Essential Oil Nanoemulsions Prepared with Natural Emulsifiers for Improved Food Safety

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I am submitting herewith a dissertation written by Jia Xue entitled "Essential Oil Nanoemulsions Prepared with Natural Emulsifiers for Improved Food Safety." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

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Essential Oil Nanoemulsions Prepared with Natural Emulsifiers for Improved Food Safety

A Dissertation Presented for the Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Jia Xue
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ABSTRACT

As natural and effective antimicrobials, essential oils (EOs) have been increasingly investigated to control foodborne pathogens and enhance food safety. It is usually difficult to achieve high antimicrobial efficacy when directly incorporating EOs in food systems due to their low water solubility. Thus, a variety of EO delivery systems have been developed. Nanoemulsions have shown many benefits to encapsulate EOs. However, most EO nanoemulsions are currently prepared with synthetic surfactants.

The overall goal of this research was to prepare EO nanoemulsions with generally-recognized-as-safe (GRAS) emulsifying agents to enhance their antimicrobial activity in foods. The first group of transparent nanoemulsions containing thymol, the major component in thyme oil, was prepared using whey protein-maltodextrin conjugate produced through the Maillard reaction using shear homogenization, with propylene glycol as a co-surfactant. Compared to free thymol, nanoemulsions showed significantly enhanced antilisterial activity in milk, which was attributed to the increased thymol solubility in milk and synergistic antimicrobial activity with propylene glycol. The second group of stable and translucent thymol nanoemulsions were prepared using the combination of gelatin and lecithin that formed complexes with the improved emulsifying activity than individual emulsifiers. The nanoemulsions were consistently more effective than free thymol against Listeria monocytogenes and Escherichia coli O157:H7 in milk and cantaloupe juice. To improve the emulsifying capacity, the last group of nanoemulsions with thyme oil was studied using the combination of sodium caseinate (NaCas) and lecithin. Co-adsorption of NaCas and lecithin on oil droplets emulsified a higher amount of thyme oil as nanoemulsions than individual emulsifiers, reduced the occurrence of creaming, coalescence, and Ostwald ripening, and resulted in a long term storage stability. The nanoemulsified thyme oil
demonstrated similar or better antimicrobial activity than free thyme oil in tryptic soy broth and 2% reduced fat milk. Therefore, the studied nanoemulsions prepared with GRAS emulsifiers have great potential for use as novel antimicrobial preservatives to improve food safety.

**Key words:** thymol and thyme oil, nanoemulsion, GRAS emulsifiers, antimicrobial activity, milk, solubility
TABLE OF CONTENTS

Chapter 1. Introduction and literature review ................................................................. 1
1.1 Introduction ...................................................................................................................... 2
1.2 Properties of EOs ........................................................................................................... 3
  1.2.1 Composition of EOs .................................................................................................. 3
  1.2.2 Antimicrobial activity of EOs .................................................................................. 4
  1.2.3 Modes of actions of EOs .......................................................................................... 6
  1.2.4 Challenges of applying EOs in foods ......................................................................... 7
1.3 EO delivery systems ...................................................................................................... 8
  1.3.1 Liposomes ................................................................................................................ 8
  1.3.2 Biopolymeric nanoparticles ...................................................................................... 9
  1.3.3 Emulsions ................................................................................................................ 11
1.4 Preparation of nanoemulsions ....................................................................................... 12
  1.4.1 Formations of nanoemulsions .................................................................................... 12
  1.4.1.1 High energy emulsification .................................................................................. 13
  1.4.1.2 Low energy emulsification ................................................................................... 14
  1.4.2 Nanoemulsions prepared with surfactant blend ....................................................... 15
  1.4.3 Natural emulsifying agents ...................................................................................... 16
  1.4.3.1 Proteins ............................................................................................................... 17
  1.4.3.2 Phospholipids ...................................................................................................... 19
  1.4.3.3 Polysaccharides .................................................................................................... 20
  1.4.4 EO nanoemulsions .................................................................................................... 21
1.5 Hypothesis and overview of dissertation research ...................................................... 22
References ........................................................................................................................... 25
Appendix .............................................................................................................................. 40
Chapter 2. Thymol nanoemulsified by whey protein-maltodextrin conjugates: the enhanced
emulsifying capacity and anti-listerial properties in milk by propylene glycol ...................... 42
  2.1 Abstract ......................................................................................................................... 42
  2.2 Introduction ................................................................................................................... 45
  2.3 Materials and Methods ................................................................................................ 48
    2.3.1 Materials ................................................................................................................ 48
    2.3.2 Preparation of WPI-MD conjugates ......................................................................... 49
    2.3.3 Preparation of nanoemulsions ............................................................................... 49
    2.3.4 Characterization of thymol nanoemulsion ............................................................... 49
    2.3.5 Determination of anti-listerial activity .................................................................... 51
    2.3.6 Solubility of thymol in solvents .............................................................................. 53
    2.3.7 Quantification of thymol dissolved in the aqueous phase of milk ......................... 53
    2.3.8 Statistical analysis .................................................................................................. 54
2.4 Results and Discussion ................................................................................................. 55
  2.4.1 Properties of thymol emulsions ............................................................................... 55
  2.4.2 MIC and MBC in TSB ............................................................................................. 57
  2.4.3 Anti-listerial activities of antimicrobials in three types of milk ................................ 58
  2.4.4 Content of thymol in the serum of milk ................................................................. 59
2.5 Conclusions .................................................................................................................... 61

vii
LIST OF TABLES

Table 1-1. Traditional or regulatory-approved food antimicrobials .................................................. 40

Table 2-1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of free thymol, nanoemulsified thymol with and without PG, and PG alone against Listeria monocytogenes Scott A in tryptic soy broth at 32 °C......................................................... 67

Table 3-1. Volume fraction-length mean diameter ($d_{4,3}$, nm) of dispersions with 0.8% w/v thymol dispersed by 0.2% w/v gelatin B and 1.0% w/v lecithin at pH 7.0 and after acidification to pH 5.0 and 6.0, with comparison to the blend (without thymol) prepared at identical conditions........... 95

Table 3-2. Percentages of total protein in the serum of dispersions with 0.8% w/v thymol dispersed by 0.2% w/v gelatin B and 1.0% w/v lecithin at pH 7.0 and after acidification to pH 5.0 and 6.0, with comparison to the blend or gelatin B only (without thymol) prepared at identical conditions............................................................ 96

Table 4-1. MIC and MBC of free thymol and thymol emulsions against E. coli O157:H7 at 37°C and L. monocytogenes at 32°C in TSB. ........................................................................................................ 125

Table 4-2. 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 in cantaloupe juice at room temperature (21 °C) after 24 h. ................................................................. 126

Table 5-1. Absorbance at 600 nm of 1 or 2% w/v thyme oil emulsified by 2.5% w/v NaCas and 0-1.0% w/v lecithin................................................................. 154

Table 5-2. Mean hydrodynamic diameters (nm) of 1% and 2% w/v thyme oil emulsified by NaCas, lecithin or both, before and after storage at room temperature for 120 days. .............. 155

Table 5-3. Zeta-potential (mV) of thyme oil (1.0% w/v) nanoemulsions prepared with NaCas with and without lecithin at pH 7.0...................................................................................... 156

Table 5-4. Volume-area mean diameter $d_{3, 2}$ (nm), polydispersity index, percentage of adsorbed NaCas, and surface load of NaCas in emulsions with 10% w/v thyme oil prepared with NaCas, lecithin, or both, before and after storage at 21 °C for 15 days.......................................................... 157

Table 6-1. Absorbance at 600 nm, mean hydrodynamic diameter, and polydispersity index of thyme oil nanoemulsions. ...................................................................................... 181

Table 6-2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of free or nanoemulsified thyme oil against Escherichia coli O157:H7, Salmonella Enteritidis at 37°C, and Listeria monocytogenes at 32°C in tryptic soy broth. .............. 182
LIST OF FIGURES

Figure 1-1. Structure of selected EO components. ................................................................. 41

Figure 2-1. Absorbance at 600 nm (A), and volume fraction-length mean particle diameter ($d_{4,3}$) (B) of thymol emulsions prepared with 7% w/v WPI-MD conjugates, 10% v/v PG, and 0-2.0% w/v thymol, before and after heating at 80 °C for 15 min. ................................................................. 68

Figure 2-2. AFM images of thymol emulsions prepared with 7% w/v WPI-MD conjugates, 1.0% w/v thymol with (A) and without (B) 10% v/v PG. The dimension of images is 2 μm x 2 μm. ................................................. 69

Figure 2-3. Zeta-potential of WPI-MD conjugate, emulsion prepared with 7% w/v WPI-MD conjugates and 1.0% v/v thymol with and without 10% v/v PG. ................................................................. 70

Figure 2-4. Time-kill assays showing the population of Listeria monocytogenes Scott A at 32 °C in skim (A), 2% reduced fat (B), and full fat milk (C) that was treated with 0.1%, 0.45%, and 0.6% w/v thymol, respectively. Emulsions of thymol were prepared with and without propylene glycol (PG). Free thymol was tested with and without 4% v/v PG, with the PG amount equivalent to the emulsion treatment. The detection limit is 1 log CFU/mL. ................................................................. 71

Figure 2-5. Thymol concentration detected in the continuous phase of skim, 2% reduced fat, and full fat milk that was mixed respectively with 0.1%, 0.45%, and 0.6% w/v thymol prepared in different forms after 0 h (A) and 48 h (B) at room temperature (21 °C). Different letters above bars indicate significant differences of the mean in the same plot ($P < 0.05$). ................................................................. 72

Figure 3-1. Volume fraction-length mean particle diameter ($d_{4,3}$) of thymol dispersions prepared with 0.2%w/v type A (A) and type B (B) gelatin and different amounts of lecithin. Size distributions are shown in C and D for samples containing 0.8% thymol emulsified by gelatin A and B, respectively. Error bars are standard deviations from duplicates. ................................................................. 97

Figure 3-2. Appearance (A), absorbance at 600 nm (B), and volume-length mean particle diameter ($d_{4,3}$) of thymol dispersions prepared with 0-0.6% w/v gelatin B, 1% w/v lecithin, and 0.8% w/v thymol. Error bars are standard deviations from duplicates. Different letters next to symbols indicate significant differences in the mean ($P < 0.05$). ................................................................. 98

Figure 3-3. TEM images of dispersions prepared with 0.8% w/v thymol and 1.0% w/v lecithin only (A), 0.2% w/v gelatin B only (B), and their blend (C and D, in two magnifications) .......... 99

Figure 3-4. Appearance of aqueous mixtures with 1.0% w/v lecithin and 0.2% w/v gelatin B adjusted to different pH. .................................................................................................................. 100

Figure 3-5. Zeta-potential of dispersions with 0.8% w/v thymol dispersed by 0.2% w/v gelatin B alone, 1.0% w/v lecithin alone, or their blend at pH 3.0-7.0. Error bars are standard deviations from triplicates. .......................................................................................................... 101
Figure 3-6. Changes of volume fraction-length mean particle diameter ($d_{4,3}$) of dispersions with 0.8%w/v thymol dispersed by 1.0%w/v lecithin alone, 0.2%w/v gelatin B alone, or their blend at pH 7.0 during storage at room temperature (21 °C) for 4 weeks. Error bars are standard deviations from triplicates. Different letters above bars indicate significant differences in the mean of the same sample ($P < 0.05$). ................................................................. 102

Figure 4-1. 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 or nanoemulsified thymol. Error bars are standard deviations (n = 2)........................................................................................................... 127

Figure 4-2. 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 or nanoemulsified thymol. Error bars are standard deviations (n = 2)........................................................................................................... 128

Figure 4-3. 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 or nanoemulsified thymol. Error bars are standard deviations (n = 2)........................................................................................................... 129

Figure 4-4. 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 or nanoemulsified thymol 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)........................................................................................................... 130

Figure 4-5. 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 or nanoemulsified thymol. Free thymol was prepared by adding crystals directly or a stock solution with 10% thymol in propylene glycol (PG). Error bars are standard deviations (n = 2)........................................................................................................... 131

Figure 5-1. Appearance of thyme oil nanoemulsions prepared with various combinations of 5% w/v NaCas, 10% v/v PG, and 1% w/v lecithin. Thyme oil concentration in each image is 0.5, 1.0, 1.5, 2.0, and 2.5% w/v from left to right.............................................................. 158

Figure 5-2. Absorbance at 600 nm of nanoemulsions with 0.5-2.5% w/v thyme oil prepared with various combinations of 5% w/v NaCas, 10% v/v PG, and 1% w/v lecithin.............................................................. 159

Figure 5-3. Appearance of nanoemulsions with 1.0% w/v (top) or 2.0% w/v (bottom) thyme oil emulsified by 2.5%w/v NaCas, 0.5 or 1%w/v lecithin, or both. All samples contained 10% v/v PG. Labels on vial caps: S - NaCas; L - lecithin; numbers – surfactant concentrations. .................. 160

Figure 5-4. AFM images of thyme oil (1.0% w/v) nanoemulsions prepared with 2.5% w/v NaCas (A), 0.5% w/v lecithin (B), or both (C). All samples contained 10%v/v PG. ....................... 161

Figure 5-5. Particle size distributions of nanoemulsions with 1.0% w/v (A and C) or 2% w/v (B and D) thyme oil emulsified by lecithin and NaCas-lecithin blend before (solid curves) and after (dashed curves) storage at room temperature (21 °C) for 4 months......................................................... 162
**Figure 6-1.** Growth of *Escherichia coli* O157:H7 at 21°C in TSB with (A) 300 or (B) 500 ppm free or nanoemulsified thyme oil. The HiE and LoE nanoemulsions were prepared with 1% thyme oil and high (4% NaCas+0.5% lecithin) and low (2% NaCas+0.25% lecithin) amounts of emulsifiers. Error bars are standard deviations (n = 3).

**Figure 6-2.** Growth of *Salmonella* Enteritidis at 21°C in TSB with (A) 300 or (B) 500 ppm free or nanoemulsified thyme oil. The HiE and LoE nanoemulsions were prepared with 1% thyme oil and high (4% NaCas+0.5% lecithin) and low (2% NaCas+0.25% lecithin) amounts of emulsifiers. Error bars are standard deviations (n = 3).

**Figure 6-3.** Growth of *Listeria monocytogenes* Scott A at 21°C in TSB with (A) 300 or (B) 500 ppm free or nanoemulsified thyme oil. The HiE and LoE nanoemulsions were prepared with 1% thyme oil and high (4% NaCas+0.5% lecithin) and low (2% NaCas+0.25% lecithin) amounts of emulsifiers. Error bars are standard deviations (n = 3).

**Figure 6-4.** Growth of *Escherichia coli* O157:H7 at 21°C in 2% reduced fat milk with (A) 0.3 or (B) 0.4% free or nanoemulsified thyme oil. The HiE and LoE nanoemulsions were prepared with 1% thyme oil and high (4% NaCas+0.5% lecithin) and low (2% NaCas+0.25% lecithin) amounts of emulsifiers. Error bars are standard deviations (n = 3).

**Figure 6-5.** Growth of *Salmonella* Enteritidis at 21°C in 2% reduced fat milk with (A) 0.3 or (B) 0.4% free or nanoemulsified thyme oil. The HiE and LoE nanoemulsions were prepared with 1% thyme oil and high (4% NaCas+0.5% lecithin) and low (2% NaCas+0.25% lecithin) amounts of emulsifiers. Error bars are standard deviations (n = 3).

**Figure 6-6.** Growth of *Listeria monocytogenes* Scott A at 21°C in 2% reduced fat milk with (A) 0.3 or (B) 0.4% free or nanoemulsified thyme oil. The HiE and LoE nanoemulsions were prepared with 1% thyme oil and high (4% NaCas+0.5% lecithin) and low (2% NaCas+0.25% lecithin) amounts of emulsifiers. Error bars are standard deviations (n = 3).

**Figure 6-7.** Percentage of crystal violet taken up by (A) *Escherichia coli* O157:H7, (B) *Salmonella* Enteritidis, and (C) *Listeria monocytogenes* after treatment with free or nanoemulsified thyme oil. The HiE and LoE nanoemulsions were prepared with 1% thyme oil and high (4% NaCas+0.5% lecithin) and low (2% NaCas+0.25% lecithin) amounts of emulsifiers. The emulsion treatments with bacteria were subtracted from the emulsions without bacteria. Error bars are standard deviations (n = 2).
Chapter 1. Introduction and literature review
1.1 Introduction

Food safety has always been on spotlight due to continued outbreaks of foodborne illnesses caused by pathogenic viruses and bacteria (McCabe-Sellers and Beattie, 2004). According to the Centers for Disease Control and Prevention (CDC 2013), approximately 48 million illnesses, 3000 deaths and 128,000 hospitalizations occur in the United States each year, which have created heavy economic burden from health losses (Scharff, 2012). The total annual medical costs of major foodborne illnesses are estimated to be between $2.3 and $4.3 billion (Buzby et al., 1996). Despite the development of novel strategies and technologies, the occurrence of foodborne illnesses is not decreasing in recent years (CDC 2011). Therefore, efforts are still needed to effectively control foodborne pathogens and improve food safety.

The conventional methods for food preservation include heat treatment, dehydration, freezing, antimicrobials, water activity, pH and modified-atmosphere packaging, which are still widely used in food industry (Rahman, 2007). During the past 30 years, hurdle technology, combinations of several preservation factors (e.g. temperature, water activity, pH, and preservatives), has been increasingly developed and used all over the world (Leistner, 2000). Of many preservation methods, food antimicrobials serve significant roles in inhibiting growth of pathogenic and spoilage microorganisms (Davidson, 2005). They can be classified as synthetically-derived or naturally-occurring antimicrobials (López-Malo et al., 2000). Application of many synthetic antimicrobials in foods such as organic acid and esters (e.g. acetic acid and acetates, benzoic acid and benzoates, lactic acid and lactates and propionic acid and
propionates), have been approved by most regulatory agencies (Davidson et al., 2005).

Compared with synthetic antimicrobials, natural antimicrobials, isolated from animal, plant and microbial sources, are more desirable to satisfy consumer demand for ‘natural’ food (Davidson et al., 2007). For example, lysozyme, lactoferrin and chitosan are animal origin antimicrobials; essential oils (EOs) are a large group of plant origin antimicrobials; nisin and natamycin produced by bacteria, are known as bacteriocins (Davidson et al., 2007; Tiwari et al., 2009).

Traditional or regulatory-approved food antimicrobials were listed in Table 1-1.

EOs have received particular attention in food preservation because they are natural antimicrobials with high antimicrobial activity and some potentials for health benefits (Davidson, 2005). They can be obtained from various parts of plants such as leaf, bark, seed, fruit, and root and are commonly produced by steam distillation (Burt, 2004). Due to their volatile and aromatic properties, EOs have been used as flavors and fragrances since antiquity (Bauer et al., 2001). They are also well-known for their antimicrobial, antiviral, antioxidant, antifungal, and insecticidal properties (Burt, 2004). The focus of this dissertation is on antimicrobial properties of EOs.

1.2 Properties of EOs

1.2.1 Composition of EOs

EOs contain about 20-60 components (Bakkali et al., 2008), which are oxygenated or non-oxygenated terpene hydrocarbons (Thormar, 2011). They are usually constituted by up to 85% major components and other trace components (Bauer et al., 2008). The major
antimicrobial components can be grouped by chemical similarities. For example, aldehydes, phenols and terpene alcohols are considered as the most active antimicrobial components (Ceylan and Fung, 2004). An example of aldehydes in EOs is cinnamaldehyde, which is a major component found in cinnamon bark oil. The antimicrobial activity of aldehydes may be related to their reactive carbonyl groups (Thormar, 2011). For terpene alcohols, they can be grouped based on their molecular structure into: linear or cyclic; mono, di- or sesquiterpenes. For example, the main component of lavender oil is linalool, which is a linear monoterpenic alcohol and has a wide spectrum of antimicrobial activity. Monoterpenes menthol and terpinen-4-ol present in mint oils and tea tree oils, respectively, are cyclic alcohols (Hüsnü et al., 2007). Phenols are aromatic compounds, and thymol, carvacrol, and eugenol found in thyme, oregano and clove oils, respectively, are well known for their excellent antimicrobial activities (Burt, 2004). The activities of alcohols and phenols are usually attributed to the hydroxyl group (Thormar, 2011). Aside from the above-mentioned groups, ketones (e.g. menthone), esters (e.g. benzyl acetate), and terpene hydrocarbons (e.g. limonene) are other groups of compounds with antimicrobial attributes (Thormar, 2011). Structure of several EO components were showed in Figure 1-1.

1.2.2 Antimicrobial activity of EOs

The antimicrobial activities of EOs and their components have been extensively studied and reviewed (Chaieb et al., 2007; Jain and Kar, 1971; Zaika, 1988). They have been widely used in food preservation, pharmaceuticals, alternative medicine and natural therapies (Lis-Balchin and Deans, 1997). Many EOs are highly effective against a broad spectrum of Gram-
positive and Gram-negative bacteria including some major foodborne pathogens such as *Listeria monocytogenes*, *Salmonella Enteritidis*, *Escherichia coli*, *Campylobacter jejuni* and *Staphylococcus aureus* (Burt, 2004; Smith-Palmer et al., 1998). Thyme, oregano, clove, black pepper, geranium and nutmeg essential oils exhibited different inhibitory effects against 25 bacteria, including animal and plant pathogens, food poisoning and spoilage bacteria (Dorman and Deans, 2000). Among the tested oils, thyme oil was found to have the widest spectrum of activity, followed by oregano, clove, and the other three oils (Dorman and Deans, 2000). Cinnamon oil and clove oil both were equally effective against Gram-positive and Gram-negative food borne pathogens such as *Bacillus cereus*, *Staphylococcus aureus*, *E. coli*, and *Pseudomonas aeruginosa* (Gupta et al., 2008). Peppermint oil and lemon oil showed more significant antimicrobial activity against Gram-positive bacteria such as *Bacillus cereus* as compared to Gram-negative bacteria (Gupta et al., 2008). The antimicrobial activity of each EO is usually related to the presence of one or two major EO components. For examples, thymol and carvacrol are two major components found in thyme oil and oregano oil (Akgül and Kivanc, 1988; Hudaib et al., 2002). Cinnamaldehyde and eugenol are the active constituents in cinnamon oil and clove oil, respectively (Bullerman et al., 1977; Chaieb et al., 2007). These EO components also have broad spectrum antimicrobial activity when used individually or more effective in combination due to some synergistic antimicrobial effects (Didry et al., 1994; García - García et al., 2011; Pei et al., 2009; Si et al., 2006).
1.2.3 Modes of actions of EOs

The exact mechanisms of antimicrobial activities of EOs are still not clear although several modes of actions have been proposed and studied (Lambert et al., 2001; Sikkema et al., 1994). A generalized model is related to the hydrophobic nature of EO components, which enable them to insert into the cell membrane, disturbing the structure and increasing its permeability, and result in the leakage of cell contents such as ions, ATP, nucleic acids and amino acids (Burt, 2004).

Different EOs or EO components may act in different ways against different microorganisms such as Gram-positive and Gram negative bacteria. For phenolic compounds, the mode of actions is largely related to the interaction of the hydroxyl group on the phenolic rings with the cell membrane (Thormar, 2011). For example, carvacrol, a major phenolic compound in oregano and thyme oil, can disrupt the out membrane of Gram-negative bacteria, releasing lipopolysaccharides and increasing the membrane permeability (Burt, 2004). It was also reported that carvacrol caused the loss of potassium ions from Bacillus cereus by exchanging its hydroxyl group (Ultee et al., 2002) and the decrease of intracellular ATP along with the increase of extracellular ATP on E. coli (Helander et al., 1998). Eugenol, a major component in clove oil, was able to inhibit the formation of some enzymes such as amylase and proteases in Bacillus cereus (Thoroski et al., 1989) and prevent enzymatic action in Enterobacter aerogenes by binding to proteins with its hydroxyl group (Wendakoon and Sakaguchi, 1995).

Cinnamaldehyde, an aldehyde component in cinnamon oil, different from phenols, did not
disintegrate the cell membrane or decrease the intracellular ATP (Helander et al., 1998). It can only cause the leakage of small ions due to the partial membrane disruption (Gill and Holley, 2004) and prevent the action of amino acid decarboxylases in Enterobacter aerogenes caused by the binding between its carbonyl group and proteins (Wendakoon and Sakaguchi, 1995).

1.2.4 Challenges of applying EOs in foods

As discussed above, the high antimicrobial efficacy of EOs and their components is mainly attributed to their hydrophobic nature. On the other hand, the hydrophobicity makes them barely soluble in water, causing non-uniform distribution in food matrices and reducing their antimicrobial effectiveness when directly incorporated in foods due to the hydrophobic binding with food components, such as proteins and lipids (Aureli et al., 1992; Tassou et al., 1995). Moreover, the sufficient nutrients in foods may enable the damaged cells to recover faster in comparison with the laboratory growth media (Gill et al., 2002). For example, very low or no antimicrobial efficiency against Salmonella or Yersinia enterocolitica and L. monocytogenes were reported when EOs were used on ground beef (Uhart et al., 2006) or barbecued chicken (Firouzi et al., 2007). Higher concentrations (up to 100-fold-greater) of EOs are needed to obtain effective antimicrobial activity in foods than in growth media (Burt, 2004; Solomakos et al., 2008). High levels of EOs may cause organoleptical problems because EOs can apparently affect the taste and flavor of foods and make them unacceptable (Gutierrez et al., 2008, 2009). Delivery systems of EOs are more promising strategies to achieve high antimicrobial efficiency of EOs in foods.
1.3 EO delivery systems

1.3.1 Liposomes

Liposomes are colloidal particles associated from amphiphilic lipids usually phospholipids as bilayer vesicles, which can be used to incorporate hydrophobic or hydrophilic bioactive components within the non-polar regions and the interior aqueous core, respectively (McClements, 2012a; São Pedro et al., 2013). They contain one or more bilayers, forming different structures with a wide range of particle dimensions (Bilia et al., 2014). Liposomes can be prepared with various methods, e.g. thin-film rehydration, reverse-phase evaporation, membrane extrusion, high-pressure homogenization, microfluidization and ultrasonication (Taylor et al., 2005). They have been widely studied in drug delivery systems due to advantages such as targeted delivery, sustained release of incorporated materials, and good ability to protect drugs (Fathi et al., 2012; Lian and Ho, 2001).

Recently, liposomes were also used to encapsulate EOs or their components. Thymol, carvacrol and their mixture all showed significantly enhanced antimicrobial activities after liposomal encapsulation (Liolios et al., 2009). Enhanced antimicrobial and antioxidant activities of EO from *Citrus limon* after encapsulation in liposomes were also reported (Gortzi et al., 2007). Eugenol loaded in nanoliposomes exhibited improved storage stability and sustained release but the antimicrobial activities of eugenol were reduced because the good protection of liposomal encapsulation also reduced the contact of antimicrobial with bacteria (Peng et al., 2015). Clove oil in liposomes showed sustained release of eugenol (its major component) and maintained good
quality and stability during storage (Akrachalanont, 2008). EO from *Zanthoxylum tingoassuiba*, showing significant antimicrobial activity against *Staphylococcus aureus*, was successfully loaded into multilamellar liposomes, which would be helpful to enhance EO targeting to bacteria cells (Detoni et al., 2009).

### 1.3.2 Biopolymeric nanoparticles

Biopolymeric nanoparticles are another group of EO delivery system. These nanoparticles usually have a biopolymeric wall and an EO core. The wall materials can be synthetic, such as poly (DL-lactide-co-glycolide) (PLGA), or natural biopolymers, such as proteins and polysaccharides (São Pedro et al., 2013). They can be fabricated using a variety of techniques such as emulsion polymerization, interfacial polymerization and emulsification/solvent evaporation (Reis et al., 2006). A significant characteristic of this system is controlled release, usually including an initial burst release and a followed prolong release (Gomes et al., 2011; São Pedro et al., 2013). Controlled release is able to reduce the EO’s organoleptic effect on food and ensure a long-term antimicrobial efficacy (Gomes et al., 2011; Hosseini et al., 2013).

Eugenol and trans-cinnamaldehyde loaded in PLGA nanoparticles prepared by emulsion evaporation were effective against *Salmonella* and *Listeria monocytogenes* (Gomes et al., 2011). These nanoparticles also demonstrated high entrapment efficiency ranging from 92% to 98% and continuous release of antimicrobials in 72 h (Gomes et al., 2011). For food applications and low
cost, food biopolymers such as chitosan and zein are more feasible options for encapsulation of EOs.

Chitosan, a deacetylated form of chitin, has been extensively used to encapsulate bioactive compounds through different methods such as spontaneous emulsification (Wilson et al., 2010) and ionic gelation (Xu and Du, 2003). Chitosan nanoparticles loaded with carvacrol were fabricated by combination of emulsification and ionic gelation, which showed effective inhibition against *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* and continued release of carvacrol in 30 days (Keawchaoon and Yoksan, 2011). Oregano EO was also successfully encapsulated in chitosan nanoparticles with similar techniques and showed a two phase release profile (Hosseini et al., 2013).

Zein, the major protein found in maize, is another widely investigated natural biopolymer in the development of delivery systems. Due to its high content (> 50%) of nonpolar amino acids, zein is insoluble in water but soluble in aqueous alcohol solution. The mostly used method to prepare zein nanoparticles is liquid-to-liquid dispersion (Zhong and Jin, 2009). Using this method, thymol and carvacrol was encapsulated in zein nanoparticles, which can be well dispersed in water and exhibited antimicrobial activity against *Escherichia coli* (Wu et al., 2012). Zein nanoparticles were also investigated to encapsulate oregano, red thyme, cassia oil and thymol through similar or modified process (Li et al., 2013; Parris et al., 2005). In these studies, sustained releases of EOs were all achieved.
1.3.3 Emulsions

Emulsions are a large group of EO delivery systems prepared with synthetic surfactants such as polysorbates or amphiphilic biopolymers such as protein. They can be classified into three categories: microemulsions, nanoemulsions and macroemulsions.

Microemulsions are thermodynamically stable systems that can be formed spontaneously, in which oil droplets are usually encapsulated in small surfactant micelles with radius less than 25 nm (Rao and McClements, 2011b). Carvacrol and eugenol encapsulated in nonionic surfactant micelles (Surfynol 465 and 485W) effectively inhibit the growth of *Escherichia coli* O157:H7 and *Listeria monocytogenes*, which was attributed to the increased solubility of EO components in the aqueous phase (Gaysinsky et al., 2005). Clove bud oil and eugenol microemulsions stabilized by Tween 20 also showed improved antioxidant and antimicrobial activities compared with free oils (Hamed et al., 2012). Microemulsions are usually easy to prepare but they require large quantities of surfactants, which may cause toxicity and increase cost (São Pedro et al., 2013).

Unlike microemulsions, nanoemulsions and macroemulsions are thermodynamically unstable systems that require a moderate amount of surfactants and can be formulated with biopolymeric emulsifiers, but formation of these emulsions require some external energy (McClements, 2012b). Nanoemulsions also have small droplets (radius < 100 nm), which make them transparent or translucent. In contrast, macroemulsions have droplets with a radius larger than 100 nm, so they usually appear turbid or opaque (Rao and McClements, 2011a). With
smaller droplets, nanoemulsions are much more stable than macroemulsions against flocculation, coalescence, sedimentation or creaming (Weiss et al., 2009). Therefore, EOs are commonly encapsulated in nanoemulsions rather than macroemulsions to achieve high stability efficacy. Eugenol nanoemulsions demonstrated antimicrobial activity against *Staphylococcus aureus* (Ghosh et al., 2014). Nanoemulsified EO components (terpenes mixture and D-limonene) exhibited enhanced antimicrobial efficacy against *Lactobacillus delbrueckii, Saccharomyces cerevisiae, Escherichia coli* in fruit juices (Donsi et al., 2011).

1.4 Preparation of nanoemulsions

As discussed above, nanoemulsions are a promising strategy to deliver EOs in foods, possessing some unique advantages as delivery systems. In this section, nanoemulsions are reviewed from several aspects, including the preparation methods, use of surfactant blend, natural emulsifying agents, and EO nanoemulsions.

1.4.1 Formation of nanoemulsions

Formation of nanoemulsions requires energy that can be obtained from mechanical device or stored chemical energy in the system (Gutiérrez et al., 2008; Maali and Mosavian, 2013). Nanoemulsion preparation can be classified as either high energy emulsification or low energy emulsification.
1.4.1.1 High energy emulsification

High energy emulsification usually involves the use of mechanical devices that can generate intense disruptive forces to reduce droplet size, such as higher pressure homogenizers, ultrasonic homogenizers, and microfluidizers (McClements, 2004a).

Given to its many advantages, e.g., easy scale up, organic solvent free and high efficiency (Maali and Mosavian, 2013), high pressure homogenizers are the most widely used emulsifying device to produce nanoemulsions in the food industry (McClements, 2004a). Conventionally, coarse emulsions produced by high shear mixers are pumped into a chamber in the homogenizer and then forced through a narrow valve at high pressure (50-200 MPa) (Maali and Mosavian, 2013), which causes intense disruptive forces such as turbulence, hydraulic shear and cavitation that are capable of breaking down large droplets into small ones (Lovelyn and Attama, 2011; McClements, 2011).

Microfluidizers are another type of commonly used high energy emulsifying device. Similar to high pressure homogenizer, microfluidizers also work at high pressure (3-134MPa) (Thakur et al., 2012). Coarse emulsions are forced into an inlet channel and then separated into two streams that impinge on each other intensely in an interaction chamber, where droplet disruption occurs under strong disruptive forces (McClements, 2011). Microfluidizers can produce fine nanoemulsions with a narrow droplet distributions (Maali and Mosavian, 2013).

Ultrasonic homogenizers produce nanoemulsions by using high-intensity ultrasonic waves (frequency > 20 kHz) that are very efficient in decreasing the droplet size but they are
more suitable for small batches and thus widely used in research laboratories (McClements, 2011). An ultrasonic probe in the device converts electrical waves into intense pressure waves, which generate intense disruptive forces as mentioned above (Kentish et al., 2008). The emulsifying efficiency significantly depends on the ultrasonication time at different amplitudes (Solans et al., 2005).

1.4.1.2 Low energy emulsification

Low energy emulsifications rely on physicochemical characteristics of surfactants and co-surfactants (Anton et al., 2008). Nanoemulsions can be spontaneously formed as the system compositions or environmental conditions are adjusted. Spontaneous emulsification and phase inversion temperature (PIT) are two most commonly used approaches (McClements and Rao, 2011).

The process of spontaneous emulsification is very simple. Nanoemulsions are formed by mixing an organic phase (consisting of oil, surfactant and a water-miscible solvent) and a pure aqueous phase at a particular temperature (Anton and Vandamme, 2009). This approach is based on the rapid diffusion of water-miscible solvent such as ethanol and acetone from the organic phase into the aqueous phase, which induces great turbulent force at the water/oil interface (Anton and Vandamme, 2009; Maali and Mosavian, 2013). A drawback of this approach is that, a high solvent/oil ratio is required to form nano-droplets, which largely reduces the oil amount in the final nanoemulsions (McClements, 2011).
PIT method takes advantage of changes in the affinities of nonionic surfactants for water and oil with temperature (Anton et al., 2008). At a low temperature, the surfactant is fully solubilized in water, which favors the formation of O/W emulsions. As temperature is gradually increased above a particular temperature (PIT), O/W emulsions invert to W/O emulsions as the surfactant becomes more soluble in oil than in water (Anton and Vandamme, 2009). Then an O/W nanoemulsion can be produced by rapidly cooling the system below the PIT. These processes are reversible, as the temperature is raised, clear nanoemulsion will become turbid again, which could be an issue in some food and beverage applications that need thermal treatments (McClements, 2011).

1.4.2 Nanoemulsions prepared with surfactant blend

It has been widely reported that surfactant blends are usually more efficient than individual uses for various applications (Gullapalli and Sheth, 1999; Peng et al., 2010; Velev et al., 1994; Vilasau et al., 2011), especially when a hydrophilic surfactant and a lipophilic surfactant such as Tween and Span were blended, which were frequently investigated (Fu et al., 2010; Griffin, 1946; Gullapalli and Sheth, 1999). These surfactant blends utilize synergism of hydrophilic and lipophilic properties of different surfactants (Bierre, 1971). Surfactant blends can reduce droplets size and increase the rigidity and strength of interfacial layer by aligning of surfactant molecules through forces such as hydrogen bonding (Fox, 1986). The blend of Tween 40 and Span 20 was found to be capable of producing optimum mineral oil emulsions (Gullapalli and Sheth, 1999). The lipophilic tail of Tween 40 penetrates between the adsorbed Span 20
molecules that are more oil soluble, and the interweaving hydrophilic chains of Tween 40 formed a gel-like structure in the aqueous phase to reduce droplet coalescence (Boyd et al., 1972).

The mixed surfactant systems have also been investigated in the formation of nanoemulsions to obtain more desirable properties in food, pharmaceutical, and cosmetic fields. For example, nanoemulsions prepared using only sucrose monopalmitate, a non-ionic food grade surfactant, was highly unstable to aggregation at low pH, which limited its applications in many commercial products (Rao and McClements, 2011a). The acidity stability of lemon oil nanoemulsions was improved by mixing sucrose monopalmitate and Tween 80 (Rao and McClements, 2012). Stable nanoemulsions formed using surfactant blends can usually be obtained by adjusting the surfactant types and their ratios based on their emulsifying properties. When water-in-octane nanoemulsions were prepared with combinations containing one hydrophilic surfactant (Tween 80 and isooctylphenol poly (ethylene glycol) ether (OP 10)) and one lipophilic surfactant (Span 80 and Span 85). The combination of Tween 80 and Span 80 exhibited the best synergistic effect on stabilizing nanoemulsions (Fu et al., 2010). Stable hexadecane nanoemulsions were also studied using phospholipid (e.g. dimyristoylphosphatidylcholine (DMPC))–surfactant (e.g. sodium tetradecylsulfate (SC14S)) mixtures (Imai et al., 2006).

1.4.3 Natural emulsifying agents

A large amount of synthetic surfactants, such as mono- and diacylglycerols, have been produced and widely used in the food industry due to their strong surface activity (Hasenhuettl
and Hartel, 2008). However, as consumers become more concerned about the potential harmful effects of synthetic food additives, demands for products formulated with natural ingredients have been increasing over the years. Some naturally derived biopolymers such as proteins and polysaccharides also have amphiphilic structures and thus are more desirable to be used as emulsifying agents for food applications.

1.4.3.1. Proteins

A variety of proteins derived from plants or animals, e.g. milk proteins (whey protein and casein), soy protein, and gelatin, have been frequently used to facilitate the formation and improve the stability of food emulsions (McClements, 2004b; Norde, 2003). These proteins with a high proportion of non-polar groups, are capable of rapidly absorbing to oil–water interfaces and form electrically charged interfacial layers during homogenization (Dickinson, 1992; Hu et al., 2003). The interfacial layers could provide some steric repulsion, while the major mechanism to prevent droplet flocculation is electrostatic repulsion (Dickinson, 1992).

Whey proteins, a major by-product of cheese manufacturing, have been widely used as food ingredients due to their well-known functional and nutritional properties (Ercelebi and Ibanoğlu, 2007; Turgeon and Beaulieu, 2001). Whey protein ingredients are composed of \( \beta \)-lactoglobulin, \( \alpha \)-lactalbumin, bovine serum albumin and some minor components such as immunoglobulins, lactoferrin (Farrell et al., 2004). \( \beta \)-lactoglobulin and \( \alpha \)-lactalbumin, make up approximately 70-80% of total whey protein and are responsible for functional properties such as emulsifying and foaming properties (Wang, 2013; Wong et al., 1996). Whey protein isolate
(WPI), containing >90% protein, is frequently used as an emulsifying agent for the preparation of oil-in-water emulsions in literatures (Klein et al., 2010; Sun et al., 2007; Thanasukarn et al., 2004). An important disadvantage of whey protein-stabilized emulsions is the thermal denaturation of these globular proteins above a certain temperature and the subsequent droplet aggregation (Euston et al., 2000). To improve heat stability, whey proteins can be conjugated with reducing saccharides, e.g. by the Maillard reaction (Neirynck et al., 2004; Shah, Davidson, et al., 2012; Shah, Ikeda, et al., 2012; Zhu et al., 2010).

Sodium caseinate (NaCas) is one of the most commonly used natural emulsifying agents in the food industry due to its distinguished emulsifying properties (Sánchez and Patino, 2005). It is produced from milk through subsequent steps of isoelectric precipitation of caseins, neutralization of the precipitated casein with NaOH, and spray drying (Liu et al., 2012). NaCas is composed of four types of caseins: $\alpha_{\text{s1}}$, $\alpha_{\text{s2}}$, $\beta$-, and $\kappa$-caseins. $\beta$- and $\alpha_{\text{s1}}$-caseins account for more than 75% of the total protein and are major surface active components (Surh et al., 2006a). Different from globular proteins such as whey proteins, the structure of casein is highly disordered, which enables their rapid absorption on oil droplet surfaces during emulsification (Dalgleish et al., 1995). The formation of a thick interfacial layer (up to 10 nm) of caseins and the strong electrostatic repulsion can effectively protect droplets against flocculation and coalescence (Dalgleish et al., 1995). Moreover, NaCas-stabilized emulsions have a higher heat stability but are more sensitive to low pH (below 4.5) when compared to emulsions stabilized by whey protein (Euston et al., 2000; Wong et al., 1996).
Gelatin is another class of proteins with many functional properties, e.g. emulsifying, foaming, gelling, and water-binding, and thus widely used in food applications (Karim and Bhat, 2009). It is a linear protein with a relatively high molecular weight (~ 40,000 to 90,000 Da) (Djagny et al., 2001). Gelatin is produced by thermal hydrolysis of animal collagens from bovine, porcine and fish, at acidic (Type A gelatin) or alkaline (Type B gelatin) pH (Baziwane and He, 2003). Type A and Type B gelatins have an isoelectric point of ~7-9 and ~5, respectively (Kobayashi, 1996), which make them have different charges at the same pH and result in different applications (Surh et al., 2006b). As an emulsifier, gelatin is capable of facilitating the formation of emulsions (Lobo, 2002; Müller and Hermel, 1994). Some studies have shown that emulsions prepared with gelatin had relatively large particle size and were not very stable (Dickinson and Lopez, 2001; Lobo, 2002). Hence, gelatin was usually hydrophobically modified (Toledano and Magdassi, 1998) or used in combination with other biopolymers such as pectin (Cheng et al., 2008) and whey protein (Taherian et al., 2011) to improve its emulsifying and stabilizing properties (Surh et al., 2006b).

1.4.3.2 Phospholipids

Phospholipids are natural, highly surface-active compounds widely used to prepare food emulsions (Cardenia et al., 2011; Kabalnov et al., 1996). The commonly used phospholipid emulsifiers include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA) (Bergenståhl, 2008). In particular, PCs are the most common phospholipids for the preparation of emulsions (Nii and Ishii, 2004).
Lecithin is a complex mixture of various phospholipids and other compounds such as fatty acids, triglycerides and carbohydrates (Wendel, 2000). It is a typical natural emulsifier with many health benefits and thus widely accepted by consumers and legislators (Dickinson and Yamamoto, 1996; Oke et al., 2010). Commercial lecithin is usually derived from vegetable oilseeds (e.g. soybeans, rapeseeds and sunflower seeds) or animal sources (e.g. egg yolk, milk and brain tissue) (Oke et al., 2010; van Nieuwenhuyzen and Tomás, 2008). Soybean is the primary source of vegetable lecithin and soy lecithin is separated from the by-product of soybean oil manufacturing (Wu and Wang, 2003). PCs, known as the most nutritionally significant phospholipids (Oke et al., 2010), are the main constituents in soy lecithin (Bylaite et al., 2001). Soy lecithin has been frequently used individually or combined with other emulsifiers such as proteins to formulate emulsions or enhance emulsion stability (Bylaite et al., 2001; Comas et al., 2006; Surh et al., 2008).

1.4.3.3 Polysaccharides

Polysaccharides also exhibit emulsifying or stabilizing properties in food applications, such as beverages (Tan et al., 2004), ice cream (Goff, 1997), sauces and dressings (Sikora et al., 2008). Many polysaccharides are effective in controlling emulsion shelf-life, but only a few polysaccharides, e.g. gum arabic, modified starches and celluloses, and some pectin and galactomannansand, can be used as emulsifiers (Dickinson, 2009). Unlike proteins, polysaccharides are less sensitive to pH, and hence emulsions stabilized by polysaccharides could be stable at a wide pH range.
Gum arabic, obtained from the natural exudation of *Acacia Senegal*, is one of the most widely used polysaccharide emulsifiers in the food industry (Dickinson, 2003), especially in flavor beverage emulsions because of its good emulsifying properties and remarkable low viscosity (Dickinson et al., 1991). It is a complex branched heteropolyelectrolyte and composed of at least three distinct high molecular weight biopolymer fractions: arabinogalactan, glycoprotein, and arabinogalactan-protein complex (Chen, 2014; Dickinson, 2003). The arabinogalactan-protein complex, containing several polysaccharide units linked to a common protein core (called ‘wattle blossom’ model) (Islam et al., 1997), is believed to be predominantly responsible for the surface-activity of gum arabic (Ray et al., 1995). The hydrophobic protein chain can firmly absorb to oil droplets surface and the hydrophilic arabinogalactan fractions protrude into the aqueous solution, preventing droplets from flocculation and coalescence through strong steric repulsion (Chanamai and McClements, 2001; Phillips and Williams, 1995).

1.4.4 EO nanoemulsions

EO nanoemulsions have been extensively developed in numerous reports. The antimicrobial activities of nano-emulsified EOs vary with composition of emulsion systems. Some studies showed effective antimicrobial activity of EO nanoemulsions. For example, compared with free oil, peppermint oil nanoemulsions stabilized with modified starch exhibited more effective and prolonged antimicrobial activities against *Listeria monocytogenes* Scott A and *Staphylococcus aureus* ATCC 25923 (Liang et al., 2012). Significant reduction of *Bacillus cereus* population was observed after treatment by cinnamon oil nanoemulsion formulated with Tween
80 prepared with ultrasonic emulsification (Ghosh, Saranya, et al., 2013). Similarly, with Tween 80 as a surfactant, carvacrol nanoemulsions formed by spontaneous emulsification were effective against *Salmonella enterica* Enteritidis and *Escherichia coli* O157:H7 (Landry et al., 2014). In contrast, some researchers reported reduced antimicrobial activity of EO nanoemulsions. Carvacrol and eugenol nanoemulsions were found less effective than macroemulsions against *Escherichia coli* and *Listeria innocua* (Terjung et al., 2012). Thyme oil nano-emulsified by ionic antimicrobial surfactant lauric arginate and sodium dodecyl sulfate demonstrated reduced antimicrobial efficacy against four strains of acidi-resistant spoilage yeasts (Ziani et al., 2011).

Currently, EO nanoemulsions in literature were commonly formulated with synthetic surfactants such as Tween 80 and Tween 20 due to their efficient emulsifying property (Chang et al., 2012; Ghosh, Mukherjee, et al., 2013; Ghosh et al., 2014).

### 1.5 Hypothesis and overview of dissertation research

The overall hypothesis of this dissertation is that EO nanoemulsions can be formulated with natural emulsifying agents as novel antimicrobial preservatives to improve food safety.

Whey protein-maltodextrin conjugate, gelatin, sodium caseinate and soy lecithin are selected for current research. Whey protein isolates have been conjugated with various polysaccharides, such as dextran (Akhtar and Dickinson, 2003), pectin (Einhorn-Stoll et al., 2005), xanthan (Benichou et al., 2007) and maltodextrin (Shah, Ikeda, et al., 2012), to improve emulsifying property.

Recent studies from our laboratory suggest that whey protein-maltodextrin conjugates can be used to encapsulate EO components thymol and eugenol through an emulsion evaporation
process, where thymol or eugenol was firstly dissolved in hexane and then emulsified with WPI-MD conjugate solution, followed by evaporation of hexane by spray drying. Compared with free oil, nano-encapsulated thymol or eugenol exhibited similar (e.g. in tryptic soy broth) or more effective (e.g. in 2% reduced fat milk) antimicrobial activities against *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella typhimurium* (Shah, 2011). The drawbacks of this method include the use of hexane and the loss of thymol or eugenol during spray drying. To facilitate EO nanoemulsion formation using shear emulsification, propylene glycol (PG) was studied as a GRAS co-surfactant (Devarajan and Ravichandran, 2011; Rao and McClements, 2011a). EO nano-emulsions as prepared can be directly used in the antimicrobial study. The first nanoemulsion system was studied in Chapter 2 using whey protein-maltodextrin conjugate to emulsify thymol. Thymol was pre-dissolved in PG and emulsified in WPI-MD conjugate solution. Thymol nanoemulsions with and without PG were compared for physicochemical properties and antilisterial activities in growth media and milk.

To test the hypothesis that a combination of a hydrophilic and a hydrophobic emulsifier is capable of producing more desirable EO nanoemulsions than individual emulsifier, Gelatin-lecithin and NaCas-lecithin blends were studied to prepare two additional EO nanoemulsions. Chapter 3 focuses on the preparation of thymol nanoemulsion with gelatin-lecithin blend and characterization of physicochemical properties. Antimicrobial activity of these thymol nanoemulsions was characterized in milk with three fat levels and cantaloupe juice in Chapter 4. Similar to Chapters 3 and 4, Chapter 5 was dedicated to formulate and characterize thyme oil
nanoemulsions with the combination of NaCas and lecithin; antimicrobial activities of thyme oil nanoemulsions were investigated in Chapter 6.
References


Thormar, H., 2011. Lipids and essential oils as antimicrobial agents. Wiley Online Library.


**Appendix**

**Table 1-1.** Traditional or regulatory-approved food antimicrobials (Davidson et al., 2005).

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th>Microbial Target</th>
<th>Primary Food Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid, acetates,</td>
<td>Yeasts, bacteria</td>
<td>Baked goods, condiments, confections, dairy products, fats/oils, meats, sauces</td>
</tr>
<tr>
<td>diacetates, dehydroacetic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzoic acid, benzoates</td>
<td>Yeasts, molds</td>
<td>Beverages, fruits products, margarine</td>
</tr>
<tr>
<td>Dimethyl dicarbonate</td>
<td>Yeasts</td>
<td>Beverages</td>
</tr>
<tr>
<td>Lactic acid, lactates</td>
<td>Bacteria</td>
<td>Meats, fermented food</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Bacteria</td>
<td>Meats</td>
</tr>
<tr>
<td>Lysozyme</td>
<td><em>Clostridium botulinum</em>, other bacteria</td>
<td>Cheese, casings for frankfurters, cooked meat, and poultry products</td>
</tr>
<tr>
<td>Natamycin</td>
<td>Molds</td>
<td>Cheese</td>
</tr>
<tr>
<td>Nisin</td>
<td><em>Clostridium botulinum</em>, other bacteria</td>
<td>Cheese, casings for frankfurters, cooked meat, and poultry products</td>
</tr>
<tr>
<td>Nitrite, nitrate</td>
<td><em>Clostridium botulinum</em></td>
<td>Cured meats</td>
</tr>
<tr>
<td>Parabens (alkyl esters (propyl, Methyl, heptyl) of $p$-hydroxybenzoic acid)</td>
<td>Yeasts, molds, bacteria (Gram positive)</td>
<td>Beverages, baked goods, syrups, dry sausage</td>
</tr>
<tr>
<td>Propionic acid, propionates</td>
<td>Molds</td>
<td>Bakery products, dairy products</td>
</tr>
<tr>
<td>Sorbic acid, sorbates</td>
<td>Yeasts, molds, bacteria</td>
<td>Most foods, beverages, wines</td>
</tr>
<tr>
<td>Sulfites</td>
<td>Yeasts, molds</td>
<td>Fruits, fruit products, potato products, wines</td>
</tr>
</tbody>
</table>
Figure 1-1. Structure of selected EO components (Chang et al., 2001; Burt, 2004).
Chapter 2. Thymol nanoemulsified by whey protein-maltodextrin conjugates: the enhanced emulsifying capacity and anti-listerial properties in milk by propylene glycol
A version of this chapter was originally published by Jia Xue, P. Michael Davidson and Qixin Zhong:


My primary contributions to this paper include sample preparation, data collection and analysis, results interpretation and writing.
2.1 Abstract

The objective of this research was to enhance the capability of whey protein isolate-maltodextrin conjugates in nanoemulsifying thymol using propylene glycol (PG) to improve anti-listerial properties in milk. Thymol was pre-dissolved in PG and emulsified in 7% conjugate solution. Transparent dispersions with mean diameters of <30 nm were observed up to 1.5% w/v thymol. In skim and 2% reduced fat milk, *Listeria monocytogenes* Scott A was reduced from ~5 log CFU/mL to below the detection limit in 6 h by 0.1% w/v and 0.45% w/v nanoemulsified thymol, respectively, contrasting with gradual reductions to 1.15 and 2.26 log CFU/mL after 48 h by same levels of free thymol. In 4% fat milk, *L. monocytogenes* was gradually reduced to be undetectable after 48 h by 0.6% w/v nanoemulsified thymol, contrasting with the insignificant reduction by free thymol. The improved anti-listerial activities of nanoemulsified thymol resulted from the increased solubility in milk and synergistic activity with PG.

**Keywords:** thymol, nanoemulsion, propylene glycol, anti-listerial properties, milk, solubility
2.2 Introduction

The occurrence of foodborne illnesses is a critical problem threatening public health around the world. It is estimated that foodborne pathogens cause 48,000,000 illnesses, 128,000 hospitalizations and 3,000 deaths in the United States each year (CDC 2013a). Although various strategies have been developed and implemented, the number of reported foodborne illness outbreaks has not decreased dramatically (CDC 2013a). *Listeria monocytogenes* is a major foodborne pathogen that can cause illness and death among susceptible populations, including pregnant women, infants, the elderly, and immunosuppressed individuals (Mastronicolis et al., 1996). Around 1,662 foodborne illnesses caused by *L. monocytogenes* occur annually in the United States (Cartwright et al., 2013). Outbreaks have been associated with contaminated milk and other dairy products, meat, fish, and vegetables (Bell and Kyriakides, 1998; Schlech and Acheson, 2000). The most recent outbreak of listeriosis in the US was linked to the imported Frescolina Marte brand Ricotta Salata cheese and caused 22 illnesses across 13 States (CDC 2013b). To effectively control *L. monocytogenes*, methods for reducing contamination by the pathogen need to be improved and intervention strategies, as additional hurdles, need to be implemented.

Naturally occurring antimicrobials such as essential oils derived from plants have received increasing attention due to their efficacy against a broad spectrum of pathogens (Burt, 2004). For example, thymol is a phenolic compound that is the major component in the essential oil extracted from the aromatic plant thyme (*Thymus vulgaris*) (Burt, 2004). Thymol exhibits
excellent antimicrobial activity because its hydroxyl groups can interact with the cell membrane of bacteria to disrupt membrane structures and cause the leakage of cellular components (Di Pasqua et al., 2007). However, direct incorporation of essential oils in aqueous food systems has many challenges. In complex food matrices, essential oil components bind with hydrophobic food components such as proteins and lipids and are therefore required to be used at concentrations much higher than what is needed in microbial growth media and simple food systems like juices (Weiss et al., 2009). These use levels can be well-above the water solubility of the essential oils which would require a mechanism to evenly distribute them in food matrices to effectively control pathogens that likely exist in the water-rich phase of food matrices (Weiss et al., 2009).

Emulsions have been studied as delivery systems for essential oils to improve their antimicrobial efficacy (Donsì et al., 2012). Nanoemulsions are those with droplets smaller than ~200 nm in diameter (McClements and Rao, 2011). The reduced droplet dimension prevents creaming and reduces turbidity. Co-surfactants such as generally-recognized-as-safe (GRAS) propylene glycol (PG) (Reproduction, 2004; ATSDR 2013) are widely used to facilitate the formulation of microemulsions (El Maghraby, 2008; Kale and Allen, 1989; Stilbs et al., 1983) and nanoemulsions (Rao and McClements, 2011). In addition, PG is used as a preservative (Barr and Tice, 1957; Kinnunen and Koskela, 1990; Olitzky, 1965). Several studies reported the enhanced antimicrobial activity of essential oils after preparation of nanoemulsions using small molecular surfactants. The antimicrobial tests however were performed in microbial growth
media (Donsì et al., 2012; Ziani et al., 2011) or fruit juices (Donsì et al., 2011). In these simple systems, complete inhibition of bacteria can be achieved below the solubility limit of essential oil components (Shah, Davidson, et al., 2012b; Shah et al., 2013a, b), and the need of a delivery system is not justified. Additionally, the reduced antimicrobial activity after preparation of essential oils to nanoemulsions was also reported which correlated well with the enhanced binding by polyoxyethylene (20) sorbitan monooleate (Tween 80) (Terjung et al., 2012). Studies reporting improved antimicrobial effectiveness of essential oils in real food matrices are scarce.

Recently, an emulsion-evaporation process to encapsulate the essential oil components thymol and eugenol in whey protein isolate-maltodextrin (WPI-MD) conjugate capsules (Shah, Davidson, et al., 2012a; Shah, Ikeda, et al., 2012) was studied in our laboratory. WPI-MD conjugates are known for their emulsifying properties (Akhtar and Dickinson, 2007) and have been used to encapsulate various lipophilic ingredients (Akhtar and Dickinson, 2007; Choi et al., 2010). The conjugate was prepared by dry heating spray-dried powder with protein and an oligosaccharide mixture resulting in the Maillard reaction. The encapsulation was enabled by spray drying coarse emulsions pre-formed with an oil phase of eugenol or thymol dissolved in hexane and an aqueous phase with the conjugate. After hydrating spray-dried emulsions, transparent and heat-stable dispersions were observed at pH 3, 5, and 7. Nanodispersed eugenol or thymol was not as effective as the free (unencapsulated) antimicrobial control when tested in tryptic soy broth (TSB) or apple cider but was more effective against *Escherichia coli* O157:H7 and *L. monocytogenes* in milk (Shah, Davidson, et al., 2012b; Shah et al., 2013a, b).
technique however has the drawbacks of using hexane and the loss of eugenol and thymol during spray drying.

The objectives of the present study were to (1) evaluate the direct preparation of thymol nanoemulsions with WPI-MD conjugates as assisted by PG, (2) characterize anti-listerial properties of thymol in growth media and milk, and (3) characterize the availability of thymol in the continuous phase of milk and its correlation with anti-listerial properties. The conditions adopted in the present study enable the preparation of food grade nanoemulsions based on GRAS ingredients that potentially can be incorporated in foods directly. In addition to showing direct relevance to the safety of real food systems, milk with various fat contents (skim, 2% reduced fat and full (~3.3%)) is an excellent model system to demonstrate protein and lipid interference with the antimicrobial activity of essential oils.

2.3 Materials and Methods

2.3.1 Materials

Thymol (99% purity) was purchased from Acros Organics (Thermo Fisher Scientific, Morris Plains, NJ). WPI was a gift from Hilmar Cheese Company (Hilmar, CA). MD 180, with an average dextrose equivalent of 18, was a product of Grain Processing Corporation (Muscatine, IA). TSB, peptone, and agar (chemical grade) were purchased from Becton, Dickinson and Company (Sparks, MD). Other chemicals, such as PG (with density of 2.62) and methanol, were obtained from Fisher Scientific (Pittsburgh, PA). Ultra high temperature (UHT) pasteurized organic milk (skim, 2% reduced fat, and full (~3.3%)) was purchased from Kroger Company.
2.3.2 Preparation of WPI-MD conjugates

WPI and MD were hydrated at 5% w/v each in deionized water overnight at room temperature (21 °C). The solution was adjusted to pH 7.0 using 10 N NaOH and spray dried using a model B-290 mini spray-dryer (Büchi Labortechnik AG, Flawil, Switzerland) at an inlet temperature of 160 °C, a recorded outlet temperature of 80-90 °C, a feed rate of 2 mL/min, a compressed air pressure of 600 kPa and an air flow rate of 35 m³/h. The collected spray-dried powder was heated at 80 °C and a relative humidity of 70% for 4 h in an environmental chamber (Yamato Scientific American, Inc. Santa Clara, CA). The conjugate was stored at -20 °C before use.

2.3.3 Preparation of nanoemulsions

The conjugate solution was prepared by dissolving 0.7 g conjugate powder in 9 mL deionized water with a resultant pH of 6.4. Thymol solution was prepared separately by dissolving various amounts in 1 mL PG. The conjugate and thymol solutions were mixed and emulsified at 15,000 rpm for 1 min using a model Cyclone I.Q.² microprocessor homogenizer (The VirTis Company, Inc., Gardiner, NY). Another set of emulsions was prepared without PG, by emulsifying thymol powder directly in the conjugate solution.

2.3.4 Characterization of thymol nanoemulsion

Thermal Stability. Emulsions were prepared with 0, 0.5, 1, 1.5, and 2% w/v thymol using conjugates or an equivalent mass of unconjugated WPI and MD powder using the above
procedures. Two mL of sample were placed in a 4 mL glass vial and heated in an 80 °C water bath for 15 min. Absorbance of the emulsions at 600 nm was measured using a UV-vis spectrophotometer (Biomate 5, Thermo Electron Corp., Woburn, MA).

Particle Size Measurement. Particle size distributions of thymol emulsions were measured using a Delas™ Nano-Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter, Inc., Brea, CA). To meet the sensitivity range of the instrument, samples were diluted 20 times using 0.01 M phosphate buffer solution at pH 7. The volume fraction-length mean particle diameter \( d_{4,3} \) was calculated from the number of particles \( n_i \) with the corresponding diameter \( d_i \) based on the following equation:

\[
d_{4,3} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}
\]

(1)

Zeta-potential. The zeta potentials of WPI-MD conjugate and emulsions containing 1.0% thymol prepared with and without PG were measured using a Delas™ Nano-Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter Inc., Brea. CA). All samples were diluted to 0.2% w/v of conjugate using deionized water and adjusted to pH 3 to 7 using 1 N NaOH or HCl before the analysis. Three replicates were tested for three times each.

Atomic Force Microscopy (AFM). The morphology of thymol particles was characterized using a Multimode VIII microscope (Bruker Corp., Santa Barbara, CA). Emulsions containing 1% thymol prepared with and without PG were diluted to 10 ppm of conjugate using 0.01 M phosphate buffer solution at pH 7. Four μL of each sample was spread evenly onto a freshly-
cleaved mica sheet that was mounted on a sample disk (Bruker Corp., Santa Barbara, CA) and air-dried for > 2 h. The samples were scanned using a rectangular cantilever probe (Bruker Nanoprobe, Camarillo, CA) with aluminum reflective coating on the backside and a quoted force constant of 2.80 N/m. The tapping mode images were collected.

2.3.5 Determination of anti-listerial activity

Culture Preparation. The L. monocytogenes strain Scott A was obtained from the culture collection of the Department of Food Science and Technology at the University of Tennessee. The culture was kept frozen at -20 °C in glycerol. Before use, 100 µL culture was inoculated in 50 mL TSB, shaken, incubated at 32 °C and then transferred at least twice in TSB with an interval of 24 h before use. The independent culture was grown for each replicate.

Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Fractional Inhibitory Concentration (FIC). The MIC was determined using a microbroth dilution assay (Davidson and Parish, 1989). The 120 µL of bacterial culture with ca. 10^5 CFU/mL L. monocytogenes was added to wells of 96-well microtiter plates followed by 120 µL of antimicrobial (free thymol, thymol emulsion with and without PG, or PG alone treatments diluted to various concentrations using TSB). The free thymol sample was prepared by adding 0.01 g thymol into 10 mL TSB and heating in a water bath at 60 °C for about 2 min until the thymol dissolved. This was then diluted to working solutions with various thymol concentrations. A negative control was prepared by adding 120 µL of TSB without culture, and a positive control was prepared by adding 120 µL of TSB and 120 µL of bacterial culture. Absorbance was read 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. Inoculated wells with an increase in absorbance of <0.05 after 24 h incubation were considered inhibited and this was defined as the MIC. For wells showing inhibition, 20 μL of the mixture was transferred to tryptic soy agar (TSA) plates. If there was no growth on the plates at 32 °C after 24 h, the thymol concentration was considered bactericidal, with the lowest concentration defined as the MBC.

A checkerboard method (Brandt et al., 2010) was used to investigate the antimicrobial interaction between thymol and PG. Wells of the 96-well microplate were filled with 60 μL of various concentrations of thymol solution and PG solution (diluted with TSB) along with 120 μL of *L. monocytogenes* (ca. 10⁵ CFU/mL). The MIC of antimicrobial combinations was determined as above and was used to calculate the FIC as eq. 2. FIC values below, equal and above 1 corresponded to synergistic, additive, and antagonistic antimicrobial activities, respectively (Davidson et al., 1993).

\[
FIC = \frac{\text{MIC of thymol in combination}}{\text{MIC of thymol alone}} + \frac{\text{MIC of PG in combination}}{\text{MIC of PG alone}}
\]  

(2)

Anti-listerial Activity in Milk. The anti-listerial activity in milk was studied in duplicate using time-kill assays (Shah et al., 2013b). Thymol nanoemulsion prepared with PG, thymol nanoemulsion prepared without PG, free thymol, and free thymol dissolved in PG were studied in this group of tests. For nanoemulsion treatments, 4 mL of nanoemulsion containing 0.25, 1.125 or 1.5% w/v thymol was mixed with 5 mL of skim, 2% reduced fat, or full fat milk, respectively. For the free thymol treatment, thymol was added directly into milk and mixed with an end-to-end
shaker (Laboratory Industries Inc., Berkeley, CA) at room temperature (21 °C) for 30 min. For free thymol dissolved in PG, 0.4 mL of thymol solution in PG was added to 5 mL milk. The volume of the free thymol treatments was increased to 9 mL using sterilized water. The control sample was prepared by mixing 5 mL of milk with 4 mL sterilized water. The overall concentration of thymol in the final mixture was 0.1, 0.45, and 0.6% w/v in skim, 2% reduced fat, and full fat milk treatments, respectively.

The milk samples after adding antimicrobials or controls were mixed with 1 mL culture that was previously diluted to ca. 1.0 × 10⁶ CFU/mL in TSB. After incubation at room temperature (21 °C) for 0, 3, 6, 24, and 48 h, samples were diluted with 0.1% peptone, and survivors enumerated by plating on TSA and incubating at 32 °C for 24 h. The detection limit of the enumeration method was 1 log CFU/mL.

2.3.6 Solubility of thymol in solvents

Thymol solubility in solvents was tested by adding 0.45% w/v thymol in deionized water and binary mixtures of water and 4% or 10% v/v PG. After hydration overnight at room temperature (21 °C) and filtration through a 0.45 μm polyvinyl difluoride (PVDF) membrane filter, thymol concentration in the permeate was quantified using HPLC as below. The experiments were conducted in triplicate.

2.3.7 Quantification of thymol dissolved in the aqueous phase of milk

Preliminary experiments showed that thymol nanoemulsions precipitated at pH 4.6, the isoelectric point of caseins. To quantify the amount of thymol dissolved in the aqueous phase of
milk, samples were prepared as in anti-listerial experiments except that 1 mL of bacterial culture was replaced with 1 mL of deionized water. To separate serum, milk samples were adjusted to pH 4.6 to precipitate caseins, followed by centrifugation at 4,629 g for 5 min using a RC-5B Plus centrifuge (Sorvall, Inc. Norwalk, CT) and filtration of the supernatant through a 0.45 µm PVDF membrane syringe filter (Fisher Scientific). The permeate was analyzed for thymol concentration using a 1200 series high performance liquid chromatography (HPLC) system (Agilent Technologies, Inc., Santa Clara, CA). A ZORBAX Eclipse Plus C18 column (4.6×150 mm, 5 µm particle size) was used. A binary solvent with different volume proportions of methanol (solvent A) and HPLC grade water (solvent B) was used as the mobile phase in the following steps: a linear increase from 20% A to 80% A within the first 20 min, an isocratic step with 80% A in 21-25 min, a linear decrease 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 throughout, and the temperature of the column was 25 °C. Ten µL of each sample was injected directly into the HPLC. The absorbance at 274 nm was measured with a UV detector. Thymol from Sigma-Aldrich Corp. (St. Louis, MO) was used as an external standard to establish a calibration curve using five standard solutions with 0.02, 0.04, 0.06, 0.08 and 0.10% w/v of thymol. Preliminary experiments showed that the elution time of thymol did not overlap the elution profile of milk serum as prepared.

2.3.8 Statistical analysis

Analysis of variance (ANOVA) test was conducted using the SPSS 16.0 statistical analysis system (SPSS Inc., Chicago, IL, USA). Least significant difference (LSD) test was used
to determine the difference of mean values at a significance level of 0.05.

2.4 Results and Discussion

2.4.1 Properties of thymol emulsions

Emulsifying properties of WPI-MD conjugates were investigated at pH 6.4. Up to 1.5% w/v of thymol can be emulsified by 7% w/v conjugates and 10% v/v PG without showing turbidity (photograph not shown), which is well above the 0.1% w/v water solubility of thymol at 20 °C reported in the literature (Seidell, 1919) and 0.095%w/v in 10%v/v PG measured in the present study. In comparison, the mixture with same amounts of unconjugated WPI, MD, and PG was only capable of emulsifying 0.5%w/v of thymol as a transparent dispersion (photograph not shown). After heating at 80 °C for 15 min, nanoemulsions prepared with conjugates and 0-1.5% w/v thymol remained transparent and fluid, while emulsions prepared with the mixture of WPI and MD formed gels shortly after heating. The improved emulsifying and stabilizing properties of WPI after glycation with MD were previously reported for orange, flavor and triglyceride oils, (Akhtar and Dickinson, 2007) due to the adsorption of more hydrophobic protein moiety onto the oil-water interface and the hydrophilic oligosaccharide protruding in the water phase providing steric hindrance against aggregation. Likewise, the MD glycated to whey proteins effectively prevents the aggregation of whey proteins during heating at various pH and ionic conditions and improves heat stability (Liu and Zhong, 2012, 2013). It was also observed that the emulsion prepared with conjugates and 2% w/v thymol and those prepared with WPI and MD mixture and 1.0-1.5% w/v thymol became clearer after heating. This is likely caused by the increased thymol
solubility at elevated temperatures (Akgün et al., 2000) that enables the redistribution of thymol to be associated with available whey protein or conjugates.

The absorbance values at 600 nm of thymol emulsions prepared with WPI-MD conjugates (Figure 2-1A) agreed with the visual appearance (photograph not shown). The increase in absorbance with the increase in thymol concentration was insignificant ($P > 0.05$) except for the 2.0% thymol treatment that was turbid before heating (photograph not shown). The particle sizes of transparent nanoemulsions, with 0-1.5% w/v thymol, were also measured before and after heating (Figure 2-1B). The $d_{4,3}$ of conjugates was about 8 nm. The $d_{4,3}$ of transparent nanoemulsions increased with an increase in thymol concentration and was smaller than 30 nm. There was no significant difference in $d_{4,3}$ changes after heating ($P > 0.05$).

Thymol emulsions prepared with 7% w/v conjugates without PG were also examined. The dispersion with 0.5% w/v of thymol without PG was transparent but that with 1.0% w/v thymol was turbid and had visible thymol precipitate shortly after the sample stood at room temperature (21 °C, image not shown). The emulsions containing 1.0% thymol, prepared with and without PG, also were imaged by AFM (Figure 2-2). The emulsion prepared with PG (Figure 2-2A) had much smaller (mean diameter = 29 nm) and more uniform particles than the emulsion without PG (mean diameter = 117 nm) (Figure 2B), which agrees with transparent and turbid appearance of the two samples (photograph not shown). Therefore, PG significantly improved the emulsifying capacity of WPI-MD conjugates.

To further study effects of PG on the formation of thymol emulsion, zeta-potentials of
WPI-MD conjugate and thymol emulsions prepared with and without PG were investigated (Figure 2-3). At pH 4, thymol emulsions with and without PG exhibited the same magnitude of zeta-potential, which was significantly higher than that of conjugates alone ($P < 0.05$). At pH 5, both emulsions demonstrated a lower magnitude of zeta-potential than conjugates, with the emulsion prepared with PG being significantly higher than that without PG ($P < 0.05$). At pH 3, 6, and 7, conjugate alone and thymol emulsions with and without PG did not show significant differences in zeta-potential. The zeta-potential data indicate no significant impact of PG on protein conformation in emulsions under the conditions studied.

2.4.2 MIC and MBC in TSB

The MIC and MBC of free thymol, thymol nanoemulsions prepared with and without PG, and PG alone against *L. monocytogenes* Scott A in TSB are listed in Table 2-1. Thymol nanoemulsified without PG demonstrated a higher MIC (0.055% w/v) and MBC (0.08% w/v) than those of free thymol. This indicates that binding between WPI-MD conjugates and thymol causes the significant reduction in antimicrobial efficacy of thymol ($P < 0.05$). In comparison, nanoemulsified thymol with PG exhibited the same MIC (0.02% w/v) and MBC (0.045% w/v) as free thymol, which may be attributed to the weakened binding between conjugate and thymol by PG. PG also exhibited slight anti-listerial activity, with an MIC and MBC of 26.2% and 52.4% w/v, respectively. The MIC of PG is consistent with a previous study (Barr and Tice, 1957). In the interactive assay with free thymol and PG, the MIC of thymol and PG when used in combination was 0.015% w/v and 20.96% w/v, respectively. This corresponded to an FIC of
0.875 which indicates that thymol and PG have potential synergistic antimicrobial activity. This agrees with another report of the enhanced antimicrobial activity of propolis extract by PG (Tosi et al., 1996).

In previous studies when nanoemulsions of thymol (Shah et al., 2013a) and eugenol (Shah et al., 2013b) were prepared with WPI-MD conjugate using the emulsion-evaporation technique, the MIC of nanoemulsified thymol/eugenol against *L. monocytogenes* Scott A in TSB was higher than that of the free thymol/eugenol. Eugenol and carvacrol also showed reduced antimicrobial activity after preparation as nanoemulsions with smaller droplets (Terjung et al., 2012). This corresponded to a reduced concentration in the continuous aqueous phase and suggested a greater extent of association with the emulsifier Tween 80. The similar MICs of the nanoemulsion prepared with PG and free thymol, lower than that of the emulsion without PG, may be physically due to the weakened binding of thymol with WPI-MD conjugate by PG due to a slight decrease in polarity and possibly due to the synergistic antimicrobial activity of thymol and PG.

### 2.4.3 Anti-listerial activities of antimicrobials in three types of milk

The anti-listerial activities of free thymol and thymol emulsions used at 0.1, 0.45, and 0.60% w/v thymol in skim, 2% reduced fat, and full fat (4% fat) milk, respectively, are shown in Figure 2-4. In skim milk (Figure 2-4A), *L. monocytogenes* Scott A was reduced by thymol nanoemulsion prepared with PG to below the detectable limit (1 log CFU/mL) within 6 h, followed by no recovery in 48 h. For free thymol, the gradual reduction of *L. monocytogenes* in
48 h was observed, and there was no significant difference \((P > 0.05)\) for treatments with and without PG. The emulsion prepared without PG was the least effective, which is consistent with the higher MIC and MBC values (Table 2-1), with the highest population after 48 h.

The trends in 2\% reduced fat milk (Figure 2-4B) were similar to those in skim milk (Figure 2-4A), except that the inactivation was at a slower rate. For the nanoemulsion prepared with PG, the \textit{L. monocytogenes} was reduced to 3.2 log CFU/mL after 3 h and below the detection limit after 6 h. For free thymol, the gradual reduction of \textit{L. monocytogenes} was observed in 48 h. For the treatment of free thymol with PG, the recovery of \textit{L. monocytogenes} was observed after 24 h. The emulsion prepared without PG showed bacteriostatic properties, with insignificant changes in 48 h \((P > 0.05)\).

In full-fat milk (Figure 2-4C), the inactivation rate was even slower than in 2\% reduced fat milk (Figure 2-4B), although at a higher level of thymol. The nanoemulsion prepared with PG gradually reduced \textit{L. monocytogenes} to below the detection limit after 48 h, while other three thymol treatments were only bacteriostatic. Overall, the anti-listerial efficacy of thymol was significantly affected by the fat content in milk \((P < 0.05)\), and the nanoemulsion prepared with PG had the highest bactericidal activities reducing \textit{L. monocytogenes} in a shorter time to a greater extent than other comparable treatments at the studied conditions.

\textbf{2.4.4 Content of thymol in the serum of milk}

The thymol concentration in the aqueous phase of milk as prepared in anti-listerial tests in Figure 4 was quantified to understand differences in anti-listerial activity of thymol treatments.
Treatments with PG had a PG content of about 4% v/v in milk. Thymol solubility in deionized water and 4% v/v aqueous PG was found to be 0.054 and 0.056% w/v, respectively. The increase of thymol solubility by 4% v/v PG was insignificant (P < 0.05).

Thymol concentrations in the milk serum (TCMS) are shown in Figure 2-5. In each type of milk, the TCMS followed the increasing order of free thymol, free thymol and PG, emulsion without PG, and emulsion with PG. In skim milk, the TCMS was well below the overall added concentration of 0.1% w/v thymol and even below the solubility in the corresponding solvent (water or 4% v/v PG in treatments with PG), which indicates the significant binding between thymol and dairy proteins. When mixed initially (Figure 2-5A), the TCMS of free thymol and the emulsion without PG treatments were not significantly different among different types of milk, even though the added thymol concentrations were 0.1, 0.45 and 0.60% w/v in skim, 2%, and full fat milk, respectively. This indicates a significant amount of thymol binding with fat globules. The nanoemulsion with PG had the highest TCMS in all treatment groups. It was also noted that PG improved the TCMS compared to free thymol. Results suggest that PG reduces binding of thymol with milk components. After 48 h (Figure 2-5B), trends in TCMS were similar to those at 0 h (Figure 2-5A). Notably, the TCMS of the free thymol treatment increased after incubation, likely due to the continued dissolution of thymol crystals. However, it was still lower than other treatments most likely due to differences in solvent polarity or dispersibility. For other treatments, incubation did not have significant impact on the TCMS.

Although a much higher level of thymol is applied in milk than in TSB, thymol
molecules binding with dairy proteins and fat globules may not be available to interaction with bacteria, causing anti-listerial properties different from those in TSB. This hypothesis can be examined by correlating TCMS with MIC and MBC estimated in TSB to interpret the anti-listerial activity in milk. The TCMS (Figure 2-5) of free thymol in milk was between MIC and MBC (Table 2-1) which agreed with the inhibition or incomplete inactivation of *L. monocytogenes* in milk (Figure 2-4). For the nanoemulsion without PG, the TCMS was below 0.045% w/v which was lower than the MIC of 0.055% w/v (Table 2-1) resulting in the weakest anti-listerial activity in milk (Figure 2-4). For the nanoemulsion with PG, the TCMS was above the MBC of 0.045% w/v (Table 2-1) and the anti-listerial properties were the best, showing complete inactivation of *L. monocytogenes* in 48 h at all studied conditions.

### 2.5 Conclusions

In summary, WPI-MD conjugates can be used as a novel emulsifier to produce thymol nanoemulsions suitable for use as preservatives in food applications. PG, as a solvent to dissolve thymol and a co-surfactant, greatly enhanced the emulsifying capacity of WPI-MD conjugate and anti-listerial properties of thymol in all types of milk. The antimicrobial efficacy of thymol in milk was directly affected by the binding with proteins and fat globules and therefore the availability to interact with bacteria, more significant at a higher fat concentration. Conditions enabling the TCMS above MBC enabled the complete inactivation of *L. monocytogenes*, while inhibition or partial inactivation was observed when TCMS was between MIC and MBC. The enhanced anti-listerial activity of nanoemulsion with PG was attributed to the increased TCMS,
the improved dispersibility of thymol, and the synergistic antimicrobial activity between thymol and PG. These nanoemulsions have great potential to increase the antimicrobial activity of essential oils in food systems.

Acknowledgements

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References


Appendix

Table 2-1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of free thymol, nanoemulsified thymol with and without PG, and PG alone against *Listeria monocytogenes* Scott A in tryptic soy broth at 32 °C.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>MIC (% w/v)</th>
<th>MBC (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free thymol</td>
<td>0.02</td>
<td>0.045</td>
</tr>
<tr>
<td>Thymol nanoemulsion with PG*</td>
<td>0.02</td>
<td>0.045</td>
</tr>
<tr>
<td>Thymol nanoemulsion without PG**</td>
<td>0.055</td>
<td>0.08</td>
</tr>
<tr>
<td>PG</td>
<td>26.2</td>
<td>52.4</td>
</tr>
</tbody>
</table>

*Thymol nanoemulsion with PG was prepared by homogenizing 9 mL of 7% WPI-MD conjugate solution and 1 mL of PG dissolved with 0.01 g thymol.

**Thymol nanoemulsion without PG was prepared by homogenizing 9 mL of 7% WPI-MD conjugate solution and 1 mL of DI water suspended with 0.01 g thymol.
Figure 2-1. Absorbance at 600 nm (A), and volume fraction-length mean particle diameter ($d_{4,3}$) (B) of thymol emulsions prepared with 7% w/v WPI-MD conjugates, 10% v/v PG, and 0-2.0% w/v thymol, before and after heating at 80 °C for 15 min.
Figure 2-2. AFM images of thymol emulsions prepared with 7% w/v WPI-MD conjugates, 1.0% w/v thymol with (A) and without (B) 10% v/v PG. The dimension of images is 2 μm × 2 μm.
Figure 2-3. Zeta-potential of WPI-MD conjugate, emulsion prepared with 7% w/v WPI-MD conjugates and 1.0% v/v thymol with and without 10% v/v PG.
**Figure 2-4.** Time-kill assays showing the population of *Listeria monocytogenes* Scott A at 32 °C in skim (A), 2% reduced fat (B), and full fat milk (C) that was treated with 0.1%, 0.45%, and 0.6% w/v thymol, respectively. Emulsions of thymol were prepared with and without propylene glycol (PG). Free thymol was tested with and without 4% v/v PG, with the PG amount equivalent to the emulsion treatment. The detection limit is 1 log CFU/mL.
Figure 2-5. Thymol concentration detected in the continuous phase of skim, 2% reduced fat, and full fat milk that was mixed respectively with 0.1%, 0.45%, and 0.6% w/v thymol prepared in different forms after 0 h (A) and 48 h (B) at room temperature (21 °C). Different letters above bars indicate significant differences of the mean in the same plot ($P < 0.05$).
Chapter 3. Blending lecithin and gelatin improves the formation of thymol nanodispersions
A version of this chapter was originally published by Jia Xue and Qixin Zhong:


My primary contributions to this paper include sample preparation, data collection and analysis, results interpretation and writing.
3.1 Abstract

Delivery systems of lipophilic antimicrobials like thymol prepared with generally-recognized-as-safe ingredients are needed to enhance the microbiological safety of low-acid (pH > 4.6) foods. Nanodispersions with particle diameters below 100 nm are particularly demanded because of the low turbidity and physical stability. In this study, thymol dispersions were prepared by gelatin and soy lecithin individually or in combination. Dispersions prepared with the lecithin-gelatin blend were translucent and stable at pH 5.0-8.0, contrasting with turbid and unstable dispersions when the emulsifiers were used individually. The synergistic surface activity of gelatin and lecithin was due to complex formation that effectively prevented particle size change due to coalescence and Ostwald ripening. Electrostatic interactions were observed to be the colloidal force responsible for preventing particle aggregation. The studied generally-recognized-as-safe nanodispersions have great potential to deliver lipophilic antimicrobials like thymol in low-acid foods to enhance food safety.

Keywords: Gelatin, lecithin, synergistic surface activity, thymol, nanodispersions, stability
3.2 Introduction

Essential oils extracted from edible, medicinal and herbal plants have been well recognized as natural antimicrobial preservatives (Holley and Patel, 2005) and are classified by the US Food and Drug Administration as generally recognized as safe (GRAS). (Weiss et al., 2009b) The high efficacy of essential oils against foodborne pathogens and spoilage microorganisms has been widely studied. (Dorman and Deans, 2000; Elgayyar et al., 2001; Hammer et al., 1999; Smith-Palmer et al., 1998) However, direct incorporation of essential oils in food systems encounters many challenges due to their low water solubility and interactive binding with food components like protein and lipids, which greatly reduce their antimicrobial activity in complex food matrices. (Chen et al., 2013) One example is thymol, the major component of essential oils from thyme (Thymus vulgaris), that exhibits excellent antimicrobial activity against both Gram-positive and Gram-negative bacteria, such as Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae and Staphylococcus aureus. (Dorman and Deans, 2000) However, the water solubility of thymol is only about 0.048%w/v at 21 °C, (Chen et al., 2013) but a concentration much higher than 0.1% is needed to achieve effective inhibition of foodborne pathogens in food systems like milk, (Shah et al., 2012) carrot juice, (Delgado et al., 2004) and ground beef. (Del Nobile et al., 2009; Juneja et al., 2006) This would require a delivery system to enable the even distribution of thymol and improve the antimicrobial effectiveness to inhibit pathogens throughout food matrices.

Emulsions are frequently studied as delivery systems of essential oils to achieve uniform
distribution of lipophilic compounds in the aqueous phase. (Donsì et al., 2012; Terjung et al., 2012) Nanoemulsions are those with mean droplet diameters below 100 nm, which, due to little scattering of visible light, enables the transparent or translucent appearance. Moreover, although nanoemulsions are thermodynamically unstable, they have better physical stability against gravitational separation than conventional emulsions. (McClements, 1999) However, little research has been reported about nanoemulsions of essential oils. (Weiss et al., 2009a) Moreover, compared to abundant studies using synthetic surfactants such as polysorbates, there is limited work in nanoemulsions based on GRAS emulsifying agents such as gelatin and lecithin.

Gelatin is produced by partial hydrolysis of collagen from animal hide, pigskin, and bone tissues. (Gioffrè et al., 2012) Depending on the hydrolysis conditions, two types of gelatin are available. Type A gelatin is obtained by acidic hydrolysis and has a high isoelectric point (pI, ~8-9). In contrast, alkaline hydrolysis yields type B gelatin that has a lower pI of ~4-5 (Gómez-Guillén et al., 2011). In the food industry, gelatin has been widely used as emulsifiers, foaming agents, colloid stabilizers, gelling agent, biodegradable packaging materials and micro-encapsulating agents due to the naturally-occurring characteristics, biodegradability, abundance, and low cost (Gómez-Guillén et al., 2011). Conversely, lecithin can be prepared from oil-bearing seeds such as soybeans, sunflower kernels and rapeseed (van Nieuwenhuyzen and Tomás, 2008) and is widely used as a natural emulsifier/surfactant in the food, cosmetic, medicine, and biotechnology industries. (Shchipunov and Schmiedel, 1996) The excellent emulsifying property of lecithin is mainly attributed to phospholipids that consist of a glycerol backbone esterified...
with two fatty acids and a phosphate group. (Arnold et al., 2012)

The aim of this work was to study the possibility of blending lecithin and gelatin to prepare stable, GRAS thymol nanodispersions. Thymol was homogenized at different concentrations using different amounts of gelatin and soy lecithin individually or in combination. To facilitate nanodispersion formation, propylene glycol (PG), a GRAS food additive used in food and tobacco products, (Karl and Rozman, 2006; Registry, 2007) was used as a co-surfactant, as in studies formulating microemulsions (El Maghraby, 2008; Kale and Allen, 1989; Stilbs et al., 1983) and nanoemulsions. (Rao and McClements, 2011).

3.3 Materials and Methods

3.3.1 Materials

Thymol (99% purity) and type A gelatin were purchased from Acros Organics (Morris Plains, NJ). Type B gelatin, soy lecithin (phosphatidylcholines) and other chemicals, such as PG and methanol, were obtained from Fisher Scientific (Pittsburgh, PA).

3.3.2 Preparation of nanodispersions

The 0-0.06 g gelatin was dissolved in 9 mL deionized water. Different amounts of lecithin (0, 0.05, 0.1 and 0.15 g) were added into the gelatin solution, followed by vortexing for 1 h at room temperature (21 °C), and the pH was 6.8. The working thymol solution was prepared separately by dissolving various amounts (0, 0.04, 0.08, 0.10, and 0.12 g) in 1 mL PG. The gelatin/lecithin sample and thymol solution were mixed and emulsified at 15,000 rpm for 2 min using a model Cyclone I.Q.² microprocessor homogenizer (The VirTis Company, Inc., Gardiner,
3.3.3 Particle size measurement

Particle size distributions of dispersions were measured using a DelaSTM Nano-Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter, Inc., Brea, CA). The volume-length mean particle diameter ($d_{4,3}$) was calculated from the number of particles ($n_i$) with the corresponding diameter ($d_i$) based on the following equation:

$$d_{4,3} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$$

(1)

3.3.4 Turbidity

The absorbance of dispersions was measured at 600 nm ($A_{bs600}$) as the indicator of turbidity using a UV-vis spectrophotometer (model Biomate 5, Thermo Electron Crop., Woburn, MA).

3.3.5 Transmission Electron Microscopy (TEM)

The morphology of thymol dispersions prepared with gelatin alone, lecithin alone and gelatin-lecithin blend was characterized using a Zeiss Libra 200 transmission electron microscope (Carl Zeiss SMT Inc., Oberkochen, Germany). Five µL of the emulsion was placed onto a copper grid and stained with a drop of 2%w/w uranyl acetate solution. After 2 min, the grid was tapped with filter paper, followed by air-drying at room temperature (21 °C) before imaging.
3.3.6 Quantification of protein in the serum of dispersions

The nanodispersions were prepared with the aqueous phase with 0.2%w/v gelatin B and 1.0%w/v lecithin at pH 7.0. After homogenizing 0.8%w/v thymol as above, nanodispersions were adjusted to pH 2.0-6.0 using 1.0 M HCl or pH 8.0 using 1.0 M NaOH. The gelatin B-lecithin blend without thymol was also prepared using the same procedures. All samples were centrifuged at 16,110 g for 30 min at 25 °C using an Optima MAX Ultracentrifuge (Beckman Coulter, Brea, CA, USA) to obtain the supernatant for protein assay. Because thymol interferes with protein assay, 200 μL of the supernatant was mixed with 6 mL of an acetone-hexane (1:1, v:v) mixture to extract thymol and precipitate protein. (Martín-Hernández et al., 2005) After vigorous mixing and centrifugation at 6,700 g for 5 min using an Eppendorf MiniSpin plus centrifuge (Hamburg, Germany), the supernatant (organic phase) was discarded, and the bottom protein precipitate was re-dissolved in 300 μL of 100 mM NaOH and was quantified using the bicinchoninic acid (BCA) method (Thermo Fisher Scientific Inc., Morris Plains, NJ). Gelatin was used as a reference protein in the BCA assay.

3.3.7 Zeta-potential

The zeta potentials of thymol dispersions were measured using a DelasTM Nano-Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter Inc., Brea, CA). Samples were diluted 100 times using deionized water and adjusted to pH 3.0-7.0 before analysis. Three dispersion replicates were tested for three times each.
3.3.8 Storage stability

Storage stability of thymol dispersions at room temperature (21 °C) was assessed over a period of one month. The dispersions were monitored weekly for changes in particle size.

3.3.9 Statistical Analysis

Analysis of variance (ANOVA) test was conducted using the SPSS 16.0 statistical analysis system (SPSS Inc., Chicago, IL, USA). The least significant difference (LSD) test was used to determine the significant difference of mean values at a $P$ level of 0.05.

3.4 Results and Discussion

3.4.1 Effects of gelatin type and lecithin concentration on particle dimension at neutral pH

The $d_{4,3}$ and particle size distribution of dispersions prepared with two types of gelatin and different amounts of lecithin are shown in Figure 3-1. The $d_{4,3}$ of dispersions prepared with gelatin alone was around 250 nm. Blending lecithin with gelatin significantly decreased the $d_{4,3}$ when compared to gelatin alone without thymol ($P < 0.05$), indicating the formation of gelatin-lecithin complexes. When thymol was present, the $d_{4,3}$ of the dispersions prepared with gelatin-lecithin blends further decreased, which may have resulted from the increased particle packing due to the strengthened intra-particle attraction by hydrophobic thymol. Generally speaking, the $d_{4,3}$ decreased with an increase in lecithin concentration until about 1% w/v, above which there was no significant further decrease in $d_{4,3}$ ($P > 0.05$). The increase of lecithin content also corresponded to the narrowed particle size distributions of dispersions containing 0.8% thymol (Figure 3-1C and D). A greater amount of lecithin enables the stabilization of a larger interfacial
area, leading to smaller particles, until the radius of droplet curvature reaching a critical value beyond which no further decrease in the particle size is enabled (Yang et al., 2011). Furthermore, a greater $d_{4,3}$ was observed at a higher thymol concentration between 0.4 and 1.2% w/v when 0.5% w/v lecithin and 0.2% w/v gelatin were used. In contrast, there was no significant change in $d_{4,3}$ when 0.4-1.2% w/v thymol was dispersed by 0.2% w/v gelatin and 1 or 1.5% w/v lecithin ($P > 0.05$). Therefore, the combination of 1% w/v lecithin and 0.2% w/v gelatin was sufficient to disperse 1.2% w/v thymol.

When two gelatin types were compared, it was observed that dispersions prepared with gelatin A had significantly bigger $d_{4,3}$ than those with gelatin B when 0 and 0.5% w/v lecithin was used ($P < 0.05$, Figure 3-1A and B), which indicates that complexes formed with gelatin B are more effective in dispersing thymol. As discussed previously, Gelatin A has pI of ~ 8-9 and has net positive charges at neutral pH. (Gómez-Guillén et al., 2011) In contrast, gelatin B has pI of ~4-5 and is net negatively charged at neutral pH. (Gómez-Guillén et al., 2011) The electrostatic attraction between gelatin A and highly negatively-charged lecithin may enable the formation of more compact complexes than those of gelatin B, which may reduce the ability to disperse thymol. When more lecithin (1 and 1.5% w/v) was used with 0.2% w/v gelatin, the $d_{4,3}$ of the dispersions prepared with gelatin B was slightly smaller than those with gelatin A, which indicates that the effect of gelatin type was not significant when a sufficient amount of lecithin was used. Furthermore, it was observed that dispersions prepared with gelatin A precipitated
after storage for two to three days at room temperature (21 °C), while those prepared with gelatin B were stable after several months. This likely is due to the net surface charge being smaller for samples with gelatin A, due to opposite charges between gelatin A and lecithin but same types of net charges for the case of gelatin B and lecithin. Based on this set of experiments, gelatin B and 1% w/v lecithin were chosen for further studies.

3.4.2 Effects of gelatin B concentration on dispersion properties at neutral pH

Appearance, $A_{560}$, and $d_{4,3}$ of thymol dispersions prepared with 1% w/v lecithin, 0.8% w/v thymol, and 0-0.6% w/v gelatin B are presented in Figure 3-2. The dispersion prepared with lecithin only was very turbid (Figure 3-2A), which became translucent after blending with gelatin B. The dispersions prepared with intermediate gelatin B concentrations (0.2-0.5% w/v) showed the lowest turbidity. The sample with 0.6% w/v gelatin B was more turbid, possibly because of the limited solubility of gelatin B. The $A_{560}$ data (Figure 3-2B) generally agreed with the visual appearance (Figure 3-2A). No obvious differences in $A_{560}$ of treatments with 0.2-0.5% w/v gelatin B indicate the 0.8% w/v thymol was well dispersed at these conditions. Conversely, a sharp decrease in $d_{4,3}$ was observed when gelatin B concentration increased from 0 to 0.1% w/v, followed by graduate increases in particle dimension as gelatin B concentration further increased. The $d_{4,3}$ was not significantly different for the treatments with 0.2-0.5% w/v gelatin B ($P > 0.05$), while that with 0.6% w/v gelatin B was significantly bigger than other treatments with gelatin B ($P < 0.05$, Figure 3-2C). In a study encapsulating drug Amphotericin B, the lipophilic drug was mixed with lecithin in an organic solvent mixture of dimethyl sulfoxide
and methanol, followed by mixing with an aqueous gelatin solution to form sub-micrometer particles. (Jain et al., 2012) Confocal laser scanning microscopy and fluorescent resonance energy transfer analyses showed that lecithin was present in the particle interior and gelatin was present on particle surface. This study suggests that the more hydrophobic lecithin is preferably in contact with the lipophilic drug, while the hydrophilic gelatin binds with lecithin on particle surface. In our study, if thymol particles are preferentially emulsified by lecithin, the particles with adsorbed gelatin B would be bigger than those with lecithin only. The expectation from this sequential adsorption of lecithin and gelatin on thymol particles is opposite to the data in Figure 3-2C. Therefore, it is more likely that gelatin B forms complexes with lecithin, which adsorb on thymol particles simultaneously during emulsification. Since 0.2%w/v of gelatin B was sufficient to form a translucent dispersion, this concentration was selected for further studies.

3.4.3 Morphology of thymol particles

The morphology of thymol particles (Figure 3-3) was studied for dispersions prepared with 0.8%w/v thymol dispersed by 0.2%w/v gelatin B alone, 1.0%w/v lecithin alone, and their combination. The dispersion prepared with the blend (Figure 3-3C and D) had mostly spherical particles with a mean diameter of 60 nm that agrees with the $d_{4,3}$ of 49 nm (Figure 3-1B) and the translucent appearance (Figure 3-2A). In contrast, bigger and irregular particles were observed for dispersions prepared with lecithin only (Figure 3-3A) and gelatin B only (Figure 3-3B), which agrees with turbid appearance of both dispersions (Figure 3-2B) for the sample dispersed with lecithin only; the other sample is not shown). The heterogeneity of particle structures
(Figure 3-3) overall agrees with the particle size distributions in Figure 3-1D. Lecithin used in this study (phosphatidylcholines) is soluble in hexane and its poor water-solubility can contribute to turbidity (Figure 3-2) and irregular structures (Figure 3-3A). In contrast, 0.2%w/v gelatin B is dissolved in water as a transparent solution, but the turbid dispersion and irregular big particles after emulsifying thymol indicate the weak emulsifying property of gelatin. In addition, the particle dimension observed by TEM was bigger than the $d_{4,3}$ values of dispersions made with gelatin B alone (250 nm, Figure 3-1B) or lecithin alone (76 nm, Figure 3-2C). The difference can be contributed by sample preparation with (in light scattering) and without (in TEM) dilution. Dilution can separate weakly flocculated particles/structures, lowering the measured particle dimension.

**3.4.4 Effects of pH on dispersion stability**

The stability of dispersions prepared with 0.8%w/v thymol, 0.2%w/v gelatin B and 1%w/v lecithin was investigated at pH 2.0-8.0. The first set of samples was prepared for aqueous mixtures of gelatin B and lecithin without thymol to study the complex formation of the two molecules. Figure 3-4 shows that samples at pH 4.0-8.0 were turbid and those at pH 2 and 3 precipitated quickly. The turbidity is due to the poor water solubility of lecithin, as discussed previously. The precipitation at pH 3.0 contrasts with stable samples with gelatin or lecithin alone (not shown). After centrifugation of the mixture at pH 3.0, the serum contained 0.0169%w/v protein (gelatin), less than 10% of the total gelatin concentration (0.2%w/v), which indicates the co-precipitation of lecithin and gelatin B.
In the second set of treatments, thymol dispersions prepared at pH 7.0 were adjusted to pH 2.0-6.0. Like the mixture (Figure 3-4), dispersions at pH 2.0 and 3.0 also showed significant precipitation. The dispersion at pH 4.0 showed some precipitated structures, while dispersions at pH 5.0-7.0 were stable. The acidification increased the $d_{4,3}$ (Table 3-1), with that at pH 5.0 being significantly bigger ($P < 0.05$). In comparison, the $d_{4,3}$ of gelatin-lecithin mixture (without thymol) was not significantly different at pH 5.0-7.0 ($P > 0.05$) but was significantly bigger than comparable samples with thymol at pH 6.0 and 7.0 ($P < 0.05$). This suggests that the adsorption of gelatin B-lecithin complexes on thymol particles resulted in much ordered structures. The inclusion of thymol in nanoparticles increases the overall hydrophobicity that can cause some particle aggregation when repulsive electrostatic interactions are weakened below pH 5.0, as discussed further below for zeta-potential data.

To further study surface composition of thymol particles, the protein content in the serum of dispersions with and without thymol after centrifugation was measured as the content of free gelatin (Table 3-2). At pH 5.0, close to the pI of gelatin B, (Gómez-Guillén et al., 2011) the protein content in the thymol dispersion serum was lower than that at pH 6.0 and 7.0 ($P < 0.05$), which was in agreement with the significantly bigger particles at pH 5.0 (Table 3-1). Conversely, similar to $d_{4,3}$ (Table 3-1), there was no significant difference in the serum protein content in the blend dispersions (without thymol) at pH 5.0-7.0 ($P > 0.05$). Because gelatin only solutions had the gradually decreased protein contents after acidification to 5.0 and centrifugation (Table 3-2), similar serum protein contents in the blend dispersions suggest the complex formation between
gelatin B and lecithin that resulted in the stability at pH 5.0-7.0. The thymol dispersion at pH 5.0 did not show visible precipitation, and the lowered serum protein content after centrifugation is likely due to the sedimentation of aggregated particles (Table 3-1).

3.4.5 Zeta-potential of thymol dispersions

To study the role of electrostatic interactions on dispersion formation and stability, zeta-potentials of thymol dispersions prepared with gelatin B alone, lecithin alone and their blend were measured (Figure 3-5). The zeta-potentials of dispersions prepared with lecithin only were highly negative at pH 3.0-7.0, with a reduced magnitude at a lower pH. This is due to the presence of ionized phosphate group of lecithin that is protonated to a greater extent at a lower pH, as previously reported. (Chuah et al., 2009) In comparison, dispersions prepared with gelatin B only showed the characteristics expected from gelatin B, with zeta-potential being zero at about pH 4.8 that corresponds to the pI of gelatin B. At pH 6.0 and 7.0, the negative zeta-potential of dispersions prepared with gelatin B only had much smaller magnitudes than those prepared with lecithin.

For dispersions prepared with gelatin B-lecithin blend, zeta-potentials at pH 5.0, 6.0 and 7.0 were close to those prepared with lecithin alone. Besides, thymol dispersion prepared with the blend exhibited similar zeta-potential at pH 5.0, 6.0 and 7.0, which contradicts with the different d_{4,3} and serum protein content of thymol nanodispersions at pH 5.0, 6.0 and 7.0 (Tables 3-1 and 2). The zeta-potential data suggest additional colloidal forces causing particle aggregation at pH 5.0. It may be caused by the increased particle hydrophobicity due to gelatin B
at the acidity close to its pI (Figure 3-5). The strong electrostatic repulsion at pH 5.0 was able to limit the particle aggregation (Table 3-1) so that the overall dispersion showed the absence of macroscopic precipitation.

At pH 4.0, zeta-potential of the thymol nanodispersion prepared by the gelatin B-lecithin blend reduced to about one-half of the dispersion prepared with lecithin only (Figure 3-5), indicating that gelatin B also had a significant influence on the droplet surface charge. At pH 4.0, gelatin B is positively charged and neutralizes negative charges of lecithin. The reduced zeta-potential at pH 4.0 may have caused some precipitation of thymol particles, as discussed previously. At pH 3.0, as gelatin B had more positive charges, the zeta-potential of the dispersion prepared with the blend decreased further in magnitude to be close to zero, which caused severe precipitation. Therefore, electrostatic interactions are the major colloidal force responsible for the stability of thymol dispersions prepared with gelatin B-lecithin blend. The stable dispersions at pH 5.0 and above are significant for their applications, as many foodborne pathogens can grow in low acid foods (> pH 4.6).

3.4.6 Storage stability of dispersions at neutral pH

The storage stability of dispersions containing 0.8%w/v thymol at neutral pH was evaluated by measuring $d_{4,3}$ weekly for one month at room temperature (21 °C). Figure 3-6 shows that the dispersion prepared with lecithin-gelatin B blend had a constant $d_{4,3}$ of around 55 nm during storage. In contrast, $d_{4,3}$ of dispersions prepared with gelatin B alone increased significantly after 4 weeks ($P < 0.05$), from 220 to 280 nm. For dispersions prepared with
lecithin alone, \(d_{4,3}\) also increased from 68 to 85 nm. Because thymol has a solubility of about 0.048% w/v in water, (Chen et al., 2013) Ostwald ripening has been observed to cause the increase of particle size in thyme oil nanodispersions emulsified by Tween 80, which can be eliminated by blending with lipids with low water solubility. (Chang et al., 2012; Ziani et al., 2011) The use of low water solubility lipids however reduced the loading and antimicrobial efficacy of essential oils. Therefore, stable thymol nanodispersions prepared by the gelatin B-lecithin blend are promising for use as antimicrobial delivery systems. Furthermore, in our recent studies, the minimum inhibitory concentration of thymol increased or remained unchanged after encapsulation in whey protein-maltodextrin conjugates (Shah et al., 2013; Xue et al., 2013) and sodium caseinate (Pan et al., 2014), respectively. Whey proteins have more ordered secondary structures and are more hydrophobic than caseins (Voutsinas et al., 1983). Since gelatin is less hydrophobic than whey proteins and caseins and does not have secondary structures (Voutsinas et al., 1983), impacts of gelatin on antimicrobial activity of thymol may be insignificant. Characterizations of antimicrobial activities of these dispersions and their impact on sensory properties of food products are underway.

3.5 Conclusions

In conclusion, gelatin B and lecithin, both GRAS emulsifiers, can be used as a blend to prepare translucent thymol nanodispersions with a mean diameter of about 50 nm at pH 5.0 and above. The synergistic surface activity of the blend is likely due to complex formation of the blend. The electrostatic repulsion, contributed mostly by lecithin, prevented particles from
aggregation. The blend complexes on thymol particle surface prevented Ostwald ripening and maintained particle dimension during storage at neutral pH. Therefore, the stable nanodispersions prepared from GRAS ingredients have great potential to incorporate lipophilic food antimicrobials like thymol in low acid foods to enhance the microbiological safety.

Acknowledgements

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References


Gioffrè, M., Torricelli, P., Panzavolta, S., Rubini, K., Bigi, A., 2012. Role of pH on stability and


Appendix

Table 3-1. Volume fraction-length mean diameter ($d_{4,3}$, nm) of dispersions with 0.8%w/v thymol dispersed by 0.2%w/v gelatin B and 1.0%w/v lecithin at pH 7.0 and after acidification to pH 5.0 and 6.0, with comparison to the blend (without thymol) prepared at identical conditions.*

<table>
<thead>
<tr>
<th></th>
<th>pH 5</th>
<th>pH 6</th>
<th>pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>With thymol</td>
<td>159.3±11.9a</td>
<td>58.9±2.0cde</td>
<td>45.3±2.1e</td>
</tr>
<tr>
<td>Without thymol</td>
<td>135.2±3.0ab</td>
<td>132.7±4.9ab</td>
<td>109.1±7.7ab</td>
</tr>
</tbody>
</table>

*Numbers are mean ± standard deviation from triplicates. Different superscript letters represent significant differences in the mean.
Table 3-2. Percentages of total protein in the serum of dispersions with 0.8% w/v thymol dispersed by 0.2% w/v gelatin B and 1.0% w/v lecithin at pH 7.0 and after acidification to pH 5.0 and 6.0, with comparison to the blend or gelatin B only (without thymol) prepared at identical conditions.*

<table>
<thead>
<tr>
<th></th>
<th>Protein% in the serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 5</td>
</tr>
<tr>
<td>Thymol dispersion</td>
<td>49.51±12.28&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gelatin B-lecithin blend</td>
<td>67.06±1.52&lt;sup&gt;cdef&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gelatin B only</td>
<td>75.32±0.82&lt;sup&gt;bcd&lt;/sup&gt;</td>
</tr>
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</table>

*Numbers are mean ± standard deviation from triplicates. Different superscript letters represent significant differences in the mean.
**Figure 3-1.** Volume fraction-length mean particle diameter ($d_{4,3}$) of thymol dispersions prepared with 0.2%w/v type A (A) and type B (B) gelatin and different amounts of lecithin. Size distributions are shown in C and D for samples containing 0.8% thymol emulsified by gelatin A and B, respectively. Error bars are standard deviations from duplicates.
Figure 3-2. Appearance (A), absorbance at 600 nm (B), and volume-length mean particle diameter ($d_{4,3}$) of thymol dispersions prepared with 0-0.6% w/v gelatin B, 1% w/v lecithin, and 0.8% w/v thymol. Error bars are standard deviations from duplicates. Different letters next to symbols indicate significant differences in the mean ($P < 0.05$).
Figure 3-3. TEM images of dispersions prepared with 0.8% w/v thymol and 1.0% w/v lecithin only (A), 0.2% w/v gelatin B only (B), and their blend (C and D, in two magnifications).
Figure 3-4. Appearance of aqueous mixtures with 1.0% w/v lecithin and 0.2% w/v gelatin B adjusted to different pH.
**Figure 3-5.** Zeta-potential of dispersions with 0.8% w/v thymol dispersed by 0.2% w/v gelatin B alone, 1.0% w/v lecithin alone, or their blend at pH 3.0-7.0. Error bars are standard deviations from triplicates.
Figure 3-6. Changes of volume fraction-length mean particle diameter ($d_{4,3}$) of dispersions with 0.8%w/v thymol dispersed by 1.0%w/v lecithin alone, 0.2%w/v gelatin B alone, or their blend at pH 7.0 during storage at room temperature (21 °C) for 4 weeks. Error bars are standard deviations from triplicates. Different letters above bars indicate significant differences in the mean of the same sample ($P < 0.05$).
Chapter 4. Inhibition of *Escherichia coli* O157:H7 and *Listeria monocytognes* growth in milk and cantaloupe juice by thymol nanoemulsions prepared with gelatin and lecithin
4.1 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. Nanoemulsions were overall more effective than free thymol in both media. Compared to the bacteriostatic effect of free thymol, nanoemulsions 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 the gelatin-B nanoemulsion. Conversely, the nanoemulsion with gelatin-B reduced *Escherichia coli* O157:H7 to below the detection limit after 8 h in 2% reduced fat 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 between nanoemulsions prepared by two types of gelatin suggest the impacts of droplet surface charge 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
4.2 Introduction

Foodborne illnesses continue to be a critical health issue according to the Centers for Disease Control and Prevention (CDC), estimated to include 48 million illnesses, 3000 deaths and 128000 hospitalizations in the United States annually that amount as much as $77.7 billion economic losses (Scharff, 2012). *Listeria monocytogenes* and *Escherichia coli* O157:H7 are two major foodborne pathogens, and their infections cost $2.3 billion and $0.7 billion annually, respectively (Gaysinsky et al., 2007). CDC estimated that 91% of hospitalizations were caused by *L. monocytogenes* infections (Jemmi and Stephan, 2006), which can lead to sever illnesses and even deaths among susceptible populations, e.g. pregnant women, infants, the elderly, and immunosuppressed individuals (Mastronicolis et al., 1996). Listeriosis has been linked to consumption of contaminated milk, cheese, meat, fish, and vegetables (Bell and Kyriakides, 1998). Each year, 73000 illnesses, 2168 hospitalizations and 61 deaths are caused by *E. coli* O157:H7 in the United States (Rangel et al., 2005). Undercooked ground beef (Mead and 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.

Novel and efficient strategies are still needed to reduce foodborne illnesses. Natural antimicrobials such as essential oils (EOs) have been intensively 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 (Burt, 2004). 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 5-50 nm. Microemulsions increase the concentration of EOs in the aqueous phase and can effective inhibited the growth of bacteria including *L. monocytogenes* and *E. coli* O157:H7 (Gaysinsky et al., 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 antilisterial activity in milk when comparing to unencapsulated (free) thymol (Pan et al., 2014; Xue et al., 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. 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 studied in our recent
study (Xue and Zhong, 2014a). 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) demonstrated synergistic surface activity with lecithin to form thymol nanoemulsions. Thymol nanoemulsions produced with the elatin-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 gelatin A-lecithin blend, which was attributed to their differences in droplet surface charges.

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 as tested as model foodborne pathogens.

4.3 Materials and Methods

4.3.1 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). Ultra high temperature (UHT)-pasteurized organic milk (skim, 2% reduced fat and full fat milk) and fresh cantaloupe was purchased from Kroger Company (Cincinnati, OH).
4.3.2 Preparation of thymol nanoemulsions

0.02 g gelatin was dissolved in 9 mL deionized water. 0.08 g soy lecithin were 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).

4.3.3 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.

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

The MIC was determined using a microbroth dilution assay (Davidson and Parish, 1989). The stock free thymol sample was prepared by diluting a 10% w/v thymol in ethanol to 0.1% w/v with TSB. The 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. 10^6 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 by preparing culture with a same volume of 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.

### 4.3.5 Antimicrobial susceptibility test in milk

The antimicrobial susceptibility in three types of milk was studied in duplicate using time-kill assays (Shah et al., 2012b). 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 110 mL.
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 ~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 enumerated by 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 log CFU/mL.

4.3.6 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 (Chen et al., 2014). 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) PVDF 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 HPLC conditions were detailed previously (Chen et al., 2014). 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.
4.3.7 Antimicrobial susceptibility test in cantaloupe juice

Preparation of cantaloupe juice. Fresh cantaloupes were washed. 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 4629g 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 10mL. 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 μL) 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 μL culture with ~ 10⁸ CFU/mL L. monocytogenes. After incubation at room temperature (21 °C) for 24h, samples were serially diluted with 0.1% peptone, and survivors enumerated by plating on TSA and incubating at 32 °C for 24 h. The detection limit was 1 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. The juice samples were prepared as above to a
final thymol concentration of 0.6 g/L. The enumeration was conducted after incubation at room temperature (21 °C) for 0, 3, 6, 24, and 48 h.

4.3.8 Statistical analysis

Analysis of variance (ANOVA) test was conducted using the SPSS 16.0 statistical analysis system (SPSS Inc., Chicago, IL). The least significant difference (LSD) test was used to determine the difference of mean values at a significance level of 0.05.

4.4 Results and Discussion

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

MICs and MBCs of free thymol and two thymol emulsions against *E. coli* O157:H7 and *L. monocytogenes* are shown in Table 4-1. The MICs and MBCs of free and nano-emulsified thymol were either identical or different only by one level in the dilution scheme. The MICs and MBCs of free thymol in Table 4-1 were in agreement with our previous studies (Chen et al., 2014; Wu et al., 2014; Xue et al., 2013). The overall similar MICs and MBCs of thymol before and after nanoemulsification indicates insignificant impacts of the studied emulsifier formulations on antimicrobial activity of nano-emulsified thymol. Previously, the binding of EO components with lecithin (Li, 2011) and polysorbate 80 (Terjung et al., 2012) was reported to reduce the antimicrobial activity. In our previous study, gelatin and lecithin were observed to co-adsorb on thymol nanoparticles (Xue and Zhong, 2014b). 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.

4.4.2 Antimicrobial activities of thymol in milk

The antimicrobial activities of free and nano-emulsified thymol against *L. monocytogenes* and *E. coli* O157:H7 in skim, 2% reduced fat and full fat milk are shown in Figure 4-1, 2, and 3, respectively. In skim milk, with 1 g/L thymol, 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 gelatin-A emulsion and no significant change for the treatment with gelatin-B emulsion after 48 h (Figure 4-1A). *E. coli* O157:H7 was less resistant than *L. monocytogenes* in skim milk (Figure 4-1B). Free thymol reduced more than 3 log CFU/mL *E. coli* O157:H7 after 24 h. The emulsions exhibited a faster reduction rate than free thymol, and the *E. coli* O157:H7 became undetectable after 24 h. 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 gelatin-A emulsion but only 2 log CFU/mL for the gelatin-B emulsion (Figure 4-1B).

In 2% reduced fat milk, 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 4-2A). 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 gelatin-A emulsion treatments but was still undetectable for the treatment with gelatin-B emulsion (Figure 4-2B).

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 4-3A). 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 4-3B). Similar to the observations in 2% reduced fat milk (Figure 4-2B), *E. coli* O157:H7 treated by the gelatin-A emulsion and free thymol recovered to 2.7 log CFU/mL but was undetectable in the gelatin-B emulsion treatment after 48 h (Figure 4-3B).

Overall, both thymol emulsions were more effective than free thymol against *L. monocytogenes* and *E. coli* O157:H7 in milk, and the gelatin-B emulsion was more effective than the gelatin-A emulsion 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). The difference between emulsions prepared with gelatin A and gelatin B in inhibition of *E. coli* O157:H7 in 2% reduced fat and full fat milk may be related to the impact of droplet surface charge on interactions with bacteria membrane.
4.4.3 Thymol concentration in the serum of milk

In our previous studies (Pan et al., 2014; Xue et al., 2013), nanoencapsulation of thymol in whey protein-maltodextrin or sodium caseinate increased the concentration of thymol in the serum of milk, which was correlated to their antimicrobial activity. When the thymol concentration in milk serum 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 concentration of thymol in milk serum was between MIC and MBC. By analyzing thymol concentration in milk serum, it is then possible to correlate antimicrobial activity in simple (TSB) and complex media (milk, with protein and lipid particles binding antimicrobials).

The concentrations of free thymol and two emulsions in milk serum are shown in Figure 4-4. Shortly after mixing with milk (0 h in Figure 4-4A), two emulsions treatments had similar thymol concentrations in the serum of 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 emulsions (Figures 4-1, 2 and 3). When the same amount (4.5 g/L) of free or emulsified thymol was added, thymol concentration in the serum of 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 (Cava et al., 2007; Chen et al., 2014) and supports the lowered antimicrobial activities of thymol in full fat milk than in 2% reduced fat milk (Figure 4-2 and 3).
After 24-h incubation at room temperature (Figure 4-4B), the thymol concentration in milk serum remained significantly higher for emulsion treatments than the free thymol treatment. After 48 h, thymol concentration in the serum of skim milk was similar (P > 0.05) in free thymol and emulsion treatments (Figure 4-4C), suggesting that free thymol (in crystal form) slowly dissolved in skim milk. In contrast, free thymol in 2% reduced fat milk showed slightly decreased thymol concentration when compared to those at 0 h and 24 h, which may indicate more binding occurred between thymol and fat globular. 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 emulsions treatments.

Releasing from thymol crystals or emulsion droplets and binding with protein and lipid particles are two competing mechanisms impacting thymol concentration in milk serum. Incubation only increased the thymol concentration in the serum of skim milk because of the weakened impacts of the low fat content. Generally, the trends of different treatments in the thymol concentration in aqueous phase of three types of milk was in agreement with their antimicrobial activities in each type of milk. For example, treatments (especially emulsions) in 2% reduced fat milk had the highest amounts of thymol in aqueous phase resulted in the most effective antimicrobial activities against *L. monocytogenes* and *E. coli* O157:H7 (Figure 4-2).

4.4.4 Antimicrobial activities of thymol in cantaloupe juice

Fruit juice is another large group of food products that can be easily contaminated by foodborne pathogens (Balla and Farkas, 2006). The outbreaks of foodborne illnesses have been
associated with unpasteurized fruit juices (Raybaudi-Massilia et al., 2009). Most fruit juices in antimicrobial studies have a pH below 5 such as apple and orange juices (Ferrante et al., 2007; Friedman et al., 2004). In this part of study, the antimicrobial assays were conducted in cantaloupe juice with a neutral pH (6.81).

Log reduction of *L. monocytogenes*

Log reductions of *L. monocytogenes* in cantaloupe juice treated by different amounts of thymol are presented in Table 4-2. Two groups of free thymol were studied: crystals added in juice directly or pre-dissolved in PG. 0.6 g/L thymol pre-dissolved in PG or nanoemulsified thymol reduced *L. monocytogenes* 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 0.19 and 0.50 log CFU/mL reduction, 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 molecules throughout the juice, which also competes with the growth of bacteria. Similarly, 0.067% carvacrol was found highly bactericidal in apple juice (Friedman et al., 2004).

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 gelatin–A emulsion, gelatin-B emulsion, 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 and Grimm, 2001). When comparing two emulsions, the more effective anti-listerial activity of gelatin-A emulsion may be attributed to the relatively weak binding between emulsifiers (gelatin A-lecithin blend) and thymol as compared to emulsion prepared with gelatin B-lecithin blend (produced smaller droplets), which was caused by the different interaction of gelatin A or B with lecithin (Xue and Zhong, 2014a).

**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 4-5). 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. 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 $\sim$3 log CFU/mL and $\sim$2 log CFU/mL, respectively. A recovery of *L. monocytogenes* to $\sim$3 log CFU/mL was also observed for the treatment with gelatin A-emulsion after 48 h, while no recovery was found in the treatment with gelatin-B emulsion. No recovery of *E. coli* O157:H7 was observed after 48 h for both emulsions. 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, and the recovery
occurred quicker (after 6 h) to a greater extent (Shah et al., 2012a). 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 4-1, 2 and 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 4-1), which resulted in quick inhibition of bacteria (Figure 4-5). The better inhibition of *L. monocytogenes* by the gelatin-B emulsion than the gelatin-A emulsion after 48 h (Figure 4-5) is in agreement with treatments in skim milk (Figure 4-1), which can be correlated to charge differences between the two types of gelatin, as discussed previously. When two emulsion treatments were compared, the better log reduction of *L. monocytogenes* after 24 h by 0.4 g/L thymol from gelatin-A emulsion than gelatin-B emulsion (Table 4-2) was opposite to the data after 48 h with 0.6 g/L. As discussed above, the binding between emulsifier and thymol in gelatin A emulsion was weaker than that in gelatin B emulsion and may be significant when the thymol level (0.4 g/L) was close to MBC (Table 4-1). There was more thymol available in gelatin A emulsion than in gelatin B emulsion, which probably led to the higher log reduction after 24 h, but the bacteria may recover after 48 h. When thymol amount was sufficient (such as 0.6 g/L) in the system, the binding between emulsifier and thymol in gelatin B emulsion may reduce the interaction of thymol with some juice components. Lastly, the consistently better inhibition of nanoemulsions than thymol pre-dissolved in PG (Table 4-2,
Figure 4-5) may result from the reduced binding between juice components and thymol after encapsulation.

4.5 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. The slight difference in antimicrobial activity between two emulsions may be attributed to their different physicochemical properties such as droplet surface charge and binding with thymol. The effectiveness of thymol nanoemulsions inhibiting pathogens in different food matrices indicate their potential as novel antimicrobial preservatives in food systems to enhance microbial safety.

Acknowledgements

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References


124
Appendix

Table 4-1. MIC and MBC of free thymol and thymol emulsions against *E. coli* O157:H7 at 37°C and *L. monocytogenes* at 32°C in TSB.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th><em>E. coli</em> O157:H7</th>
<th><em>L. monocytogenes</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (g/L)</td>
<td>MBC (g/L)</td>
</tr>
<tr>
<td>Free thymol</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Gelatin-A emulsion</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Gelatin-B emulsion</td>
<td>0.20</td>
<td>0.20</td>
</tr>
</tbody>
</table>
Table 4-2. 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 in cantaloupe juice at room temperature (21 °C) after 24 h.

<table>
<thead>
<tr>
<th>Thymol concentration (g/L)</th>
<th>Thymol crystals reduction (log CFU/mL)*</th>
<th>Thymol pre-dissolved in PG reduction (log CFU/mL)*</th>
<th>Gelatin-A emulsion reduction (log CFU/mL)*</th>
<th>Gelatin-B emulsion reduction (log CFU/mL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>-1.63 ± 0.09d</td>
<td>1.02 ± 0.34c</td>
<td>4.23 ± 0.49a</td>
<td>2.69 ± 0.05b</td>
</tr>
<tr>
<td>0.6</td>
<td>-1.37 ± 0.07d</td>
<td>&gt;4.58 ± 0.00a</td>
<td>&gt;4.58 ± 0.00a</td>
<td>&gt;4.58 ± 0.00a</td>
</tr>
<tr>
<td>0.8</td>
<td>0.19 ± 0.61c</td>
<td>&gt;4.58 ± 0.00a</td>
<td>&gt;4.58 ± 0.00a</td>
<td>&gt;4.58 ± 0.00a</td>
</tr>
<tr>
<td>1.0</td>
<td>0.50 ± 0.05c</td>
<td>&gt;4.58 ± 0.00a</td>
<td>&gt;4.58 ± 0.00a</td>
<td>&gt;4.58 ± 0.00a</td>
</tr>
</tbody>
</table>

* Numbers are mean ± standard deviation from duplicates. Different superscript letters represent significant differences in the mean (*P* < 0.05).
**Figure 4-1.** 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 or nanoemulsified thymol. Error bars are standard deviations (n = 2).
Figure 4-2. 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 or nanoemulsified thymol. Error bars are standard deviations (n = 2).
Figure 4-3. 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 or nanoemulsified thymol. Error bars are standard deviations (n = 2).
Figure 4-4. 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 or nanoemulsified thymol 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 4-5. 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 or nanoemulsified thymol. Free thymol was prepared by adding crystals directly or a stock solution with 10% thymol in propylene glycol (PG). Error bars are standard deviations (n = 2).
Chapter 5. Thyme oil nanoemulsions co-emulsified by sodium caseinate and lecithin
A version of this chapter was originally published by Jia Xue and Qixin Zhong:


My primary contributions to this paper include sample preparation, data collection and analysis, results interpretation and writing.
5.1 Abstract

Many nanoemulsions are currently formulated with synthetic surfactants. The objective of the present work was to study the possibility of blending sodium caseinate (NaCas) and lecithin to prepare transparent thyme oil nanoemulsions. Thyme oil was emulsified using NaCas and soy lecithin individually or in combination at neutral pH by shear homogenization. The surfactant combination improved the oil content in transparent/translucent nanoemulsions, from 1.0% to 2.5% w/v for 5% NaCas with and without 1% lecithin, respectively. Nanoemulsions prepared with the NaCas-lecithin blend had hydrodynamic diameters smaller than 100 nm and had significantly smaller and more narrowly-distributed droplets than those prepared with NaCas or lecithin alone. Particle dimension and protein surface load data suggested the co-adsorption of both surfactants on oil droplets. These characteristics of nanoemulsions minimized destabilization mechanisms of creaming, coalescence, and Ostwald ripening, as evidenced by no significant changes in appearance and particle dimension after 120-day storage at 21 °C.

Keywords: Thyme oil, nanoemulsion, sodium caseinate, lecithin, synergistic surface activity
5.2 Introduction

Nanoemulsions are frequently studied as delivery systems of functional lipophilic compounds such as flavors (Rao and McClements, 2011a), vitamins (Relkin et al., 2009) and antimicrobials (Donsì et al., 2011) due to their advantages over other oil-containing systems. For example, compared with microemulsions, nanoemulsions can be formulated using a large variety of food grade ingredients such as proteins and polysaccharides and require relatively lower surfactant concentrations (McClements, 2004a). With small droplets (<100 nm in radius), nanoemulsions can be transparent or translucent and are more stable than conventional emulsions against gravitational separation, flocculation, and coalescence (McClements, 2004a). Therefore, nanoemulsions have great potential for use as delivery systems in the food industry.

Essential oils are well recognized as natural antimicrobial preservatives (Holley and Patel, 2005) and are classified by the US Food and Drug Administration as generally recognized as safe (GRAS) (Weiss et al., 2009). Their high efficacy against foodborne pathogenic and spoilage microorganisms has been widely reported (Elgayyar et al., 2001; Smith-Palmer et al., 1998). Like other lipophilic bioactive compounds, colloidal systems such as nanoemulsions are needed to disperse essential oils in aqueous food products (Weiss et al., 2009). Despite tremendous progresses in recent years (Donsì et al., 2012), much work is needed to prepare low-cost, safe, and scalable nanoemulsions for food applications (Kralova and Sjöblom, 2009).

Sodium caseinate (NaCas) and lecithin are two commonly-studied water-soluble and water-insoluble GRAS surfactants, respectively. NaCas is produced from milk through sequential...
steps of isoelectric precipitation of caseins (at ~pH 4.6), resuspension and neutralization of the precipitated casein with sodium hydroxide, and spray drying (Liu et al., 2012). NaCas has excellent emulsifying and stabilizing properties when used to prepare emulsions (Dickinson, 1999). Different from globular food proteins like whey proteins, caseins have highly disordered structures (Dickinson et al., 1998; Surh et al., 2006) that enable the rapid adsorption on the droplet surface during homogenization to form an interfacial layer as thick as 10 nm (Dalgleish et al., 1995; Dickinson and McClements, 1996). The interfacial casein layer not only protects emulsion droplets against flocculation and hence coalescence by providing repulsive electrostatic and steric interactions (Hu et al., 2003) but also preserves the stability of emulsions during heating, due to heat stability of caseins and elasticity of the interface (Hunt and Dalgleish, 1995; McClements, 2004b). Conversely, lecithin derived from oil-bearing seeds such as soybeans, sunflower kernels and rapeseed contains phospholipids that consist of a glycerol backbone esterified with two fatty acids and a phosphate group (van Nieuwenhuyzen and Tomás, 2008). The amphiphilic structure of phospholipids is responsible for the excellent emulsifying properties of lecithin, and the negative charges of phosphate groups provide repulsive electrostatic interactions important to the stability of emulsion droplets (Arnold et al., 2013). The combination of casein and egg yolk lecithin was observed to have better ability than individual ones to prepare soybean oil emulsions (Fang and Dalgleish, 1993) but has not been studied for essential oils.

The objective of the present work was to study emulsions of thyme oil prepared with
NaCas and soy lecithin individually or in combination by using shear homogenization. Thyme oil was selected due to its excellent antimicrobial activity against various microorganisms (Gaysinsky et al., 2008). Propylene glycol (PG), a GRAS food additive (Reproduction, 2004), was used as a co-surfactant because it facilitates small molecular surfactants to form microemulsions (El Maghraby, 2008; Kale and Allen, 1989) and nanoemulsions (Rao and McClements, 2011b). Our recent study also showed that PG greatly improved the capacity of whey protein-maltodextrin conjugates in emulsifying thymol, the major component in thyme oil, and antimicrobial properties of nanodispersions (Xue et al., 2013).

5.3 Materials and Methods

5.3.1 Materials

Thyme oil was purchased from Sigma-Aldrich Corp. (St. Louis, MO). NaCas was obtained from American Casein Co. (Burlington, NJ). Soy lecithin (phosphatidylcholines) and other chemicals were products of Fisher Scientific (Pittsburgh, PA).

5.3.2 Preparation of emulsions

NaCas was hydrated at 5% w/v in deionized water overnight at room temperature (21 °C). Four sets of thyme oil emulsions were prepared. In the first set, different amounts of thyme oil (0.5, 1.0, 1.5, 2.0, and 2.5% w/v) were directly emulsified in 10 mL of NaCas solution (9 mL 5% NaCas and 1 mL deionized water) at 15,000 rpm for 3 min using a model Cyclone I.Q.² microprocessor homogenizer (The VirTis Company, Inc., Gardiner, NY). In the second set, same amounts of thyme oil were dissolved in 1 mL PG that was then mixed with 9 mL of the 5% w/v
NaCas solution and emulsified as above. In the third set, 0.1 g lecithin was added into 9 mL of the 5% w/v NaCas solution, vortexed for 30 min, and then mixed with thyme oil and homogenized as in the first set. In the fourth set, lecithin was mixed with the NaCas solution as in the third set, and thyme oil pre-dissolved in PG was homogenized as in the second set. Emulsions as prepared in the fourth set were also studied for NaCas (2.5% and 5% w/v) and lecithin (0, 0.5% and 1.0% w/v) concentrations in the aqueous phase.

5.3.3 Turbidity

The absorbance of thyme oil emulsions was measured at 600 nm ($Abs_{600}$) as an indicator of turbidity using a UV-vis spectrophotometer (model Biomate 5, Thermo Electron Corp., Woburn, MA).

5.3.4 Particle size and zeta potential measurements

The dynamic light scattering experiments were conducted using a DelaTM Nano-Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter, Inc., Brea, CA). The emulsion samples were diluted 20 times in 0.01 M phosphate-buffered saline (PBS, pH 7) at ambient temperature prior to analysis. The zeta potentials of thyme oil emulsions were measured using the same instrument for samples diluted 100 times in deionized water and adjusted to pH 7.0. Two emulsion replicates were tested for three times each.

5.3.5 Atomic Force Microscopy (AFM)

The morphology of thyme oil emulsions was characterized using a Multimode VIII microscope (Bruker Corp., Santa Barbara, CA). Emulsions containing 1% thyme oil prepared
with NaCas and lecithin were diluted $10^5$ times using 0.01 M PBS at pH 7. Four μL of each sample was spread evenly onto a freshly-cleaved mica sheet that was mounted on a sample disk (Bruker Corp., Santa Barbara, CA) and air-dried for >2 h. The samples were scanned using a rectangular cantilever probe (Bruker Nanoprobe, Camarillo, CA) with aluminum reflective coating on the backside and a quoted force constant of 2.80 N/m. The topography images were collected at the tapping mode.

5.3.6 Quantification of protein surface load

The amount of NaCas on thyme oil droplet surface was quantified to investigate the effect of lecithin on surface adsorption of NaCas. To facilitate the separation of oil droplets, an excess amount (10% w/v) of thyme oil was directly emulsified in the aqueous phase with various combinations of NaCas (0, 2.5, and 5% w/v) and lecithin (0, 0.5, and 1.0% w/v). Particle sizes were measured right after homogenization and after storage at room temperature (21 °C) for 15 days. Emulsions before and after storage were centrifuged at 12,550g for 60 min using an Eppendorf MiniSpin plus centrifuge (Hamburg, Germany). The bottom aqueous phase was filtrated through a 0.22 μm polyvinyl difluoride membrane filter. To minimize the interference of thyme oil compounds such as thymol on protein assay, 50 μL of the permeate was mixed with 500 μL of an acetone-hexane (1:1, v:v) mixture (McClements et al., 2014) to extract thyme oil and precipitate protein. After vigorous mixing and centrifugation at 6,700g for 10 min, the supernatant (organic phase) was discarded, and the bottom protein precipitate was re-dissolved in 600 μL of 100 mM NaOH and was quantified using the bicinchoninic acid (BCA) method.
(Thermo Fisher Scientific Inc., Morris Plains, NJ). NaCas was used as a reference protein in the BCA assay. The surface load ($\Gamma_s$, mg/m²) of protein and the volume-area ($d_{3,2}$) mean diameters were calculated using eq 1 and 2, respectively.

$$\Gamma_s = \frac{M_s d_{3,2}}{6V_{oil}}$$  \hspace{1cm} (1)

where $M_s$ is the mass of NaCas adsorbed on oil droplets, and $V_{oil}$ is the volume of thyme oil. The thymol oil density used in calculation was 0.932 g/mL (Wu et al., 2014).

$$d_{3,2} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2}$$  \hspace{1cm} (2)

where $d_i$ is the diameter of the $i^{th}$ group of droplets and $n_i$ is the corresponding number of droplets.

### 5.3.7 Statistical analysis

Two independent emulsion replicates were studied throughout. Analysis of variance (ANOVA) test was conducted using the SPSS 16.0 statistical analysis system (SPSS Inc., Chicago, IL). The least significant difference (LSD) test was used to determine the significant difference of mean values at a $P$ level of 0.05.

### 5.4 Results and Discussion

#### 5.4.1 Turbidity of emulsions

Photographs of thyme oil emulsions prepared with various combinations of 5% w/v NaCas, 1% w/v lecithin and 10% v/v PG are shown in Figure 5-1. The treatments with 5% w/v NaCas alone and its combination with 10% v/v PG were only capable of emulsifying 1.0% w/v
thyme oil as transparent or translucent dispersions. In comparison, after adding 1% w/v lecithin, emulsions with up to 2.5% w/v thyme oil were translucent when prepared with 5% w/v NaCas, and the clarity further improved with 10% v/v PG. The appearance generally agreed with the $\text{Abs}_{600}$ (Figure 5-2). Since PG is fully miscible with water, pre-dissolving thyme oil in PG before emulsification likely reduces the oil/water interfacial tension, which is a main mechanism of co-surfactants facilitating the formation of nanoemulsions (McClements and Rao, 2011).

Furthermore, PG had no significant impact on emulsion formation by NaCas but facilitated emulsion formation for the NaCas-lecithin blend. This is likely due to the impacts on solubility of surfactants: NaCas is water-soluble, while lecithin (phosphatidylcholines) is not.

Subsequently, two concentrations (1.0 and 2.0% w/v) of thyme oil were emulsified using various combinations of NaCas (2.5% w/v) and lecithin (0.5 or 1% w/v) concentrations. Photographs are shown in Figure 5-3, while the $\text{Abs}_{600}$ is compiled in Table 1. With 1.0% w/v thyme oil, emulsions prepared with combinations of NaCas and lecithin were all transparent, while those with 2.5% NaCas or (0.5 or 1.0% w/v) lecithin alone were turbid. As thyme oil concentration increased to 2.0% w/v, all samples were turbid. The $\text{Abs}_{600}$ of thyme oil emulsions in Table 5-1 showed that addition of lecithin significantly decreased the turbidity of thyme oil emulsions prepared with NaCas ($P < 0.05$).

5.4.2 Hydrodynamic diameters of emulsions

Mean hydrodynamic diameters of thyme oil emulsions are compiled in Table 5-2.

Emulsions prepared with NaCas-lecithin blend had significantly smaller particles than those
prepared with NaCas or lecithin alone ($P < 0.05$). For example, compared to treatments with 1.0% w/v thyme oil emulsified by 1.0% w/v lecithin (mean diameter = 179.7 nm) or 5.0% w/v NaCas alone (mean diameter = 105.5 nm), the corresponding nanoemulsion prepared with the NaCas-lecithin blend had much smaller droplets with a mean diameter of 66.6 nm ($P < 0.05$). These results were consistent with the visual appearance (Figure 5-3) and $Abs_{600}$ (Table 5-1). Similar results have been reported for emulsions made with blends of NaCas and sucrose esters (with lipids being mono, di-, and tristearate or palmitate) (Courthaudon et al., 1991). Small molecular weight surfactants may co-adsorb with NaCas or displace NaCas on droplet surfaces and therefore alter the composition and properties of interfaces, and the reduction of interfacial tension may facilitate the formation of smaller droplets (Tual et al., 2006).

5.4.3 Emulsion structures studied by AFM

Emulsions containing 1.0% w/v thyme oil prepared with 2.5% w/v NaCas, 0.5% w/v lecithin, or both were imaged using AFM (Figure 5-4). Relatively bigger particles were observed for the emulsions prepared with NaCas or lecithin alone (Figure 5-4A and 4B) when compared to the treatment prepared with the surfactant blend (Figure 5-4C). The average particle dimension estimated in AFM was 134.3, 147.3, and 76.9 nm for emulsions prepared with NaCas only, lecithin only, and both, respectively. These results were generally in agreement with those obtained using dynamic light scattering (Table 5-2).
5.4.4 Zeta-potential of emulsions

Zeta-potentials of emulsions prepared with 1.0% w/v thyme oil emulsified by NaCas alone, lecithin alone, and their blend were measured at pH 7 (Table 5-3). The emulsion prepared with 1% w/v lecithin only had a highly negative zeta-potential (-58.2 ± 1.2 mV), which is attributed to the phosphate group of lecithin (Chuah et al., 2009). In comparison, the zeta-potential of the emulsion prepared with 2.5% w/v NaCas only had a significantly smaller magnitude (-25.8 ± 6.8 mV) than that with lecithin only ($P < 0.05$). When 5% w/v NaCas was used in homogenization, the zeta-potential magnitude (-39.7±1.2 mV) was higher than that prepared with 2.5% NaCas and was close to the zeta-potential of NaCas solution (Chen and Zhong, 2014), which may suggest a significant amount of free NaCas in the aqueous phase. For the nanoemulsion prepared with 2.5% w/v NaCas and 1% w/v lecithin, the magnitude of zeta-potential (-44.6 ± 2.6mV) was between those prepared with lecithin and NaCas alone. Since free NaCas was not removed, the zeta-potential data in Table 5-3 did not provide straightforward information about interfacial structures. It however should be noted that the electrostatic attraction between negatively charged phosphate group of lecithin and positively charged amine groups of NaCas is still possible at neutral acidity despite both are overall negatively charged, as previously demonstrated for pectin and NaCas (Surh et al., 2006).

5.4.5 Effect of lecithin on surface adsorption of NaCas

The protein content on unit surface area of emulsion droplets ($\Gamma_s$, mg/m²) is commonly determined to study the surface activity of proteins and the impacts by competing or co-
adsorbing surfactants. For 10% thyme oil emulsified by 2.5% w/v NaCas and 0-1% w/v lecithin, an increase in lecithin content decreased the droplet dimension but not the amount of adsorbed NaCas ($P > 0.05$), resulting in the reduced $\Gamma_s$ (Table 5-4). Because a larger surface area (smaller droplets) requires the coverage by a greater amount of surfactants, the data suggest the co-adsorption of NaCas and lecithin. The $\Gamma_s$ did not change significantly after 15-day storage at 21 °C, suggesting there was no detachment of NaCas. With the gradual increase in lecithin content, the droplet dimension decreased to near those prepared with lecithin only. Therefore, lecithin is more surface-active than NaCas in emulsifying thyme oil. For emulsions prepared with 5% w/v NaCas, the droplet size ($P < 0.05$) and the percentage of adsorbed NaCas ($P > 0.05$) were smaller than those prepared with 2.5% NaCas, corresponding to similar $\Gamma_s$ ($P > 0.05$). The $\Gamma_s$ of treatments prepared with 5% w/v NaCas and 0 and 0.5% lecithin decreased ($P < 0.05$) after storage for 15 days. Because the droplet dimension of these two emulsions did not change after storage, the results indicated the detachment of NaCas. Conversely, the treatment prepared with 5% NaCas and 1% lecithin showed insignificant changes in both droplet dimension and $\Gamma_s$ ($P < 0.05$).

The competitive surface adsorption and possible displacement of NaCas by more surface-active lecithin are dependent on their overall concentrations and molar ratios (Courthaudon et al., 1991; Fang and Dalgleish, 1993, 1996). In a study co-emulsifying 20% w/w soybean oil by 0.2-2% w/w NaCas and lecithin at lecithin: casein molar ratios of 0.7:1 - 49:1, the gradual reduction of $\Gamma_s$ of NaCas with increases in lecithin concentration was similar to the present study (Table 5-
4), but their $I'_s$ increased with increases in NaCas concentration and was all below 3.5 mg/m$^2$.

The $I'_s$ in the present study was much higher than the reference (Fang and Dalgleish, 1993) and was not significantly different at two (2.5 and 5% w/v) NaCas concentrations (Table 5-4) although the lecithin:casein molar ratios (2.8-11.4:1) in this work were within their range. The differences in their and the present studies can result from differences in the droplet dimension (300-800 nm in the reference (Fang and Dalgleish, 1993)), emulsification conditions, polarity of oil, and overall NaCas and lecithin concentrations. As for the interfacial structure, Fang and Dalgleish (Fang and Dalgleish, 1993) proposed that the thickness of adsorbed casein layer could increase when lecithin concentration increases gradually and, when there is a sufficient amount of lecithin, the oil droplets are directly covered mostly by lecithin that can be adsorbed by caseins protruding to the continuous aqueous phase. At their studied conditions, Fang and Dalgleish (Fang and Dalgleish, 1993) did not observe surface displacement of NaCas by lecithin, which is mostly in agreement with the present study (Table 5-4). However, in our case, $I'_s$ decreased for nanoemulsions prepared with 5% w/v NaCas and 0 and 0.5% w/v lecithin that also showed a decrease in droplet dimension after storage (Table 5-4). Since the thickness of NaCas layer increases with its bulk concentration (Fang and Dalgleish, 1993), the observations from these two emulsions can result from the detachment of caseins rather than the displacement by lecithin. The speculation however requires experimental verifications.
5.4.6 Emulsion stability

One common concern about essential oil nanoemulsions is their stability against Ostwald ripening because of the water solubility of essential oil components being higher than lipids composed of medium- or long-chain fatty acids (McClements and Rao, 2011). Physically, compounds in smaller particles have a higher solubility, which results in the continuous dissolving and eventually disappearance of smaller particles, and the dissolved compounds join bigger particles that grow during storage (Taylor, 1998). Nonpolar substances such as corn oil (Ziani et al., 2011) can be blended with essential oils in emulsion preparation to serve as inhibitors of Ostwald ripening (McClements and Rao, 2011). Ripening inhibitors however lower the loading level and antimicrobial activity of essential oils.

Emulsions with 1 and 2% thyme oil were studied for storage stability at 21 °C for 120 days. Creaming was observed for emulsions prepared with NaCas alone after 2- or 3-day storage at room temperature and became more significant after longer storage. Creaming can be contributed by increases in overall particle dimension due to depletion flocculation (by unadsorbed NaCas) or particle growth due to coalescence and/or Ostwald ripening (McClements, 2004a). In contrast, nanoemulsions prepared with 1 and 2% thyme oil and NaCas-lecithin blend appeared transparent or translucent throughout storage.

When particle size distributions of emulsions prepared with lecithin only or NaCas-lecithin blend were compared before and after 4-month storage at room temperature (Figure 5-5), several trends were observed. Emulsions prepared with NaCas-lecithin blend exhibited smaller
particles with narrower distributions than that prepared with lecithin only, indicating the better emulsifying activity of the blend. For these emulsions, the interfacial layer next to thyme oil is likely composed of both lecithin and NaCas, different from the model of sequential layers of lecithin and NaCas as proposed by Fang and Dalgleigh (Fang and Dalgleish, 1993) that would otherwise result in bigger droplets for emulsions prepared with both surfactants. The growth of particle size was observed for all emulsions, and the growth was much smaller for emulsions prepared with the blend than those with lecithin alone, suggesting the better stabilizing ability of the blend. The growth of average hydrodynamic diameter (Table 5-2) however was overall not statistically significant ($P > 0.05$).

In addition to Ostwald ripening discussed above, coalescence can also cause the growth of particles during storage, particularly for small molecular surfactants such as lecithin with a small head group (Pan et al., 2002). Coalescence is initiated by the aggregation of two oil droplets controlled by colloidal interactions. In the present study, emulsions prepared with lecithin only had a higher magnitude of zeta-potential (Table 5-3) than those with the NaCas-lecithin blend. Since emulsions prepared with lecithin only and the NaCas-lecithin blend had insignificant increase in mean particle diameter (Table 5-2), it may suggest the electrostatic repulsion is effective in preventing particle aggregation. The protrusion of NaCas in the continuous phase of emulsions prepared with the surfactant blend may have provided additional steric repulsion that resulted in smaller net growth of droplets after storage than emulsions prepared with lecithin only (Table 5-2). Similar phenomena were reported for orange oil
emulsions prepared with lysolecithin and sucrose monopalmitate (McClements et al., 2014).

Therefore, it is likely Ostwald ripening is the major mechanism responsible for particle growth of emulsions in the present study, and the narrower particle size distribution and thicker interface of emulsions prepared with the NaCas-lecithin blend both resulted in less significant particle size changes than those prepared with NaCas or lecithin only.

The stability of emulsions prepared with a mixture of proteins and small molecular surfactants has been widely studied. In some cases, the interaction or displacement between emulsifiers resulted in the significantly decreased stability. For example, combinations of caseinate and Tween® 20 at certain ratios led to severe flocculation and creaming, which was attributed to the depletion flocculation by unabsorbed protein (Dickinson et al., 1999). Combinations of lecithin with other small molecular surfactants also have been shown to improve the stability of emulsions. Lecithin added in emulsions stabilized by sucrose monopalmitate strengthened the electrostatic repulsion at acid conditions and resulted in the excellent stability of emulsions against Ostwald ripening, flocculation, and coalescence (Choi et al., 2011). Emulsifying soybean oil by combinations of lecithin and non-ionic steric surfactants such as polyethylene glycol hexadecyl ether (Brij®) increased the stability of emulsions at increased ionic strengths when compared to those prepared with lecithin only (De Vleeschauwer and Van der Meeren, 1999). This is because, although the surfactant blend reduced the zeta-potential magnitude of droplets when compared to those stabilized by lecithin, Brij® provided steric repulsion to stabilize emulsions (De Vleeschauwer and Van der Meeren, 1999).
5.5 Conclusions

In summary, the technological advancement has been shown in the present work for stable and transparent thyme oil nanoemulsions prepared using combinations of GRAS emulsifiers NaCas and lecithin. These transparent nanoemulsions had mean diameters smaller than ~100 nm at neutral pH. The clearer and smaller droplets of nanoemulsions prepared with the NaCas-lecithin blend than those with individual surfactants suggested that NaCas and lecithin synergistically emulsified thyme oil rather than the preferential adsorption and displacement of NaCas by more surface-active lecithin. The interface composed of both NaCas and lecithin provided repulsive electrostatic and also likely steric interactions against destabilization mechanisms of creaming, flocculation, and coalescence and, together with narrow particle size distributions, minimized Ostwald ripening. These transparent nanoemulsions prepared from GRAS ingredients have great potential to incorporate lipophilic antimicrobials such as thyme oil in transparent beverages to enhance the microbiological safety.

Acknowledgements

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**Appendix**

**Table 5-1.** Absorbance at 600 nm of 1 or 2% w/v thyme oil emulsified by 2.5% w/v NaCas and 0-1.0% w/v lecithin.*

<table>
<thead>
<tr>
<th>Thyme oil (% w/v)</th>
<th>Lecithin (% w/v)</th>
<th>Absorbance at 600 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0</td>
<td>1.48±0.14c</td>
</tr>
<tr>
<td>1.0</td>
<td>0.5</td>
<td>0.30±0.01d</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>0.27±0.01d</td>
</tr>
<tr>
<td>2.0</td>
<td>0</td>
<td>2.44±0.10a</td>
</tr>
<tr>
<td>2.0</td>
<td>0.5</td>
<td>2.21±0.16b</td>
</tr>
<tr>
<td>2.0</td>
<td>1.0</td>
<td>1.35±0.01c</td>
</tr>
</tbody>
</table>

*All samples contained 10% v/v PG. Numbers are mean ± standard deviation from duplicates.

Different superscript letters represent significant differences in the mean.
Table 5-2. Mean hydrodynamic diameters (nm) of 1% and 2% w/v thyme oil emulsified by NaCas, lecithin or both, before and after storage at room temperature for 120 days.*

<table>
<thead>
<tr>
<th>Thyme oil (% w/v)</th>
<th>Lecithin (% w/v)</th>
<th>Hydrodynamic diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0% NaCas</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>0.5</td>
<td>177.5±3.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>188.5±2.4&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0</td>
<td>179.7±2.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>203.6±5.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.0</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>0.5</td>
<td>170.3±2.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>184.3±6.0&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0</td>
<td>177.7±4.6&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>196.3±11.5&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*All samples contained 10% v/v PG. Numbers are mean ± standard deviation from duplicates. Different superscript letters represent significant differences in the mean.
Table 5-3. Zeta-potential (mV) of thyme oil (1.0% w/v) nanoemulsions prepared with NaCas with and without lecithin at pH 7.0.*

<table>
<thead>
<tr>
<th>Lecithin (%) w/v</th>
<th>Zeta-potential (mV)</th>
<th>0% NaCas</th>
<th>2.5% w/v NaCas</th>
<th>5.0% w/v NaCas</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>X</td>
<td>-25.8±6.8</td>
<td>-39.7±1.2 b</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>-58.2±1.2 a</td>
<td>-44.6±2.6 b</td>
<td>-41.7±2.4 b</td>
<td></td>
</tr>
</tbody>
</table>

*All emulsions contained 10% v/v PG. Numbers are mean ± standard deviation from duplicates.

Different superscript letters represent significant differences in the mean.
Table 5-4. Volume-area mean diameter $d_{3,2}$ (nm), polydispersity index, percentage of adsorbed NaCas, and surface load of NaCas in emulsions with 10% w/v thyme oil prepared with NaCas, lecithin, or both, before and after storage at 21 °C for 15 days.*

<table>
<thead>
<tr>
<th>NaCas (% w/v)</th>
<th>Lecithin (% w/v)</th>
<th>$d_{3,2}$ (nm)</th>
<th>Polydispersity index</th>
<th>Adsorbed NaCas (%)</th>
<th>Protein surface load ($\Gamma_s$, mg/m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 15</td>
<td>Day 0</td>
<td>Day 15</td>
</tr>
<tr>
<td>0</td>
<td>0.5</td>
<td>175.28±6.97$^c$</td>
<td>182.37±7.32$^c$</td>
<td>0.28±0.02$^a$</td>
<td>N/A</td>
</tr>
<tr>
<td>1.0</td>
<td>163.14±15.22$^c$</td>
<td>161.38±27.09$^c$</td>
<td>0.23±0.00$^c$</td>
<td>0.22±0.02$^c$</td>
<td>N/A</td>
</tr>
<tr>
<td>2.5</td>
<td>0</td>
<td>307.96±10.54$^a_b$</td>
<td>350.80±12.12$^a$</td>
<td>0.31±0.02$^a$</td>
<td>69.82±3.36$^a$</td>
</tr>
<tr>
<td>0.5</td>
<td>282.66±12.89$^b$</td>
<td>350.48±8.65$^a$</td>
<td>0.32±0.01$^a$</td>
<td>0.30±0.01$^a$</td>
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</tr>
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<td>0.27±0.01$^{bc}$</td>
<td>0.28±0.02$^{ab}$</td>
<td>54.64±7.31$^{bcd}$</td>
</tr>
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</table>

* Numbers are mean ± standard deviation from duplicates. Different superscript letters represent significant differences in the mean.
Figure 5-1. Appearance of thyme oil nanoemulsions prepared with various combinations of 5% w/v NaCas, 10% v/v PG, and 1% w/v lecithin. Thyme oil concentration in each image is 0.5, 1.0, 1.5, 2.0, and 2.5% w/v from left to right.
Figure 5-2. Absorbance at 600 nm of nanoemulsions with 0.5-2.5% w/v thyme oil prepared with various combinations of 5% w/v NaCas, 10% v/v PG, and 1% w/v lecithin.
Figure 5-3. Appearance of nanoemulsions with 1.0% w/v (top) or 2.0% w/v (bottom) thyme oil emulsified by 2.5% w/v NaCas, 0.5 or 1% w/v lecithin, or both. All samples contained 10% v/v PG. Labels on vial caps: S - NaCas; L - lecithin; numbers – surfactant concentrations.
**Figure 5-4.** AFM images of thyme oil (1.0% w/v) nanoemulsions prepared with 2.5% w/v NaCas (A), 0.5% w/v lecithin (B), or both (C). All samples contained 10% v/v PG.
**Figure 5-5.** Particle size distributions of nanoemulsions with 1.0% w/v (A and C) or 2% w/v (B and D) thyme oil emulsified by lecithin and NaCas-lecithin blend before (solid curves) and after (dashed curves) storage at room temperature (21 °C) for 4 months.
Chapter 6. Antimicrobial activity of thyme oil co-nanoemulsified with sodium caseinate and lecithin
A version of this chapter was submitted to *International Journal of Food Microbiology* by Jia Xue, P. Michael Davidson and Qixin Zhong.

My primary contributions to this manuscript include sample preparation, data collection and analysis, results interpretation and writing.
6.1 Abstract

Stable thyme oil nanoemulsions can be prepared using combinations of sodium caseinate (NaCas) and soy lecithin. The objective of the present research was to study the antimicrobial activity of nanoemulsions with 1% w/v thyme oil emulsified by (A) 4% w/v NaCas and 0.5% w/v lecithin or (B) 2% w/v NaCas and 0.25% w/v lecithin. Combination A resulted in a transparent emulsion with smaller droplets than the turbid emulsion of Combination B. Nanoemulsified oil exhibited similar or better antimicrobial activity than free thyme oil in tryptic soy broth and 2% reduced fat milk, due to the improved dispersibility of thyme oil. However, the increased concentration of emulsifiers in Combination A apparently reduced the antimicrobials available to alter bacteria membrane permeability as tested by the crystal violet assay at low antimicrobial concentrations and short time (1 h). The findings suggest nanoemulsions can be used to incorporate thyme oil for use as antimicrobial preservatives in foods.

**Keywords:** Thyme oil nanoemulsion, sodium caseinate and lecithin, antimicrobial activity, membrane permeability
6.2 Introduction

Essential oils (EOs) from plants are naturally occurring antimicrobials and have been extensively investigated for their antimicrobial efficacy against foodborne pathogenic and spoilage microorganisms (Elgayyar et al., 2001; Hammer et al., 1999; Smith-Palmer et al., 1998). When incorporated into food systems, their low water solubility and hydrophobic binding to food components, e.g., proteins and lipids, reduce their activity. Encapsulation systems are a potential method to improve their antimicrobial activity. Therefore, delivery systems for EOs have been studied that include both micro- and nanoemulsions formulated with small molecular weight surfactants (Gaysinsky et al., 2005b), biopolymers (Keawchaoon and Yoksan, 2011; Parris et al., 2005), or liposomes (Liolios et al., 2009). These delivery systems have been shown to promote uniform distribution of EOs in complex food matrices and reduce the negative impacts of binding by food matrix components (Donsì et al., 2011; Donsì et al., 2012). Enhanced antimicrobial activity of encapsulated EOs has been demonstrated against various Gram positive and Gram negative bacteria (Gaysinsky et al., 2005a; Guarda et al., 2011; Jo et al., 2014; Wu et al., 2012). However in some instances, binding between EOs and their encapsulating emulsifiers may be so great that it reduces the amount of EOs available to inactivate bacteria and thus the antimicrobial activity of encapsulated EOs is less than the unencapsulated EOs (Shah et al., 2013; Terjung et al., 2012; Weiss et al., 2009). Therefore, delivery systems for EOs need to be comprehensively characterized for their fundamental antimicrobial properties in microbiological growth media and complex food matrices.

Nanoemulsions have several benefits as delivery systems for EOs. With droplets smaller than 200 nm in diameter, nanoemulsions are relatively stable against gravitational sedimentation or creaming (Weiss et al., 2009). Nanoemulsions can be prepared with food grade ingredients
such as proteins or polysaccharides instead of synthetic surfactants making them “label-friendly” for food applications (McClements, 2004). For example, thymol nanoemulsions prepared with whey protein-maltodextrin conjugate and propylene glycol (PG, as a co-surfactant) demonstrated enhanced antilisterial activity in skim, 2% reduced fat, and whole fat milk (Xue et al., 2013). However, conjugates produced by the Maillard reaction can have undesirable color and flavor (Liu and Zhong, 2014). Some studies have reported reduced antimicrobial activity of EOs after nanoemulsification. For example, a carvacrol nanoemulsion prepared with pea protein and soy lecithin did not effectively inhibit *Escherichia coli* (Donsì et al., 2012). This was attributed to little increase in aqueous-phase concentration of carvacrol which has been shown to be a mechanism for improved activity (Donsì et al., 2012). Thyme oil, nanoemulsified by the cationic antimicrobial surfactant lauric arginate (LAE), showed reduced antimicrobial activity compared to free thyme oil due to partitioning of LAE between the thyme oil droplet surfaces and bacterial membranes (Ziani et al., 2011). Thus, continued research is needed to identify nanoemulsions that require the least amount of emulsifiers and at the same time deliver maximum antimicrobial activity.

In recent work, we found that combinations of two commonly used food emulsifiers sodium caseinate (NaCas) and soy lecithin facilitated the formation of transparent and stable thyme oil nanoemulsions (Xue and Zhong, 2014). The formation of NaCas-lecithin complexes improved the surface activity and emulsion stability when compared with treatments applying the two emulsifiers individually. A combination of 5.0% w/v NaCas and 1.0% w/v lecithin emulsified 2.5% w/v thyme oil as a transparent nanoemulsion using shear homogenization. In contrast, 5% w/v NaCas only emulsified 1.0% w/v thyme oil as a translucent emulsion, while 1.0% lecithin was unable to form transparent or translucent nanoemulsions. The objective of the
present study was to determine the antimicrobial properties of stable thyme oil nanoemulsions prepared with NaCas and lecithin combinations against *E. coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* in tryptic soy broth (TSB) and 2% reduced fat milk. Furthermore, changes in the bacterial membrane permeability after treatment with free thyme oil or nanoemulsions was compared (Devi et al., 2010).

6.3 Materials and Methods

6.3.1 Materials

Thyme oil was purchased from Sigma-Aldrich Corp. (St. Louis, MO). NaCas (>90% purity) was obtained from American Casein Co. (Burlington, NJ). Tryptic soy broth (TSB), peptone, and agar (chemical grade) were purchased from Becton, Dickinson and Company (Sparks, MD). Soy lecithin (phosphatidylcholines) (laboratory grade), crystal violet, and other chemicals were obtained from Fisher Scientific (Pittsburgh, PA). Ultra high temperature (UHT) pasteurized organic milk (2% reduced fat) was purchased from Kroger Company (Cincinnati, OH).

6.3.2 Preparation and characterization of thyme oil nanoemulsions

One nanoemulsion containing 1% w/v thyme oil was prepared with 4% w/v NaCas and 0.5% w/v lecithin, and the second emulsion was prepared at the same thyme oil concentration but with one-half the emulsifiers. To prepare the nanoemulsions, NaCas was first hydrated in deionized water overnight at room temperature (21°C). After adding lecithin, the mixture was mixed by vortexing for 30 min. Thyme oil was then added at 1% w/v in the mixture, followed by blending at 15,000 rpm for 3 min using a model Cyclone I.Q.² microprocessor homogenizer (The VirTis Company, Inc., Gardiner, NY). The nanoemulsions as prepared had a pH of 6.8. The
absorbance of thyme oil nanoemulsions was measured at 600 nm using a UV-vis spectrophotometer (model Biomate 5, Thermo Electron Crop., Woburn, MA). After diluting the nanoemulsions 20 times in 0.01 M phosphate-buffered saline (PBS, pH 7) at room temperature (21°C), the mean hydrodynamic diameter and polydispersity index of emulsions were measured (Delas™ Nano-Zeta Potential and Submicron Particle Size Analyzer, Beckman Coulter, Inc., Brea, CA).

6.3.3 Culture preparation

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

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

A microbroth dilution assay was used to determine the MIC of free and encapsulated thyme oil (Davidson and Parish, 1989). To wells of sterile 96-well microtiter plates was first added 120 µL of bacterial culture (ca. 10⁶ CFU/mL bacteria) followed by 120 µL of the antimicrobial sample. Free thyme oil stock solution was prepared by dissolving 10% w/v thyme oil in ethanol and then diluting to 0.1% w/v with TSB. From the stock solution, the TSB was further diluted to prepare the working solutions with 400, 500, 600, 700, 800, and 900 ppm thyme oil. Nanoemulsion samples were directly diluted to the same thyme oil concentrations with TSB. TSB with and without culture was used as a positive and negative control, respectively. Absorbance of wells at 630 nm was measured using an Elx800 Universal Microplate Reader.
(Bio-Tek Instruments, Inc., Winooski, VT) before and after 24 h incubation at 32°C (for *L. monocytogenes*) or 37°C (for *E. coli* O157:H7 and *S. Enteritidis*). The MIC was defined as the thyme oil concentration that allowed less than or equal to a 0.05 increase in absorbance after 24 h incubation. To determine MBC, 20 µL of the mixture from wells showing no growth was transferred to tryptic soy agar (TSA) plates. Following incubation of TSA at 32°C (for *L. monocytogenes*) or 37°C (for *E. coli* O157:H7 and *S. Enteritidis*) for 24 h, the lowest thyme oil concentration corresponding to no bacterial growth was defined as the MBC.

6.3.5 *Growth kinetics of bacteria in TSB or 2% reduced fat milk*

The antimicrobial activity in TSB was further studied using time-kill assays (Shah et al., 2013). Each treatment was repeated in three independent replications. Two thyme oil concentrations (300 and 500 ppm) were studied in this group of tests. Free thyme oil was prepared at 10 g/L by dissolving in 95% ethanol and then diluting with TSB. 300 or 500 µL of free thyme oil solution or an emulsion were added in 9.7 or 9.5 mL TSB, respectively. Then, 100 µL culture with ~10⁷ CFU/mL bacteria was added into each sample. After incubation at room temperature (21°C) for 0, 4, 8, 24, 48 and 72 h, samples were serially diluted with 0.1% peptone, plated on TSA, incubated at 32°C (for *L. monocytogenes*) or 37°C (for *E. coli* O157:H7 and *S. Enteritidis*) for 24 h, and survivors enumerated. The enumeration method as used had a detection limit of 1 log CFU/mL.

The growth kinetics of bacteria in 2% reduced fat milk were studied similarly by substituting TSB for milk. The overall thyme oil concentration in milk was 0.3 and 0.4%. This was prepared by mixing 3 or 4 mL of an emulsion sample with 6 mL milk. Treatments with free thyme oil were prepared by mixing 0.03 or 0.04 g thyme oil directly with 6 mL milk. Sterilized
water was added to make up the total volume to 10 mL. After adding antimicrobials or controls (mixture of sterilized water and milk), all milk samples were mixed with 100 μL of each bacterial culture (~10⁷ CFU/mL). The enumeration was conducted as above. Each treatment was studied in three independent replications.

6.3.6 Crystal violet assay

Potential disruption of the bacterial membrane permeability by antimicrobial treatments was evaluated using the crystal violet assay (Devi et al., 2010). Cells of *E. coli* O157:H7, *S. Enteritidis* and *L. monocytogenes* were harvested from their suspensions in TSB (ca. 10⁹ CFU/mL) by centrifugation at 4,300 x g for 4 min at 25°C (Sorvall Legend 26R; Thermo Scientific, Waltham, MA). The harvested cells were washed twice with PBS and resuspended at 10⁹ CFU/mL in 0.1 M PBS (pH 7.4). Free thyme oil was prepared by first dissolving in 95% ethanol and then diluting to 0.4, 0.6, 1.0, 1.4 and 1.8 g/L with TSB as working solutions. Emulsions were diluted with TSB directly. The working solutions were then mixed with equal volume (500 μL) of cell suspensions. Negative controls were prepared for the two emulsions without bacteria. Harvested cells resuspended in PBS were used as a positive control. After incubation for 1 h at 37°C (for *E. coli* O157:H7 and *S. Enteritidis*) or 32°C (*L. monocytogenes*), cells were harvested by centrifugation at 6,700 x g for 5 min using an Eppendorf MiniSpin plus centrifuge (Hamburg, Germany) and resuspended in PBS containing 10 μg/mL of crystal violet. After 15 min at 37°C or 32°C, suspensions were centrifuged at 13,400 x g for 15 min and the absorbance of the supernatant (without cells) was measured at 590 nm. The absorbance of the PBS with 10 μg/mL crystal violet was also measured at 590 nm, and the percentage of crystal violet taken up by cells was calculated using the following equation:
\[
\text{Uptake}(\%) = \left(1 - \frac{\text{Absorbance of the supernatant}}{\text{Absorbance of the crystal violet solution}}\right) \times 100\% \tag{1}
\]

6.3.7 Statistical analysis

Three independent emulsion replicates were studied for the antimicrobial tests. The analysis of variance (ANOVA) test was conducted using the SPSS 16.0 statistical analysis system (SPSS Inc., Chicago, IL). The least significant difference (LSD) test was used to determine the difference of mean values at a significance level of 0.05.

6.4 Results and Discussion

6.4.1 Physical properties of thyme oil nanoemulsions

A combination of 4% w/v NaCas and 0.5% w/v lecithin resulted in a transparent sample with 1% w/v thyme oil, while that produced with 2% w/v NaCas and 0.25% w/v lecithin was turbid (image not shown). For simplicity, they are henceforth referred to as HiE (high emulsifier concentration) or LoE (low emulsifier concentration). The HiE emulsion had both significantly lower \((P < 0.05)\) absorbance at 600 nm and droplet hydrodynamic diameter (82.5 vs. 125.5 nm) than the LoE emulsion (Table 6-1). The higher polydispersity index (more broadly distributed droplet dimensions) of the HiE nanoemulsion may have resulted from excess emulsifier in the aqueous phase (Table 6-1).

6.4.2 MIC and MBC in TSB

Table 6-2 summarizes the MIC and MBC of free and nanoemulsified thyme oil against \textit{E. coli O157:H7}, \textit{S. Enteritidis} and \textit{L. monocytogenes} in TSB. The MIC and MBC of free thyme oil against the three bacterial pathogens are consistent with previous work (Wu et al., 2014). Overall, there was little or no difference in MICs and MBCs of free and nanoemulsified thyme oil. One
possible reason for any reduced inhibition by nanoemulsified versus free EOs is binding by one or both of the emulsifiers, reducing antimicrobial activity. In previous studies, binding between lecithin (Li, 2011) or Tween 80 (Terjung et al., 2012) and EO components after nanoemulsification was correlated to the reduced antimicrobial activity because of the lowered availability of antimicrobials.

6.4.3 Growth kinetics of bacteria in TSB after thyme oil treatments

The growth kinetics of bacteria in TSB after treatments by the two thyme oil nanoemulsions and free thyme oil was studied at thyme oil levels of 300 and 500 ppm. At 300 ppm, the same as the MBC of *E. coli* O157:H7 (300-350 ppm), near the MBC for *S. Enteritidis* (350 ppm) and below the MBC of *L. monocytogenes* (450 ppm), thyme oil was more effective against *E. coli* O157:H7 (Figure 6-1A) and *L. monocytogenes* (Figure 6-3A) than *S. Enteritidis* (Figure 6-2A). It took less than 4 or 8 h for emulsions and free oil, respectively, to reduce *E. coli* O157:H7 (Figure 6-1A and B) populations to be below the detection limit at 300 or 500 ppm. Similar to *E. coli* O157:H7, both the emulsions and free thyme oil rapidly reduce the viable population of *L. monocytogenes* to below the detection limit (Figure 6-3A and B). In contrast, *S. Enteritidis* was the most resistant to 300 ppm thyme oil, showing an initial reduction of population by the two emulsions followed by a recovery to ~4 log CFU/mL after 24 h incubation. In comparison, the population of *S. Enteritidis* treated with free thyme oil gradually increased to ~8 log CFU/mL after 48 h incubation, followed by a small reduction after 72 h. At 500 ppm, the *S. Enteritidis* was reduced to below the detection limit (1 log CFU/mL) at 4 h with free thyme oil and the emulsions (Figure 6-2B). Overall, thyme oil emulsions were as or more effective than free oil against the three pathogens in TSB, which may be attributed to the improved dispersibility of thyme oil.
Different trends were observed in the MBC compared to the growth kinetic assays. The MBC was highest for *L. monocytogenes* while *S. Enteritidis* was the most resistant in the growth kinetic tests. This may have been due to the different temperatures used for incubation in two tests. The optimum growth temperature in MIC and MBC vs. room temperature (21°C) in the growth kinetics assays.

### 6.4.4 Growth kinetics of bacteria in 2% reduced fat milk after thyme oil treatments

Nanoemulsions and free thyme oil were mixed with 2% reduced fat milk at overall thyme oil concentrations of 0.3 and 0.4%. Higher concentrations of thyme oil were used because the food components reduce the relative antimicrobial activity of the compounds. The growth of *E. coli* O157:H7 is shown in Figure 6-4 A and B. With 0.3% thyme oil (Figure 6-4A), there were initial reductions for all antimicrobial treatments, and the reduction rate of free thyme oil treatment was slower than that of emulsions. After 24 h incubation, a recovery was observed for all thyme oil treatments. The thyme oil treated samples recovered to around the initial population at 48 h but this was around 3-4 logs lower than the controls. When the overall thyme oil concentration increased to 0.4%, the complete inactivation of *E. coli* O157:H7 was observed in 4 h for all thyme oil treatments (Figure 6-4B). Inhibition by 0.3% thyme oil against *S. Enteritidis* was characterized by a slightly longer lag phase (free) or slight decline in viable cells (emulsions) through 12 h followed by rapid growth. The final cell numbers were lower than the control. With 0.4% thyme oil (Figure 6-5B), emulsions exhibited up to 5 log reductions in 24 h followed by recovery which reached only the initial population after 72 h. Both emulsions were more effective than the free thymol over the first 24 h. For *L. monocytogenes* at 0.3% thyme oil, there a lag phase extension (free) or slight reduction in viable cells (emulsions) over 24 h similar to that shown by *S. Enteritidis* (Figure 6-6A). With 0.4% thyme oil (Figure 6-6B), the HiE
emulsion initially reduced *L. monocytogenes* by 2.5 logs while the free thyme oil treatment reduced the viable population by only 0.5-1.0 log after 12 h. The LoE emulsion caused an initial 0.5 log reduction followed by ~1 log reduction throughout the incubation compared to the free thyme oil. Overall, it appeared that *S. Enteritidis* was more resistant than either *E. coli O157:H7* or *L. monocytogenes*.

The emulsified thyme oil generally demonstrated greater reduction of bacterial numbers initially (4 and 8 h) in 2% reduced fat milk when compared to free thyme oil. This may be attributed to a more even distribution of emulsified thyme oil droplets and hence facilitation of their contact with bacteria surfaces. The observations in the present study are slightly different from previous studies using whey protein-maltodextrin conjugates and NaCas only to prepare thymol emulsions (Pan et al., 2014; Xue et al., 2013). In those studies, the emulsified thymol demonstrated consistently more effective antilisterial activity than free thymol in milk with various fat contents. The difference may be attributed to the binding between emulsifiers and thyme oil because lecithin, used in the present study is more hydrophobic than whey protein-maltodextrin conjugate and NaCas. The stronger hydrophobic binding between NaCas-lecithin and thyme oil may reduce the antimicrobial activity.

### 6.4.5 Uptake of crystal violet by bacteria after treatment with thyme oil

The antimicrobial mechanisms of EOs are complex and many have speculated as to the type or types of mechanisms involved (Carson et al., 2002; Rhayour et al., 2003; Skandamis et al., 2001). One important and well recognized mechanism is an increase in membrane permeability. This is because hydrophobic EO compounds, such as thymol, eugenol and carvacrol, can partition into the phospholipid bilayer of bacterial membrane (Burt, 2004; Sikkema et al., 1994). This partitioning reportedly reduces the integrity of the membrane,
increases its permeability, and results in the leakage of intracellular contents (Burt, 2004; Sikkema et al., 1994). To evaluate whether this was occurring, the uptake of crystal violet by the test bacteria was measured in the presence of emulsified and free thyme oil.

Without thyme oil, the uptake of crystal violet by *E. coli* O157:H7, *S. Enteritidis* and *L. monocytogenes* was 24.4%, 23.4% and 29.3%, respectively (Figure 6-7). The uptake of crystal violet by the Gram positive *L. monocytogenes* in the presence of free thyme oil or emulsions was consistently higher than for the two Gram negative bacteria. Compared with Gram negative bacteria, the reduced antimicrobial resistance of Gram positive bacteria may be attributed to a lack of an outer cell membrane thus increasing the binding between antimicrobials and phospholipids and phosphate groups in the cytoplasmic membrane leading to damage (Li et al., 2014). For Gram negative bacteria, the outer membrane may provide a stronger barrier for the permeation of EOs because the lipopolysaccharides help reduce the penetration of charged and lipophilic compounds (Schop et al., 2000). Thus Gram positive bacteria are more vulnerable to lipophilic antimicrobials which enhance membrane permeability and this results in higher uptake of crystal violet.

The same trends were observed for three bacteria. Free thyme oil resulted in the highest uptake of crystal violet, followed by the LoE emulsion and the HiE emulsion (Figure 6-7). Because emulsion particles also bind with crystal violet, the net crystal violet uptake percentages by bacteria were reported after subtraction by the uptake of emulsions. With increased thyme oil concentration, the rate of crystal violet uptake was higher for free thyme oil than the LoE emulsion, while the rate was negative for the HiE emulsion. The HiE emulsion, with a larger quantity of emulsifiers, bound a greater amount of crystal violet than the turbid emulsion (not shown). This portion of binding (13.5% to 19.6% at 200-900 ppm thyme oil) was significant
compared to the uptake by bacteria. The increased crystal violet uptake by bacteria with increased free thyme oil and the LoE emulsion indicates an impact on membrane integrity. Crystal violet uptake was not correlated to either the MIC/MBC (Table 6-2) or growth kinetics (Figure 6-1, 2 and 3). This can possibly be attributed to different test conditions (e.g., reaction time, antimicrobial concentration, bacterial population, test media) or possibly other mechanisms contributing to the observed antimicrobial activities.

6.5 Conclusions

In conclusion, thyme oil nanoemulsions prepared with combinations of GRAS emulsifiers NaCas and lecithin had similar or slightly better antimicrobial activity when tested against bacterial pathogens in TSB and milk. Biophysically, the binding between emulsifiers and EO components is significant to antimicrobial activity in a short time and at low EO concentrations. This fundamental mechanism became insignificant in activity assays of nanoemulsified thyme oil possibly due to the improved dispersibility of thyme oil in TSB and milk and a longer time to reach equilibrium of antimicrobial distribution. Therefore, the nanoemulsions studied can potentially be used as antimicrobial preservatives to enhance food safety.

Acknowledgements

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References


Appendix

Table 6-1. Absorbance at 600 nm, mean hydrodynamic diameter, and polydispersity index of thyme oil nanoemulsions.*

<table>
<thead>
<tr>
<th>Emulsifier combination</th>
<th>Absorbance at 600nm</th>
<th>Hydrodynamic Diameter (nm)</th>
<th>Polydispersity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>4% NaCas+0.5% Lecithin</td>
<td>0.37±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82.5±3.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2% NaCas+0.25% Lecithin</td>
<td>1.80±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>125.5±2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.22±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Numbers are mean ± standard deviation from duplicates. Different superscript letters represent significant differences in the mean.
Table 6-2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of free or nanoemulsified thyme oil against *Escherichia coli* O157:H7, *Salmonella* Enteritidis at 37°C, and *Listeria monocytogenes* at 32°C in tryptic soy broth.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>MIC (ppm)</th>
<th>MBC (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free oil</td>
<td>HiE# emulsion</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>350</td>
<td>350</td>
</tr>
<tr>
<td><em>S. Enteritidis</em></td>
<td>350</td>
<td>350</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>350</td>
<td>300</td>
</tr>
</tbody>
</table>

* 1% thyme oil was emulsified by 2% NaCas and 0.25% lecithin.

* 1% thyme oil was emulsified by 4% NaCas and 0.5% lecithin.
Figure 6-1. Growth of *Escherichia coli* O157:H7 at 21°C in TSB with (A) 300 or (B) 500 ppm free or nanoemulsified thyme oil. The HiE and LoE nanoemulsions were prepared with 1% thyme oil and high (4% NaCas+0.5% lecithin) and low (2% NaCas+0.25% lecithin) amounts of emulsifiers. Error bars are standard deviations (n = 3).
Figure 6-2. Growth of *Salmonella* Enteritidis at 21°C in TSB with (A) 300 or (B) 500 ppm free or nanoemulsified thyme oil. The HiE and LoE nanoemulsions were prepared with 1% thyme oil and high (4% NaCas+0.5% lecithin) and low (2% NaCas+0.25% lecithin) amounts of emulsifiers. Error bars are standard deviations (n = 3).
Figure 6-3. Growth of *Listeria monocytogenes* Scott A at 21°C in TSB with (A) 300 or (B) 500 ppm free or nanoemulsified thyme oil. The HiE and LoE nanoemulsions were prepared with 1% thyme oil and high (4% NaCas+0.5% lecithin) and low (2% NaCas+0.25% lecithin) amounts of emulsifiers. Error bars are standard deviations (n = 3).
Figure 6-4. Growth of *Escherichia coli* O157:H7 at 21°C in 2% reduced fat milk with (A) 0.3 or (B) 0.4% free or nanoemulsified thyme oil. The HiE and LoE nanoemulsions were prepared with 1% thyme oil and high (4% NaCas+0.5% lecithin) and low (2% NaCas+0.25% lecithin) amounts of emulsifiers. Error bars are standard deviations (n = 3).
Figure 6-5. Growth of *Salmonella* Enteritidis at 21°C in 2% reduced fat milk with (A) 0.3 or (B) 0.4% free or nanoemulsified thyme oil. The HiE and LoE nanoemulsions were prepared with 1% thyme oil and high (4% NaCas+0.5% lecithin) and low (2% NaCas+0.25% lecithin) amounts of emulsifiers. Error bars are standard deviations (n = 3).
Figure 6-6. Growth of *Listeria monocytogenes* Scott A at 21°C in 2% reduced fat milk with (A) 0.3 or (B) 0.4% free or nanoemulsified thyme oil. The HiE and LoE nanoemulsions were prepared with 1% thyme oil and high (4% NaCas+0.5% lecithin) and low (2% NaCas+0.25% lecithin) amounts of emulsifiers. Error bars are standard deviations (n = 3).
Figure 6-7. Percentage of crystal violet taken up by (A) *Escherichia coli* O157:H7, (B) *Salmonella* Enteritidis, and (C) *Listeria monocytogenes* after treatment with free or nanoemulsified thyme oil. The HiE and LoE nanoemulsions were prepared with 1% thyme oil and high (4% NaCas+0.5% lecithin) and low (2% NaCas+0.25% lecithin) amounts of emulsifiers. The emulsion treatments with bacteria were subtracted from the emulsions without bacteria. Error bars are standard deviations (n = 2).
Chapter 7. Concluding remarks and future work
Conclusions

This dissertation demonstrated that stable EO nanoemulsions can be prepared with GRAS emulsifying agents such as whey protein-maltodextrin conjugates, gelatin-lecithin or NaCas-lecithin blend. All EO nanoemulsions were effective against both Gram positive and Gram negative foodborne pathogens such as *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella Typhimurium* in TSB and model food systems of milk and cantaloupe juice.

The emulsifying capacity of whey protein-maltodextrin conjugates and anti-listerial properties of thymol were greatly enhanced by pre-dissolving thymol in a co-surfactant PG. The antimicrobial efficacy of thymol in milk was directly affected by the binding with milk components such as proteins and fat globules. The enhanced anti-listerial activity of thymol nanoemulsions was attributed to the increased thymol concentration in aqueous phase and the synergistic antimicrobial activity between thymol and PG.

Combination of gelatin B and soy lecithin can be used to prepare GRAS thymol nanodispersions, which were stable at pH 5.0 and above. The electrostatic repulsion, contributed mostly by highly negatively charged lecithin, prevented particles from aggregation and maintained particle dimension during storage at neutral pH. Therefore, the stable nanodispersions prepared from GRAS emulsifiers was suitable to deliver lipophilic food antimicrobials like thymol in low acid foods to improve the microbiological safety. Antimicrobial activities of thymol in milk and cantaloupe juice were greatly enhanced after nano-emulsification by the gelatin-lecithin blend.

The clearer and smaller droplets of thyme oil nanoemulsions prepared with the NaCas-lecithin blend than those with individual emulsifiers suggested the synergistic surface activity between NaCas and lecithin. The interfacial layer composed of both NaCas and lecithin provided
strong electrostatic repulsion and also likely steric interactions against creaming, flocculation, and coalescence. These nanoemulsions had similar or slightly better antimicrobial activity than free oil when tested against bacterial pathogens in TSB and milk. Emulsifiers can bind EO and reduce the effect on bacteria membrane permeability in a short time and at low EO concentrations. But this effect became insignificant in activity assays of nanoemulsified thyme oil possibly due to the improved solubility of oil and a longer time to reach equilibrium of antimicrobial distribution. Therefore, these studied EO nanoemulsions can potentially be used as novel antimicrobial preservatives to enhance food safety.
Future work

Current studies showed the feasibility of using GRAS emulsifiers to prepare EO nanoemulsions as effective food antimicrobials. There are still some issues can be further explored. For example, NaCas-lecithin blend exhibited excellent emulsifying properties in the preparation of thyme oil nanoemulsions at neutral pH, but after acidification, these nanoemulsions became highly unstable due to the collapse of casein structure, which limits their applications in low acid foods. To resolve this problem, some biopolymers such as gelatin type A that are positively charged at acidic pH can be added to interact with casein molecules, providing electrostatic repulsion and thus preventing casein aggregation. These studied GRAS emulsifiers were capable of preparing stable EO nanoemulsions, but some antimicrobial results indicated the interference of emulsifiers on the antimicrobial activity of EOs. Therefore, it is necessary to further investigate the binding between emulsifiers and EOs, which may reduce the antimicrobial efficiency of EOs. In addition, food systems tested in this dissertation are juice and milk, both of them are liquid, so different types of foods such as ground beef, can be studied in the future.
VITA

Jia Xue was born in Weinan, Shaanxi, China, on October 25, 1986. She entered China Agricultural University (Beijing, China) in 2005 to pursue a Bachelor of Science degree in Horticulture Science. After receiving her B.S. degree, she continued to study at the university and work on her Master of Engineering degree in Agricultural Products Processing and Storage Engineering. In August 2011, she began her doctoral program in the Department of Food Science and Technology at the University of Tennessee, Knoxville, under the direction of Dr. Qixin Zhong. Her dissertation research was focused on the development of essential oil nanoemulsions with natural emulsifiers to enhance the antimicrobial activity of essential oils in food matrices and thus effectively control foodborne pathogens and improve food safety. She will soon be working in the College of Food Engineering and Nutritional Science at Shaanxi Normal University as a lecturer, in Xi’an, China.