Physical and antimicrobial properties of cinnamon bark oil co-nanoemulsified by lauric arginate and Tween 80

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Abstract

Lauric arginate (LAE) is a water-soluble cationic surfactant which has antimicrobial activity against a broad spectrum of foodborne pathogens. Some spice essential oils are effective lipophilic antimicrobials. Combining both antimicrobials may reduce their usage levels and possible negative sensory impacts when applied in complex food matrices. The objective of this study was to combine a nonionic surfactant (Tween 80) with LAE to form stable nanoemulsions with cinnamon bark essential oil (CBO) and to characterize the antimicrobial activity of these nanoemulsions. CBO was homogenized at 1% w/w in the aqueous phase with 3% w/w Tween 80 and 0.05-0.375% w/w LAE, followed by heating at 90 °C for 30 min to obtain final emulsions. With 0.125% and higher LAE, transparent emulsions with ~100 nm in hydrodynamic diameter were observed to be stable during 30-day storage at 21°C. Antimicrobial activities of the nanoemulsion prepared with Tween 80 and 0.375% w/w LAE were studied. The respective minimum inhibitory concentrations (MICs) of the nanoemulsion in tryptic soy broth (TSB) were 12, 7, and 8 ppm LAE for *Salmonella* Enteritidis, *Escherichia coli* O157:H7, and *Listeria monocytogenes*, while those of free LAE were 11, 6, and 6 ppm, respectively. MICs of CBO were 400 ppm for the tested bacteria and Tween 80 at 6% w/w did not show inhibitory effect. Growth kinetics of the bacteria in TSB treated with the nanoemulsion or individual components at concentrations corresponding to the MICs of free LAE showed that binding among the LAE and Tween 80 and CBO components resulted in the antibacterial activity of nanoemulsion being lower than same concentrations of free LAE and CBO. Conversely, little difference was observed for the individual antimicrobials and the nanoemulsion in 2% reduced fat milk, and 750 ppm LAE and 2000 ppm CBO were observed to be the dominant antimicrobial against Gram-positive and Gram-negative bacteria, respectively. The growth of *L. monocytogenes* in 2% reduced fat milk at 4°C was not observed when treated by the nanoemulsion corresponding to 187.5 ppm

43 LAE and 500 ppm CBO. Therefore, stable and transparent nanoemulsions of EOs can be
44 prepared with the combination of LAE and Tween 80 without compromising antimicrobial
45 activities.

46 **Keywords:** Lauric arginate, cinnamon bark oil, polysorbate 80, nanoemulsion, antimicrobial
47 activity

48

1 Introduction

A big challenge for the food industry is to ensure the microbiological safety and quality of food products. Essential oils (EOs) are volatile and aromatic oily liquids obtained from plants such as thyme, basil, oregano and cinnamon (Oussalah, Caillet, Saucier, & Lacroix, 2007). They are gaining interest for their potential use as preservatives because they are generally recognized as safe (GRAS), have activities against various foodborne spoilage and pathogenic microorganisms, and are widely accepted by consumers (Burt, 2004; Elgayyar, Draughon, Golden, & Mount, 2001). Emulsions are used to incorporate EOs in aqueous food matrices, and nanoemulsions, with droplets smaller than 100 nm in diameter, are needed in applications requiring optical transparency. The small dimension of droplets can also reduce destabilization mechanisms such as gravitational separation and aggregation.

Nanoemulsions can be prepared by high energy or low energy methods, with the former applying intense mechanical energy to break up droplets in unit operations, such as high pressure or high shear homogenization, and microfluidization (Rao & McClements, 2010; Solè, Maestro, González, Solans, & Gutiérrez, 2006). To overcome some of the drawbacks of the high energy methods, low energy approaches such as the phase inversion temperature (PIT) method have been studied (Forgiarini, Esquena, González, & Solans, 2001). The PIT method is usually based on temperature-dependent molecular geometry of nonionic surfactants (Shinoda & Arai, 1964). When heated to above the PIT, surfactants with good affinity for water and oil mixtures form bicontinuous or lamellar structures because of the ultralow interfacial tension (Rao & McClements, 2010). After rapid cooling through the PIT with continuous stirring, stable nanoemulsions can be formed as a result of redistribution of surfactants between water and oil phases (Roger, Cabane, & Olsson, 2010). The coalescence of droplets after nanoemulsion formation is one of the major problems associated with the PIT method (Izquierdo et al., 2002). To improve the kinetic stability of nanoemulsions

formed by the PIT method, a nonionic surfactant, polysorbate 80 (Tween 80), or an anionic surfactant, sodium dodecyl sulfate (SDS), was used in combination with polyoxyethylene-(4)-lauryl ether (Brij[®] 30) to change the optimum curvature of the oil/water interface and to increase repulsive colloidal interactions between the droplets (Rao & McClements, 2010). However, droplet growth due to Ostwald ripening still can occur because EO components have some water-solubility (Chen, Davidson, & Zhong, 2014). Physically, Ostwald ripening effect is due to the higher solubility of lipophilic compounds in smaller particles, which results in molecules dissolving out of smaller droplets to join bigger droplets, causing the gradual reduction and eventual disappearance of smaller droplets and the continual growth of bigger droplets (Capek, 2004).

Although nanoemulsions have been reported to enhance antimicrobial activity of EOs by increasing their solubility in water (Donsì, Annunziata, Vincensi, & Ferrari, 2012), opposite trends have also been observed. For example, the activity against *Listeria innocua* was lower after carvacrol was emulsified by Tween 80 as smaller droplets (Terjung, Löffler, Gibis, Hinrichs, & Weiss, 2012). It was determined that the concentration of carvacrol in the aqueous phase decreased with a decrease in the droplet dimension and carvacrol was preferentially located at the oil-water interface. The binding between EOs and surfactants used in emulsification therefore may reduce the availability of EOs and subsequently antimicrobial activity.

It is also known that EOs used as antimicrobials have strong flavors which are often not compatible with foods for which they might be applied. Increasing activity is one method for overcoming that problem and the other is to use other antimicrobials in combination with EOs to reduce their concentration required to inhibit microbial growth. For this reason, lauric arginate (N^α-lauroyl-L-arginine ethyl ester monohydrochloride, LAE) is particularly interesting because it is a cationic surfactant and is GRAS for use as an antimicrobial

preservative in certain food applications (Bakal & Diaz, 2005; USDA, 2005). Recently, synergistic antimicrobial activity against *Listeria monocytogenes* was observed for the combinations of LAE and cinnamon leaf oil or eugenol, while the activity was additive for the combination of LAE and thymol (Ma, Davidson, & Zhong, 2013). Conversely, an antagonistic effect was found for the same combinations against *Escherichia coli* O157:H7 and *Salmonella* Enteritidis.

Utilization of LAE as an antimicrobial is not without its challenges. Anionic compounds in food matrices, such as proteins in milk, can bind with cationic LAE and greatly increase the concentration required to achieve similar inhibitions in microbial growth media, such as tryptic soy broth (TSB) (Ma et al., 2013). Binding between LAE and anionic biopolymers (mucin) in the mouth also leads to a bitter taste (Bonnaud, Weiss, & McClements, 2010). LAE was observed to form mixed micelles with nonionic polyethylene glycol sorbitan monolaurate (Tween 20), and the reduction of zeta-potential from +37 to +6 mV was found to potentially reduce the bitter taste of LAE (Asker, Weiss, & McClements, 2011). Recently, a combination of 0.1% LAE and 0.9% Tween 80 was demonstrated to be more feasible than 1% Tween 80 in forming stable turbid nanoemulsions with 10% oil phase with different mass ratios of thyme oil and corn oil by high pressure homogenization (Chang, McLandsborough, & McClements, 2015). Corn oil effectively inhibited Ostwald ripening of EO emulsions but drastically reduced antimicrobial activity of EOs (Chang, McLandsborough, & McClements, 2012).

Therefore, much work is needed to study EO nanoemulsions prepared with the combination of LAE and nonionic surfactants that could utilize the activities of both antimicrobials to reduce their usage levels to eventually improve sensory characteristics. The objective of the present work was to study physical and antimicrobial properties of emulsions prepared with non-ionic surfactants Tween 20 and Tween 80 and different concentrations of

LAE. The PIT method was used for nanoemulsion formation. Cinnamon bark oil (CBO) was chosen as a model EO because it is one of the most effective EOs against common foodborne pathogens (Cava-Roda, Taboada-Rodríguez, Valverde-Franco, & Marín-Iniesta, 2012; Chang, Chen, & Chang, 2001). Cinnamaldehyde is the major component (50-80%) of CBO, while other minor constituents include eugenol (10%), safrole (0-11%), linalool (10-15%), and camphor (Jian Qin, 2003). The antimicrobial properties of nanoemulsions and the same concentrations of free antimicrobials were compared in TSB and 2% reduced fat milk. Milk is used as a model food matrix because anionic proteins and hydrophobic fat globules can interfere with antimicrobial activities of cationic LAE and lipophilic EOs (Ma et al., 2013; Chen, Davidson, & Zhong, 2014). This information can then be used to guide the application of nanoemulsions in specific food matrices.

2 Materials and Methods

2.1. Chemicals

LAE was purchased from Vedeqsa Inc. (New York, NY). The commercial product with a name of Mirenat-TT contained $15 \pm 0.5\%$ w/w LAE. Tween 20 and Tween 80 were purchased from Acros Organics (Morris Plains, NJ). TSB, agar and yeast extract were purchased from Thermo Fisher Scientific Inc. (Pittsburgh, PA). The ultra-pasteurized 2% reduced fat milk (Simple Truth Organic, San Diego, CA) was purchased from a local grocery store. CBO was purchased from Sigma-Aldrich Corp. (St. Louis, MO).

2.2. Emulsion preparation

CBO was mixed at 1% w/w with the aqueous phase containing 3% w/w Tween 80 or Tween 20 and 0-0.375 % w/w LAE. The mixtures were homogenized at 15000 rpm for 4 min (model Ultra Turrax, IKA Works Inc., Wilmington, NC). These emulsions were also heated at 90 °C for 30 min, followed by quenching in ice/water (Forgiarini et al., 2001).

2.3. Droplet size and turbidity of emulsions

The hydrodynamic diameter (d_h) of emulsion droplets was measured with a DelsaNano C particle analyzer from Beckman Coulter Inc. (Brea, CA). The emulsion consisting of 0.05% w/w LAE was diluted in deionized water at a mass ratio of 1:10, while this mass ratio was 1:5 for other samples to meet the instrument sensitivity requirement. In addition to d_h , polydispersity indexes (PDIs) of emulsions were reported because PDI indicates the heterogeneity of droplet dimension and an increase of PDI indicates coalescence and/or Ostwald ripening in emulsions (McClements, 2004). The turbidity of emulsions was measured without dilution for absorbance at 600 nm using a BioMate 5 UV-Vis spectrophotometer from Thermo Fisher Scientific Inc. (Pittsburgh, PA).

2.4. Bacterial culture

One Gram-positive bacterium, *L. monocytogenes* Scott A, and two Gram-negative bacteria, *E. coli* O157:H7 ATCC 43895, and *S. Enteritidis*, were obtained from the culture collection of the Department of Food Science and Technology at the University of Tennessee in Knoxville. All strains were maintained at -20°C in 20% glycerol. Each strain was transferred at least 2 times in TSB with an interval of 24 h before use. Except growth kinetics experiments, *L. monocytogenes* was incubated at 32°C while *E. coli* O157:H7 and *S. Enteritidis* were incubated at 37°C.

2.5. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC was determined using the microbroth dilution method (Branen & Davidson, 2004). The bacterial culture was diluted to about 10^6 CFU/mL in TSB. 120 μ L of the diluted culture was added into wells of a 96-well microtiter plate. Antimicrobial stock solution was diluted in TSB in series from 10 to 40 ppm LAE. Free CBO samples were prepared by dissolving at 10% w/v in ethanol, followed by dilution in TSB to concentrations of 200, 400, 800, 1200, 1600, and 2000 ppm. Tween 80 stock solution was prepared at 20% w/w in water

by overnight stirring and filtered using a sterile 0.45 μm polyvinylidene difluoride (PVDF) membrane (Fisher Scientific Inc., Pittsburgh, PA) to remove possible microorganisms and particulates. The Tween 80 stock solution was diluted in TSB to concentrations of 1%, 2%, 4%, 6%, 8%, 10% and 12% w/w. Aliquots of 120 μL each of an antimicrobial solution and the bacterial culture were added in each well which halved the overall LAE, CBO, and Tween 80 concentrations to 5-20 ppm, 100-1000 ppm, and 5000-60000 ppm, respectively. The plates were incubated for 24 h at appropriate temperatures. The MIC was determined as the lowest antimicrobial concentration corresponding to an optical density change at 630 nm ($\Delta\text{OD}_{630\text{ nm}}$) of < 0.05 after incubation. The 100 μL aliquots from negative wells (i.e., $\Delta\text{OD}_{630\text{ nm}} < 0.05$) were spread on tryptic soy agar (TSA). Following incubation for 48 h, MBCs were determined as antimicrobial concentrations corresponding to at least a 3 log reduction of viable cells (Branen & Davidson, 2004).

2.6. Microbial growth kinetics in tryptic soy broth and 2% reduced fat milk

The growth of *L. monocytogenes* Scott A, *E. coli* O157:H7 ATCC 43895, and *S. Enteritidis* was studied in both TSB and 2% reduced fat milk. For TSB treatments, nanoemulsions and solutions with same concentrations of a mixture of CBO, LAE, and Tween 80 were added to TSB. The same volume of distilled water was used as a positive control. For *L. monocytogenes* and *E. coli* O157:H7, 16 μL of the antimicrobial samples were mixed with 8,984 μL of TSB. For *S. Enteritidis*, 29.3 μL of the antimicrobial samples was mixed with 8970.7 μL of TSB. For milk treatments, 2 mL of nanoemulsions, the mixture of CBO, LAE, and Tween 80, or distilled water (as a positive control) were added to 7 mL milk. The 9 mL mixtures of TSB or milk were then inoculated with 1 mL of culture (ca. 10^7 CFU/mL) to obtain a final bacterial population of ca. 10^6 CFU/mL. The final LAE concentration in TSB treatments was the MIC of each bacterium. In milk treatments, the respective overall concentrations of LAE, CBO, and Tween 80 were 750 ppm, 2000 ppm, and

6000 ppm. Viable bacteria were enumerated after 0, 4, 8, 24 and 48 h incubation at ambient conditions (21°C) using the spread plate method on TSA and incubated at the respective optimum growth temperature of bacteria for 48 h.

The growth of *L. monocytogenes* Scott A in 2% reduced fat milk was also investigated at suboptimal growth temperature of 4°C. The used nanoemulsion was composed of 3% Tween 80, 2% CBO, and 0.75% LAE. For the control treatment, 10 mL milk and 100 µL culture with ca. 10⁸ CFU/mL bacteria were mixed. For the antimicrobial treatment, the mixture contained 9.75 mL milk, 0.25 mL of the nanoemulsion, and 100 µL of the culture, corresponding to overall antimicrobial concentrations of 187.5 ppm LAE and 500 ppm CBO. Viable bacteria were enumerated after incubation at 4°C for 0, 4, 8, 24, 48, and 72 h using the same method as the 21°C treatments.

2.7. Data and statistical analyses

Means and standard deviations were calculated for each experiment. Three independent replicates were used in all experiments, and each replicate was tested in duplicate. Biological replicates were conducted using cultures grown separately. The turbidity data were analyzed with one-way ANOVA Tukey's test using SPSS 21 (IBM, Armonk, NY). The growth kinetics and particle size data were analyzed using SAS 9.3 (SAS Institute, Cary, NC).

3 Results

3.1. Physical properties of nanoemulsions

The photographs of emulsions prepared with 1% w/w CBO emulsified by 3% w/w Tween 80/20 and 0.05-0.375% w/w LAE before and after heating at 90 °C for 30 min are presented in Figure 1. All treatments prepared with Tween 20 were turbid before and after heating. Conversely, treatments using 3% w/w Tween 80 and 0.125-0.375% w/w LAE were translucent before heating and became transparent after heating. After storage at ambient

conditions (21°C) for 30 days, all samples prepared with Tween 20 showed sedimentation of CBO, which was observed in a shorter time for samples containing a lower amount of LAE, e.g., after 1 day for the 0.05% w/w LAE treatment (photos not shown). In comparison, the Tween 80 treatments, except the sample with 0.05% w/w LAE, showed a good stability with no sedimentation, and the turbidity of transparent samples decreased significantly ($P < 0.05$) after 30-day storage (Table 1), with noticeable improvement in visual clarity (not shown). Samples prepared with a lower concentration of LAE had higher absorbance that varied more significantly among replicates, especially after 30-day storage. The difference of absorbance at 600 nm was insignificant for treatments prepared with $\geq 0.125\%$ LAE ($P > 0.05$). The rest of this paper is focused on treatments prepared with Tween 80 only.

The mean d_h of emulsions during 30-day storage at 21°C are presented in Figure 2. At the lowest LAE concentration (0.05% w/w), the growth of d_h was observed (Figure 2A), but the big variations of replicates resulted in no significant difference before and after heating, and during storage ($P > 0.05$). Errors were smaller for samples prepared with 0.125-0.375% w/w LAE. On day 1, except for the emulsion prepared with 0.05% w/w LAE, heating significantly ($P < 0.05$) reduced the d_h , which agrees with the improved sample clarity (Figure 1, Table 1). After 30-day storage, d_h decreased for all samples, but the reductions were not statistically significant ($P > 0.05$). No significant difference ($P > 0.05$) was observed for the PDI of all samples, before and after 30-day storage (Table 2). Overall, the heated samples with 0.125% w/w and higher LAE had mean droplet diameters of about 100 nm that did not differ significantly with LAE concentrations. A nanoemulsion prepared with 1% w/w CBO, 3% w/w Tween 80 and 0.375% w/w LAE was chosen for microbiological experiments.

3.2. MIC and MBC of antimicrobials and the prepared nanoemulsion

The MIC and MBC of the nanoemulsion in LAE concentration compared to a free LAE solution are shown in Table 3. The MIC of the nanoemulsion was 1 ppm higher than LAE

solution for *E. coli* O157:H7 and *S. Enteritidis*, and 2 ppm higher for *L. monocytogenes*.
MBCs were generally higher than MICs, but a higher (1 ppm) MBC for the nanoemulsion compared to free LAE was only observed for *L. monocytogenes*. No inhibition was observed for Tween 80 treatments up to 60,000 ppm, while the MIC (400 ppm) and MBC (600 ppm) of free CBO were the same for the three bacteria.

3.3. Growth kinetics of bacteria in TSB

The growth kinetics of bacteria in TSB was studied at the MIC of free LAE for the corresponding bacterium (Table 3) at room temperature (21°C). Growth was studied in the presence of the nanoemulsion, free LAE, a mixture of LAE and CBO, and a mixture of Tween80 and CBO (Figure 3). For Gram-negative *E. coli* O157:H7 and *S. Enteritidis*, in the presence of the antimicrobial systems with LAE and CBO, there generally was an initial reduction of bacterial numbers followed by recovery and growth for all treatments. The trends were similar for *E. coli* O157:H7 and *S. Enteritidis*, showing more rapid recovery by bacteria in the presence of nanoemulsion > LAE+CBO > LAE (Figure 3B,C). For Gram-positive *L. monocytogenes*, significant inhibition was only observed for the treatment with LAE and CBO (Figure 3A).

3.4. Growth kinetics of bacteria in 2% reduced fat milk

Figure 4 shows the growth kinetics of bacteria in 2% reduced fat milk at 21°C and treatments containing the nanoemulsion (6000 ppm Tween 80, 2000 ppm CBO, and 750 ppm LAE) or various combinations of these compounds in the solution. LAE concentrations were chosen based on our previous study where 750 ppm LAE gradually reduced *L. monocytogenes* by 3 log CFU/mL (from ~ 6 log CFU/mL) and inhibited the growth of *E. coli* O157:H7 and *S. Enteritidis* during incubation at 21°C for 48 h (Ma et al., 2013).

The quickest kill of *L. monocytogenes* was observed for the mixture of CBO and LAE (complete inhibition in 4 h), followed by the nanoemulsion (in 8 h) and LAE only (in 24 h)

treatments (Figure 4A). No recovery was observed for any of the three treatments. The CBO/Tween 80 mixture was the least effective and only gradually reduced the bacterial population by around 1 log CFU/mL after 48 h.

For *E. coli* O157:H7 (Figure 4B), the mixture of CBO and LAE reduced the bacteria to below the detection limit (1 log CFU/mL) after 8 h, with no recovery after 48 h. The nanoemulsion had a slightly slower kill rate than CBO/LAE but complete inactivation was also observed after 8 h. The mixture of CBO and Tween 80 had slower inactivation than the nanoemulsion at earlier time points, but also achieved inactivation to the level of detection by 24 h. LAE alone was the least effective antimicrobial treatment against *E. coli* O157:H7 showing about 3.5 log CFU/mL reduction after incubation for 24 and 48 h.

For *S. Enteritidis* (Figure 4C), no difference was observed for the treatments with the nanoemulsion and the mixtures of CBO/Tween 80 and CBO/LAE in 24 h, but the difference was observed at 48 h, with the nanoemulsion showing recovery to about 3 log CFU/mL. As with *E. coli* O157:H7 (Figure 4B), the LAE only treatment was the least effective, showing bacteriostatic activity through 24 h followed by recovery to the level of the control after 48 h.

Figure 5 shows the growth kinetics of *L. monocytogenes* in 2% reduced fat milk at 4°C. There was a slight increase of bacterial population for the control sample initially, while there was a slight decrease for the antimicrobial treatment with 187.5 ppm LAE and 500 ppm CBO. Differences between the two treatments were significant ($P < 0.05$) at 8, 24, and 48 time points, with the biggest difference of ca. 1 log CFU/mL observed at 48 h.

4 Discussion

4.1. Nanoemulsion properties

Nanoemulsions prepared with 0.125–0.375% w/w LAE and 3% w/w Tween 80 were transparent and stable during storage for 30 days. The synergistic surface activity of LAE

with Tween 80 was shown previously for the oil phase with different mass ratios of thyme oil and corn oil (Chang et al., 2015). Nanoemulsions as prepared in the present study did not require corn oil that inhibited Ostwald ripening but greatly reduced antimicrobial activities (Chang et al., 2012). The samples prepared with Tween 20 and all concentrations of LAE showed high turbidity and sedimentation during storage (Figure 1). The oily yellow precipitate in the 0.05% w/w LAE treatment appeared to be the demulsified cinnamaldehyde and eugenol that have a density of 1.05 and 1.07 g/mL at 20°C, respectively (NIH, 2015). LAE can form mixed micelles with Tween 20 (Asker et al., 2011), and the tail of LAE has a closer similarity to the laurate moiety of Tween 20 than the oleate of Tween 80 (McClements, 2004). The better surface activity synergy between LAE and Tween 80 than that of Tween 20 can possibly result from the lower hydrophilic-lipophilic balance (HLB) of Tween 80 (HLB = 15) than Tween 20 (HLB = 16.7) when CBO is emulsified and after heating and quenching (Lv, Wang, Cai, & Zhang, 2014; Rao, Aghav, Sukre, & Kumar, 2014). Both Ostwald ripening and coalescence can contribute to sedimentation of droplets observed for the emulsions prepared with Tween 20, due to growth of droplets during storage which was not measured due to instability of emulsions. The stable appearance, dimension (Figure 2) and PDI (Table 2) of emulsions prepared with Tween 80 suggest that the interfacial layer formed with both Tween 80 and LAE is robust against emulsion destabilization mechanisms.

4.2. Activities of free antimicrobials and their nanoemulsion in TSB

The MIC of the nanoemulsion was higher than that of free LAE solution for all three bacteria (Table 3). The MICs of CBO against *L. monocytogenes* Scott A, *E. coli* O157:H7 and *S. Enteritidis* were all 400 ppm (Table 3), which are much higher than 21, 19, and 32 ppm CBO in the corresponding nanoemulsion at the MICs in the present study. Previously, we showed the activity of LAE and EO/EO component combinations was synergistic or additive against Gram-positive *L. monocytogenes* but was antagonistic against Gram-negative

E. coli O157:H7 and *S. Enteritidis* (Ma et al., 2013). Therefore, the MIC of the nanoemulsion in Table 3 likely is contributed mostly by LAE.

Two possible factors can result in the higher MICs of the nanoemulsion than free LAE. First, the content of free LAE is reduced after adsorbing onto CBO droplets to form nanoemulsions. However, the concentrations of the CBO components cinnamaldehyde, eugenol, and linalool are below their solubility (Chen et al., 2014; Jian Qin, 2003) at the MICs of the nanoemulsion, and no droplets are expected. The second possible factor is the complex formation between LAE and Tween 80 reducing the antibacterial activity. The respective critical micelle concentrations of Tween 80 and LAE are 22 and 2100 ppm (Asker, Weiss, & McClements, 2009; Mandal, Nair, & Ramaswamy, 1988). Therefore, the concentration of LAE at the MICs (Table 3) is too low to form micelles, but Tween 80 can form its micelles and co-micelles with LAE (Asker et al., 2011). The formed complex micelles with possible inclusion of hydrophobic components of CBO can reduce the availability of LAE to react with bacteria, which increases the MIC. The MBCs are higher than the MICs and the impact of binding between LAE and the other compounds can be less pronounced (Table 3).

In the growth kinetics assay (Figure 3), the mixture of CBO and Tween 80 did not show inhibition against any of the test microorganisms which is because the CBO concentration was below the MIC (400 ppm) and Tween 80 had no antibacterial activity up to 6% w/w (Table 3). However, the same concentration of CBO in combination with LAE caused an apparent antagonistic effect against Gram-negative bacteria compared to the free LAE and an apparent synergistic effect against Gram-positive bacteria. The latter was previously reported by Ma et al. (2013). Reduced inhibition by the nanoemulsion compared to the same concentrations of free LAE and CBO may be due to complex formation as discussed above. Furthermore, bacterial growth was observed after 24 h when *L. monocytogenes* was treated

by free LAE at the concentration of MIC (Figure 3A), which contrasts with no bacterial growth in the MIC assay. The discrepancy likely resulted from the higher (about twice) bacterial population used in the growth kinetics assay than in the MIC assay. Different assay temperatures in these two methods can also cause differences in these two sets of data (Table 3, Figure 3).

4.3. Growth kinetics of bacteria in 2% reduced fat milk

The concentration of antimicrobials required to inhibit bacterial growth in milk (Figure 4) was much higher than that in TSB (Figure 3), likely due to binding between antimicrobials and milk proteins and lipid particles (Chen et al., 2014; Ma et al., 2013). Under the conditions studied, the mixture of Tween 80 and CBO was the least effective against *L. monocytogenes* but *L. monocytogenes* was completely inactivated to the level of detection by all treatments containing LAE. In contrast, free LAE was the least effective against *E. coli* O157:H7 and *S. Enteritidis*. The data can be the result of the different antimicrobial mechanisms of the two types (cationic vs. lipophilic) of antimicrobials. Cationic antimicrobials like LAE can cause disturbance of membrane structure, leakage of cytoplasm constituents, alteration of efflux pumps, and structural changes of bacteria (Rodríguez, Seguer, Rocabayera, & Manresa, 2004). Whereas, lipophilic antimicrobials like CBO can accumulate in the lipid bilayer of bacteria membrane and impact lipid-protein interactions (Burt, 2004). EOs can also interact directly with hydrophobic areas of proteins (Burt, 2004). Different antimicrobial mechanisms of LAE and EOs are indirectly indicated by the magnitude of their MICs and MBCs (Table 3). For the Gram-positive *L. monocytogenes* (Figure 4A), all LAE-containing treatments resulted in the complete inactivation indicating that LAE is the dominant contributor of antimicrobial activity in milk. Conversely, CBO was more effective than LAE for Gram-negative *E. coli* O157:H7 and *S. Enteritidis* (Figure 4B,C). Negatively-charged lipopolysaccharides on the outer membrane of Gram-negative bacteria can result in the

significant binding with LAE, reducing its activity compared to Gram-positive *L. monocytogenes* (Lovitt & Wright, 1999).

In complex food matrices, the interpretation of antimicrobial activities becomes more complicated. Proteins and lipids are major factors interfering with antimicrobial activities of EOs (Kühn, Considine, & Singh, 2006). For that reason, milk is a good model food system to study the influences of proteins and lipids on activities of antimicrobials. With a hydrophilic surface, bacteria are expected to be present in the aqueous phase (Gaysinsky, Davidson, Bruce, & Weiss, 2005), and the antimicrobial activity is physically dependent on the amounts of antimicrobials available in the aqueous phase. LAE is cationic and can bind with negatively charged proteins and fat globules in milk (Asker et al., 2011), thereby requiring a much higher concentration than in TSB to inhibit bacterial growth. When studied in milk, EO nanoemulsions typically inactivate bacteria more quickly and/or extensively when compared to free antimicrobials, because nanoemulsions can increase the solubility of EOs in the aqueous phase of the milk (Pan, Chen, Davidson, & Zhong, 2014; Xue, Davidson, & Zhong, 2013). This phenomenon was not observed when comparing with the mixture of free LAE and CBO and the nanoemulsion (Figure 4), because LAE is both an antimicrobial and an emulsifier and its availability and antibacterial activity are affected by Tween 80, as discussed previously.

The experiments conducted at 4°C showed the survival of *L. monocytogenes* in milk, as previously reported (Petran & Zottola, 1989). The inclusion of the nanoemulsion at 187.5 ppm LAE and 500 ppm CBO reduced the cell counts when compared to the control, but the reduction was much less than the treatment in Figure 4A. Besides the lower antimicrobial concentrations, the higher fat content of the mixture at 4°C (1.95%, vs. 1.4% at 21°C) is another contributor of the reduced antimicrobial efficiency (Chen *et al.*, 2014).

Finally, anchoring both LAE and Tween 80 on CBO nanodroplets is expected to reduce the overall positive charge when compared to LAE alone. The weakened electrostatic attraction between the cationic nanodroplets and anionic proteins and lipid particles in milk might then be expected to increase the availability and therefore antibacterial activity of LAE. However, the mixture of free LAE and CBO performed similarly or better than the nanoemulsion in milk. This mechanistic understanding requires future studies using various antimicrobial concentrations and comparing nanoemulsions prepared with LAE only, Tween 80, and both, as well as their distributions in milk.

5 Conclusions

A combination of cationic LAE and nonionic Tween 80 was observed to enhance surface activity in forming nanoemulsions of CBO, and the PIT method further reduced droplet dimension and emulsion turbidity. The interface with the surfactant mixture was effective in maintaining emulsion stability without the use of an Ostwald ripening inhibitor. When the nanoemulsion with 0.375% LAE, 3% Tween 80, and 1% CBO was evaluated in antimicrobial testing, the binding between LAE and Tween 80 and CBO components resulted in the antibacterial activity of the nanoemulsion being lower than same concentrations of free LAE and CBO in TSB. In 2% reduced fat milk, the mixture of free LAE and CBO had better or similar activity as the nanoemulsion at 21°C, and LAE and CBO were observed to be the dominant antimicrobial against Gram-positive and Gram-negative bacteria, respectively. At 4°C, the growth of *L. monocytogenes* in 2% reduced fat milk was not observed in both the control and the nanoemulsion treatment corresponding to 187.5 ppm LAE and 500 ppm CBO. Since EOs are to be prepared as emulsions for use in aqueous food systems, findings from the present work demonstrate that LAE can be used in combination with Tween 80 to prepare stable nanoemulsions without compromising antimicrobial activities when applied in

424 complex food matrices such as milk. However, sensory properties are to be studied at
425 conditions mimicking practical food applications.

426

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539

Figure captions

Figure 1. Appearance of emulsions prepared with 1% w/w CBO, 3% w/w Tween 80 (A and B) or Tween 20 (C and D) and 0.05, 0.125, 0.25 and 0.375 % w/w LAE (left to right in each image). Samples are compared before (A and C) and after (B and D) heating at 90 °C for 30 min.

Figure 2. Hydrodynamic diameter of emulsions during storage at 21 °C for 30 days.

Emulsions containing 1% w/w CBO were prepared with 3% w/w Tween 80 and (A) 0.05%, (B) 0.125%, (C) 0.25%, and (D) 0.375% w/w LAE with and without heating at 90 °C for 30 min. Error bars are standard deviations from triplicates, each tested two times (n = 6). Treatments between heated and unheated samples showing significant differences ($P < 0.05$) are marked with asterisks.

Figure 3. The growth kinetics of (A) *Listeria monocytogenes*, (B) *Escherichia coli* O157:H7, and (C) *Salmonella* Enteritidis at room temperature (21°C) in TSB mixed with a nanoemulsion containing LAE, CBO, and Tween 80 ($\diamond$), or the same concentration of free LAE ($\square$), a mixture of CBO and LAE ($\triangle$), or a mixture of CBO and Tween 80 ($\times$). The control ($\circ$) was the treatment without antimicrobials. The overall LAE concentrations were 6 (A), 6 (B), or 11 (C) ppm; CBO concentrations were 16 (A), 16 (B), or 29.33 (C) ppm; and Tween 80 concentrations were 48 (A), 48 (B), or 88 (C) ppm. Error bars are standard deviations from three independent replicates, each tested in duplicate.

Figure 4. The growth kinetics of (A) *Listeria monocytogenes*, (B) *Escherichia coli* O157:H7, and (C) *Salmonella* Enteritidis at room temperature (21°C) in 2 % reduced fat milk mixed with a nanoemulsion containing 750 ppm LAE, 2000 ppm CBO, and 6000 ppm Tween 80 ($\diamond$), or same concentrations of free LAE ($\square$), a mixture of CBO and LAE ($\triangle$), or a mixture of CBO and Tween 80 ($\times$). The control ($\circ$) was the treatment without antimicrobials. The

detection limit was 1 log CFU/mL. Error bars are standard deviations from three independent replicates, each tested in duplicate.

Figure 5. The growth kinetics of *Listeria monocytogenes* Scott A at 4°C in 2 % reduced fat milk mixed with a nanoemulsion corresponding to overall concentrations of 187.5 ppm LAE, 500 ppm CBO, and 750 ppm Tween 80 (∇). The control ($\square$) was the treatment without antimicrobials. Error bars are standard deviations from three independent replicates, each tested in duplicate. Differences between the two treatments are significant at 8, 24, and 48 h ($P < 0.05$).

Table 1. Absorbance at 600 nm of emulsions prepared with 3% w/w Tween 80, 1% w/w cinnamon bark oil, and 0-0.375 % w/w LAE, with and without heating at 90 °C for 30 min, before and after storage for 30 days.*

LAE% w/w	Without heating		With heating	
	Day 0	Day 30	Day 0	Day 30
0	1.88 ± 0.03 ^b	N/D [#]	N/D	N/D
0.05	1.26 ± 0.03 ^c	2.34 ± 0.69 ^{ab}	0.50 ± 0.01 ^{def}	2.52 ± 0.55 ^a
0.125	0.80 ± 0.07 ^{cd}	0.78 ± 0.66 ^{cde}	0.55 ± 0.05 ^{def}	0.49 ± 0.16 ^{def}
0.25	1.13 ± 0.04 ^c	0.30 ± 0.08 ^{def}	0.73 ± 0.04 ^{cde}	0.24 ± 0.05 ^{ef}
0.375	1.19 ± 0.19 ^c	0.25 ± 0.05 ^{ef}	0.81 ± 0.16 ^{cd}	0.15 ± 0.05 ^f

* Numbers are mean ± standard deviation from triplicates, each tested two times (n = 6).

Different superscript letters indicate significant difference among all treatments ($P < 0.05$).

[#] Not determined because the sample was destabilized.

Table 2. Polydispersity index (PDI) of emulsions prepared with 3% w/w Tween 80, 1% w/w cinnamon bark oil, and 0.05-0.375 % w/w LAE, with and without heating at 90 °C for 30 min, during storage for 30 days.*

LAE (%)	PDI of emulsions prepared without heating			
	Day 1	Day 3	Day 7	Day 30
0.05	0.273 ± 0.015 ^{ab}	0.257 ± 0.022 ^{ab}	0.256 ± 0.066 ^{ab}	0.265 ± 0.097 ^{ab}
0.125	0.291 ± 0.061 ^{ab}	0.293 ± 0.063 ^{ab}	0.280 ± 0.055 ^{ab}	0.278 ± 0.045 ^{ab}
0.25	0.262 ± 0.045 ^{ab}	0.300 ± 0.045 ^{ab}	0.336 ± 0.042 ^a	0.313 ± 0.046 ^{ab}
0.375	0.289 ± 0.027 ^{ab}	0.313 ± 0.039 ^{ab}	0.326 ± 0.043 ^{ab}	0.327 ± 0.024 ^{ab}
	PDI of emulsions prepared with heating			
	Day 1	Day 3	Day 7	Day 30
0.05	0.261 ± 0.010 ^{ab}	0.274 ± 0.018 ^{ab}	0.268 ± 0.009 ^{ab}	0.266 ± 0.048 ^{ab}
0.125	0.275 ± 0.039 ^{ab}	0.277 ± 0.041 ^{ab}	0.246 ± 0.013 ^{ab}	0.233 ± 0.075 ^b
0.25	0.316 ± 0.015 ^{ab}	0.314 ± 0.032 ^{ab}	0.310 ± 0.026 ^{ab}	0.302 ± 0.010 ^{ab}
0.375	0.318 ± 0.015 ^{ab}	0.299 ± 0.079 ^{ab}	0.333 ± 0.020 ^a	0.292 ± 0.032 ^{ab}

*Numbers are mean ± standard deviation from triplicates, each tested two times (n = 6).

Different superscript letters indicate significant difference among all treatments ($P < 0.05$).

586 Table 3. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)
 587 of Tween 80, free CBO, free LAE, and a nanoemulsion containing 1% w/w CBO, 3% w/w Tween 80
 588 and 0.375% w/w LAE against bacteria in TSB.*

Bacteria**	MIC (ppm)				MBC (ppm)			
	LAE	CBO	Tween	Nano-	LAE	CBO	Tween	Nano-
			80	emulsion			80	emulsion
<i>L. monocytogenes</i>	6	400	>60,000	8	9	600	>60,000	10
<i>E. coli</i> O157:H7	6	400	>60,000	7	7	600	>60,000	7
<i>S. Enteritidis</i>	11	400	>60,000	12	16	600	>60,000	15

589 *The nanoemulsion and free LAE were diluted in TSB. The numbers represent LAE
 590 concentrations.

591 ** *L. monocytogenes* was incubated at 32°C while *E. coli* O157:H7 and *S. Enteritidis* were
 592 incubated at 37°C.

593

(A)

(B)

(C)

(D)

Figure 1.

Figure 2.

Figure 3.

Figure 4.

615

616 Figure 5.