Inhibition of *Escherichia coli* O157:H7 and *Listeria monocytogenes* growth in milk and cantaloupe juice by thymol nanoemulsions prepared with gelatin and lecithin

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Abstract

Thymol is an effective antimicrobial and is to be encapsulated for use in liquid foods. Previously, novel thymol nanoemulsions have been prepared with the emulsifier combination of gelatin-A (acid-hydrolyzed) or -B (base-hydrolyzed) and lecithin. The objective of this study was to characterize the antimicrobial activity of thymol nanoemulsified by gelatin and soy lecithin, using milk and cantaloupe juice as two model food systems at 21 °C. Nanoemulsions were overall more effective than free thymol in both media. Compared to the bacteriostatic effect of free thymol, 4.5 g/L nanoemulsified thymol gradually reduced *Listeria monocytogenes* by 5 and 3 log CFU/mL in 2% reduced fat and full fat milk after 48 h, respectively. In skim milk, the nanoemulsion with gelatin-A was overall more effective against *L. monocytogenes* than that with gelatin-B. Conversely, the nanoemulsion with gelatin-B reduced *Escherichia coli* O157:H7 to below the detection limit after 8 h in 2% reduced fat milk and 48 h in full fat milk, contrasting with the bacteria recovery for the nanoemulsion with gelatin-A after 48 h. The improved antimicrobial activities of thymol nanoemulsions were supported by the quantified increase of thymol concentration in the serum of milk. The nanoemulsion prepared with gelatin-B was more effective than that of gelatin-A in cantaloupe juice. The slight difference in bacterial recovery after treatment by nanoemulsions prepared with two types of gelatin suggests the impacts of droplet surface properties on interactions with bacteria membrane. The studied nanoemulsions have great potential for use as novel antimicrobial preservatives to improve food safety.

Keywords: thymol nanoemulsion, gelatin-lecithin, antimicrobial activity, milk, cantaloupe juice

Introduction

Listeria monocytogenes and *Escherichia coli* O157:H7 are two major foodborne pathogens, and their infections cost \$2.8 billion and \$0.2 billion annually, respectively (USDA ERS, 2014). Listeriosis has been linked to consumption of contaminated milk, cheese, meat, fish, and vegetables (Barancelli *et al.*, 2014; Bell & Kyriakides, 1998; Jesus *et al.*, 2016). The ability of *L. monocytogenes* to form biofilms is another concern (Lee, Cappato, Corassin, Cruz, & Oliveira, 2016). Undercooked ground beef (Mead & Griffin, 1998), unpasteurized milk (Rangel, et al., 2005), and fruit juice (Besser, et al., 1993) are food matrices that have been associated with outbreaks of illnesses caused by *E. coli* O157:H7.

Despite tremendous progresses in improving microbiological food safety, novel and effective strategies are still needed to reduce foodborne illnesses. Natural antimicrobials such as essential oils (EOs) have been extensively studied because they are highly effective against a broad spectrum of pathogens and are generally-recognized-as-safe (GRAS) according to the Food and Drug Administration of the United States (Weiss, Gaysinsky, Davidson, & McClements, 2009). However, poor water solubility and hydrophobic binding with food components such as proteins and lipids strongly reduce the antimicrobial activity of EOs, which may be improved by encapsulation in colloidal systems.

Various colloidal systems have been studied as delivery systems to achieve the antimicrobial efficacy of EOs in foods. Synthetic surfactants such as polysorbates are studied to form thermodynamically stable systems - microemulsions that have a droplet size of 2-100 nm (McClements & Rao, 2011). Microemulsions increase the concentration of EOs in the aqueous phase and can effectively inhibit the growth of bacteria including *L. monocytogenes* and *E. coli* O157:H7 (Gaysinsky, Davidson, Bruce, & Weiss, 2005; Gaysinsky, et al., 2007). The potential

toxicity of synthetic surfactants however limits their usage in food. GRAS food biopolymers, such as proteins and polysaccharides can also be used as emulsifying agents to prepare emulsions and nanoemulsions of EOs. For example, thymol emulsified by whey protein-maltodextrin conjugates and sodium caseinate exhibited the enhanced antilisteril activity in milk when comparing to unencapsulated (free) thymol (Pan, Chen, Davidson, & Zhong, 2014; Xue, Davidson, & Zhong, 2013). Gum arabic, pectin, gelatin, and soy protein are other biopolymers that can be used to prepare EO emulsions (Dickinson, 2009; Luo, et al., 2014). Compared to microemulsions, emulsions and nano-emulsions are only kinetically stable but can be prepared with less surfactants to disperse a certain amount of EOs. With droplets smaller than 200 nm in diameter, nanoemulsions are much more stable than conventional emulsions against gravitational sedimentation or creaming (Weiss, et al., 2009). Therefore, nanoemulsions prepared with GRAS food biopolymers have advantages as delivery systems for applications in foods.

To improve the emulsifying activity of gelatin, soy lecithin was used in combination with gelatin in our recent study (Xue & Zhong, 2014). Both acid- (gelatin-A, with an isoelectric point (pI) of around pH 8-9) and base-hydrolyzed products (gelatin-B, with a pI around pH 4-5) (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011) demonstrated synergistic surface activity with lecithin to form thymol nanoemulsions. Thymol nanoemulsions produced with the gelatin-lecithin blend were translucent and stable, while those prepared with individual emulsifiers were turbid and less stable. In addition, nanoemulsions prepared with the gelatin B-lecithin blend had smaller droplets and better stability than those with the gelatin A-lecithin blend.

The objective of the present study was to characterize the antimicrobial activity of thymol nanoemulsified by blends of gelatin and soy lecithin. Milk with three fat levels (skim, 2%

reduced fat, and full fat) and cantaloupe juice were studied as model food systems. *E. coli* O157:H7 ATCC 43895 and *L. monocytogenes* Scott A were tested as model foodborne pathogens. Cantaloupe juice was chosen because its neutral acidity (FDA, 2015) may minimize the interference of juice acidity during assessment of antimicrobial activity of EO emulsions (Shah, Davidson, & Zhong, 2012).

Materials and Methods

Materials

Thymol (99% purity) and gelatin-A were purchased from Acros Organics (Morris Plains, NJ). Gelatin-B, soy lecithin (phosphatidylcholines), propylene glycol (PG), and methanol were obtained from Fisher Scientific (Pittsburgh, PA). Tryptic soy broth (TSB), peptone, and agar (chemical grade) were purchased from Becton, Dickinson and Company (Sparks, MD). Ultrahigh temperature (UHT)-pasteurized organic milk (skim, 2% reduced fat, and full fat) (Kroger Company, Cincinnati, OH) and fresh cantaloupe were purchased from a grocery store.

Preparation and characterization of thymol nanoemulsions

Thymol nanoemulsions were prepared as in our previous recent study (Xue & Zhong, 2014). 0.02 g gelatin was dissolved in 9 mL deionized water. 0.08 g soy lecithin was added into the gelatin solution, followed by vortexing for 15 min at room temperature (21 °C), and the pH was 6.8. The working thymol solution was prepared separately by dissolving 0.1 g thymol in 1 mL PG. The gelatin/lecithin sample and thymol solution were mixed and emulsified at 12,000 rpm for 2 min using a model IKA 25 digital Ultra Turrax homogenizer (IKA Works, Inc., Wilmington, NC). Hereafter, nanoemulsions prepared with lecithin in combination with gelatin A and B are abbreviated as nEgA and nEgB, respectively.

The mean hydrodynamic diameter and polydispersity index of nanoemulsions were determined using a DelasTM Nano-Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter, Fullerton, CA). The zeta-potential was measured using a model Zetasizer instrument (Malvern Instruments, Worcestershire, UK). Nanoemulsions were diluted 20 and 30 times in 0.01 M phosphate-buffered saline (PBS, pH 7) before the particle size and zeta potential measurement, respectively.

Culture preparation

E. coli O157:H7 ATCC 43895 and *L. monocytogenes* Scott A were obtained from the culture collection of the Department of Food Science and Technology at the University of Tennessee (Knoxville, TN). All strains were kept at -20 °C in glycerol. Each strain was transferred at least twice in TSB with an interval of 24 h before use.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in TSB

The MIC was determined using a microbroth dilution assay (Davidson & Parish, 1989). The stock free thymol solution was prepared by diluting a thymol solution (10% w/v in ethanol) to 0.1% w/v with TSB. The 0.1% w/v stock solution was further diluted with TSB to working solutions with various thymol concentrations. Emulsion samples were directly diluted to the same thymol concentrations with TSB. A culture with ca. 10⁶ CFU/mL bacteria and an antimicrobial solution were added sequentially at 120 µL each in wells of 96-well microtiter plates. A negative control was prepared using 240 µL TSB, and a positive control was prepared by substituting an antimicrobial treatment with TSB. Absorbance was determined at 630 nm using an Elx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) at time 0 and after 24 h incubation at 32 °C (for *L. monocytogenes*) or 37 °C (for *E. coli* O157:H7).

Inoculated wells with an increase in absorbance of <0.05 after 24 h incubation were considered inhibited and the lowest thymol concentration showing inhibition was defined as the MIC. For wells showing inhibitions, 20 μL of the mixture was transferred to tryptic soy agar (TSA) plates. If there was no growth on the TSA plates after 24-h incubation at 32 °C (for *L. monocytogenes*) or 37 °C (for *E. coli* O157:H7), the lowest thymol concentration was determined as the MBC.

Antimicrobial susceptibility test in milk

The antimicrobial susceptibility in three types of milk was studied in duplicate using time-kill assays (Shah, Davidson, & Zhong, 2013). For skim milk, 1 mL thymol nanoemulsions were added into 9 mL milk. For 2% reduced fat and full fat milk, 4.5 mL nanoemulsions and 5.5 mL milk were mixed. For the free thymol treatment, 0.01 g or 0.045 g thymol was added directly into milk with a volume identical to nanoemulsion treatments and mixed with an end-to-end shaker (Laboratory Industries Inc., Berkeley, CA) at room temperature (21 °C) for 30 min, followed by increasing the volume to 10 mL using sterilized deionized water. The control sample was prepared by mixing the same volume of milk with sterilized deionized water to a total volume of 10 mL. The overall concentration of thymol in the final mixture was 1, 4.5, and 4.5 g/L in skim, 2% reduced fat, and full fat milk, respectively. All milk samples were then mixed with 100 μL culture with $\sim 10^8$ CFU/mL bacteria. After incubation at room temperature (21 °C) for 0, 4, 8, 24, and 48 h, samples were diluted with 0.1% peptone, and survivors were enumerated after plating on TSA and incubating at 32 °C (for *L. monocytogenes*) or 37 °C (for *E. coli* O157:H7) for 24 h. The detection limit was 1.0 log CFU/mL.

Quantification of thymol dissolved in the serum of milk

Quantification of (truly-dissolved) thymol in the serum of milk followed the protocol in our early work (Xue et al., 2013). Milk (without bacteria culture) and antimicrobials were

prepared as above. The mixture was acidified to pH 4.6 using 1.0 M HCl to precipitate caseins, followed by centrifugation at 4629 ×g for 5 min using a RC-5B Plus centrifuge (Sorvall, Inc. Norwalk, CT). The supernatant was filtered through a 0.45 µm poly (vinyl difluoride) syringe membrane filter (Fisher Scientific). Thymol concentration in the permeate was analyzed using a 1200 series high performance liquid chromatography (HPLC) system (Agilent Technologies, Inc., Santa Clara, CA). A ZORBAX Eclipse Plus C18 column with a coupling guard column (4.6 mm ×12.5 mm; 5 µm; Agilent, Palo Alto, CA) was used. The mobile phase was a binary solvent with different proportions of methanol (solvent A) and HPLC grade water (solvent B) in the following steps: a linear gradient from 20% A to 80% A within 20 min, an isocratic step with 80% A in 21–25 min, a linear gradient from 80% A to 20% A in 26–30 min, and an isocratic step with 20% A in 31–35 min. The flow rate was 0.5 mL/min, and the temperature of the column was 25 °C. 10 µL of each sample was injected directly into the HPLC. The absorbance at 274 nm was measured with a UV detector. A calibration curve was established using five standard solutions with 0.02, 0.04, 0.06, 0.08, and 0.10% w/v of thymol.

Antimicrobial susceptibility test in cantaloupe juice

Preparation of cantaloupe juice. Fresh cantaloupes were washed with tap water. After removing rinds, the flesh was cut into pieces and blended using a Hamilton Beach 58148A Power Elite Multi-Function Blender (Hamilton Beach Brands, Inc. Southern Pines, NC). The obtained puree was centrifuged at 4629 ×g for 20 min using a RC-5B Plus centrifuge (Sorvall, Inc. Norwalk, CT) and the supernatant was collected and autoclaved. After centrifuging the autoclaved sample in 50 mL sterilized tubes at 8000g for 5 min at 25°C (Sorvall Legend 26R; Thermo Scientific, Waltham, MA), the obtained supernatant was used as the working juice sample in antimicrobial experiments.

Log-reduction. Different volumes (0.4, 0.6, 0.8, and 1.0 mL) of emulsions with 10 g/L thymol were mixed with 9 mL cantaloupe juice, followed by adding sterilized water to a total volume of 10 mL. Two types of free thymol were studied. In the first type, thymol crystals (0.004, 0.006, 0.008, 0.01 g) were added in the 9 mL juice directly. In the second type, different volumes (40, 60, 80, and 100 μ L) of a thymol stock solution (pre-dissolved at 10% w/v in PG) were mixed with the 9 mL juice. The 10 mL juice samples were mixed with 100 μ L culture with $\sim 10^8$ CFU/mL *L. monocytogenes*. After incubation at room temperature (21 °C) for 24 h, samples were serially diluted with 0.1% peptone, and survivors enumerated after plating on TSA and incubating at 32 °C for 24 h. The detection limit was 1.0 log CFU/mL.

Time-kill assay. Growth kinetics of *L. monocytogenes* and *E. coli* O157:H7 at 21°C in cantaloupe juice after antimicrobial treatments was studied following the protocol used in the above milk treatments. The juice samples were prepared to a final thymol concentration of 0.6 g/L. After incubation at room temperature (21 °C) for 0, 3, 6, 24, and 48 h, bacteria were enumerated as above.

Statistical analysis

Analysis of variance (ANOVA) test was conducted using SAS software (version 9.3; SAS Institute, Cary, NC). Differences between pairs of means were analyzed using a Tukey test at a significance level of 0.05.

Results and Discussion

Characteristics of thymol nanoemulsions

The mean hydrodynamic diameter, polydispersity index, and zeta-potential of two thymol emulsions at neutral pH are shown in Table 1. nEgB had a significantly smaller droplet diameter

and polydispersity index, and higher magnitude of zeta-potential than nEgA ($P < 0.05$), which may be attributed to different interactions between two types of gelatin and lecithin due to their different pI values (Xue & Zhong, 2014).

MICs and MBCs of free and nano-emulsified thymol in TSB

MICs and MBCs of free and nanoemulsified thymol against *E. coli* O157:H7 and *L. monocytogenes* are shown in Table 2. The MICs and MBCs of free and nanoemulsified thymol were either identical or different only by one level in the dilution scheme. The MICs and MBCs of free thymol in Table 2 were in agreement with our previous studies (Chen, Davidson, & Zhong, 2014; Wu, Lin, & Zhong, 2014; Xue, et al., 2013). The overall similar MICs and MBCs of thymol before and after nanoemulsification indicate insignificant impacts of the studied emulsifier formulations on antimicrobial activity of nanoemulsified thymol. Previously, the binding of EO components with lecithin and polysorbate 80 was reported to reduce the antimicrobial activity (Li, 2011; Terjung, Löffler, Gibis, Hinrichs, & Weiss, 2012). In our previous study, gelatin and lecithin were observed to co-adsorb on thymol nanoparticles (Xue & Zhong, 2014). The presence of water-soluble gelatin on nanoparticles can weaken the overall hydrophobic binding between emulsifiers and thymol, which may have resulted in similar MICs and MBCs of free and nanoemulsified thymol in the present study.

Antimicrobial activities of thymol in milk

The antimicrobial activities of free and nanoemulsified thymol against *L. monocytogenes* and *E. coli* O157:H7 in skim, 2% reduced fat, and full fat milk are shown in Figures 1, 2, and 3, respectively. In skim milk, 1 g/L free thymol was only bacteriostatic against *L. monocytogenes* within 48 h. The two nanoemulsions demonstrated a similar inactivation rate within 24 h, followed by further reduction for the treatment with nEgA and no significant change for the

treatment with nEgB after 48 h (Figure 1A). *E. coli* O157:H7 was less resistant than *L. monocytogenes* in skim milk (Figure 1B). Free thymol reduced more than 3 log CFU/mL *E. coli* O157:H7 after 24 h, while nanoemulsions exhibited a faster reduction rate than free thymol, showing undetectable *E. coli* O157:H7 after 24 h for the nEgB treatment. After 48 h, a recovery of *E. coli* O157:H7 was observed for all treatments, which was ~ 4 log CFU/mL for free thymol and nEgA treatments but only 2 log CFU/mL for the nEgB treatment (Figure 1B).

In 2% reduced fat milk (Figure 2A), 4.5 g/L free thymol was also bacteriostatic against *L. monocytogenes* even though the level of thymol was higher than in skim milk (1 g/L). In contrast, the two emulsions effectively inhibited the growth of *L. monocytogenes* and exhibited the same reduction rate within 48 h (Figure 2A). For *E. coli* O157:H7, the bacteria population was reduced by two emulsions to below the detection limit (1 log CFU/mL) within 4 h, while it took 24 h for free thymol to reduce *E. coli* O157:H7 to 1.2 log CFU/mL. After 48 h, the population of *E. coli* O157:H7 was 1.5 log CFU/mL for the free thymol and nEgA emulsion treatments but was still undetectable for the treatment with nEgB (Figure 2B).

In full fat milk, the two emulsions applied at 4.5 g/L thymol gradually reduced *L. monocytogenes* to 2.8 log CFU/mL in 48 h, contrasting with a ~ 1 log CFU/mL reduction in the first 4 h and no subsequent changes in 48 h by the same concentration of free thymol (Figure 3A). The two emulsions also showed similar reduction rates against *E. coli* O157:H7 within 24 h, which were faster than the treatment with free thymol (Figure 3B). Similar to the observations in 2% reduced fat milk (Figure 2B), *E. coli* O157:H7 treated by the nEgA emulsion and free thymol recovered to 2.7 log CFU/mL but was undetectable in the nEgB treatment after 48 h (Figure 3B).

Overall, both thymol emulsions were more effective than free thymol against *L. monocytogenes* and *E. coli* O157:H7 in milk, and the nEgB was more effective than the nEgA in

inhibiting the recovery of *E. coli* O157:H7 after 48 h. The increased thymol concentration required to inhibit bacteria in milk with a higher fat content is due to binding with lipid particles, as previously reported (Chen, et al., 2014). The enhanced antimicrobial activity of thymol in milk was also observed in our recent study after nanoemulsification in whey protein-maltodextrin conjugate and sodium caseinate (Pan, et al., 2014; Xue, et al., 2013).

Thymol concentration in the serum of milk (TCSM)

In our previous studies (Pan, et al., 2014; Xue, et al., 2013), nanoencapsulation of thymol in whey protein-maltodextrin or sodium caseinate increased the TCSM, which was correlated to their antimicrobial activity in TSB. When the TCMS was higher than MBC observed in TSB, complete inhibition of *L. monocytogenes* was observed. Conversely, bacteriostatic or partial inhibition of *L. monocytogenes* was found when the TCMS was between MIC and MBC. By analyzing TCMS, it is then possible to correlate antimicrobial activity in simple (TSB) and complex media (milk, with protein and lipid particles binding antimicrobials).

The TCMSs in milk with free or nanoemulsified thymol are shown in Figure 4. Shortly after mixing with milk (0 h in Figure 4A), two emulsion treatments had a similar TCMS in all three types of milk ($P > 0.05$) and were significantly higher than free thymol treatments ($P < 0.05$). The results agreed with the quicker initial reduction rate of bacteria by nanoemulsions (Figures 1-3). When the same amount (4.5 g/L) of free or nanoemulsified thymol was added, the TCMS in full fat milk was significantly lower than that of 2% reduced fat milk ($P < 0.05$). This confirms the significant interaction between hydrophobic EO components and milk fat globules (Chen, et al., 2014) and supports the lower antimicrobial activities of thymol in full fat milk than in 2% reduced fat milk (Figure 2 vs. 3).

After 24-h incubation at room temperature (21°C), the TCMS remained significantly

higher for nanoemulsion treatments than the free thymol treatment (Figure 4B). After 48 h, the TCMS in skim milk was similar ($P > 0.05$) in free thymol and emulsion treatments (Figure 4C), suggesting that free thymol (in crystal form) slowly dissolved in skim milk. In contrast, free thymol in 2% reduced fat milk showed a significantly lower TCMS when compared to those at 0 h and 24 h ($P < 0.05$), which may indicate more binding occurred between thymol and fat globules. In full fat milk, the difference between free thymol and two emulsion treatments became insignificant ($P > 0.05$) after 48 h due to the slowly decreasing thymol concentration for nanoemulsions treatments ($P > 0.05$). Releasing from thymol crystals or emulsion droplets and binding with protein and lipid particles are two competing mechanisms impacting TCMS. Compared with nanoemulsions, the effect of incubation on the TCMS was more significant for free thymol treatments, showing the significantly increased and decreased TCMS in skim and 2% reduced fat milk ($P < 0.05$), respectively, which further supports the influence of fat globules binding with the gradually dissolved thymol in the latter treatment. Gradual decreases of TCMS were also observed for nanoemulsion treatments and the reductions were not significant ($P > 0.05$), indicating the reduced interference by milk fat globules after emulsification.

The TCMS can be correlated with MIC and MBC in TSB to elucidate the antimicrobial activity in milk. In skim milk with *L. monocytogens*, the TCMS of free thymol treatment (Figure 4) was between the MIC and MBC (Table 2), which agreed with the bacteriostatic activity (Figure 1A). For nanoemulsion treatments in skim milk, the TCMS was just a little higher than their MBC in TSB (Table 2), corresponding to incomplete inactivation (Figure 1A) which may be related to the slower antimicrobial diffusion rate in milk at a lower temperature than in TSB (21 vs. 32 °C). In skim milk with *E. coli*, the TCMSs of three treatments were all above their MBC, especially for two emulsions, which can be correlated to the significant initial reductions

(Figure 1B). In 2% reduced fat milk with *E. coli*, the TCMSs of all treatments were even higher than those in skim milk, resulting in faster inactivation rates (Figure 2B). For *L. monocytogens* in 2% reduced fat milk, the free thymol treatment had a TCMS slightly higher than the MBC and the bacteriostatic activity, while the nanoemulsion treatments had a TCMS much higher their MBC, corresponding to the significant reductions of pathogens (Figure 2A). In full fat milk, all treatments had a TCMS close to or above their MBC against *E. coli*, but below their MBC against *L. monocytogens*, which explained different antimicrobial activities observed for the two bacteria (Figure 3). The higher TCMS in full fat milk was observed for the two nanoemulsion treatments than free thymol, which also agreed with their differences in antimicrobial activities.

When comparing two nanoemulsion treatments against *E. coli* in skim milk, nEgB was more effective than nEgA after 48h, while the opposite was observed for *L. monocytogens* (Figure 1). Because the two nanoemulsions had a similar TCSM (Figure 4), different trends after 48 h may be related to physical properties of thymol droplets. Compared to nEgA, nEgB had smaller droplets (Table 1), which would favor their contact with bacteria, and overall more negatively-charged droplets, which may reduce the contact with highly negatively charged bacteria (Briandet et al., 1999) due to stronger electrostatic repulsion. It however is to be reminded that zeta-potential is a reflection of overall particle properties, and the detailed surface charge properties can result in binding of two colloidal particles of overall negative charges due to the presence of surface cationic amino acid residues (Dickinson, 2003). At neutral pH, gelatin-A is overall positively charged, while gelatin-B has more negative changes than positive ones (Gómez-Guillén, et al., 2011). Therefore, droplets in nEgA and nEgB have different local surface structures that may result in different interactions with Gram-negative and Gram-positive bacteria to induce different extents of injury which became noticeable after 48 h.

Antimicrobial activity of thymol in cantaloupe juice

Fruit juices are another large group of food products that can be easily contaminated by foodborne pathogens (Balla & Farkas, 2006). The outbreaks of foodborne illnesses have been associated with unpasteurized fruit juices (Raybaudi-Massilia, Mosqueda-Melgar, Soliva-Fortuny, & Martín-Belloso, 2009). Most fruit juices in antimicrobial studies have an acid pH, such as apple and orange juices (Ferrante, Guerrero, & Alzamora, 2007; Friedman, Henika, Levin, & Mandrell, 2004). In this part of study, the antimicrobial assays were conducted in cantaloupe juice with pH 6.81 as measured in the present study.

Log reduction of L. monocytogenes

Log reductions of *L. monocytogenes* in cantaloupe juice treated by different amounts of thymol are presented in Table 3. Two groups of free thymol were studied: crystals added in juice directly or pre-dissolved in PG. With 0.4 g/L thymol, *L. monocytogenes* population was reduced by 4.23, 2.69, and 1.02 log CFU/mL for treatments of nEgA, nEgB and thymol pre-dissolved in PG, respectively, and the differences among these three treatments were significant ($P < 0.05$). A lower log reduction by thymol pre-dissolved in PG may be related to binding between thymol and some organic compounds such as esters and aldehydes in cantaloupe juice (Beaulieu & Grimm, 2001). For 0.6 g/L thymol pre-dissolved in PG or nanoemulsified thymol, *L. monocytogenes* was reduced to below the detection limit after 24 h incubation at 21°C. In contrast, the same level (0.6 g/L) of thymol crystals did not inhibit the growth of *L. monocytogenes*, showing a 1.37 log CFU/mL increase. Increasing thymol crystal levels to 0.8 and 1.0 g/L only achieved a reduction of 0.19 and 0.50 log CFU/mL, respectively. The significant difference in anti-listerial activities between thymol crystals and nanoemulsified or pre-dissolved thymol can be attributed to the greatly enhanced thymol solubility in juice after

mixing nanoemulsified or pre-dissolved thymol with juice. Conversely, thymol crystals precipitate in juice and require the dissolution process to distribute thymol molecules throughout the juice, which also competes with the growth of bacteria.

Growth kinetics of L. monocytogenes and E. coli O157:H7

The growth kinetics of *L. monocytogenes* and *E. coli* O157:H7 in cantaloupe juice was further investigated at 0.6 g/L thymol (Figure 5). The trends against two bacteria are similar. For the thymol crystal treatment, the bacteria population did not change much within 24 h but increased slightly after 48 h ($P > 0.05$). In contrast, two emulsions and thymol pre-dissolved in PG reduced *L. monocytogenes* and *E. coli* O157:H7 to below the detection limit within 3 h. After 48 h, *L. monocytogenes* and *E. coli* O157:H7 treated by thymol pre-dissolved in PG recovered to ~3 log CFU/mL and ~2 log CFU/mL, respectively. No recovery of *E. coli* O157:H7 was observed after 48 h for both emulsions. A recovery of *L. monocytogenes* to ~3 log CFU/mL was also observed for the nEgA treatment after 48 h, while no recovery was found in the treatment with nEgB, which was in contradiction to observations in skim milk (Figure 1A). In addition to differences in droplet surface structures of the two nanoemulsions, as discussed previously, different compositions of milk and cantaloupe juice can cause a different extent of bacterial injury at the studied conditions to result in differences in the recovery of *L. monocytogenes*. The recovery of both bacteria treated by 0.3 and 0.5 g/L nano-dispersed thymol in apple juice adjusted to pH 5.5 was reported previously (Shah, Davidson, & Zhong, 2012). Differences in that study and the present study can result from different pH and composition of juices and thymol levels.

Different from thymol in milk (Figures 1-3), a much lower level (0.6 g/L) of nano-emulsified or pre-dissolved thymol can completely inhibit both bacteria in cantaloupe juice.

Because cantaloupe juice has less interfering compounds (protein and fat globules in milk), 0.6 g/L of nano-emulsified or pre-dissolved thymol is mostly available to interact with bacteria and can easily exceed the MBC (Table 2), which resulted in quick inhibition of bacteria (Figure 5). Lastly, the consistently better inhibition of nanoemulsions than thymol pre-dissolved in PG (Table 3, Figure 5) may result from the reduced binding between juice components and thymol after encapsulation.

Conclusions

In conclusion, antimicrobial activities of thymol in milk and cantaloupe juice were greatly enhanced after nano-emulsification by the gelatin and lecithin blend due to the significantly increase of truly dissolved thymol and reduced interference by food components. The two emulsions showed some differences in inhibiting the recovery of pathogens after long time storage that may be attributed to their different surface structures. The present study showed the effectiveness of thymol nanoemulsions inhibiting pathogens in different food matrices indicating their potential as novel antimicrobial preservatives to enhance microbial safety. Much work however is needed to address practicality of EO nanoemulsions such as **cost analysis**, sensory properties, and to understand biophysical mechanisms underlying antimicrobial activities of these nanoemulsions and the interference by food matrix.

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Figure captions

Figure 1. Growth kinetics of *Listeria monocytogenes* Scott A (A) and *Escherichia coli* O157:H7 (B) at 21°C in skim milk with 1 g/L free thymol and thymol emulsified with lecithin and gelatin A or B. Error bars are standard deviations (n = 2).

Figure 2. Growth kinetics of *Listeria monocytogenes* Scott A (A) and *Escherichia coli* O157:H7 (B) at 21°C in 2% reduced fat milk with 4.5 g/L free thymol and thymol emulsified with lecithin and gelatin A or B. Error bars are standard deviations (n = 2).

Figure 3. Growth kinetics of *Listeria monocytogenes* Scott A (A) and *Escherichia coli* O157:H7 (B) at 21°C in full fat milk with 4.5 g/L free thymol and thymol emulsified with lecithin and gelatin A or B. Error bars are standard deviations (n = 2).

Figure 4. Thymol concentration detected in the serum of skim, 2% reduced fat, and full fat milk that was mixed respectively with 1, 4.5, and 4.5 g/L free thymol and thymol emulsified with lecithin and gelatin A or B after 0 h (A), 24 h (B) and 48 h (C) at room temperature (21 °C). Different letters above bars indicate significant differences of the mean in the same plot ($P < 0.05$). Error bars are standard deviations (n = 2).

Figure 5. Growth kinetics of *Listeria monocytogenes* Scott A (A) and *Escherichia coli* O157:H7 (B) at 21°C in cantaloupe juice with 0.6 g/L free thymol and thymol emulsified with lecithin and gelatin A or B. Free thymol was prepared by adding crystals directly or a stock solution with 10% thymol pre-dissolved in propylene glycol (PG). Error bars are standard deviations (n = 2).

502 Table 1. Mean hydrodynamic diameter, polydispersity index, and zeta-potential of thymol
 503 emulsions prepared with lecithin and gelatin A or B at neutral pH.*

	Hydrodynamic diameter (nm)	Polydispersity index	Zeta-potential (mV)
Gelatin A-emulsion	257.97±52.23 ^a	0.32±0.03 ^a	-21.73±0.91 ^a
Gelatin B-emulsion	84.70±1.86 ^b	0.25±0.01 ^b	-35.44±2.21 ^b

504 * Numbers are mean ± standard deviations (n = 2). Different superscript letters in the same column
 505 indicate significant differences of the mean ($P < 0.05$).

506

507 Table 2. MIC and MBC of free thymol and thymol emulsions prepared with lecithin and gelatin
 508 A or B against *E. coli* O157:H7 at 37°C and *L. monocytogenes* at 32°C in TSB.

Antimicrobial	<i>E. coli</i> O157:H7		<i>L. monocytogenes</i>	
	MIC (g/L)	MBC (g/L)	MIC (g/L)	MBC (g/L)
Free thymol	0.25	0.25	0.25	0.30
Gelatin-A emulsion	0.25	0.25	0.25	0.35
Gelatin-B emulsion	0.20	0.20	0.25	0.35

509

510

Table 3. Reduction of *L. monocytogenes* Scott A (initial population of 5.58 log CFU/mL) treated with thymol crystals, thymol pre-dissolved in PG, and thymol emulsions prepared with lecithin and gelatin A or B in cantaloupe juice at room temperature (21 °C) after 24 h.

Thymol concentration (g/L)	Reduction (log CFU/mL)*			
	Thymol crystals	Thymol pre-dissolved in PG	Gelatin-A emulsion	Gelatin-B emulsion
0.4	-1.63 ± 0.09 ^d	1.02 ± 0.34 ^c	4.23 ± 0.49 ^a	2.69 ± 0.05 ^b
0.6	-1.37 ± 0.07 ^d	>4.58 ± 0.00 ^a	>4.58 ± 0.00 ^a	>4.58 ± 0.00 ^a
0.8	0.19 ± 0.61 ^c	>4.58 ± 0.00 ^a	>4.58 ± 0.00 ^a	>4.58 ± 0.00 ^a
1.0	0.50 ± 0.05 ^c	>4.58 ± 0.00 ^a	>4.58 ± 0.00 ^a	>4.58 ± 0.00 ^a

* Numbers are mean ± standard deviation from duplicates. Different superscript letters represent significant differences in the mean ($P < 0.05$).

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.