JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Thymol Nanoemulsified by Whey Protein-Maltodextrin Conjugates: The Enhanced Emulsifying Capacity and Antilisterial Properties in Milk by Propylene Glycol

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ABSTRACT: The objective of this research was to enhance the capability of whey protein isolate-maltodextrin conjugates in nanoemulsifying thymol using propylene glycol to improve antilisterial properties in milk. Thymol was predissolved 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 full 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 antilisterial activities of nanoemulsified thymol resulted from the increased solubility in milk and synergistic activity with propylene glycol.

KEYWORDS: *thymol, nanoemulsion, propylene glycol, antilisterial properties, milk, solubility*

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 3000 deaths in the United States each year.[1] Although various strategies have been developed and implemented, the number of reported foodborne illness outbreaks has not decreased dramatically.[1] *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.[2] Around 1662 foodborne illnesses caused by *L. monocytogenes* occur annually in the United States.[3] Outbreaks have been associated with contaminated milk and other dairy products, meat, fish, and vegetables.[4,5] The most recent outbreak of listeriosis in the United States was linked to the imported Frescolina Marte brand Ricotta Salata cheese and caused 22 illnesses across 13 states.[6] 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.[7] For example, thymol is a phenolic compound that is the major component in the essential oil extracted from the aromatic plant thyme (*Thymus vulgaris*).[7] 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.[8] 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.[9] 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.[9]

Emulsions have been studied as delivery systems for essential oils to improve their antimicrobial efficacy.[10] Nanoemulsions are those with droplets smaller than ∼200 nm in diameter.[11] The reduced droplet dimension prevents creaming and reduces turbidity. Cosurfactants such as generally recognized-as-safe (GRAS) propylene glycol (PG)[12,13] are widely used to facilitate the formulation of microemulsions[14−16] and nanoemulsions.[17] In addition, PG is used as a preservative.[18−20] 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[10,21] or fruit juices.[22] In these simple systems, complete inhibition of bacteria can be achieved below the solubility limit of essential oil components,[23−25] and the need for 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).[26] 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[27,28] was

Received: September 27, 2013
Revised: November 19, 2013
Accepted: December 3, 2013
Published: December 3, 2013

studied in our laboratory. WPI-MD conjugates are known for their emulsifying properties[29] and have been used to encapsulate various lipophilic ingredients.[29,30] 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 preformed 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.[23−25] The 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 antilisterial 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 antilisterial 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.

MATERIALS AND METHODS

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%) fat) was purchased from Kroger Company (Cincinnati, OH).

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 m^3/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.

Preparation of Nanoemulsions. The conjugate solution was prepared by dissolving 0.7 g of conjugate powder in 9 mL of deionized water with a resultant pH of 6.4. Thymol solution was prepared separately by dissolving various amounts in 1 mL of 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.

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. A 2 mL portion of sample was 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 Crop., Woburn, MA).

Particle Size Measurement. Particle size distributions of thymol emulsions were measured using a Delas Nano-Zeta Potential and Submicrometer 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:

$$d_{4,3} = \frac{\sum_{i=1} n_i d_i^4}{\sum_{i=1} n_i d_i^3} \quad (1)$$

Zeta Potential. The zeta potentials of WPI-MD conjugate and emulsions containing 1.0% thymol prepared with and without PG were measured using a DelasTM Nano-Zeta Potential and Submicrometer 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 3−7 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. A 4 μL portion 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.

Determination of Antilisterial 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 of culture was inoculated in 50 mL of 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.[31] 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 of 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[32] 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. 10^5 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 to, and above 1 corresponded to synergistic, additive, and antagonistic antimicrobial activities, respectively.[33]

$$\text{FIC} = \frac{\text{MIC of thymol in combination}}{\text{MIC of thymol alone}} + \frac{\text{MIC of PG in combination}}{\text{MIC of PG alone}} \quad (2)$$

Antilisterial Activity in Milk. The antilisterial activity in milk was studied in duplicate using time-kill assays.[34] 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 of 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 of 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 of culture that was previously diluted to ca. 1.0×10^6 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.

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.

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 antilisterial 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 4629*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 mm × 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. A 10 μL portion 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.

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

RESULTS AND DISCUSSION

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[35] and 0.095% w/v in 10% v/v PG measured in the present study. In comparison, the mixture with the 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,[29] 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.[36,37]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[38] 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 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 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). The emulsion prepared with PG (Figure 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 3). At pH 4, thymol emulsions with and without PG

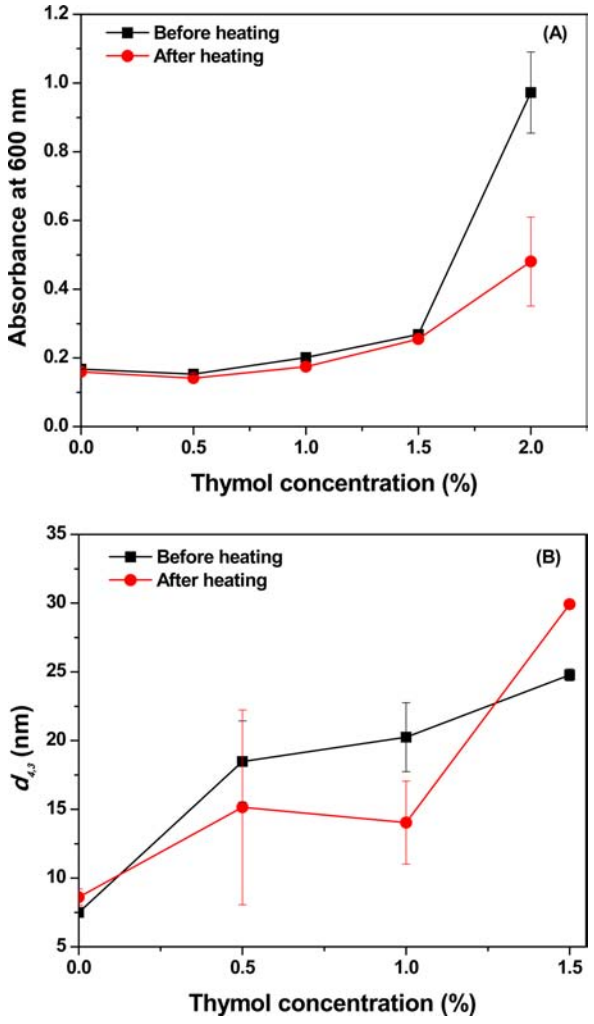

Figure 1. Absorbance at 600 nm (A) and volume fraction-length mean particle diameter ($d_{4,3}$) (B) of thymol emulsions prepared with 7% w/v WPI-MD conjugates, 10% v/v PG, and 0−2.0% w/v thymol, before and after heating at 80 °C for 15 min.

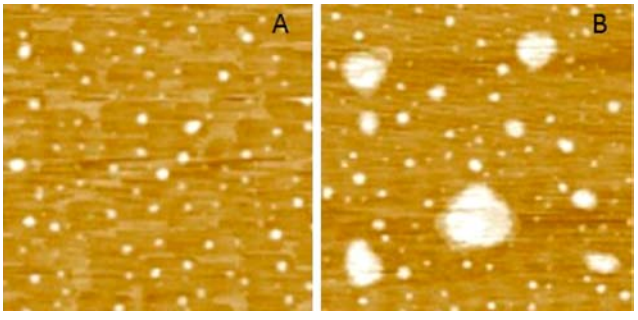

Figure 2. AFM images of thymol emulsions prepared with 7% w/v WPI-MD conjugates, 1.0% w/v thymol with (A) and without (B) 10% v/v PG. The dimension of images is 2 μm × 2 μm.

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

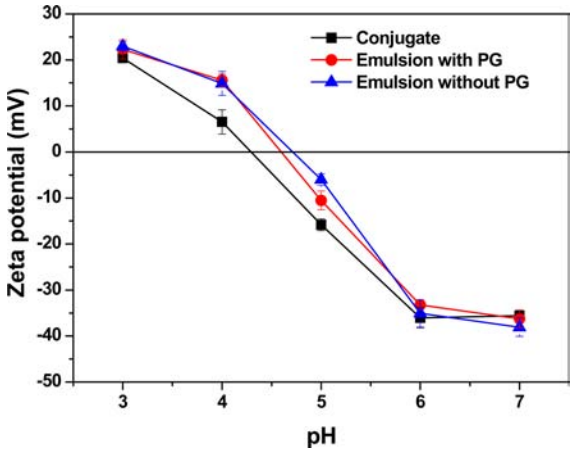

Figure 3. Zeta-potential of WPI-MD conjugate, emulsion prepared with 7% w/v WPI-MD conjugates, and 1.0% v/v thymol with and without 10% v/v PG.

of PG on protein conformation in emulsions under the conditions studied.

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 1. Thymol nanoemulsified without PG demonstrated a

Table 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

antimicrobial	MIC (% w/v)	MBC (% w/v)
free thymol	0.02	0.045
thymol nanoemulsion with PG[a]	0.02	0.045
thymol nanoemulsion without PG[b]	0.055	0.08
PG	26.2	52.4

[a]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. [b]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.

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 antilisterial 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.[18] 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.[39]

In previous studies when nanoemulsions of thymol[24] and eugenol[23] were prepared with WPI-MD conjugate using the emulsion-evaporation technique, the MIC of nanoemulsified

dx.doi.org/10.1021/jf4043437 | J. Agric. Food Chem. 2013, 61, 12720−12726

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.[26] 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.

Antilisterial Activities of Antimicrobials in Three Types of Milk. The antilisterial 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 4. In skim milk (Figure 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 1), with the highest population after 48 h.

The trends in 2% reduced fat milk (Figure 4B) were similar to those in skim milk (Figure 4A), except that the inactivation was at a slower rate. For the nanoemulsion prepared with PG, the *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 *L. monocytogenes* was observed in 48 h. For the treatment of free thymol with PG, the recovery of *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 4C), the inactivation rate was even slower than in 2% reduced fat milk (Figure 4B), although at a higher level of thymol. The nanoemulsion prepared with PG gradually reduced *L. monocytogenes* to below the detection limit after 48 h, while other three thymol treatments were only bacteriostatic. Overall, the antilisterial 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 *L. monocytogenes* in a shorter time to a greater extent than other comparable treatments at the studied conditions.

Content of Thymol in the Serum of Milk. The thymol concentration in the aqueous phase of milk as prepared in antilisterial tests in Figure 4 was quantified to understand differences in antilisterial 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 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

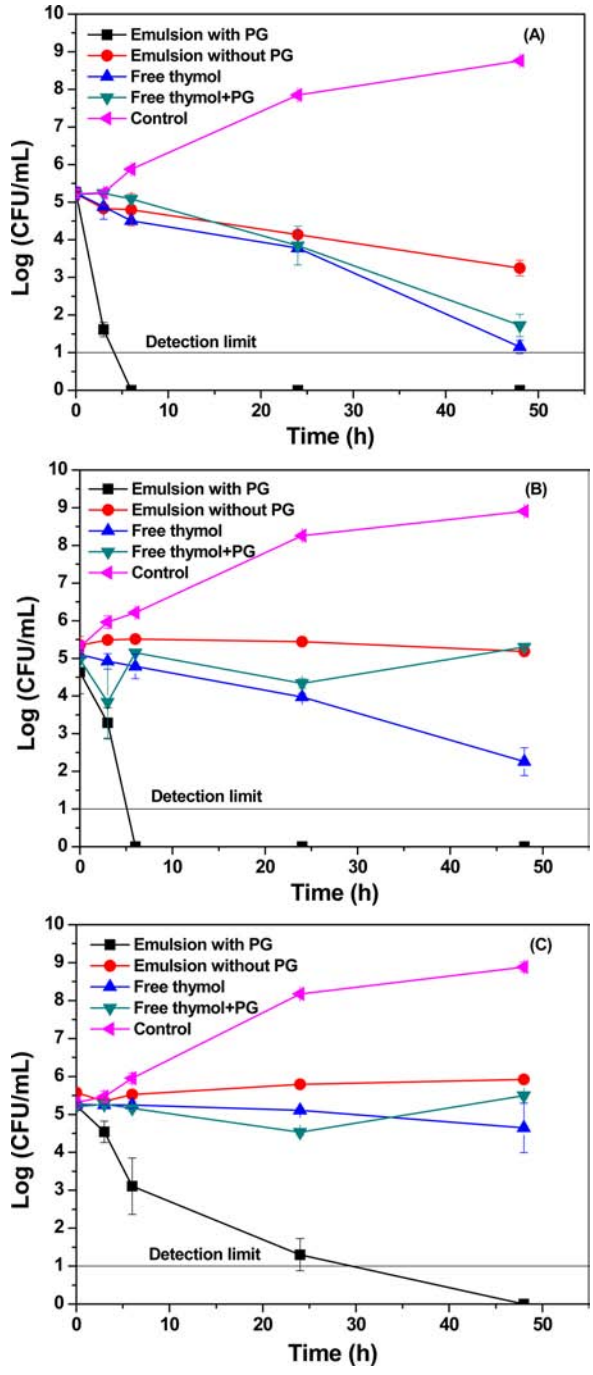

Figure 4. Time-kill assays showing the population of *Listeria monocytogenes* Scott A at 32 °C in skim (A), 2% reduced fat (B), and full fat milk (C) that was treated with 0.1%, 0.45%, and 0.6% w/v thymol, respectively. Emulsions of thymol were prepared with and without propylene glycol (PG). Free thymol 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.

indicates the significant binding between thymol and dairy proteins. When mixed initially (Figure 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

dx.doi.org/10.1021/jf4043437 | *J. Agric. Food Chem.* 2013, 61, 12720−12726

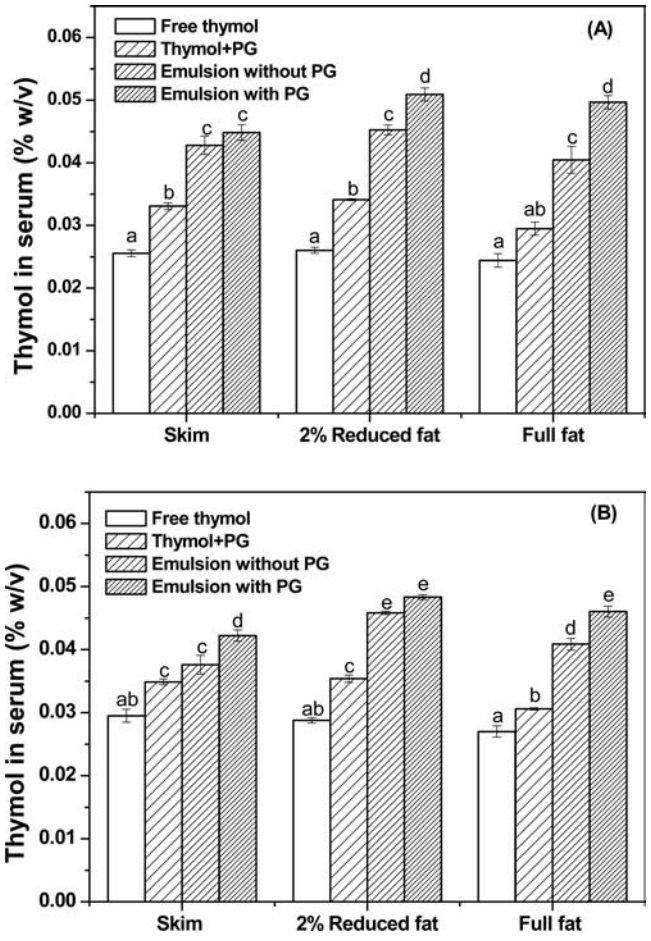

Figure 5. Thymol concentration detected in the continuous phase of skim, 2% reduced fat, and full fat milk that was mixed respectively with 0.1%, 0.45%, and 0.6% w/v thymol prepared in different forms after 0 h (A) and 48 h (B) at room temperature (21 °C). Different letters above bars indicate significant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

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 5B), trends in TCMS were similar to those at 0 h (Figure 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 antilisterial properties different from those in TSB. This hypothesis can be examined by correlating TCMS with MIC and MBC estimated in TSB to interpret the antilisterial activity in milk. The TCMS (Figure 5) of free thymol in milk was between MIC and MBC (Table 1) which agreed with the inhibition or incomplete inactivation of *L. monocytogenes* in milk (Figure 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 1) resulting in the weakest antilisterial activity in milk (Figure 4). For the nanoemulsion with PG, the TCMS was above the MBC of 0.045% w/v (Table 1) and the antilisterial properties were the best, showing complete inactivation of *L. monocytogenes* in 48 h at all studied conditions.

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 cosurfactant, greatly enhanced the emulsifying capacity of WPI-MD conjugate and antilisterial 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 antilisterial 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.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the University of Tennessee and the USDA National Institute of Food and Agriculture under Project TEN02010-03476.

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