and 55% for free LAE, eugenol nanoemulsion, and thymol nanoemulsion, respectively. After 8h, the release of LAE was slower in all samples. Overall, the free LAE solution passed through the dialysis tube more rapidly and to a greater extent under the conditions studied.

3.4. MICs and MBCs in tryptic soy broth

No inhibition of the test bacteria by lecithin was observed, indicating MICs greater than 1,500ppm (Table 1). The mixture of LAE and lecithin, LAE alone, and nanoemulsions had the same MICs of 11.8ppm LAE against *L. monocytogenes* Scott A and *E. coli*

O157:H7 ATCC43895 and 23.5ppm against *S. Enteritidis*. When LAE was used alone, the MBC was 11.8ppm for *L. monocytogenes* and *E. coli* O157:H7 and was 23.5ppm for *S. Enteritidis*. The MBCs against *E. coli* O157:H7 and *S. Enteritidis* were the same (in LAE concentration) after mixing LAE with lecithin or preparing nanoemulsions. However, the MBCs of the mixture and nanoemulsions increased to 23.5ppm (one dilution higher) when tested against *L. monocytogenes*.

3.5. Microbial growth kinetics in 2% reduced fat milk

Growth curves of Gram-positive *L. monocytogenes* Scott A and Gram-negative *E. coli* O157:H7 ATCC43895 in 2% reduced fat milk at 21°C treated by antimicrobials at an overall LAE concentration of 750ppm are shown in Fig. 5. For *L. monocytogenes*, a continuous reduction of viable cells was observed for nanoemulsion and LAE only treatments, reaching below the detection limit (1.0 log CFU/mL) after 24 h. The reduction of *L. monocytogenes* by the LAE-lecithin mixture was the slowest, and viable cells were still detected after 72 h. Except for the untreated control, a decrease of *E. coli* O157: H7 population was observed for all treatments in the first 8 h, with the eugenol nanoemulsion treatment demonstrating the least effectiveness. After 8 h, recovery of *E. coli* O157: H7 was observed for all treatments except LAE alone. The relative effectiveness in increasing order was eugenol nanoemulsion < thymol nanoemulsion < LAE-lecithin mixture < LAE alone. This group of studies demonstrated the negative effect of lecithin on the antimicrobial activity of LAE.

4. Discussion

Findings from this work showed the improved emulsification properties for EOCs when LAE and lecithin were used in combination than either one used alone (Fig. 1). LAE has a high water solubility (>247g/kg at 20°C) (EFSA, 2007). Lecithin is a natural zwitterionic surfactant

consisting of various phospholipids (Salve, Fernandez, Lopez, & Jato, 1998), which results in a hydrophile-lipophile-balance (HLB) value of about 9.2-9.5 (Kunieda & Ohyama, 1990) and an overall lipophilic property. As reported in many studies, surfactants with a proper HLB value are required to form stable emulsions (Peng, Liu, Kwan, & Huang, 2010; Sagitani, 1981). Thus, the mixture of LAE with a probably high HLB value and lecithin with a low HLB value may favor the formation of EOC nanoemulsions. Based on zeta-potential data (Fig. 3), the mixture of overall anionic lecithin and cationic LAE has a similar zeta-potential as LAE, which indicates the two surfactants form complexes with the surface being predominantly hydrophilic LAE (Fig. 3B). The complex can be formed through electrostatic attraction between opposite charges of LAE and lecithin or hydrophobic attraction. Similar zeta potentials of LAE and LAE-lecithin mixture suggest hydrophobic attraction is the major mechanism. Complexes were also previously found to favor the preparation of EOC nanoemulsions when lecithin and gelatin (Xue & Zhong, 2014a) and other surfactants (Gullapalli & Sheth, 1999; Porras, Solans, Gonzalez, & Gutierrez, 2008) were used in combination.

No creaming or precipitation of nanoemulsions prepared with 1% w/w EOC, 0.93% w/w LAE, and 1% w/w lecithin was observed during 30-day storage. The stable hydrodynamic diameters and particle size distributions of nanoemulsions (Fig. 2A and B) suggested the negligible Ostwald ripening and coalescence at the studied conditions. The high magnitude of positive zeta-potential (Fig. 3) provides strong electrostatic repulsion to prevent the aggregation and thus coalescence of emulsion droplets. As discussed previously, LAE and lecithin form complexes that have similar zeta-potential at pH 4.0-7.0 as the eugenol nanoemulsion, which suggests the adsorption of complexes on droplets and the lipophilic lecithin being in contact with the oil phase (Jain, Valvi, Swarnakar, & Thanki, 2012), as illustrated in Fig. 3B. The complexes

on droplet surfaces may be effective in preventing droplet dimension changes due to Ostwald ripening.

The formation of LAE-lecithin complexes likely reduced the inhibition by LAE of *E. coli* O157: H7 and *L. monocytogenes* in milk (Fig. 5). Milk was used because the binding between proteins and/or fats and antimicrobials is significant and can indicate the influence of food components on antimicrobial activity of compounds. LAE is an arginine-based cationic antimicrobial that inhibits microorganisms by first binding with negatively charged bacteria surfaces (Castillo et al., 2004; Pattanayaiying, Aran, & Cutter, 2014). Binding with lecithin reduced the amount of free LAE thus reducing the availability to interact with the bacteria and the lowered antimicrobial activity (Fig. 5). In contrast, no significant difference was found for inhibition of *L. monocytogenes* with the same concentrations of free LAE and nanoemulsion LAE in milk (Fig. 5B). Because, as discussed previously, while complexing of LAE with lecithin reduced antimicrobial activity, similar activity of free and nanoemulsion LAE likely resulted from the complementary effect of the synergistic activity of LAE and eugenol or thymol inhibiting *L. monocytogenes* (Ma et al., 2013). Conversely, the antagonistic effects of LAE-EO/EOC combinations against *E. coli* O157:H7 found in our previous study (Ma et al., 2013) together with the effect of lecithin-LAE complex formation resulted in the most rapid recovery by the Gram-negative bacteria in milk treated with the nanoemulsion (Fig. 5).

No differences were detected in the MICs of free LAE, LAE-lecithin mixture, and nanoemulsions for the three tested bacteria (Table 1). There was also no difference in the MBCs of the above antimicrobials when tested for Gram-negative bacteria (Table 1). The contradiction between MICs/MBCs and growth in 2% reduced fat milk can be the result of several factors. The end-point assay to determine MIC and MBC is done at a single time, 24 h. No kinetics is

determined in the end-point test so the level of growth or inactivation is unknown. Thus, MICs/MBCs can only give a limited picture of the antimicrobial efficacy and are therefore must be used together with dynamic assay such as growth curves to comprehensively analyze antimicrobial properties. Additionally, the two assays were conducted at different temperatures and different media. As presented previously, 2% reduced fat milk has proteins and fat globules that can bind with both LAE and EOCs, which impacts the mass transfer and distribution of antimicrobials among different phases (oil droplets, continuous water phase, proteins, fat globules, and bacteria) in milk.

The release kinetics of LAE from nanoemulsions (Fig. 4) was in good agreement with the growth kinetics of *E.coli* O157: H7 or *L. monocytogenes* in milk (Fig. 5). The high release rate of LAE in the first 8 h corresponded to the rapid reduction of *E.coli* O157: H7 and *L. monocytogenes*. The lesser increases of LAE release during longer time points agreed with less reduction of *L. monocytogenes* or recovery of *E.coli* O157: H7 treated by nanoemulsions. For free LAE, it was not bound by lecithin and hydrophobic EOCs and therefore had the greatest activity against *E.coli* O157: H7. However, even 750 ppm LAE was insufficient to completely inhibit *E.coli* O157:H7 which gradually recovered over 120 h.

While lecithin was observed to reduce the antimicrobial activity of LAE, it effectively enhanced the ability of LAE to emulsify EOCs and stabilize nanoemulsions (Figs. 1 and 2). Previously, Ostwald ripening was observed for EO nanoemulsions prepared with Tween 80, and non-volatile lipids such as medium chain triglycerides (MCTs) and corn oil are needed to inhibit Ostwald ripening (Chang, McLandsborough, & McClements, 2012, 2015). The incorporation of these lipids in nanoemulsions however greatly reduced antimicrobial activities of EOs. For example, for thyme oil-in-water nanoemulsions prepared with Tween 80, >50% w/w of MCT

or >60% w/w of corn oil in the oil phase was needed to inhibit Ostwald ripening(Chang, McLandsborough, & McClements, 2012).The minimum amount of thyme oil required to inhibit yeast strain *Zygosaccharomyces bailii*(ca.10⁴ CFU/mL) increased from 375 to >6000 µg/mL in malt extract broth when the concentration of corn oil in the lipid phase increased from 60% to 90% w/w (Chang, McLandsborough, & McClements, 2012). In another study, at least 30% w/w of corn oil in the lipid phase was needed to stabilize thyme oil nanoemulsionswith 10% w/w lipid phase prepared with 0.1% w/w LAE and 0.9% w/w Tween 80 using high pressure homogenization (Chang, McLandsborough, & McClements, 2015).The negative effect of corn oil on the antimicrobial activity of nanoemulsions was also observed, with the MIC against *Z. bailii* in broth media increasing by2 times in thyme oil concentration and more than 4 times in LAE concentration when the corn oil concentration in the oil phase increased from 30% to 70% w/w (Chang, McLandsborough, & McClements, 2015). In contrast, lecithin had insignificant effect on MICs in the present study (Table 1). Therefore,lecithin may be more suitable than Tween 80 as a co-emulsifier of LAE to prepare stable GRAS nanoemulsions of EOs/EOCs without adding a ripening inhibitor to preserve the activities of antimicrobials.

5. Conclusions

In the present study, nanoemulsions of EOCs were successfully preparedwith positively-charged LAE and overall negatively-charged lecithin. LAE and lecithin formed complexes that improved the ability to emulsify EOCs and stabilize emulsion droplets during storage. Complex formation reduced the availability of LAE and the antimicrobial activity in milk.Similar antilisterialactivities of nanoemulsions and free LAE were observed in milk. Conversely, compared to free LAE, much reduced inhibition of Gram-negative *E.coli* O157:H7 in milk by

329 nanoemulsions was detected. Overall, the combination of LAE and lecithin provided an effective
330 approach to nano-emulsify EO_Cs for incorporation in food products, especially those requiring
331 optical transparency. However, it did not improve the activity of LAE and EO_Cs to effectively
332 inhibit Gram-negative pathogens like *E. coli* O157:H7.

333

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Figure and Table captions

Table 1. Minimum inhibitory concentration (MICs, in LAE concentration, ppm) and minimum bactericidal concentrations (MBCs, in LAE concentration, ppm) of antimicrobials against *Listeria monocytogenes* at 32 °C and *Escherichia coli* O157:H7 and *Salmonella* Enteritidis at 37 °C in tryptic soy broth.

Fig. 1. Appearance of emulsions with 1% w/w eugenol emulsified by (A) 0.47-1.09% w/w lauric arginate (LAE), (B) 0.47-1.09% w/w LAE with 1% w/w lecithin, or (C) 1% w/w lecithin alone, and (D) absorbance of eugenol emulsions at 600 nm ($OD_{600\text{ nm}}$) prepared by LAE with and without 1% w/w lecithin.

Fig. 2. Hydrodynamic diameter of thymol and eugenol nanoemulsions during storage at 21 °C (A), particle size distributions of the nanoemulsions before and after 30-day storage (B), and AFM topography images of fresh nanoemulsions prepared with eugenol (C) or thymol (D). Nanoemulsions were prepared with 1% w/w EOC, 0.93% w/w LAE, and 1% w/w lecithin. Error bars are standard deviations (n=6).

Fig. 3. (A) Zeta-potential of LAE, lecithin, LAE-lecithin mixture, and a nanoemulsion with 1% w/w eugenol emulsified by 0.93% w/w LAE and 1% w/w lecithin at pH 4.0-7.0 and 25 °C. Error bars are standard deviations (n=9). (B) Schematic illustration of droplet structure co-emulsified by lauric arginate (in gray) and lecithin (in orange).

Fig. 4. Kinetics of LAE releasing from a LAE solution or nanoemulsions contained in a dialysis tubing to bulk water at 21 °C. Nanoemulsions were prepared with 1% w/w EOC, 0.93% w/w LAE, and 1% w/w lecithin. Error bars are standard deviations (n=3).

Fig. 5. Growth curves of *L. monocytogenes* Scott A (A) and *E. coli* O157:H7 ATCC 43895 (B) in 2% reduced fat milk at 21 °C after treatment by antimicrobials containing 750 ppm lauric arginate

475 (LAE). The LAE+lecithin mixture had same LAE and lecithin concentrations as in
476 nanoemulsions. Error bars are standard deviations ($n = 3$).

477 Table 1. Minimum inhibitory concentration (MICs, in LAE concentration, ppm) and minimum
 478 bactericidal concentrations (MBCs, in LAE concentration, ppm) of antimicrobials against *Listeria*
 479 *monocytogenes* at 32 °C and *Escherichia coli* O157:H7 and *Salmonella* Enteritidis at 37 °C in
 480 tryptic soy broth.

	Bacteria	Lecithin	LAE	LAE+lecithin	Nanoemulsion of eugenol*	Nanoemulsion of thymol*
MICs	<i>L. monocytogenes</i>	>1500	11.8	11.8	11.8	11.8
	Scott A					
	<i>E. coli</i> O157:H7	>1500	11.8	11.8	11.8	11.8
	ATCC43895					
	<i>S. Enteritidis</i>	>1500	23.5	23.5	23.5	23.5
MBCs	<i>L. monocytogenes</i>	>1500	11.8	23.5	23.5	23.5
	ScottA					
	<i>E. coli</i> O157:H7	>1500	11.8	11.8	11.8	11.8
	ATCC43895					
	<i>S. Enteritidis</i>	>1500	23.5	23.5	23.5	23.5

481 *Nanoemulsions were prepared with 1%w/w eugenol or thymol and 0.93% w/w LAE and 1% w/w
 482 lecithin.

483

Fig.1. Appearance of emulsions with 1% w/w eugenol emulsified by (A) 0.47-1.09% w/w lauric arginate (LAE), (B) 0.47-1.09% w/w LAE with 1% w/w lecithin, or (C) 1% w/w lecithin alone, and (D) absorbance of eugenol nanoemulsions at 600 nm (OD_{600 nm}) prepared by LAE with and without 1% w/w lecithin ($n \geq 2$).

Fig.2. Hydrodynamic diameter of thymol and eugenol nanoemulsions during storage at 21 °C (A), particle size distributions of the nanoemulsions before and after 30-day storage (B), and AFM topography images of fresh nanoemulsions prepared with eugenol (C) or thymol (D).

497 Nanoemulsions were prepared with 1% w/w EOC, 0.93% w/w LAE, and 1% w/w lecithin. Error
498 bars are standard deviations (n=6).

499

500

(A)

501

502

(B)

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504 w/w eugenol emulsified by 0.93% w/w LAE and 1% w/w lecithin at pH 4.0-7.0 and 25°C. Error
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