Impact of starch-based emulsions on the antibacterial efficacies of nisin and thymol in cantaloupe juice

Preetam Sarkar¹, Arun K. Bhunia², Yuan Yao^{1*}

¹Whistler Center for Carbohydrate Research, Department of Food Science, Purdue University, West Lafayette, IN, 47907

²Molecular Food Microbiology Laboratory, Department of Food Science, Purdue University, West Lafayette, IN, 47907

*Corresponding author: Tel: 765-494-6317; Email address: yao1@purdue.edu

Abstract

The use of antimicrobial compounds to prevent foodborne pathogens from contaminating fresh-cut produce has received broad attentions; however, the applications of these compounds are hindered by their rapid depletion in foods. To prolong their efficacies, the use of delivery systems is essential. In this study, oil-in-water emulsions formed using starch octenyl succinate (starch-OS) were used to stabilize nisin and thymol in cantaloupe juice-containing fluid. *Listeria monocytogenes* V7 and *Salmonella enterica* serovar Typhimurium were used as model pathogens to evaluate the antimicrobial activities of nisin and thymol formulations in cantaloupe juice. The results showed that the emulsions had much greater capability to retain nisin and thymol over the storage and displayed much greater effect to inhibit *Listeria* and *Salmonella* than non-emulsion, aqueous formulations. Starch-OS based emulsions not only retained nisin and thymol activities separately, but also exhibited their cooperative antibacterial effects.

Keywords: Nisin, thymol, starch-OS emulsion, cantaloupe, prolonged antibacterial efficacy

1. Introduction

Cantaloupe has an important economic role among fresh and fresh-cut produce, and it is also a primary source for human pathogen outbreak (Martíñon, Moreira, Castell-Perez, & Gomes, 2014). Cantaloupe has been associated with contaminations of various pathogenic organisms such as *Listeria* and *Salmonella enterica* (Fang, Liu, & Huang, 2013; Ismail, Chan, Mariod, & Ismail, 2010; Mahmoud, 2012). Various thermal and non-thermal processing techniques are utilized for improving the shelf-life of fresh-cut fruits (Martíñon, Moreira, Castell-Perez, & Gomes, 2014). Meanwhile, the food industry has constantly been exploring novel and cost-effective technologies for minimally processed foods. For fresh and fresh-cut produce including cantaloupes, the use of natural antimicrobial compounds can be a feasible approach.

Natural antimicrobials such as essential oils (e.g. thymol) and bacteriocins (e.g. nisin) have strong antibacterial efficacies and are also generally considered as safe for food application (Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Burt, 2004). Nisin is an antimicrobial peptide generated by *Lactococcus lactis* and inhibits Gram-positive bacteria through pore-forming on cytoplasmic membrane. In general, it is ineffective against Gram-negative bacteria (Breukink & de Kruijff, 2006; Breukink, Ganz, de Kruijff, & Seelig, 2000). Thymol (2-isopropyl-5-methylphenol) is a natural phenolic compound found in thyme (*Thymus vulgaris*) essential oil and has shown inhibitory effects against both Gram-positive and negative bacteria (Burt, 2004). The antibacterial efficacy of thymol is due to the interactions between its phenolic structure and the phospholipids within the cytoplasmic membrane (Burt, 2004).

However, antimicrobial compounds may undergo quick depletion within foods. For example, nisin is susceptible to depletion due to its physical diffusion within the food system or

degradation by proteases. In addition, components within foods such as glutathione, sodium metabisulphite, and titanium dioxide can react with nisin, thereby reducing its effective concentration (Bhatti, Veeramachaneni, & Shelef, 2004; Jung, Bodyfelt, & Daeschel, 1992; Mahadeo & Tatini, 1994; Rose, Sporns, Stiles, & McMullen, 1999). Thymol is chemically stable, however, it may be easily lost due to evaporation. Thymol may also interact with hydrophobic food components, which reduce its concentration in the aqueous phase (Shah, Davidson, & Zhong, 2012, 2013). To protect antimicrobials from depletions in foods, the use of protective delivery agents could be very beneficial.

Delivery system of different types have been designed for the protection of antimicrobial compounds. For example, antimicrobial molecules within essential oils have been encapsulated within protein or polysaccharide carriers (Baranauskienė, Venskutonis, Dewettinck, & Verh, 2006; Beristain, Garca, & Vernon-Carter, 2001) and emulsions (Suriyarak & Weiss, 2014). Different matrices such as β -cyclodextrin (Cevallos, Buera, & Elizalde, 2010), zein nanoparticles (Zhang et al., 2014), whey protein isolate-maltodextrin conjugates (Shah, Ikeda, Davidson, & Zhong, 2012), and liposomes (Liolios, Gortzi, Lalas, Tsaknis, & Chinou, 2009) have been used for the encapsulation of thymol. Films, nanoparticles, and liposomes have been used to deliver nisin (Prombutara, Kulwatthanasal, Supaka, Sramala, & Chareonpornwattana, 2012; Xiao, Gmmel, Davidson, & Zhong, 2011). Research in our lab has shown that phytoglycogen octenyl succinate in non-emulsion and emulsion systems can be used to protect nisin (Bi, Yang, Bhunia, & Yao, 2011; Bi, Yang, Narsimhan, Bhunia, & Yao, 2011).

Various types of antimicrobial compound such as carvacrol, thymol, β -resorcylic acid, and caprylic acid have been used to inhibit the growth of pathogens on cantaloupes (Upadhyay, Upadhyaya, Mooyottu, Kollanoor-Johny, & Venkitanarayanan, 2014). Recently, thymol-based

emulsions were prepared to wash organic cantaloupes to improve the microbial safety (Zhang, Ma, Critzer, Davidson & Zhong, 2016). In our lab, we have been studying the impact of delivery system on the efficacies of antimicrobial compounds, using cantaloupe fresh-cut and whole fruit as models. In the current study, the goal was to evaluate the efficacies of nisin and thymol in the presence of emulsion-based delivery systems against *Listeria monocytogenes* V7 and *Salmonella enterica* serovar Typhimurium. Cantaloupe juice was used as a model, since it usually covers the surface of fresh-cut cantaloupe and contains proteases that may negatively affect nisin activity. The overall hypothesis was that, starch octenyl succinate (starch-OS) stabilized emulsion was able to substantially prolong the efficacy of nisin and thymol, either individually or in combination, against both model pathogens.

2. Materials and methods

2.1 Materials

Starch octenyl succinate (starch-OS) was obtained from a food ingredient company. Our analysis showed that it had a degree of substitution (DS) of 0.022 (molar ratio of octenyl succinate groups to glucosyl groups), with weight-average molecular mass of 7.63×10^5 g/mole. Tween-20 was purchased from Sigma Aldrich (St. Louis, MO). Soybean oil was purchased from local grocery store. Nisin powder (2.5% nisin) and thymol ($\geq 99.5\%$) were purchased from Sigma Aldrich (St. Louis, MO). Brain heart infusion (BHI) powder and Bacto agar were purchased from BD Biosciences (Franklin Lakes, NJ).

2.2 Thymol and nisin formulations

2.2.1 Thymol and nisin in non-emulsion, aqueous dispersions

Stock solution of thymol was prepared by adding 2.0 g of thymol crystals to 1.0 L of sterile sodium acetate buffer (pH 5.5, 50 mM). The solution was gently stirred in dark for 24 h at 25 °C. Thereafter, the solution was centrifuged at $7,800 \times g$ for 15 min twice to obtain a thymol concentration of 978.3 $\mu\text{g/mL}$ determined using HPLC analysis. Nisin stock solution was prepared by dispersing 1.2 g of nisin powder in 3.00 mL sterile sodium acetate buffer (pH 5.5, 50 mM). The dispersion was slowly agitated on a rotary shaker (Lab-Line, Max/Rotator, USA) for 15 h at 6 °C. The nisin dispersion was centrifuged at $5000 \times g$ for 10 min to obtain nisin stock solution at 10.0 mg/mL. In order to prepare nisin and thymol combined solution, nisin stock solution was added to thymol stock solution to obtain a final nisin concentration of 400 $\mu\text{g/mL}$.

2.2.2 Thymol and nisin in emulsions

Starch-OS, soybean oil, thymol, and sodium acetate buffer (50 mM, pH5.5) were used to prepare thymol-containing oil-in-water emulsion. To prepare the aqueous phase, starch-OS was dissolved in the sodium acetate buffer. To prepare the oil phase, thymol was dissolved in soybean oil. Thereafter, the water and oil portions were mixed and subjected to high-speed homogenization (Ultra Turrax, IKA T25 digital, NC, USA) at 18,000 rpm for 1 min. In the next step, high-pressure homogenization (GEA, PandaPlus, Italy) at 10,000 psi for 3 cycles was performed to obtain the final emulsions. In the generated emulsion (stock emulsion), the concentrations of starch-OS and oil were each 10.0 mg/mL. To determine thymol concentration in the stock emulsion, 100 μL emulsion was added to 900 μL ethanol, and the mixture was vortexed for 10 min. Thereafter, the mixture was centrifuged at $12,000 \times g$ for 15 min to remove precipitated starch materials. The supernatant was used for thymol quantification with HPLC as described later. The thymol concentration in the emulsion was 972.4 $\mu\text{g/mL}$.

To prepare nisin in emulsion, similar method was followed without thymol addition to oil. Nisin stock solution (10.0 mg/mL) was added to the formed emulsion to achieve 400 µg/mL, and the mixture was incubated at 4 °C for 24 h on a rotary shaker. To prepare emulsion that contained both nisin and thymol, nisin stock solution was added to the thymol-containing emulsion and the mixture was incubated at 4 °C for 24 h. Different emulsions preparations (nisin, thymol, and combination of nisin and thymol) were immediately transferred to 50-mL tubes, capped and protected from light to prevent thymol evaporation and degradation. All formulated emulsions were sterilized in boiling-water bath for 3 min before proceeding with further tests.

2.3 Preparation of cantaloupe juice

Cantaloupe juice was extracted from cantaloupe fruits purchased from a local grocery store. The cantaloupe pulp was separated and crushed with a commercial blender (Waring Inc, USA). The pulp homogenate was centrifuged at $7,800 \times g$ for 20 min (Beckman Coulter, CA). The supernatant was collected as cantaloupe juice, which was filter-sterilized using a 0.2 µm filter (Millipore, MA) and termed “cantaloupe juice”.

2.4 Protease activity measurement of cantaloupe juice

A protease fluorescent detection kit (Sigma Aldrich, St. Louis, MO) was used to determine the protease activity of cantaloupe juice. In this procedure, 20 µL of incubation buffer, 20 µL of FITC-casein substrate and 10 µL solution (test, control, or blank) were mixed together. In this assay, the concentration of the control solution varied between 0.3125 to 20.0 µg/mL. The mixtures were incubated at 37 °C for 4 h in the dark. Incubation was performed again at 37 °C for 30 min after adding 150 µL of 0.6 N trichloroacetic acid (TCA). Centrifugation of the mixture was performed at $10,000 \times g$ for 10 min and supernatant (10 µL) was withdrawn. This supernatant was then mixed with 1.0 mL assay buffer. Aliquots (200 µL) were transferred from

this mixture to a black 96-well plate for quantification of protease activity using fluorescence intensity. All measurements were performed in triplicates.

2.5 Antimicrobial efficacy evaluations

Antimicrobial activity of different preparations was measured in two groups: (1) model group (in the presence of depletion factors) and (2) reference group (in the absence of depletion factors). In the model group, proteolytic enzymes in the cantaloupe juice and chamber headspace for evaporation were depletion factors of nisin and thymol, respectively. For the reference group, the proteolytic enzymes in the juice were deactivated through heating (boiling-water bath, 20 min), and the preparations were placed in full volume in 50-mL tubes to eliminate headspace evaporation.

Associated with various formulations and storage environments, the individual treatments are labeled in the format of “X-Y-Z”. The position “X” indicates the type of active ingredient; “N” represents nisin, “T” represents thymol, and “NT” represents a combined use of nisin and thymol. The position “Y” indicates the type of fluid; “A” represents non-emulsion, aqueous fluid whereas “E” represents emulsion. The position “Z” indicates the storage environment; “model” represents the model group in which each formulation was mixed with non-treated cantaloupe juice (1:1, v/v) and placed in a 1-liter sterile glass bottle (with large headspace, **Fig. 1** of Supplementary Material) whereas “ref” represents the reference group in which each formulation was mixed with heat-treated juice (1:1, v/v) and placed in full in a 50-mL tube (with no headspace). With both model and reference groups, the preparations were incubated at 4 °C for 11 days. For example, “N-A-ref” represents the nisin-containing non-emulsion, aqueous fluid incubated with heat-treated juice in full in a 50-mL tube, and “NT-E-model” represents the

emulsion containing both nisin and thymol was incubated with non-treated cantaloupe juice in a 1-liter glass bottle.

A 12-inch sterile needle (**Fig. 1** of Supplementary Material) was used to withdraw fluid aliquots from glass bottles. To maintain sterility in the bottles, elastic polytetrafluoroethylene film was used to seal the punctured caps. After 30 min and 1, 3, 5, 7, and 11 days of incubation, 1.5 mL fluid was withdrawn from each glass bottle (model) or 50-mL tube (reference) and was then divided into two portions. One portion was subjected to immediate antibacterial efficacy tests; another portion was heated at 80 °C for 15 min to deactivate enzymes in juice and then stored in an -80 °C freezer for later characterizations.

2.6 Bacterial inoculation

To 1.0 mL of the each withdrawn fluid transferred to a culture tube, 10 µL of inoculum from the *Listeria. monocytogenes* V7 culture (1×10^8 CFU/mL) were added to obtain a starting bacterial load of 1×10^6 CFU/mL. Similar procedure was followed with *Salmonella enterica* serovar. Typhimurium to obtain a starting bacterial load of 1×10^6 CFU/mL. To obtain the fresh cultures, the *L. monocytogenes* V7 and *S. Typhimurium* stocks maintained using glycerol at -80 °C were transferred to BHI broth and the cultures were grown overnight.

After inoculation, the culture tubes were incubated at 4 °C for 20 h on a rotary shaker at 60 rpm (Lab-Line, Max Rotator, USA). The 4 °C incubation was used to simulate the growth of foodborne pathogens under low temperature of food storage. After incubation, 100 µL culture fluid was withdrawn and decimally diluted in PBS, pour plated on BHI agar, incubated at 37 °C for overnight, and enumerated. The colony counts were reported as mean $\log_{10}$ CFU/mL from three replicates.

2.7 Thymol quantification in aqueous and emulsion formulations

Thymol content in aliquots collected after 30 min, 1, 3, 5, 7 and 11 days of storage were analyzed using HPLC technique. The HPLC system was equipped with a diode array detector, a quaternary pump, and a Supelcosil LC-18-DB column (3 μ m, 15 $\times$ 4.6 mm). HPLC grade water mixed with acetonitrile (50:50 v/v) was used as the mobile phase in an isocratic mode at a flow rate of 1.00 mL/min. The sample injection volume was 10 μ L. UV spectra were recorded at a wavelength of 274 nm. The data were analyzed using Chemstation software Rev. B.01.03 (Agilent Technologies, CA). For calibration, 10 to 500 μ g/mL of thymol standards were prepared in a solvent that was generated by mixing 90% ethanol and 10% sodium acetate buffer (50 mM, pH 5.5) by volume.

To determine the total thymol concentration in preparations, 100 μ L of each heat-treated fluid (solution or emulsion) was mixed with 900 μ L of ethanol. The mixture was vortexed for 30 min and centrifuged at 12,000 $\times$ g for 15 min to remove precipitates (mostly starch-OS). The supernatant obtained was subjected to HPLC analysis. To determine thymol amount in the aqueous phase of emulsions, the fluids were centrifuged (75,000 $\times$ g, 15 min) and the serums were collected. From each serum, 100 μ L was withdrawn, mixed with 900 μ L ethanol, and centrifuged. The supernatant was analyzed using HPLC to determine thymol amount. Thymol quantifications for all aqueous and emulsion formulations were performed in triplicates.

2.8 Nisin quantification

Nisin quantification for preparations that did not contain thymol was conducted using the agar diffusion bioassay with *L. monocytogenes* V7 as the testing strain. For thymol containing preparations, the fluids were initially subjected to nitrogen gas flushing to completely remove thymol before conducting nisin diffusion bioassay. Thymol removal was essential to making sure

that the inhibitory ring in the diffusion assay was attributed to nisin only. Reacti-Vap iii module (Thermo Fisher Scientific, PA) was used to perform nitrogen flushing using nitrogen gas (1 psi) through stainless steel needles. The flushing was performed for 50 min, each flushed solution was added with sodium acetate buffer (50 mM, pH 5.5) to make up its original weight. After this process, the fluids were assayed for nisin activity along with those without thymol in formulation.

The procedure of BHI agar diffusion assay of nisin is as following. BHI medium (18.5 g) was dispersed in 500.0 mL of distilled water and hydrogen chloride was used to adjust to pH5.5. To this dispersion, 3.75 g agar and 5.0 g Tween-20 were added, and the mixture was autoclaved and thereafter transferred to square-Petri-plates (10 × 10 cm). Meanwhile, autoclaved BHI agar was also prepared in glass tubes and added with overnight grown *L. monocytogenes* (10^8 CFU/mL) culture. This inoculated BHI-agar mixture was poured on the surface of the BHI-agar plates to form a layer. After the solidification of BHI-agar system, a 7-mm cork borer was used to form wells in the petri plates. For each fluid, 100.0 µL were transferred in the well in triplicates. The loaded plates were then incubated at 25 °C for 24 h and the diameter of inhibitory zone (D) was measured. To quantify ring thickness, three diameter readings (D) were taken and the size (thickness) of inhibitory ring (x) was calculated using the equation: $x = (D-7)/2$. From three readings of x for each of triplicate testing wells, the average value was used for further calculations.

2.9 Stability of emulsions during storage

Average particle size of emulsions in both reference and model groups were measured using a Zetasizer (ZS90, Malvern, Worcestershire, UK) after 100 volumes dilution with sodium acetate buffer (pH 5.5, 50 mM).

2.10 Statistical analysis

All data are expressed as mean $\pm$ SD with sample size (n) = 3. A probability value (p-value) of 0.05 was used for statistical significance for all experiments. ANOVA analysis was performed using SPSS software (version 21, IBM). Tukey's multiple HSD comparison test was performed to evaluate significant differences among treatments.

3. Results

3.1 Retention of nisin activity by starch-OS emulsion

For the reference group (with no depletion factors for nisin and thymol), nisin aqueous preparations (N-A-ref and NT-A-ref) showed negligible depletion of nisin activity (**Fig. 1A**). Over the entire storage period (30 min to 7 days), the size of inhibitory ring of N-A-ref maintained at around 5.87 mm, and for NT-A-ref the ring size slightly decreased from 6.11 to 5.87 mm. This shows a high stability of nisin activity in the absence of depletion factors. In the presence of cantaloupe juice (with model group), however, both N-A-model and NT-A-model showed quick depletion of nisin activity in the first 3 days of storage, leading to the disappearance of inhibitory ring from the original 6.34 mm for N-A-model and 6.97 mm for NT-A-model.

For emulsion preparations (**Fig. 1B**), the retained nisin activity in the aqueous phase was determined as the size of inhibitory ring for the serum collected after centrifugation of emulsion (and thymol removal for the thymol-containing preparations). For N-E-ref and NT-E-ref, the ring size maintained around 4.40 mm over 7 days. Compared with the aqueous system (N-A-ref and

NT-A-ref), the ring size was smaller for emulsions presumably due to a partial adsorption of nisin with the oil droplets in emulsion. In the model group (with cantaloupe juice), size of inhibitory ring for N-E-model declined from 4.32 mm (30 min) to 2.76 mm (7 days), and ring size of NT-E-model declined from 4.72 mm (30 min) to 4.08 mm (7 days). Evidently, the use of starch-OS emulsion reduced nisin depletion in the presence of cantaloupe juice. **Fig. 2** of the Supplementary Material provided a direct comparison of nisin activity retention between aqueous and emulsion preparations in model groups. The emulsion preparations performed much better than aqueous preparations in retaining nisin activity, regardless the presence of thymol or not.

3.2 Retention of thymol by starch-OS emulsion

For the aqueous thymol preparation in the reference group (T-A-ref), thymol concentration slightly decreased from 493 $\mu\text{g/mL}$ (30 min) to 438 $\mu\text{g/mL}$ (11 days) (**Fig. 2A**). For NT-A-ref, the change of thymol concentration was negligible over 11 days of storage. In contrast, thymol activity was substantially reduced in the model groups. For both T-A-model and NT-A-model, thymol concentration reduced to a negligible level after 5 days, suggesting a nearly complete evaporation of thymol from aqueous preparations.

For T-E-ref and NT-E-ref, the total thymol concentration (TTC) and serum thymol concentration (STC) were both determined. As shown in **Fig. 2B** and **2C**, the total (TTC) and serum thymol concentrations (STC) did not show substantial change over the 11-day period due to minimal thymol volatilization. For example, STC of T-E-ref and NT-E-ref decreased from 167 to 125 $\mu\text{g/mL}$ and 183 to 156 $\mu\text{g/mL}$, respectively (**Fig. 2C**).

Emulsion significantly enhanced the retention of thymol in model systems (**Fig. 2B** and **2C**). For example, after 7 days T-E-model retained 87 µg/mL thymol in total (TTC) and 40 µg/mL thymol in serum (STC), and NT-E-model retained 118 µg/mL thymol in total (TTC) and 51 µg/mL in serum (STC). Evidently, the emulsion preparations performed much better than the aqueous preparations in retaining thymol from evaporation, regardless the presence of nisin or not.

3.3 Efficacy retention against *Listeria monocytogenes* V7

The non-emulsion, aqueous formulations in the reference group demonstrated strong antibacterial efficacy against *L. monocytogenes* during the 11-day storage period (**Fig. 3A**). The negative control maintained *Listeria* count between 6 to 6.32 log₁₀ CFU/mL. In the reference group, all formulations showed a minimum of 3.0 log₁₀ CFU/mL reduction. Over the 11-day incubation, N-A-ref and T-A-ref recovered from 1 to 3.26 log₁₀ CFU/mL and 1.9 to 3.10 log₁₀ CFU/mL, respectively. For NT-A-ref, *Listeria* increased from 1.125 to 2.93 log₁₀ CFU/mL during the same storage period. In general, NT-A-ref (a combination of nisin and thymol) demonstrated a greater anti-listerial efficacy compared with N-A-ref and T-A-ref (**Fig. 3A**), suggesting a cooperative effect between nisin and thymol.

In the non-emulsion, model group (**Fig. 3B**), the storage of N-A-model (nisin only) and T-A-model (thymol only) led to a quick reduction of their efficacy to inhibit bacterial growth, showing a rapid listerial population recovery in the first 3 days. In contrast, NT-A-model (combined use of nisin and thymol) led to a slower listerial recovery, which used 5 days to reach a similar level to that of positive control (Buffer-model).

For emulsion preparations in the reference group, the listerial population could be effectively suppressed by preparations subjected to up to 11 days of storage (**Fig. 3C**). For example, at day-11, the N-E-ref and T-E-ref could suppress *Listeria* at 2.79 and 2.98 log₁₀ CFU/mL, respectively, which were similar to that of non-emulsion systems. The combined use of nisin and thymol in emulsion (NT-E-ref) showed similar effect at day-11, even though it was seemingly more effective in the first 3 days (**Fig. 3C**).

Compared with non-emulsion preparations in the model group (N-A-model and T-A-model), the retained anti-listerial efficacies of nisin (N-E-model) and thymol (T-E-model) in emulsion preparations were much higher in the first 5 days of storage (**Fig. 3B** and **3D**). For example, after 3 days of storage, both N-A-model and T-A-model did not show any anti-listerial effect when compared with the control (Buffer-model, about 6.0 log₁₀ CFU/mL); in contrast, N-E-model and T-E-model were able to reduce *Listeria* to about 3.5 and 4.2 log₁₀ CFU/mL, respectively. The combined use of nisin and thymol in emulsion (NT-E-model) showed the highest efficacy among individual preparations in the model group, showing an anti-listerial efficacy till at least day-7 (3.9 log₁₀ CFU/mL), substantially stronger than the individual uses of nisin and thymol in emulsion (N-E-model and T-E-model) and the combined use of nisin and thymol in non-emulsion system (NT-A-model) (**Fig. 3B** and **3D**).

3.4 Efficacy retention against Salmonella Typhimurium

As shown in **Fig. 4A** and **4C**, nisin-only preparations (N-A-ref and N-E-ref) were ineffective in inhibiting *S. Typhimurium*. *S. Typhimurium* is a Gram-negative bacterium with the presence of lipopolysaccharides in its outer membrane, which is resistant to nisin (Helander & Mattila-Sandholm, 2000). On the other hand, thymol-containing preparations, such as T-A-ref, NT-A-ref, T-E-ref, and NT-E-ref, demonstrated strong antibacterial efficacy. For example, T-A-

ref and NT-A-ref still maintained their substantial anti-salmonella efficacy after the 11-day storage, leading to $\log_{10}$ CFU/mL values below 3.84 as compared with about 7.8 for the control (Buffer-ref) and nisin-only (N-A-ref). Similar scenario was also evident for emulsion preparations in the reference group, even though the thymol-containing emulsion preparations (T-E-ref and NT-E-ref) seemed to be slightly more effective than their non-emulsion counterparts (**Fig. 4A** and **4C**).

For the model group, the capability of emulsion preparations to retain anti-salmonella efficacy was evident when the values presented in **Fig. 4B** were compared with **Fig. 4D**. For thymol-only emulsion (T-E-model), it was able to show significant anti-salmonella activity after 5 days of storage (about $5.5 \log_{10}$ CFU/mL), whereas the thymol-only non-emulsion (T-A-model) led to $6.4 \log_{10}$ CFU/mL similar to the buffer control. The anti-salmonella activity was most evident for the combined use of nisin and thymol in emulsion (NT-E-model), which was able to suppress *Salmonella* to about $4.1 \log_{10}$ CFU/mL at day-7 (**Fig. 4D**) as compared with over $6.0 \log_{10}$ CFU/mL for all other emulsion and non-emulsion preparations. This highlights the capability of emulsion to realize a cooperative effect between nisin and thymol against *S. Typhimurium*. Such a nisin-thymol combined effect was only slightly seen in non-emulsion preparation (NT-A-model, day-1 in **Fig. 4B**).

3.5 Stability of emulsions

As shown in **Fig. 5**, the particle sizes of emulsions in the model and reference groups did not show substantial variation over the 11-day storage period. In the model group, the particle sizes for N-E-model, T-E-model, and NT-E-model emulsions were 510 - 482 nm, 491 - 501 nm, and 478 - 498 nm, respectively. For the reference group, the particle sizes for N-E-ref, T-E-ref, and NT-E-ref emulsions were 525 - 625 nm, 528 - 606 nm, and 547 - 607 nm, respectively. The

minor difference in particle sizes over the 11-day storage period demonstrated that starch-OS could form highly stable emulsions in the presence of fresh or heat-treated cantaloupe juice.

4. Discussion

The transportation of nisin or thymol in a food system depends on its concentration difference between the “depot” and “sink”. In a non-emulsion system, the solution is the depot of nisin or thymol. In the emulsion system, on the other hand, the oil droplets and interfacial layer served as the primary depot of thymol and nisin, respectively, in addition to the aqueous phase of emulsion. In a cantaloupe juice system, the depletions of thymol and nisin are associated with multiple sinks: (1) nisin degradation by the proteolytic enzymes in juice, (2) thymol evaporation to the chamber headspace, and (3) adsorption of nisin and thymol with bacterial cell membrane.

In the absence of bacterial cells, sinks (1) and (2) affect the rate of nisin or thymol depletions. Nisin can be rapidly degraded through the hydrolysis by proteolytic enzymes in cantaloupe juice. Proteolytic enzymes such as the alkaline serine protease cucumisin in melons (e.g. honeydews and cantaloupes), thiol protease actinidin in kiwi fruit, and cysteine protease bromelain in pineapples are highly predominant in fruits (Katsaros, Katapodis, & Taoukis, 2009; Raimbault, Zuily-Fodil, Soler, Mora, & de Carvalho, 2013; Yamagata, Masuzawa, Nagaoka, Ohnishi, & Iwasaki, 1994). Proteases from such fruit sources can cause peptic cleavage or comprehensive proteolysis, thus can readily degrade antimicrobial peptides such as nisin. Thymol, in the presence of sufficient headspace with the container, can be depleted via evaporation due to its high volatility. With the same juice components and storage conditions, the rates of nisin and thymol depletion are primarily affected by their concentrations in the aqueous phase. Compared with the non-emulsion solutions, in emulsions most nisin or thymol molecules are associated with oil droplets, and their lower concentrations in aqueous phases lead

to lower rate of degradation (for nisin) and evaporation (for thymol). For both antimicrobial compounds, lower depletion over storage results in their higher retentions.

Once bacterial contamination occurs (e.g. inoculation induced in this study), bacterial cells act as a major sink for the transportations of nisin or thymol from their depots. Positively charged nisin molecules transport to negatively charged cell membrane through electrostatic interactions. The motion of thymol molecules towards cell membranes is driven by hydrophobic interactions (Burt, 2004). For non-emulsion preparations, the low retention of nisin or thymol after a prolonged storage is not sufficient to bring appreciable antimicrobial activity. In contrast, high retentions of nisin and thymol in emulsions allow for significant activities against the bacteria tested in this study.

The combined antibacterial action between nisin and thymol in non-emulsion or emulsion systems deserve additional discussions. In general, nisin acts against Gram-positive bacteria by creating transient pore-like structures in the cell membrane. Thymol, on the other hand, inhibits bacterial growth by interacting with the phospholipid bi-layer of cell membranes, resulting in metabolite dissipation (Ettayebi, Yamani, & Rossi-Hassani, 2000). In this study, the result showed the aggregated effect between nisin and thymol against *L. monocytogenes* in both non-emulsions (Fig. 3B) and emulsions (Fig. 3D), and against *S. Typhimurium* mostly in emulsion (Fig. 4D). The combined, aggregated effects of nisin and thymol against Gram-positive bacteria is possibly due to their simultaneous action on the bacterial cell membrane that leads to greater disintegration of the phospholipid bi-layer. On the other hand, the seemingly aggregated effects against Gram-negative bacteria are considered to be related to the disintegration of the outer membrane caused by thymol, which leads to enhanced sensitivity of cells towards nisin. In the studies by Rasooli et al. (2006) and Pol & Smid (1999), it was shown that the essential oil

components could improve nisin efficacy by prolonging the timescale of the nisin pores and increasing their size or quantity, thereby leading to aggregated antibacterial effects.

It is important to note that the combined effect of nisin and thymol against *S. Typhimurium* was much more evident in emulsion than in non-emulsion. This leads to our consideration that the presence of a carrier, which may prolong the release of nisin and thymol, can substantiate their collaborative interactions with cell membranes. Xiao et al. (2011) reported another type of delivery system for nisin and thymol, i.e. the spray-dried zein capsules, showing the superiority of this system in terms of antibacterial efficacy against food-borne pathogens. Conceivably, to effectively realize an aggregated effect between nisin and thymol, adjustments of release amount and timing could be an important factor.

5. Conclusion

This study demonstrated the potential of using starch-based emulsions to protect antimicrobial compound nisin and thymol in a cantaloupe juice system and thus prolong their efficacy against *L. monocytogenes* and *S. Typhimurium*. The primary finding was that the use of emulsions may greatly reduce the proteolytic degradation of nisin and the evaporation of thymol, both leading to prolonged antibacterial efficacy compared with the non-emulsion preparations. With *L. monocytogenes*, a combined use of nisin and thymol showed appreciable higher inhibitory effects than their individual uses, with the emulsion showing the greatest cooperative effect between these two compounds. With *S. Typhimurium*, the use of nisin only did not show any antibacterial effect, regardless the use of emulsion or not. In the presence of thymol in emulsion, however, the additional use of nisin substantially enhanced the antibacterial activity compared with thymol only. This finding suggested a synergistic effect between nisin and

thymol against Gram-negative bacteria that can be achieved by the use of emulsion as the delivery system. We hope that this study can provide an exploratory methodology in formulating antimicrobial compounds to reduce the pathogen load for fresh-cut cantaloupe and other produce.

Acknowledgement

This study was supported by a USDA-NIFA grant (GRANT11282711) to Dr. Yuan Yao and Dr. Arun Bhunia.

References

1. Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils—a review. *Food and Chemical Toxicology*, 46(2), 446-475.
2. Baranauskienė, R., Venskutonis, P. R., Dewettinck, K., & Verhé, R. (2006). Properties of oregano (*Origanum vulgare* L.), citronella (*Cymbopogon nardus* G.) and marjoram (*Majorana hortensis* L.) flavors encapsulated into milk protein-based matrices. *Food Research International*, 39(4), 413-425.
3. Beristain, C. I., Garcia, H. S., & Vernon-Carter, E. J. (2001). Spray-dried encapsulation of cardamom (*Elettaria cardamomum*) essential oil with mesquite (*Prosopis juliflora*) gum. *LWT-Food Science and Technology*, 34(6), 398-401.
4. Bhatti, M., Veeramachaneni, A., & Shelef, L. A. (2004). Factors affecting the antilisterial effects of nisin in milk. *International Journal of Food Microbiology*, 97(2), 215-219.

5. Bi, L., Yang, L., Bhunia, A. K., & Yao, Y. (2011). Carbohydrate nanoparticle-mediated colloidal assembly for prolonged efficacy of bacteriocin against food pathogen. *Biotechnology and Bioengineering*, 108(7), 1529-1536.
6. Bi, L., Yang, L., Narsimhan, G., Bhunia, A. K., & Yao, Y. (2011). Designing carbohydrate nanoparticles for prolonged efficacy of antimicrobial peptide. *Journal of Controlled Release*, 150(2), 150-156.
7. Breukink, E., & de Kruijff, B. (2006). Lipid II as a target for antibiotics. *Nature Reviews Drug Discovery*, 5(4), 321-323.
8. Breukink, E., Ganz, P., De Kruijff, B., & Seelig, J. (2000). Binding of nisin Z to bilayer vesicles as determined with isothermal titration calorimetry. *Biochemistry*, 39(33), 10247-10254.
9. Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods - a review. *International Journal of Food Microbiology*, 94(3), 223-253.
10. Cevallos, P. A. P., Buera, M. P., & Elizalde, B. E. (2010). Encapsulation of cinnamon and thyme essential oils components (cinnamaldehyde and thymol) in β -cyclodextrin: Effect of interactions with water on complex stability. *Journal of Food Engineering*, 99(1), 70-75.
11. Ettayebi, K., El Yamani, J., & Rossi-Hassani, B.-D. (2000). Synergistic effects of nisin and thymol on antimicrobial activities in *Listeria monocytogenes* and *Bacillus subtilis*. *FEMS Microbiology Letters*, 183, 191-195.
12. Fang, T., Liu, Y., & Huang, L. (2013). Growth kinetics of *Listeria monocytogenes* and spoilage microorganisms in fresh-cut cantaloupe. *Food microbiology*, 34(1), 174-181.

13. Helander, I. M., & Mattila-Sandholm, T. (2000). Permeability barrier of the Gram-negative bacterial outer membrane with special reference to nisin. *International Journal of Food Microbiology*, 60(2), 153-161.
14. Ismail, H. I., Chan, K. W., Mariod, A. A., & Ismail, M. (2010). Phenolic content and antioxidant activity of cantaloupe (*Cucumis melo*) methanolic extracts. *Food Chemistry*, 119(2), 643-647.
15. Jung, D. S., Bodyfelt, F. W., & Daeschel, M. A. (1992). Influence of fat and emulsifiers on the efficacy of nisin in inhibiting *Listeria monocytogenes* in fluid milk. *Journal of Dairy Science*, 75(2), 387-393.
16. Katsaros, G. I., Katapodis, P., & Taoukis, P. S. (2009). Modeling the effect of temperature and high hydrostatic pressure on the proteolytic activity of kiwi fruit juice. *Journal of Food Engineering*, 94(1), 40-45.
17. Liolios, C. C., Gortzi, O., Lalas, S., Tsaknis, J., & Chinou, I. (2009). Liposomal incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus* L. and in vitro antimicrobial activity. *Food Chemistry*, 112(1), 77-83.
18. Mahadeo, M., & Tatini, S. R. (1994). The potential use of nisin to control *Listeria monocytogenes* in poultry. *Letters in Applied Microbiology*, 18(6), 323-326.
19. Mahmoud, B. S. M. (2012). Effects of X-ray treatments on pathogenic bacteria, inherent microflora, color, and firmness on whole cantaloupe. *International Journal of Food Microbiology*, 156(3), 296-300.

20. Martiñon, M. E., Moreira, R. G., Castell-Perez, M. E., & Gomes, C. (2014). Development of a multilayered antimicrobial edible coating for shelf-life extension of fresh-cut cantaloupe (*Cucumis melo* L.) stored at 4 °C. *LWT-Food Science and Technology*, 56(2), 341-350.
21. Pol, I., & Smid, E. (1999). Combined action of nisin and carvacrol on *Bacillus cereus* and *Listeria monocytogenes*. *Letters in Applied Microbiology*, 29, 166-170.
22. Prombutara, P., Kulwatthanasal, Y., Supaka, N., Sramala, I., & Chareonpornwattana, S. (2012). Production of nisin-loaded solid lipid nanoparticles for sustained antimicrobial activity. *Food Control*, 24(1), 184-190.
23. Raimbault, A. K., Zuily-Fodil, Y., Soler, A., Mora, P., & de Carvalho, M. H. C. (2013). The expression patterns of bromelain and AcCYS1 correlate with blackheart resistance in pineapple fruits submitted to postharvest chilling stress. *Journal of Plant Physiology*, 170(16), 1442-1446.
24. Rasooli, I., Rezaei, M. B., & Allameh, A. (2006). Ultrastructural studies on antimicrobial efficacy of thyme essential oils on *Listeria monocytogenes*. *International Journal of Infectious Diseases*, 10, 236-241.
25. Rose, N. L., Sporns, P., Stiles, M. E., & McMullen, L. M. (1999). Inactivation of nisin by glutathione in fresh meat. *Journal of Food Science-Chicago*, 64(5), 759-762.
26. Shah, B., Davidson, P. M., & Zhong, Q. (2012). Nanocapsular dispersion of thymol for enhanced dispersibility and increased antimicrobial effectiveness against *Escherichia coli* O157: H7 and *Listeria monocytogenes* in model food systems. *Applied and environmental microbiology*, 78(23), 8448-8453.

27. Shah, B., Davidson, P. M., & Zhong, Q. (2013). Antimicrobial activity of nanodispersed thymol in tryptic soy broth. *Journal of Food Protection*, 76(3), 440-447.
28. Shah, B., Ikeda, S., Davidson, P. M., & Zhong, Q. (2012). Nanodispersing thymol in whey protein isolate-maltodextrin conjugate capsules produced using the emulsion–evaporation technique. *Journal of Food Engineering*, 113(1), 79-86.
29. Suriyarak, S., & Weiss, J. (2014). Cutoff Ostwald ripening stability of alkane-in-water emulsion loaded with eugenol. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 446, 71-79.
30. Upadhyay, A., Upadhyaya, I., Mooyottu, S., Kollanoor-Johny, A., Venkitanarayanan, K. (2014). Efficacy of plant-derived compounds combined with hydrogen peroxide as antimicrobial wash and coating treatment for reducing *Listeria monocytogenes* on cantaloupes. *Food Microbiology*, 44, 47-53.
31. Xiao, D., Davidson, P. M., & Zhong, Q. (2011). Spray-dried zein capsules with coencapsulated nisin and thymol as antimicrobial delivery system for enhanced antilisterial properties. *Journal of Agricultural and Food Chemistry*, 59(13), 7393-7404.
32. Xiao, D., Gömmel, C., Davidson, P. M., & Zhong, Q. (2011). Intrinsic Tween 20 improves release and antilisterial properties of co-encapsulated nisin and thymol. *Journal of Agricultural and Food Chemistry*, 59(17), 9572-9580.
33. Yamagata, H., Masuzawa, T., Nagaoka, Y., Ohnishi, T., & Iwasaki, T. (1994). Cucumisin, a serine protease from melon fruits, shares structural homology with subtilisin and is generated from a large precursor. *Journal of Biological Chemistry*, 269(52), 32725-32731.

34. Zhang, Y., Niu, Y., Luo, Y., Ge, M., Yang, T., Yu, L. L., & Wang, Q. (2014). Fabrication, characterization and antimicrobial activities of thymol-loaded zein nanoparticles stabilized by sodium caseinate–chitosan hydrochloride double layers. *Food Chemistry*, 142, 269-275.
35. Zhang, Y., Ma, Q., Critzer, F., Davidson, P.M., Zhong, Q. (2016). Organic thyme oil emulsion as an alternative washing solution to enhance the microbial safety of organic cantaloupes. *Food Control*, 67, 31-38.

Figure captions

Figure 1. Inhibitory ring size showing residual nisin activity over 7 days' storage for preparations containing nisin in model and reference groups. A: non-emulsion, aqueous preparations; B: emulsion preparations. Time point "0" indicates 30 min after combining formulations with juice. Mean values are shown with error bars of standard deviations (n= 3).

Figure 2. Thymol concentration over the 11-day storage of preparations in model and reference groups. A: thymol concentration in non-emulsion, aqueous systems; B: total thymol concentration (TTC) in emulsion systems; C: serum thymol concentration (STC) after centrifugation of emulsion. Time point "0" indicates 30 min after combining formulations with juice. Mean values are shown with error bars of standard deviations (n= 3).

Figure 3. Capability to inhibit the growth of *Listeria monocytogenes* V7 by various preparations subjected to storage for different periods of time. A: non-emulsion, aqueous preparations in reference group; B: non-emulsion, aqueous preparations in model group; C: emulsion preparations in reference group; D: emulsion preparations in model group. Time point "0" indicates 30 min after the combination of formulations with juice. Mean values are shown with error bars of standard deviations (n= 3).

Figure 4. Capability to inhibit the growth of *Salmonella* Typhimurium by various preparations subjected to storage for different periods of time. A: non-emulsion, aqueous preparations in

reference group; B: non-emulsion, aqueous preparations in model group; C: emulsion preparations in reference group; D: emulsion preparations in model group. Time point “0” indicates 30 min after the combination of formulation with juice. Mean values are shown with error bars of standard deviations (n= 3).

Figure 5. Particle size of different emulsion preparations in the model (A) and reference (B) groups. In (A), “Control” sample indicates starch-OS emulsion mixed with cantaloupe juice without nisin and thymol. In (B), “Control” sample indicates starch-OS emulsion mixed with heat-treated cantaloupe juice without nisin and thymol. Mean values are shown with error bars of standard deviations (n= 3).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5