1 **Antimicrobial Properties of Microemulsions Formulated with Essential Oils,**
2 **Soybean Oil, and Tween 80**

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13
14 **Abstract**

15 It was previously found that blending soybean oil with cinnamon bark oil (CBO),
16 eugenol or thyme oil, Tween 80, and equal masses of water and propylene glycol could
17 be used to prepare microemulsions. In the present study, the objective was to determine
18 the antimicrobial activity of the microemulsions in tryptic soy broth (TSB) and 2%
19 reduced fat milk. In TSB, the minimum inhibitory concentration (MIC) of CBO
20 solubilized in microemulsions was up to 625 ppm against cocktails of *Listeria*
21 *monocytogenes*, *Salmonella enterica* or *Escherichia coli* O157:H7, which was equal to or
22 higher in concentration than free CBO dissolved in ethanol. However, MICs of eugenol
23 or thyme oil in microemulsions were much higher than that of free antimicrobials.

Therefore, microemulsions of CBO were chosen to do further study. Inactivation curves of *L. monocytogenes* or *E. coli* O157:H7 in TSB or 2% reduced fat milk were tested and fitted using the Weibull model. In TSB, a gradual decrease in cell viability of *L. monocytogenes* and *E. coli* O157:H7 was observed with the microemulsion treatments at 625 ppm CBO, which was in contrast to the more rapid and greater inactivation by free CBO. Gradual inactivation of *L. monocytogenes* in 2% reduced fat milk was also observed in the treatment with 10,000 ppm free or microemulsified CBO. When fitted using the Weibull model, the predicted time to obtain a 3-log decrease of *L. monocytogenes* and *E. coli* O157:H7 in TSB or 2% reduced fat milk increased with an increased amount of soybean oil in microemulsions. Additionally, increasing the amount of Tween 80 in mixtures with different mass ratios of Tween 80 and essential oils significantly decreased the log reductions of *L. monocytogenes* in TSB. Our study showed that microemulsions can be used to dissolve EOs and control the rate of inactivating bacteria, but the composition of microemulsions is to be carefully chosen to minimize the reduction of antimicrobial activities.

Keywords: microemulsion; essential oil; antimicrobial activity; soybean oil; Tween 80.

1. Introduction

Essential oils (EOs) play important roles in mediating plant-environment interactions, such as defense, plant pollination and plant-plant communication (Burt, 2004; Croteau et al., 2000; Lewinsohn et al., 1991). In recent years, studies on EOs as potential antimicrobial food preservatives have become an active research area because many EOs and their components have demonstrated antimicrobial efficacy against food-borne pathogenic and spoilage microorganisms (Burt, 2004; Klein et al., 2013; Ma et al., 2013). In addition, EOs have been approved by the United States Food and Drug Administration as generally-recognized-as-safe food additives (FDA, 2014). However, the practical application of EOs and their components in/on food products has several challenges, including low water solubility, organoleptic properties, and high volatility.

Colloidal systems such as nano- and microemulsions can effectively disperse EOs in water and the nanometer-sized oil droplets may preserve the appearance of food products (Ma and Zhong, 2015; Xue and Zhong, 2014). This interesting feature has inspired studies preparing nanoemulsions of EOs (Wu et al., 2014; Xue et al., 2015; Xue and Zhong, 2014). However, nanoemulsions are thermodynamically unstable and can be destabilized by mechanisms such as Ostwald ripening, coalescence, and aggregation, resulting in alterations of particle size distribution and possibly visible creaming/sedimentation and phase separation during storage (McClements, 2012). Furthermore, the formation of nanoemulsions usually needs high-energy input, such as high-speed or high-pressure homogenization (McClements, 2012; Xue et al., 2015). Unlike nanoemulsions, microemulsions are thermodynamically stable under a specific combination of compositional and environmental conditions and can be formed by

64 mixing water, oil and surfactants with little external energy, such as stirring or heating
65 (McClements, 2012). Thus, microemulsions offer advantages of shelf-stability and
66 reduced production cost.

67 Due to a high concentration of surfactants used in preparation and the limited
68 dilutability of microemulsions (McClements, 2012), there are only limited studies on the
69 antimicrobial properties of EOs dissolved in microemulsions. Zhang et al. (2009) studied
70 the antimicrobial activity of glycerol monolaurate in microemulsions prepared with
71 polyethylene glycol sorbitan monooleate (Tween 80) against *Escherichia coli* and
72 *Staphylococcus aureus*. A reduction of ca. 6 logs of both bacteria was observed in 1 min
73 and 60 min in a nutrient broth with a microemulsion containing 9.38% w/w glycerol
74 monolaurate and the 10-fold diluted microemulsion, respectively. In a separate study
75 conducted by the same research group, antimicrobial activity of glycerol monolaurate in
76 microemulsions was improved compared to free glycerol monolaurate on Luria-Bertani
77 plates (Zhang et al., 2007). In contrast, the antimicrobial activity of eugenol in
78 microemulsions was shown to be equal to or less effective as an inhibitor than
79 unencapsulated eugenol when testing against *Listeria monocytogenes* and *E. coli* O157:
80 H7 in ultrahigh-temperature pasteurized milk (Gaysinsky et al., 2007). In a recent study,
81 Deng et al. (2015) reported the antimicrobial activity of microemulsions comprised of
82 thymol/ethanol, Tween 80, and water against *E. coli* and *Staphylococcus aureus*. The
83 authors observed that the antimicrobial activity of microemulsions decreased as the
84 aqueous phase content increased. Thus, antimicrobial properties of microemulsions are
85 dependent on the composition and the media being tested.

In a recent study, Ma and Zhong (2015) formulated microemulsions of EOs using Tween 80 as the surfactant and equal masses of water and propylene glycol (PG) as the polar phase. The incorporation of soybean oil (SBO) greatly expanded the conditions for forming microemulsions with a wide range of EO concentrations (Ma and Zhong, 2015). Incorporating SBO in the oil phase may also contribute to a reduction in the loss of volatile EOs during shelf storage (Zhang et al., 2015). Thus, the objective of this study was to characterize antimicrobial properties of the EO microemulsions formulated with various concentrations of SBO and Tween 80. To study the impact of food components on antimicrobial properties, 2% reduced fat milk was studied because protein and fat globules have been shown to interfere antimicrobial activities of EOs (Chen et al., 2014). An additional objective was to study the impact of Tween 80 level on the antimicrobial activity of EOs.

2. Materials and methods

2.1. Materials

Cinnamon bark oil (CBO), eugenol, and thyme oil were purchased from Sigma-Aldrich Corp. (St. Louis, MO). PG, SBO, tryptic soy broth (TSB), and Tween 80 were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA). Ultra-pasteurized 2% reduced fat milk (Simple Truth[®] brand, Kroger Co., Cincinnati, OH) was purchased from a local grocery store.

2.2. Bacterial cultures

To be more representative, bacterial cocktails were used in this study, which were formulated with strains mainly isolated from produce products associated with outbreaks of foodborne illnesses, as in our previous studies (Ma et al., 2016; Zhang et al., 2015). Each bacterial cocktail consisted of 5 test strains as follows: (1) *L. monocytogenes* cocktail: LM1, LM2, 310, Scott A, and V7; (2) *Salmonella enterica* cocktail: *S. Agona*, *S. Montevideo*, *S. Gaminara*, *S. Michigan*, and *S. Saint Paul*; (3) *E. coli* O157: H7 cocktail: H1730, F4546, K3995, 658, and 932. Before mixing, each strain was cultured in TSB for 24 h at 32°C (*L. monocytogenes*) or 37°C (*S. enterica* and *E. coli* O157:H7) and transferred at least 2 consecutive times with an interval of 24 h. Then 2 mL culture from each strain was mixed together to yield the cocktail before testing.

2.3. Preparation of microemulsions

Microemulsions of EOs (CBO, eugenol, or thyme oil) with or without SBO were prepared as described previously (Ma and Zhong, 2015). Tween 80 and EO were mixed at a mass ratio of 5:1, SBO was incorporated into the oil phase with mass ratios of EO and SBO at 1:0, 2:1, or 4:1, then the polar phase with equal masses of water and PG was added to the oil/surfactant mixture, followed by mixing on a magnetic stirring plate to obtain microemulsions. The overall concentration of Tween 80 and EO was 60% w/w, and that of the polar phase and SBO was 40% w/w.

2.4. Determination of minimum inhibitory concentration (MIC)

The MIC of EOs solubilized in microemulsions were compared with free EO that was dissolved in ethanol at a concentration of 5% w/w as a stock solution before diluting

in TSB to different EO concentrations (Thongson et al., 2005). The antimicrobial samples were added into 60 mL melted tryptic soy agar (TSA) to obtain a final overall EO concentration from 156 to 5,000 ppm. The mixtures were stirred vigorously until the TSA was just about to solidify, followed by addition of equal volumes to 3 petri dishes. Bacterial cultures (10 μ L) with ca. 10^4 CFU/mL were inoculated at 3 spots on TSA plates. MIC was defined as the lowest concentration of EO corresponding to no growth of bacteria after 2-day incubation at 32°C for *L. monocytogenes* or 37°C for *S. enterica* and *E. coli* O157:H7.

2.5. Bacterial inactivation end point analysis in 2% reduced fat milk

One mL of each cocktail was added into 9 mL 2% reduced fat milk to obtain a final population of about 10^6 CFU/mL. The CBO treatments with various concentrations of EO solubilized in microemulsions or ethanol were added to the inoculated milk. A treatment without antimicrobial addition was used as a control. Viable cells were enumerated before and after incubation at room temperature (21°C) for 24 h using either surface (*S. enterica*, *E. coli* O157: H7) or pour plating (*L. monocytogenes*). Log reductions were calculated based on the control at 0 h. Experiments were conducted at least in triplicate.

2.6. Microbial inhibition kinetics in TSB or 2% reduced fat milk

Survivals of bacteria in TSB or 2% reduced fat milk after antimicrobial treatments were studied as described previously (Ma et al., 2013). One mL culture with ca. 10^7 CFU/mL *L. monocytogenes* or *E. coli* O157:H7 was added to 9 mL TSB or 2% reduced fat milk. Free CBO or a CBO microemulsion was added into the bacteria-milk mixture at

an overall CBO concentration of 625 ppm and incubated at room temperature (21°C). The same concentration of ethanol as in the free CBO treatment was used as an ethanol control. A microemulsion control, based on the formulation with a CBO:SBO mass ratio of 1:0 (with the highest PG content), was prepared with same amounts of Tween 80 and PG (50% and 20% w/w specifically) but without CBO and SBO; distilled water was used to replace the portion of CBO and SBO. Viable cells were enumerated after 0, 24, 48, 72, 96, and 120 h using the pour plating method for *L. monocytogenes* or surface plating method for *E. coli* O157:H7. The detection limit was 1 log CFU/mL. Experiments were repeated at least twice using independent cultures and microemulsions.

The Weibull model (Eq. 1) was used to analyze the inactivation kinetics of *L. monocytogenes* and *E. coli* O157:H7 based on the initial bacterial population (N_0) and the survival population (N) after a reaction time of t (Mafart et al., 2002). When fitting the data to the model, a concave upward, a linear, and a concave downward curve corresponds to a shape parameter (p) of <1 , 1 , and >1 , respectively. Besides estimating parameters in the model, the time needed to obtain a 3-log reduction of bacterial population ($t_{3\text{-log}}$) by each treatment was calculated based on the obtained model.

$$\text{Log}_{10} N = \text{Log}_{10} N_0 - (t/\delta)^p \quad (1)$$

where δ is the time needed to reduce the bacterial population to 10% of N_0 , i.e., the first 1-log reduction.

2.7. Effect of Tween 80 content on the antimicrobial activity of EOs

To investigate the role of Tween 80 on antimicrobial activity of EOs, Tween 80 and EO (CBO, eugenol or thyme oil) were mixed at mass ratios of 1:1, 5:1 and 9:1 on a

magnetic stirring plate. *L. monocytogenes* cocktail was added to TSB to obtain a final population of about 10^6 CFU/mL, followed by the addition of CBO (313 ppm), eugenol (625 ppm) or thyme oil (625 ppm) and with or without Tween 80. The control for Tween 80 had the same concentration as the Tween 80:EO mass ratio of 9:1 (the highest Tween 80 concentration). A viability control was TSB with no EOs or Tween 80 and inoculated with *L. monocytogenes*. Mixtures were incubated at 32°C for 24 h and the pour plate method was used to detect the viable cells at 0 h for the negative control and 24 h for all samples. The experiments were performed at least in triplicate.

2.8. Statistical analysis

One way ANOVA Tukey test in SPSS 22 software (IBM, Armonk, NY) was performed to analyze the log reduction data at a 5% significance level. The estimation of parameters in the Weibull model was enabled with the R statistical software (version 3.2.2, 2015; The R Foundation for Statistical Computing, Vienna, Austria).

3. Results and Discussions

3.1. MICs of microemulsions

MICs of microemulsions and free EOs (dissolved in ethanol) are presented in Table 1. MIC of free CBO against *L. monocytogenes* was 313 ppm, while MIC of CBO microemulsions was 625 ppm, one step higher in the dilution scheme. Equivalent MICs (625 ppm) for free CBO and CBO solubilized in microemulsions were observed for *S. enterica*. For *E. coli* O157:H7, MICs of CBO in microemulsions were one concentration step higher than free CBO. The concentration of SBO in microemulsions did not impact the MIC of CBO in TSB. As for eugenol and thyme oil, MICs of microemulsions

increased remarkably from those of free EOs, from 313-625 ppm to $\geq 2,500$ ppm for eugenol microemulsions and to $> 5,000$ ppm for thyme oil microemulsions. Moreover, MICs of eugenol microemulsion with a 2:1 mass ratio of eugenol and SBO against *S. enterica* and *E.coli* O157:H7 (5,000 ppm) were higher than that of the microemulsion without SBO (2,500 ppm). These findings indicated that microemulsions of CBO were more effective than those of eugenol and thyme oil, and that the microemulsion preparation had the least influence on antimicrobial activity of CBO. Therefore, microemulsions of CBO were chosen for further study.

The lower MICs of CBO microemulsions compared to those of eugenol and thyme oil may be related to water solubility of the EOs. *Trans*-cinnamaldehyde, the major component of CBO, has higher solubility in TSB (1.51 g/L) than eugenol (1.42 g/L) and thymol (0.43 g/L), the major components of thyme oil (Chen et al., 2014). Therefore, it should be easier for CBO to migrate from microemulsion micelles into the aqueous phase to act on bacteria. The higher hydrophilicity of *trans*-cinnamaldehyde than eugenol and thymol also may have resulted in the weaker binding between CBO and Tween 80/SBO, which also explained the less effect of SBO on the MICs of CBO than those of eugenol (Table 1).

3.2. Microbial survivability end point analysis in 2% reduced fat milk

Viable bacterial cells were enumerated after treatment by various concentrations of free CBO or microemulsions of CBO in 2% reduced fat milk after 24-h incubation. For *L. monocytogenes*, 3000, 4000 and 5000 ppm of free or microemulsified CBO were observed to produce no significant log reductions (Table 2). A maximum of a 0.19 log

217 reduction of *L. monocytogenes* was detected among all the treatments suggesting the low
218 antimicrobial efficacy of CBO in inhibiting *L. monocytogenes* in 2% reduced fat milk
219 under the conditions studied. Treatments with 625 ppm free or microemulsified CBO
220 reduced *S. enterica* by 0.08-0.26 log CFU/mL and *E. coli* O157:H7 by 0.14-0.19 log
221 CFU/mL (Table 2). Treatments by 1,250 ppm CBO resulted in a 0.48-0.68 log CFU/mL
222 reduction of *S. enterica*, and a 0.33-0.45 log CFU/mL reduction of *E. coli* O157:H7. No
223 significant differences were detected among treatments. Much higher log reductions of
224 the two Gram-negative bacteria were detected when increasing the concentration of CBO
225 to 2,500 ppm. For *S. enterica*, about a 3.5-4.1 log CFU/mL reduction was found and,
226 again, no significant differences were observed between treatments of 2,500 ppm free and
227 microemulsified CBO. For *E. coli* O157:H7, about a 3.5 log CFU/mL reduction was
228 detected in the treatment of 2,500 ppm free CBO, which was significantly higher than
229 treatments with a same amount of CBO solubilized in microemulsions (2.33-2.58 log
230 CFU/ml reduction).

231 Similar results have been shown in other studies. A high concentration (9000 ppm)
232 of *trans*-cinnamaldehyde was needed to generate a bactericidal effect against *L.*
233 *monocytogenes* in skim milk at 21°C (Chen et al., 2014). Compared to the antimicrobial
234 activity of CBO in TSB (Table 1), the difference in antimicrobial efficacy of CBO
235 against *L. monocytogenes* and the two Gram-negative bacteria was much more
236 pronounced in 2% reduced fat milk (Table 2). This may be caused by the differential
237 protective effects of milk components on the different bacteria. When milk component
238 casein was studied for its influence on the attachment of *L. monocytogenes* and *S.*
239 Typhimurium to stainless steel and Buna-N, it was found that *L. monocytogenes* cells

pre-treated with 1% casein almost completely lost its attachment ability to stainless steel and Buna-N, while pre-treating *S. Typhimurium* with 1% casein had no effect on its attachment to stainless steel and limited effect on its attachment to Buna-N (Helke et al., 1993). The results suggested that binding between casein and bacteria cell surface interferes more with attachment of Gram-positive *L. monocytogenes* than attachment of Gram-negative *S. Typhimurium*. This may be caused by the much stronger interaction of casein with *L. monocytogenes* cell surface or that the change in hydrophobicity of the *L. monocytogenes* cell surface caused by casein was more pronounced than that of *S. Typhimurium*. In the present study, milk components may also have interacted more with *L. monocytogenes* than the two Gram-negative bacteria, which further affected the interaction of CBO molecules with *L. monocytogenes* or protected *L. monocytogenes* more than the two Gram-negative bacteria. Similar results were reported for the microemulsion of eugenol being more effective in inhibiting Gram-negative bacteria *E. coli* O157:H7 than against *L. monocytogenes* in milk (Gaysinsky et al., 2007).

3.3. Inhibition of bacteria in TSB and 2% reduced fat milk as analyzed with the Weibull model

Viable cells of *L. monocytogenes* and *E. coli* O157:H7 treated by free CBO or CBO microemulsions in TSB for 24-120 h are shown in Fig. 1 according to the Weibull model (Eq. 1). No inhibition of bacteria was observed for the microemulsion control, and the ethanol control at an ethanol level identical to the free CBO treatments also did not show the bacterial inhibition (data not shown). The Weibull model is commonly used to quantitatively analyze inactivation kinetics of bacteria by antimicrobials (de Oliveira et al., 2013; Sanz-Puig et al., 2016). The estimated values of model parameters are

263 presented in Table 3. For *L. monocytogenes* in TSB, the treatment with 625 ppm free
 264 CBO had a concave upward curve (Fig. 1A), corresponding to a p value of 0.36. In
 265 contrast, all p values of microemulsion treatments with 625 ppm CBO were close to 1,
 266 which indicates the first-order kinetics of inactivating *L. monocytogenes* (Fig. 1A). The δ
 267 by CBO in microemulsions ranged from 134 to 140 min and was higher at a higher
 268 content of CBO (Table 3). The $t_{3\text{-log}}$ was only about 25 min for the treatment of 625 ppm
 269 free CBO, while this time was from 368 to 378 min for microemulsion treatments. For *E.*
 270 *coli* O157:H7, all curves were concave upward (Fig. 1B), with p values ranging from
 271 0.35 to 0.84. The treatment of 625 ppm free CBO had the smallest δ (0.92 min) and p
 272 (0.35) values (the steepest slope in Fig. 1B), indicating the fastest initial inactivation of
 273 the bacteria. The $t_{3\text{-log}}$ of *E. coli* O157:H7 was 22 min for the free CBO treatment, while
 274 up to 81 min of $t_{3\text{-log}}$ was needed for microemulsion treatments that also showed a longer
 275 $t_{3\text{-log}}$ for a higher SBO content (Table 3).

276 For *L. monocytogenes* in 2% reduced fat milk (Fig. 2A), the treatment with
 277 10,000 ppm of free CBO had a p value of 0.87, while p values of microemulsion
 278 treatments were greater than 1, corresponding to concave downward curves. The δ value
 279 was the smallest for the free CBO treatment (16.74 min), suggesting the quicker initial
 280 inactivation of the bacteria by free CBO than CBO microemulsions. The $t_{3\text{-log}}$ of *L.*
 281 *monocytogenes* in milk also followed the increasing order of free CBO (~59 min) <
 282 microemulsion of CBO without SBO (~73 min) < microemulsion with a CBO:SBO mass
 283 ratio of 4:1 (~88 min) < microemulsion with a CBO:SBO mass ratio of 2:1 (~105 min).
 284 For *E. coli* O157:H7, p values for all treatments with 2,500 ppm CBO were less than 1,

with that of the free CBO treatment (0.13) being the smallest (Fig. 2B). The $t_{3-\log}$ of *E. coli* O157:H7 followed the same order as that of *L. monocytogenes*.

When microemulsions are mixed with milk inoculated with bacteria, CBO is expected to partition between swollen micelles (if no structural changes) and the continuous aqueous phase (milk serum), and the presence of a higher amount of SBO favors the retention of CBO in oil droplets. CBO in the milk serum further binds bacterial cells and milk protein and fat globules. Because the concentration of EOs in the milk serum is positively correlated to the inactivation rate of bacteria (Chen et al., 2014), results in Fig. 2 indicate that incorporation of SBO in microemulsions slowed the release of CBO into the milk serum. The retention of EOs in swollen micelles may be a useful feature when applied on food products, such as antimicrobial coatings or films, because the reduction of EO evaporation is critical to maintain a sufficient concentration of antimicrobials to inhibit pathogens during the shelf-life storage of a product (Zhang et al., 2015). Future biophysical understandings of microemulsion structures, energies involved with binding between CBO and microemulsion and milk components, as well as bacteria cells, and modeling the mass transfer dynamics of CBO will greatly enhance the understanding of results in the present study. Such biophysical studies will also facilitate the formulation of microemulsions so that the release of EOs to the continuous phase of food matrices and eventually the effective concentration available to inactivate bacteria can be maximized.

3.4. Effect of Tween 80 content on the antimicrobial activity of EOs

Since a relatively high concentration of Tween 80 was used to prepare microemulsions of EOs, effects of Tween 80 on the antimicrobial activity of EOs were investigated. In this study, Tween 80 and EOs were simply mixed at different mass ratios. Tween 80 alone used at a concentration corresponding to the highest amount (9:1 mass ratio of Tween 80 and CBO) showed no antimicrobial effect (Table 4). Without Tween 80, 313 ppm of CBO reduced *L. monocytogenes* by 1.98 log CFU/mL, and 625 ppm of eugenol and thyme oil resulted a reduction of 0.26 and 5.28 log CFU/mL, respectively. At 1:1 mass ratio of Tween 80 and EO, the reduction of *L. monocytogenes* significantly decreased when compared to free EOs except for the thyme oil treatment, showing only a 1.20 log CFU/mL reduction in the treatment of 313 ppm CBO and bacteria growth in the treatment of 625 ppm eugenol. Overall, as the concentration of Tween 80 increased, the viable cells of *L. monocytogenes* significantly increased, which indicated the negative effect of Tween 80 on the antimicrobial activity of EOs. Similar results have been reported in other studies that Tween 80 can reduce the antimicrobial activity of EOs (Hammer et al., 1999; Inouye et al., 2001; Remmal et al., 1992). This may be due to the hydrophobic binding between Tween 80 and EOs reducing the amount of EOs available to interact with bacteria. No significant differences in antimicrobial efficacy were detected between the treatment with equal mass of Tween 80 and eugenol and the control without EOs, indicating the impact of Tween 80 on eugenol was more pronounced than on thyme oil and CBO which may be due to the stronger binding between Tween 80 and eugenol.

The improvement in antimicrobial efficacy of EOs dissolved in microemulsions can be attributed to the increased antimicrobial concentration in the continuous phase, the enhanced bacteria cell membrane permeability to allow better access of antimicrobials, and the enhanced interaction between bacteria cell membrane components and antimicrobials (Gaysinsky et al., 2007). Specifically, the antimicrobial efficacy of EOs in microemulsions can be affected by the structure and quantity of surfactants, the composition of oil phase, and the composition of media. Shaaban and Edris (2015) showed that carvacrol microemulsion formulated with cationic cetylpyridinium chloride had a significantly higher antimicrobial activity than that formulated with Tween 20. Glycerol monolaurate microemulsions prepared with ethoxylated sorbitan esters and sucrose esters were observed to have an improved antimicrobial activity than free glycerol monolaurate (Zhang et al., 2007). However, in our study, Tween 80 used to solubilize CBO and SBO used to enhance the dilutability of CBO (Ma and Zhong, 2015) reduced the antimicrobial activity of CBO. Therefore, although microemulsions can improve the water solubility of EOs, the formulation should be chosen carefully to prevent any negative effect on antimicrobial activities.

4. Conclusions

Overall, microemulsions can be used to solubilize EOs but are only able to deliver a certain EO such as CBO without comprising antimicrobial activity significantly. Tween 80 had negative effects on the antimicrobial activity of EOs, and SBO used to facilitate the solubilization of EOs in microemulsions additionally lowered the antimicrobial

activity of EOs. These observations point to the need of biophysical studies to illustrate fundamental phenomena underlying the distribution of EOs in application matrices and the correlation to their antimicrobial activities. A large amount of surfactants used to prepare microemulsions and the potential sensory effects of EOs are additional barriers preventing their use in real food systems. The more practical applications of the studied microemulsions may be to coat semi-solid foods such as cantaloupes whose surface is not consumed. These applications also utilize the ability of SBO in microemulsions to retain EOs so as to reduce the evaporation of EOs and enable the gradual release of EOs to achieve long-term efficacy during shelf-storage of food products.

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Figure Captions

Fig. 1. Survival curves corresponding to the inactivation of *Listeria monocytogenes* (A) and *Escherichia coli* O157:H7 (B) cocktail in tryptic soy broth at room temperature (21 °C) by 625 ppm cinnamon bark oil (CBO) dissolved in ethanol (diamond) or microemulsions prepared with an oil phase with CBO and soybean oil at mass ratios of 1:0 (square), 2:1 (triangle) and 4:1 (circle). Curves were fitted using the Weibull model (Mafart et al., 2002).

Fig. 2. Survival curves corresponding to the inactivation of *Listeria monocytogenes* (A) and *Escherichia coli* O157:H7 (B) cocktails in 2% reduced fat milk at room temperature (21 °C) by 2,500 ppm (for *E. coli* O157:H7) or 10,000 ppm (for *L. monocytogenes*) cinnamon bark oil (CBO) dissolved in ethanol (diamond) or microemulsions prepared with an oil phase containing CBO and soybean oil at mass ratios of 1:0 (square), 2:1(triangle) and 4:1 (circle). Curves were fitted using the Weibull model (Mafart et al., 2002).

458 Table 1. Minimum inhibitory concentration (MIC, ppm) of free or microemulsified
 459 cinnamon bark oil (CBO), eugenol, or thyme oil.

Antimicrobials	<i>L. monocytogenes</i>	<i>S. enterica</i>	<i>E. coli</i> O157: H7
Free EO (dissolved in ethanol)			
CBO	313	625	313
Eugenol	625	625	625
Thyme oil	625	313	313
Microemulsions with mass ratios of EO:soybean oil in parentheses			
CBO (1:0)	625	625	313-625
CBO (2:1)	625	625	625
CBO (4:1)	625	625	625
Eugenol (1:0)	>5000	2500	2500
Eugenol (2:1)	>5000	5000	5000
Eugenol (4:1)	>5000	2500	2500
Thyme oil (1:0)	>5000	>5000	>5000
Thyme oil (2:1)	>5000	>5000	>5000
Thyme oil (4:1)	>5000	>5000	>5000

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Table 2. Log reduction of *Listeria monocytogenes*, *Salmonella enterica* and *Escherichia coli* O157:H7 cocktails* after treatment with free or microemulsified cinnamon bark oil (CBO) in 2% reduced fat milk at room temperature (21°C) for 24 h.

Antimicrobial concentration (ppm)	Reduction (log CFU/mL)**		
	<i>L. monocytogenes</i>	<i>S. enterica</i>	<i>E. coli</i> O157:H7
Free CBO (dissolved in ethanol)			
625 (3000)***	0.08 ± 0.02 ^a	0.26 ± 0.06 ^b	0.19 ± 0.13 ^c
1250 (4000)	0.11 ± 0.05 ^a	0.68 ± 0.16 ^b	0.45 ± 0.17 ^c
2500 (5000)	0.18 ± 0.05 ^a	4.08 ± 0.48 ^a	3.53 ± 0.45 ^a
Microemulsion of CBO:soybean oil (SBO) mass ratio of 1:0			
625 (3000)	0.11 ± 0.04 ^a	0.14 ± 0.01 ^b	0.15 ± 0.11 ^c
1250 (4000)	0.10 ± 0.03 ^a	0.58 ± 0.11 ^b	0.40 ± 0.11 ^c
2500 (5000)	0.11 ± 0.02 ^a	3.84 ± 0.49 ^a	2.58 ± 0.19 ^b
Microemulsion of CBO:SBO mass ratio of 2:1			
625 (3000)	0.09 ± 0.05 ^a	0.08 ± 0.08 ^b	0.15 ± 0.14 ^c
1250 (4000)	0.13 ± 0.04 ^a	0.48 ± 0.07 ^b	0.36 ± 0.09 ^c
2500 (5000)	0.19 ± 0.02 ^a	3.53 ± 0.50 ^a	2.33 ± 0.15 ^b
Microemulsion of CBO:SBO mass ratio of 4:1			
625 (3000)	0.10 ± 0.04 ^a	0.19 ± 0.06 ^b	0.14 ± 0.10 ^c
1250 (4000)	0.16 ± 0.07 ^a	0.51 ± 0.13 ^b	0.33 ± 0.10 ^c
2500 (5000)	0.17 ± 0.04 ^a	3.91 ± 0.56 ^a	2.51 ± 0.20 ^b

* Average initial population of *L. monocytogenes*, *S. enterica* and *E. coli* O157:H7 was 5.64, 6.09 and 6.00 log CFU/mL, respectively.

**Numbers are mean ± standard error (n ≥ 4). Different superscript letters in the same column indicate mean values differ significantly ($p < 0.05$).

***Different antimicrobial concentrations were tested for different bacteria; those in parentheses were used for the *L. monocytogenes* cocktail only.

472 Table 3. Parameters in the Weibull model estimated from inactivation curves of *Listeria monocytogenes* or *Escherichia coli* O157:H7
473 exposed to cinnamon bark oil (CBO) or microemulsified CBO in tryptic soy broth (TSB) or in 2% reduced fat milk, and the predicted
474 time required for a 3-log reduction ($t_{3\text{-log}}$).

Media	Bacteria	Treatment and CBO concentration (ppm)	p	δ	Log N_0	$t_{3\text{-log}}$
TSB	<i>L. monocytogenes</i>	Free CBO (625)	0.36	1.22	5.92	24.88
		Microemulsion, 1:0 (625)	1.09	133.71	6.20	367.50
		Microemulsion, 2:1 (625)	1.11	139.82	6.21	375.95
		Microemulsion, 4:1 (625)	1.08	137.09	6.21	378.36
	<i>E. coli</i> O157:H7	Free CBO (625)	0.35	0.92	5.99	21.97
		Microemulsion, 1:0 (625)	0.81	14.92	5.95	57.87
		Microemulsion, 2:1 (625)	0.72	17.33	6.07	80.51
		Microemulsion, 4:1 (625)	0.84	18.35	5.92	67.53
Milk	<i>L. monocytogenes</i>	Free CBO (10,000)	0.87	16.74	6.30	59.10
		Microemulsion, 1:0 (10,000)	1.25	30.21	6.37	72.83
		Microemulsion, 2:1 (10,000)	2.04	61.37	6.15	105.11
		Microemulsion, 4:1 (10,000)	1.61	44.29	6.24	87.72
	<i>E. coli</i> O157:H7	Free CBO (2,500)	0.13	0.00	6.02	1.58
		Microemulsion, 1:0 (2,500)	0.26	0.17	6.01	12.27
		Microemulsion, 2:1 (2,500)	0.29	0.36	6.04	15.47
		Microemulsion, 4:1 (2,500)	0.26	0.16	6.07	11.72

Table 4. Log reduction of *Listeria monocytogenes* cocktail* after treatment by mixtures with different mass ratios of Tween 80 and essential oil (EO) in tryptic soy broth at 32°C for 24 h.

Mass ratio of Tween 80:EO	Reduction (log CFU/mL)**		
	CBO	Eugenol	Thyme oil
0:1	1.98 ± 0.62 ^a	0.26 ± 0.25 ^a	5.28 ± 0.05 ^a
1:1	1.20 ± 0.49 ^b	-3.06 ± 0.02 ^b	5.25 ± 0.04 ^a
5:1	-1.00 ± 0.21 ^c	-3.07 ± 0.07 ^b	-2.72 ± 0.01 ^b
9:1	-1.36 ± 0.28 ^c	-2.99 ± 0.02 ^b	-2.85 ± 0.00 ^{bc}
1:0	-3.03 ± 0.06 ^d	-3.03 ± 0.06 ^b	-3.03 ± 0.06 ^d
Control	-2.97 ± 0.03 ^d	-2.97 ± 0.03 ^b	-2.97 ± 0.03 ^{cd}

* Average initial population was 6.30 log CFU/mL.

** Negative values indicate the increased population of bacteria after 24 h. Numbers are mean ± standard error (n ≥ 3). Different superscript letters in the same column indicate mean values differ significantly ($p < 0.05$).

Fig. 1. Survival curves corresponding to the inactivation of *Listeria monocytogenes* (A) and *Escherichia coli* O157:H7 (B) cocktail in tryptic soy broth at room temperature (21 °C) by 625 ppm cinnamon bark oil (CBO) dissolved in ethanol (diamond) or microemulsions prepared with an oil phase with CBO and soybean oil at mass ratios of 1:0 (square), 2:1 (triangle) and 4:1 (circle). Curves were fitted using the Weibull model (Mafart et al., 2002).

Fig. 2. Survival curves corresponding to the inactivation of *Listeria monocytogenes* (A) and *Escherichia coli* O157:H7 (B) cocktails in 2% reduced fat milk at room temperature (21 °C) by 2,500 ppm (for *E. coli* O157:H7) or 10,000 ppm (for *L. monocytogenes*) cinnamon bark oil (CBO) dissolved in ethanol (diamond) or microemulsions prepared with an oil phase containing CBO and soybean oil at mass ratios of 1:0 (square), 2:1 (triangle) and 4:1 (circle). Curves were fitted using the Weibull model (Mafart et al., 2002).