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Intrinsic Tween 20 Improves Release and Antilisterial Properties of Co-encapsulated Nisin and Thymol

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ABSTRACT: Antimicrobial delivery systems have been proposed as potential solutions to improve effectiveness of antimicrobials in food matrixes by shielding antimicrobials from contacting food matrix components and releasing them continuously. In this work, spray-dried capsules were produced from zein solutions with the same concentrations of nisin and thymol but with varying Tween 20 contents for characterization of release kinetics of antimicrobials and antilisterial properties. At intermediate levels of Tween 20, sustained and more complete release of antimicrobials was observed at pH 6.0 and 8.0. Most capsule samples were more effective than free antimicrobials against *Listeria monocytogenes* in 2% reduced fat milk, and the best capsule treatment reduced the bacterial population by 2 log CFU/mL more than comparable free antimicrobials after 4 h incubation at 25 °C. Our work demonstrated that nonionic surfactant can be conveniently used to modulate characteristics of delivery systems to effectively improve antimicrobial functions in food systems.

KEYWORDS: nisin, thymol, Tween 20, release kinetics, antilisterial, milk

INTRODUCTION

USDA's Food Safety and Inspection Service (FSIS) and the U.S. Food and Drug Administration classify the foodborne pathogenic bacteria, *Listeria monocytogenes* (*Lm*), as a "zero tolerance" organism in ready-to-eat (RTE) foods.[1] Despite enormous efforts by the government and food industry, there are still sporadic cases and outbreaks of listeriosis due to consumption of RTE foods, mostly due to cross-contamination post production.[2−4] The CDC in its *Healthy People 2010 Objectives* set a goal to reduce listeriosis incidence from 5 cases per million population in 1997 to 2.5 cases per million population in 2010.[1] In 2009, a major outbreak of listeriosis occurred in Canada associated with luncheon and deli meats and resulted in 20 deaths.[5] The continued threat from the potent pathogen demands the need for additional postprocess hurdle technologies such as antimicrobial delivery systems to control *Lm* and improve food safety.

Nisin is a well-known bacteriocin effective against a broad spectrum of gram positive bacteria, including *Lm*. However, numerous studies reported much reduced antimicrobial effectiveness of nisin when applied in foods rather than in a growth medium. Mahadeo and Tatini[6] reported that 2.5 μg/mL of nisin was effective against *Lm* in scald water but not on the turkey skin. A reduction of nisin activity was reported because of nonspecific binding of nisin with lipids and proteins.[7−9] Rose et al.[10] found that the compromised antimicrobial activity of nisin in fresh meat was caused by the complexation with glutathione.

Incorporation of nisin within capsules of edible polymers may reduce the interaction of nisin with food components and improve its activity in foods.[11−13] For example, Salmaso et al.[14] demonstrated that sustained release of nisin from poly(L-lactide) nanocapsules inhibited the growth of *Lactobacillus delbrueckeii* over 45 days, in comparison to ca. 4 days for unencapsulated nisin. However, the authors used an expensive process based on antisolvent precipitation in supercritical carbon dioxide, and the carrier polymer poly(L-lactide) is not generally recognized-as-safe (GRAS). Much work is needed to utilize GRAS, sustainable, and inexpensive ingredients as delivery systems of antimicrobials and low-cost and scalable processes.

Recently, we studied release kinetics of hen egg white lysozyme from spray-dried zein capsules and demonstrated sustained release of lysozyme when the solution used in spray drying contained thymol, a naturally occurring antimicrobial extracted from the thyme plant, used at 2% mass of zein.[15] Zein is a class of alcohol-soluble storage protein (prolamins) extracted from maize kernels, is available in large quantity, and can be produced as a byproduct of bioethanol industry. Therefore, our work demonstrated the feasibility to manufacture low-cost GRAS delivery systems of antimicrobials. However, antibacterial properties of the capsules were not tested.

The objective of this work was to establish new formulations allowing sustained release of nisin and improved antilisterial properties of antimicrobials in food matrixes. The rationale of such study was that formulations previously established for lysozyme did not enable sustained release of nisin. To manipulate release kinetics of the encapsulated antimicrobials, we adopted a nonionic surfactant polysorbate 20 (Tween 20) whose amphiphilic property may control interactions between the carrier biopolymer and the encapsulated antimicrobials. Previously, we demonstrated that capsules with Tween 20[16] or thymol[17] alone did not show sustained release of nisin, but the combination of both compounds in capsules at an appropriate ratio enabled more complete and controlled release of nisin in this work. Because Tween 20 is a capsule component, we used the phrase "intrinsic" to differentiate our work from studies where the surfactant is used in the bulk phase to manipulate release properties. In addition, we

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Table 1. Encapsulation Performance of Samples Spray-Dried from Nisin Solutions with Different Amounts of Tween 20[a]

sample	Tween 20 (% w/v)	mass yield (%)[b]	total solids (%)	nisin EE (%)[c]	nisin SA ratio (%)[d]	thymol EE (%)[e]	thymol content change (%)[f]
A	0	31.67 ± 7.07c	93.33 ± 3.64b	35.96 ± 8.03c	121.67 ± 4.75a	7.56 ± 0.58c	25.14 ± 6.48c
B	0.05	37.67 ± 4.24b	93.68 ± 3.05b	38.94 ± 4.39bc	110.36 ± 3.59ab	15.05 ± 1.78b	39.65 ± 0.16b
C	0.1	43.23 ± 5.02b	93.52 ± 0.64b	45.48 ± 5.28b	112.41 ± 0.77ab	17.04 ± 1.99b	40.30 ± 0.33b
D	0.5	57.71 ± 4.44a	97.80 ± 1.50a	69.14 ± 5.32a	122.49 ± 1.87a	26.02 ± 2.27a	45.50 ± 0.27a

[a] Solutions used in spray drying contained 2% w/v zein, 1% thymol, same concentration of nisin, but varying Tween 20 contents in 70% v/v aqueous ethanol. Values in a column sharing the same letters are not statistically different. [b] As defined in eq 1. [c] Encapsulation efficiency (EE) as defined in eq 2. [d] Specific activity (SA) ratio as defined in eq 4. [e] As defined in eq 3. [f] As defined in eq 5.

demonstrated improved efficacy of the studied GRAS antimicrobial delivery system as compared to free antimicrobials in inhibiting the growth of *Lm* in 2% reduced fat milk. The practical GRAS antimicrobial capsules may be used as ingredients incorporated in foods to enhance microbial food safety.

MATERIALS AND METHODS

Materials. The nisin preparation, containing approximately 2.5% nisin and labeled with a nisin concentration of 1000 IU/mg solids, was procured from MP Biomedicals, LLC (Solon, OH). Ethanol (200 proof) and zein were products of Acros Organics (Morris Plains, NJ). Materials used in microbial tests, including tryptic soy broth (TSB), yeast extract (YE), peptone, and agar, were purchased from Becton, Dickinson and Co. (Sparks, MD). 2% reduced fat milk was an ultrahigh-temperature-processed product (Farmland Dairies, LLC, Wallington, NJ) purchased from a grocery store. Other chemicals were from Fisher Scientific (Pittsburgh, PA).

Preparation of Capsules by Spray Drying. Aqueous ethanol was used to extract the 2.5% nisin preparation to achieve partial purification using conditions optimized previously.[18] The extraction was performed by agitating 6 mg of nisin solids per mL in 50% v/v aqueous ethanol for 6 h using a stirring plate. The slurry was centrifuged at 1520*g* for 5 min (model Tabletop Centrifuge E9, Beckman, Palo Alto, CA) to obtain supernatant that was then adjusted to 70% ethanol (v/v) and dissolved with 2% w/v zein, 1% w/v thymol, and Tween 20 to a concentration of 0, 0.05, 0.1, and 0.5% w/v (Table 1). The final solution was spray-dried using a benchtop spray dryer (mini spray dryer B-290, BÜCHI Corp., Flawil, St. Gallen, Switzerland) using a feed rate of 5.26 mL/min, an aspirator setting of 100%, and an inlet temperature of 105 °C that corresponded to an outlet temperature of 68−69 °C. The collected powder was stored in a −20 °C freezer until analysis. To simplify discussion, capsules prepared from the solutions containing 0.05, 0.1, and 0.5% w/v Tween 20 are referred to as low, medium, and high levels of Tween 20, respectively, hereafter.

Estimation of Encapsulation Parameters. Each spray drying experiment was evaluated for the following parameters previously used for lysozyme capsules,[15] with modification. Two replicates from separate spray drying experiments were tested for each formulation in Table 1.

Total Solids Content of Capsules. The total solids content of spray-dried powders was determined using vacuum drying[19] at 100 °C and 500 mmHg under pressure until a constant weight. Sample mass before and after drying was used to determine the total solids content.

Mass Yield. The mass yield of a spray drying experiment was defined as in eq 1:

$$\text{massyield}\ \% = \frac{\text{massofcollectedproduct}}{\text{nonsolventmassinthefeed}} \times 100\% \quad (1)$$

where nonsolvent mass was based on the formulation used in spray drying.

Encapsulation Efficiency (EE). A powdered sample was dissolved in 70% ethanol and a methanol−water−acetic acid ternary mixture at a volume ratio of 60:40:2 for determination of nisin (IU/mg) and thymol (%w/w) contents, respectively, using the methods detailed below. EE of nisin and thymol was then determined according to eqs 2 and 3 by comparing the total amount of antimicrobial in a spray-dried product and that used in spray drying.

$$\text{EEofnisin}\ (\%) = \frac{\text{totalnisinunitsinthecollectedproduct}}{\text{totalnisinunitsinthefeed}} \times 100\% \quad (2)$$

$$\text{EEofthymol}\ (\%) = \frac{\text{totalthymolmassinthecollectedproduct}}{\text{totalthymolmassinthefeed}} \times 100\% \quad (3)$$

Because only a small amount of material (150 mL, with ∼3 g of solutes) was used in each encapsulation experiment and significant errors occurred in collecting powders from spray drying chambers, encapsulation efficiency as defined in eqs 2 and 3 may not reflect the encapsulation process accurately as the sample collection errors impact the numerators. Analogous to protein purification, specific activity (SA) of nisin was estimated to be the number of nisin units per unit mass (IU/mg) in a spray-dried product or nonsolvent mass in the spray drying solution and was used to compare SA ratios before and after spray drying:

$$\text{SAratio}\ \% = \frac{\text{SA in spray-dried capsules}}{\text{SAinthefeed}} \times 100\% \quad (4)$$

Similarly, thymol content changes were compared as in eq 5:

$$\text{thymolcontentchange}\ (\%) = \frac{\text{thymol}\ \% \text{ in spray-dried capsules}}{\text{thymol}\% \text{ in nonsolvent mass of the feed}} \times 100\% \quad (5)$$

Scanning Electron Microscopy (SEM). A LEO 1525 SEM microscope (LEO Electron Microscopy, Oberkochen, Germany) was used to collect images of capsules. An adhesive tape mounted on the specimen stub was used to fix spray-dried powders directly. In separate experiments, particles were fractured using a sharp knife before being mounted onto the tape, and the sample was scanned to collect images showing exposed internal particle structures.[15] All samples were sputter-coated with a gold layer of ca. 5 nm thickness before imaging.

In Vitro Release Kinetics of Antimicrobials. The pH of food products can be as low as 2.0 (for lemon juice) and as high as 7.96 (for egg white).[20] Therefore, characterization of release kinetics of antimicrobials was conducted using 20 mM sodium phosphate buffers adjusted to pH 2.0, 6.0, and 8.0. To establish release profiles of nisin, 4 mg of spray dried powder was suspended in 1 mL of phosphate buffer contained in a 1.5 mL microcentrifuge tube. Microcentrifuge tubes were attached to an end-to-end shaker (Lab Industries Inc., Berkeley, CA) and continuously mixed at room temperature. At a designated time point, suspensions were centrifuged at 14 500*g* for 5 min (model MiniSpin, Eppendorf AG, Hamburg, Germany), and 700 μL of the supernatant was transferred for

determination of nisin activity. After supplementing 700 μL of the corresponding fresh phosphate buffer to the remaining suspension, capsules were resuspended for continued tests. The cumulative release of nisin was calculated by the following equation:

$$R_{t_i}(\%) = \frac{\sum_{n=1}^{i-1} a_n + \frac{10}{7}a_i}{U_o} \times 100\% \quad (6)$$

where R_{t_i} (%) is the cumulatively released nisin at time t_i, the ith time of sampling; a_i is the nisin (IU/mL) concentration at the sampling time t_i; and U_o is the theoretical 100% release from 4 mg capsules, equivalent to total nisin activity units. U_o was determined separately by dissolving 4 mg of spray-dried capsules in 1 mL of 70% v/v aqueous ethanol (that dissolved zein capsules completely) for the nisin assay below. The prefactor 10/7 results from derivations based on mass balance and reflects the sampling practice when 700 μL of the supernatant was taken from a total volume of 1000 μL.

In vitro release of thymol was studied similarly to nisin samples. For the 700 μL supernatant withdrawn, 400 μL of the supernatant was transferred for mixing with 600 μL of methanol and 20 μL of acetic acid, and the solution was used to quantify thymol concentration using the HPLC protocol below. The total thymol content in capsules was determined from HPLC assay of a solution prepared by dissolving 4 mg of a powdered sample in a mixture of 1 mL of 60% aqueous methanol and 20 μL of acetic acid. Cumulative release of thymol was calculated analogously to that of nisin (eq 6).

Quantification of Nisin Activity. The standard agar diffusion assay[21] was used to determine nisin activity of samples by using *Micrococcus luteus* ATCC 10240 as a test microorganism. Samples containing solvents of deionized water or 70% ethanol were determined from different standard curves (taking the form in eq 7) established using standard nisin solutions with the corresponding solvent, because nisin and 70% ethanol demonstrated synergistic antimicrobial activity in the argar diffusion assay.[18] Two replicates were used for each sample, and each replicate was loaded in four well replicates in an agar gel. Nisin activity of each sample was then estimated on the basis of eq 7, where the average of eight well replicates was used as the inhibition zone diameter.

$$D = a \log_{10}[\text{nisin}] + b \quad (7)$$

where D is the diameter (cm) of the inhibition zone after baseline subtraction, [nisin] is the concentration of standard nisin solutions in IU/mL, and a and b are the slope and intercept from the linear regression, respectively.

Quantification of Thymol by HPLC. Quantification of thymol was performed using a literature HPLC protocol.[22] The Agilent Technologies (Waldbronn, Germany) 1200 series chromatography system was composed of a quaternary pump module, a degasser, an autosampler, a temperature-controlled column chamber, and an Agilent diode array and multiple wavelength detector. Signal acquisition and analysis were enabled by Chemstation software. An Agilent ZORBAX Eclipse Plus C-18 (4.6 mm × 150 mm, 5 μm stationary phase) column was placed in a temperature-controlled chamber equilibrated at 25 °C. The mobile phase was a methanol−water−acetic acid ternary mixture at a volume ratio of 60:40:2, and the isocratic separation was performed at a flow rate of 0.5 mL/min. The sample injection volume was 25 μL, and the detector wavelength was 274 nm. Thymol concentration in unknown samples was determined from a calibration curve established from a series of thymol solutions serving as external standards.

Antilisterial Properties in 2% Reduced Fat Milk. Tryptic soy broth-yeast extract (TSB-YE) was inoculated with *Lm* Scott A culture and incubated at 30 °C for 24 h. The culture was transferred to fresh TSB-YE and incubated for another 24 h at 30 °C, with a population of ca. 9.23 log CFU/mL based on plate count. The 2% reduced fat milk was confirmed for sterility before use. The culture was diluted to 7.0 log CFU/mL with TSB-YE, and 2 mL of the culture was mixed with 18 mL of milk to a population of 6.0 log CFU/mL. Free antimicrobials, used individually or in combination, and antimicrobial-loaded capsules were incorporated in the inoculated milk to an equivalent nisin concentration of 400 IU/mL. For free antimicrobial treatments, thymol concentration was 0.44 mg/mL, corresponding to 100% release of the encapsulated thymol from samples B, C, and D, and Tween 20 was used at 0.54 mg/mL, corresponding to equivalent Tween 20 concentration for the high Tween level capsule treatment. The mixture was vortexed before incubation at 25 °C for 0, 4, 8, 12, 24, and 48 h. At each time point, milk was sampled and the *Lm* population enumerated by the plate count method.

Statistical Analysis. All experiments were completed in duplicate. Significant differences were analyzed with a least-significant-difference ($P < 0.05$) mean separation method from duplicate samples. The Statistical Analysis Software (V9.2, SAS Institute, Cary, NC) was used to carry out the statistical analysis.

RESULTS AND DISCUSSION

Encapsulation Performance and Capsule Structure. Table 1 lists encapsulation performance for the parameters defined above. Mass yield, total solids %, and nisin EE of treatments significantly increased with an increase in Tween 20 usage level, which coincided with easier collection of powders observed during experiments at a higher Tween 20 level. EE of thymol was much lower than that of nisin. Thymol content change was all smaller than 50%, suggesting that a significant amount of thymol was lost during spray drying. Nisin SA ratios were all above 100%. Nisin is relatively heat stable,[23] and spray drying is a high-temperature-short-time process. Therefore, the loss of nisin activity is expected to be low or minimum. A SA ratio higher than 100% likely results from the loss of thymol during spray drying that corresponds to an increased nisin mass concentration in spray-dried capsules as compared to that according to the spray drying formulation, that is, a greater numerator than the denominator in eq 4.

Surface morphology and exposed wall structures of capsules are shown in Figure 1. Overall, fewer concave collapsed capsules and more heterogeneous capsule walls were observed at a higher Tween 20 level, especially for the high level Tween 20 treatment. Tween 20 is a nonionic surfactant, and surfactants enhance the dispersion of zein in polar solvents such as water.[24−27] In addition, Tween 20 showed plasticization function in production of insulin-loaded poly(lactide-*co*-glycolide) capsules by spray drying.[28] Tween 20 may have facilitated the precipitation of zein during spray drying, corresponding to thicker and more heterogeneous capsule wall structures at a higher Tween 20 level. The quicker precipitation may in turn have enabled less sticky particles and thus improved mass yield and encapsulation performance of antimicrobials. Characterizations of interactions between zein molecules as affected by Tween 20 and plasticization properties of Tween 20 require future work to better illustrate observations in Table 1.

Release Kinetics of Nisin. Release kinetics of nisin is shown in Figure 2 for capsules prepared with different levels of Tween 20. At pH 2.0, all samples showed 100% release at the first time point of 30 min. At pH 6.0, the treatment without Tween 20 showed ~25% release of nisin at the first time point of 30 min, followed by no apparent increase afterward. For the sample with the low level of Tween 20 (sample B in Table 1), nisin was gradually released from 20% to 72% in 96 h, followed by no further increase. The sample containing the medium level of Tween 20 showed a release profile of nisin (Figure 2C) similar to that with

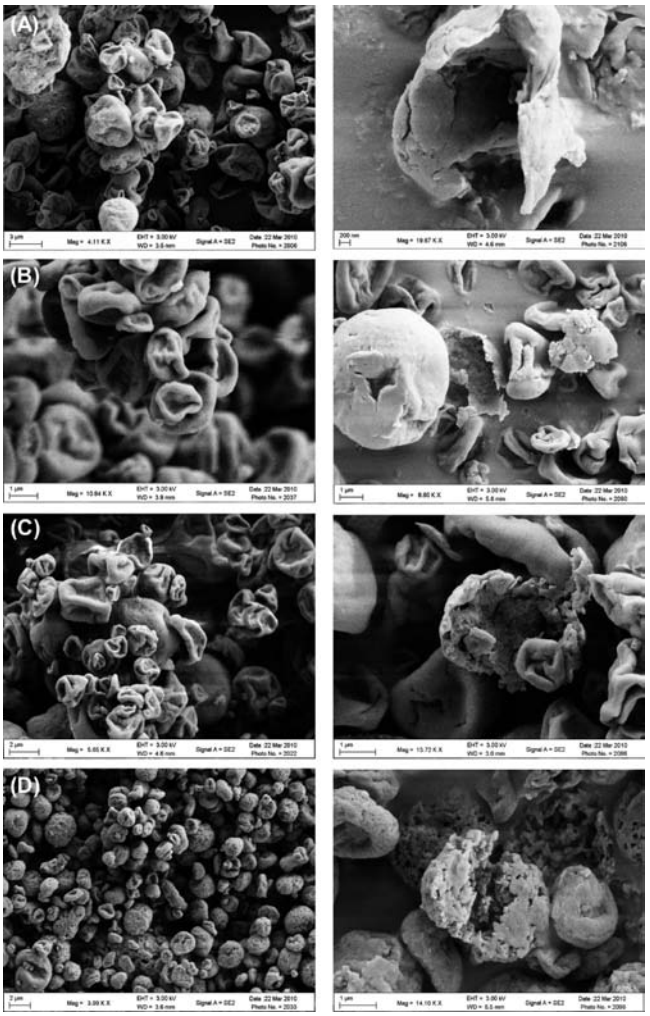

Figure 1. SEM images of zein capsules produced by spray drying nisin solutions with 2% w/v zein, 1% thymol, and different concentrations of Tween 20: (A) 0, (B) 0.05, (C) 0.1, and (D) 0.5% w/v. Left and right images are for surface morphology and internal structures, respectively.

the low level of Tween 20 (Figure 2B), with a lower equilibrium release to 60%. The equilibrium release of nisin for the capsule sample with the high level of Tween 20 was similar to the other two Tween 20 treatments, but this sample showed a much higher burst release (50%) than the ~20% of other samples (Figure 2D vs A−C). At pH 8.0, release of nisin was very limited for the sample without Tween 20 and was increased moderately to <20% in 144 h when Tween 20 was used at low and medium levels. When Tween 20 was used at the high level, no difference in nisin release was observed at pH 6.0 and 8.0 (Figure 2D), and the release at pH 8.0 was much higher than in the other three treatments (Figure 2A−C).

A reduced percentage of nisin release at a higher pH between 2.0 and 8.0 from the same capsule sample can be interpreted by impacts of molecular interactions between nisin and carrier polymer zein. The isoelectric point (pI) of nisin is 8.8,[29] and therefore nisin is overall more positively charged at a lower pH between 2.0 and 8.0. For the carrier polymer zein that has a pI of 6.8,[30] its overall charge is positive at pH 2.0, slightly positive at pH 6.0, and negative at pH 8.0. Therefore, electrostatic interactions between nisin and carrier zein are repulsive at pH 2.0, slightly repulsive at pH 6.0, and attractive at pH 8.0. In addition to electrostatic interactions, hydrophobic interactions may also impact release kinetics of nisin. Zein is practically insoluble in water at pH 2−8 and thus hydrophobic. Nisin is more hydrophobic when pH is closer to its pI of 8.8, and therefore the hydrophobic attraction by zein is stronger. When both electrostatic and hydrophobic interactions are taken into consideration, stronger attraction is expected between nisin and zein at a higher pH between 2.0 and 8.0.

In addition, Figure 2 indicates that burst and sustained release properties of nisin are strongly impacted by the level of Tween 20 in capsules. Nisin is a relatively hydrophobic peptide, and its hydrophobicity is expected to be higher when pH is increased from 2.0 to 8.0, that is, closer to its pI of 8.8. It is well studied that lipophilic tails of surfactants bind with zein.[26] Likewise, binding between surfactants and nisin is expected. Molecular bindings by Tween 20 likely impact zein−zein and zein−nisin interactions that are important to structural formation from atomized droplets during spray drying and thus microstructures of capsules and distribution of nisin in capsules. Because Tween 20 is a nonionic surfactant, its binding with nisin and zein weakens hydrophobic attraction[24−27] and improves completeness of nisin release from capsules (Figure 2). Moderately reduced hydrophobic attraction between nisin and zein may have enabled sustained release of nisin at pH 6.0 and 8.0 for low and medium levels of Tween 20 treatments, and much weakened hydrophobic attraction enabled quick release of nisin at pH 6.0 and 8.0 for the high-level Tween 20 treatment. Exact mechanisms resulting in release properties at different Tween 20 usage levels however require future work.

Impacts of surfactant intrinsic in microcapsules on release properties of encapsulated compounds were reported in a few studies where a double-emulsion solvent evaporation technology was used to encapsulate whey proteins in poly(lactide-*co*-glycolic acid) (PLGA) microcapsules. When Tween 20 was used at a 10% and 50% molar percentage of the encapsulated β-lactoglobulin, the burst release (in 2 min) reduced to 16% and 1%, respectively, from 23% for the treatment without Tween 20.[31] In another study,[32] different types of surfactants, that is, zwitterionic, anionic, and nonionic, were used at different levels to study encapsulation and release properties of bovine serum albumin (BSA). The EE of BSA and percentages of its release over time were observed to increase, decrease, or be unaffected with an increase in surfactant level used at 0.1−1% mass of PLGA, depending on the specific surfactant. When cholesterol was used, the most complete and sustained release of BSA was observed when cholesterol was used at 0.1% mass of PLGA. In contrast, the most complete and sustained release was observed when ceramide and cardiolipin were used at 0.5% and 1.0% mass of PLGA, respectively. The authors however did not interpret their observations.

Release Kinetics of Thymol. Figure 3 presents release kinetics of thymol from capsules. Sustained release of thymol in 144 h was observed for the treatment without Tween 20 and those with low and medium levels of Tween 20 that generally showed a higher percentage of release at a higher level of Tween 20. For the sample with the high level of Tween 20, release of thymol appeared to have reached equilibria quickly. A higher percentage of thymol at the first time point of 30 min for treatments with Tween 20 may indicate that a higher percentage of thymol was present on the surface and/or the areas close to the capsule surface. Thymol has a limited solubility in water, 1 mg/mL at 20 °C.[33]

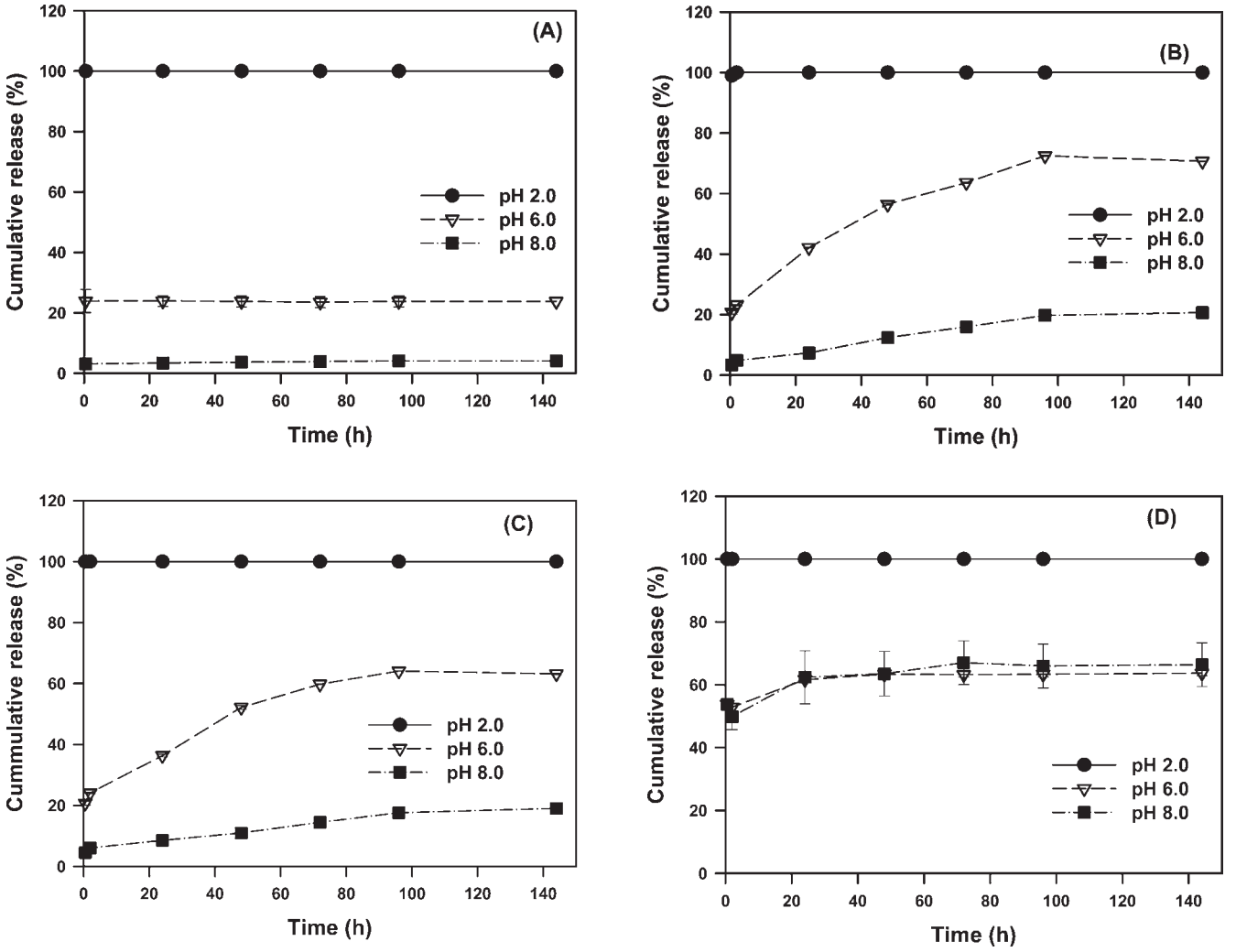

Figure 2. Release kinetics of nisin from zein capsules produced by spray drying nisin solutions with 2% w/v zein, 1% thymol, and different concentrations of Tween 20: (A) 0, (B) 0.05, (C) 0.1, and (D) 0.5% w/v. Error bars represent standard deviations from two replicates.

The hydrophobic nature of thymol implies that its release from zein capsules is expected to be impacted by hydrophobic attraction of zein and other microcapsule constituents. The reduced hydrophobic interactions by addition of Tween 20 corresponded to more complete release of thymol when Tween 20 was used at low and medium levels (Figure 3B,C vs A). At the high Tween 20 level (Figure 3D), thymol was released to the equilibrium concentration in a short time but to a lower level. As compiled in Table 2, the maximum volumetric concentration of cumulatively released thymol at pH 6.0 was 0.54 mg/mL after 144 h, which is much lower than the solubility limit of thymol (1 mg/mL in water at 20 °C).[33] Therefore, the incomplete release of thymol for the high level Tween 20 treatment was not due to its solubility limit. Further, more complete release of thymol is expected when hydrophobic attraction is weakened by more abundant Tween 20 in capsules, opposite from our observation. One possible mechanism is that thymol may be dissolved in micelles of Tween 20 in situ during hydration, which is significant at the high level Tween 20 treatment. The dimension of micelles, much bigger than individual thymol molecules, limits the diffusion through capsule matrix, corresponding to a lower percentage of thymol release. As for the impact of pH on release profiles of thymol, there did not appear to be a trend. Yousef and El-Eswed[34] studied the adsorption of phenols and corresponding phenolates onto zeolite. Phenols, with no ionizable group, showed pH-independent adsorption onto hydrophobic sites of zeolite, while the adsorption of phenolates onto hydrophilic sites of zeolite was pH-dependent. In our study, it is likely that hydrophobic attraction between zein and thymol, a phenolic compound, is pH-independent, and therefore no trend was observed for pH-dependence of thymol release profiles.

Antilisterial Properties of Free versus Encapsulated Antimicrobials. Figure 4A shows growth of *Lm* after treatments of 0.44 mg/mL free thymol, 400 IU/mL free nisin, 0.54 mg/mL Tween 20, or combinations of these three compounds at the same concentrations. Concentrations of free thymol and Tween 20 were based on estimations in the high level Tween capsules dispersed at 400 IU/mL nisin in 2% reduced fat milk. There was no difference between the negative control and free thymol treatment, showing the ineffectiveness of thymol at the studied conditions. As compared to the free nisin treatment, a combination of free nisin and free thymol improved antilisterial properties, but a more significant improvement was observed for combinations of Tween 20 and free nisin, with and without thymol, that

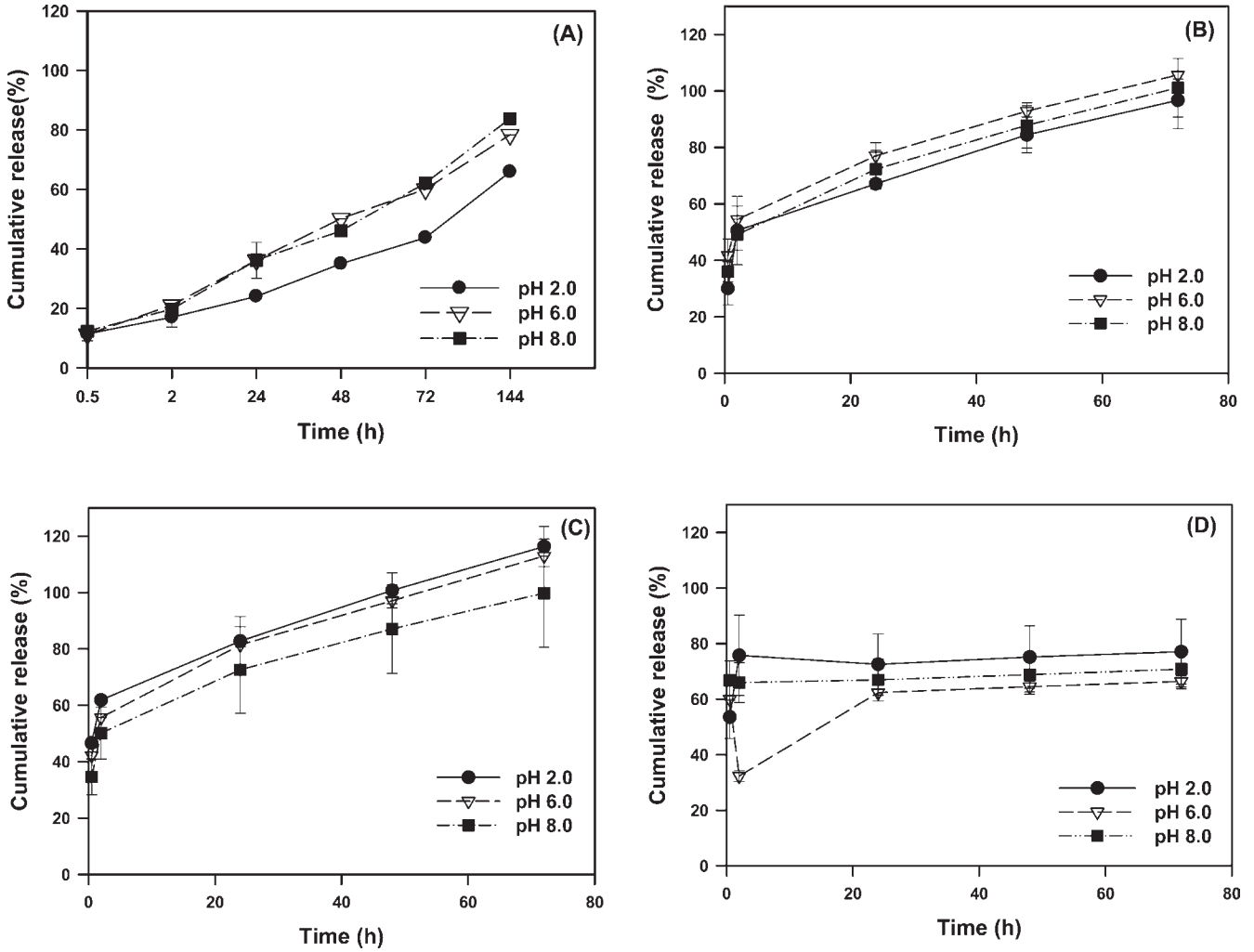

Figure 3. Release kinetics of thymol from zein capsules produced by spray drying nisin solutions with 2% w/v zein, 1% thymol, and different concentrations of Tween 20: (A) 0, (B) 0.05, (C) 0.1, and (D) 0.5% w/v. Error bars represent standard deviations from two replicates.

Table 2. Summary of Thymol Release Properties[a]

sample in Table[1]	thymol %(w/w) in dry particles	cumulative release after 144 h (mg/mL)[b]	thymol % released after 144 h[b]
A	7.70 ± 0.74c	0.20 ± 0.29b	62.00 ± 0.05c
B	13.51 ± 0.05b	0.54 ± 0.42a	104.60 ± 1.46b
C	13.73 ± 0.11a	0.54 ± 0.15a	112.95 ± 0.00a
D	13.61 ± 0.08ab	0.35 ± 0.3c	66.36 ± 2.04d

[a] Values in a column sharing the same superscript letters are not statistically different. [b] Results are from capsules suspended at 4.0 mg/mL in the pH 6.0 phosphate buffer.

were approximately 1 log CFU/mL more effective throughout 48 h incubation than nisin treatments without the surfactant.

Synergistic antimicrobial activities of nisin and thymol are well-known.[35−38] In our earlier publication,[17] we compared antilisterial properties of individual and combined free nisin (100 IU/mL) and thymol (0.02 mg/mL) in Tryptic soy broth-yeast extract (TSB-YE) medium adjusted to pH 6.0. During incubation at 30 °C, the population of *Lm* was reduced by free nisin from ~6 log CFU/mL to an undetectable level in ~<2 h, but started to recover after 12 h, eventually reaching a population similar to the negative control after 72 h. Free thymol reduced the *Lm* population by ca. 3 log CFU/mL for about 12 h, before reaching a population similar to that of the negative control after 72 h. In contrast, no *Lm* was detected for the combination of free nisin and free thymol during 144 h incubation. The synergistic antilisterial properties of free nisin and thymol were not apparent in Figure 4A, although both antimicrobials were used at a much higher level, apparently due to binding by milk components. The treatments with Tween 20 showed better antilisterial properties in milk, possibly because of reduced binding between antimicrobials and milk components.

The literature studies applying free (unencapsulated) nisin in milk demonstrate that antilisterial effectiveness of nisin is affected by the application level of nisin, milk composition, milk preparation conditions, other extrinsic compounds, *Lm* strain, and incubation temperature.[7,9,39−41] Therefore, it is not straightforward to compare results from different research papers. The fat content in milk significantly impacts antilisterial properties of nisin that is usually more effective at a lower fat level.[7,39] Bhatti et al.[7] showed that *Lm* growth at 5 °C in whole (3.5% fat) milk treated by 125 IU/mL nisin was strongly affected by the prior processing (raw, homogenized, pasteurized, or homogenized and pasteurized). For homogenized and pasteurized milk, the inhibition by 125 IU/mL nisin was only <2 log CFU/mL for an initial

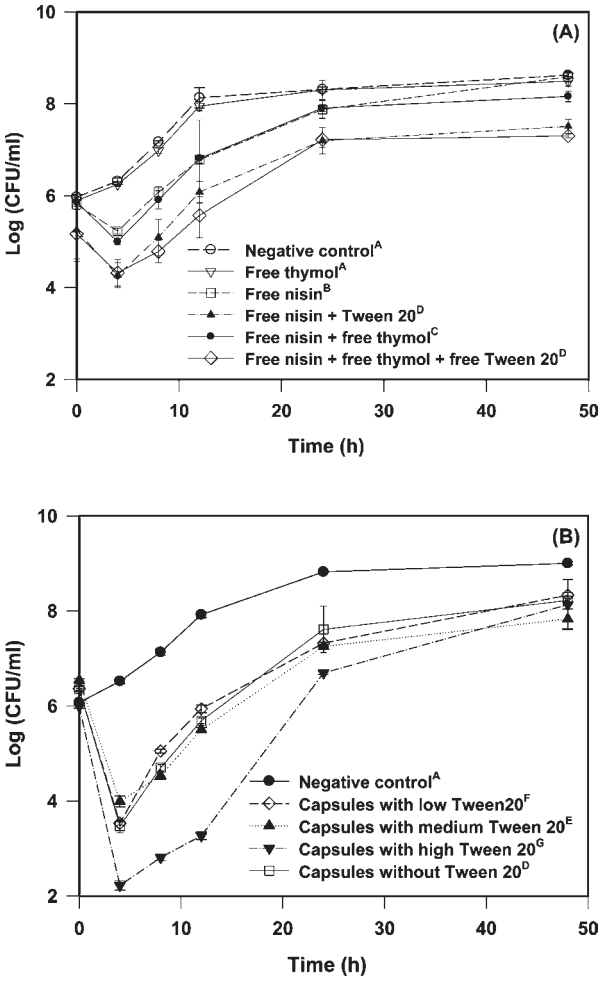

Figure 4. Comparison of antilisteria properties of (A) free antimicrobials and (B) nisin-loaded zein capsules in 2% reduced fat milk. Treatments with nisin were conducted with 400 IU/mL nisin, in free or encapsulated form. Free thymol in Figure A was used at a concentration of 0.44 mg/mL, corresponding to 100% release of encapsulated thymol from Tween 20-containing samples after 144 h (Table 2). Tween 20 in (A) was used at 0.54 mg/mL, identical to the overall content in the high level Tween 20 treatment in (B). Capsules without Tween 20 and with low, medium, and high Tween 20 contents were produced by spray drying a nisin solution with 2% w/v zein, 1% w/v thymol, and 0, 0.05, 0.1, or 0.5% w/v Tween 20, respectively. Error bars represent standard deviations from two replicates. Different superscripts in the legend represent statistical differences ($P < 0.05$) for 4 h data points.

Lm population of ~4.3 log CFU/mL, contrasting an undetectable *Lm* population in 15 days for the combination of 5 μg/mL Tween 80 and 125 IU/mL nisin. Interestingly, antilisterial properties of nisin were not improved by lecithin (a zwitterionic surfactant). The mechanisms of surfactant impacting antilisterial properties of nisin in milk however are still unknown.

Antilisterial properties of capsules with coencapsulated nisin and thymol at different levels of Tween 20 are presented in Figure 4B. Overall, antilisterial properties were similar for the treatment without Tween 20 and those with low and medium levels of Tween 20, all of which showed improvements from the free nisin treatments at shorter time points but a marginal improvement after 48 h. For the sample with the high level of Tween 20, the initial reduction of *Lm* population at the 4 h time point was 1 log CFU/mL more than all other capsule treatments, and the *Lm* population was always lower than other capsule treatments during incubation until at the last time point of 48 h when no significant difference was observed among antimicrobial treatments.

As discussed above, Tween 20 is a nonionic surfactant whose hydrophobic tail likely binds with hydrophobic patches of antimicrobials and other microcapsule components. Further, Tween 20 is water-soluble and likely diffuses out of microcapsules to impact bindings between released antimicrobials and milk components and thus antilisterial properties. In contrast to the high level Tween 20 treatment, antilisterial properties of treatments with low and medium Tween 20 levels were similar to the treatment without Tween 20 possibly because a significant portion of Tween 20 bound with other capsule components such as more hydrophobic zein, resulting in no improvement in antilisterial properties of released nisin. Nonetheless, our results in Figure 4 show that encapsulation enhances activities of antimicrobials in complex food matrixes such as milk, but the extent of enhancement highly depends on characteristics of capsules.

Correlation between release properties of nisin and antilisterial properties in TSB-YE was observed in our earlier work for spray-dried capsules containing nisin, thymol, and different levels of glycerol.[17] At an overall nisin concentration of 100 IU/mL and 30 °C, complete inhibition of *Lm* was observed for capsules showing gradual release of nisin, before the recovery of *Lm* after the release of nisin reached equilibrium. In this work, antilisterial properties of all capsules (Figure 4B) were much reduced in 2% reduced fat milk, despite at a higher concentration of nisin (400 IU/mL) and a temperature (25 °C) lower than the optimum growth temperature of *Lm* (30−37 °C). The difficulty of correlating release properties of nisin and antilisterial properties in milk may result from the short time scale of binding between antimicrobials and milk components, for example, seconds or milliseconds, contrasting hours between time intervals for data points in release profiles and microbial growth curves. The much improved antilisteial property for the high Tween 20 treatment (Figure 4B), in comparison to the free antimicrobial treatments with Tween 20 (Figure 4A), suggests the need of encapsulation to minimize binding between antimicrobials and food components before antimicrobials are released from capsules and the need of an appropriate mechanism such as surfactants intrinsic in capsules to minimize the binding for released antimicrobials.

The role of Tween 20 in antimicrobial treatments also requires clarification. Tween 20 alone does not have antimicrobial activity but is commonly used to control interactions between bacteria and food matrices, for example, for enhanced sanitization of fresh produce[42] and detachment of bacteria for detection.[43] In our recent study, although not directly relevant to this work, we observed that sucrose monolaurate, a nonionic surfactant, did not have antimicrobial activity and did not improve effectiveness of chlorine against *Escherichia coli* O157:H7 in growth media, while the surfactant was able to detach *E. coli* O157:H7 from spinach surface and enhanced sanitization performance of chlorine on spinach.[44] Therefore, surfactants can impact interactions between preservatives (sanitizers, antimicrobials) and bacteria (that are interacting with food matrices). Studies are urgently needed at the interface between food chemistry and microbiology to solvent challenging microbial food safety issues, such that the integrated knowledge can be used to develop novel intervention strategies.

One concern of incorporating thymol in food products is its impact on organoleptic properties. The acceptable thymol level in a food product varies with food matrixes. The threshold of thymol was reported to be 1.1−1.3 mg/kg in honey[45] and 124 mg/kg in sunflower oil.[46] There is currently no report about acceptability of thyme/thymol in milk. A thymol concentration of 1500 mg/kg in Fior di Latte cheese was acceptable by consumers.[47] Another study reported acceptance of 1% thyme (with 10−64% mass being thymol [48]) in white herby cheese.[49] In this work, the high level Tween sample, with the best antilisterial property, was used at an overall level of 0.44 mg/mL or about 440 mg/kg in milk. The sensory acceptability of this amount of thymol in encapsulated form is to be studied in the future.

There are several studies in the literature comparing antilisterial properties of nisin in food systems before and after encapsulation. In one study,[39] nisin was encapsulated in liposomes, and, at a level of 500 IU/mL, antilisterial properties of capsules were observed to be less effective than the same level of free nisin in microbial growth medium and skim milk incubated at 30 °C. At 500 IU/mL, no difference was observed for capsule and free nisin treatments at an incubation temperature of 6−8 °C. The improved antilisterial properties of nisin in Figure 4 show promise for our approach in developing delivery systems of antimicrobials. In the future, we wish to study other variables such as incubation temperature and milk fat content impacting antilisterial properties of capsules, in addition to improving capsule formulations by studying other types and levels of surfactants and/or coencapsulated antimicrobials.

In conclusion, our study showed that spray drying is a practical technology to produce capsules of antimicrobials whose release kinetics can be manipulated by incorporating surfactants such as Tween 20. The addition of intrinsic surfactant impacts microstructure of capsules and release properties of the encapsulated antimicrobials by impacting interactions among capsule constituents. Encapsulation of antimicrobials improved their antilisterial properties in milk, possibly due to reduced binding with milk components. Our approach based on scalable processes and low-cost, GRAS food ingredients may be practically used by the food industry to develop effective intervention strategies to improve microbiological food safety.

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