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Spray-Dried Zein Capsules with Coencapsulated Nisin and Thymol as Antimicrobial Delivery System for Enhanced Antilisterial Properties

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ABSTRACT: Food grade antimicrobial delivery systems were studied in this work to enhance the effectiveness of antimicrobials inhibiting the growth of *Listeria monocytogenes* during storage. Corn zein was used as a carrier biopolymer and nisin and thymol as antimicrobials. Capsules produced by spray drying demonstrated different microstructures and release characteristics of nisin at different usage levels of thymol. Better release profiles were achieved when glycerol was additionally used to prepare capsules. Capsules showing sustained release of significant amounts of both antimicrobials effectively inhibited the growth of *L. monocytogenes* at pH 6.0 and 30 °C in the growth medium. Capsules were also more effective than free antimicrobials in inhibiting the growth of *L. monocytogenes* in 2% reduced fat milk at 25 °C. Our work showed that engineered delivery systems have promise to fulfill the antimicrobial effectiveness during shelf life storage of foods to ensure microbiological safety.

KEYWORDS: nisin, zein, encapsulation, spray drying, thymol, glycerol, *Listeria monocytogenes*, milk

INTRODUCTION

Despite enormous developments in processing technologies by the food industry, there are still cases and outbreaks of foodborne illnesses due to consumption of ready-to-eat (RTE) foods, mostly due to postprocess recontamination.[1−3] Food antimicrobials are used in addition to processing technologies to serve as an effective combination of hurdles to inactivate and/or inhibit pathogenic and spoilage microorganisms to improve microbiological safety and quality of food products. When incorporated in food matrices, antimicrobials specifically and/or nonspecifically bind with food components such as proteins and lipids, resulting in reduced availability and thus effectiveness of antimicrobials to act against microorganisms in food matrices.[4−7] This technological challenge has recently attracted much interest in the scientific community to develop antimicrobial delivery systems for improved biological functions in foods.

The delivery system in this work was particulate structures based on generally recognized-as-safe (GRAS) biopolymers with encapsulated antimicrobials that can be incorporated in food matrices. The model antimicrobial was nisin, a well-studied GRAS peptide effective against a broad spectrum of Gram positive bacteria, including *Listeria monocytogenes* (*Lm*).[8] *Lm* is classified by the USDA's Food Safety and Inspection Service and the U.S. Food and Drug Administration as a "zero tolerance" organism in RTE foods[9] because it causes fetal death, septicemia of newborns, and meningitis of the elderly and immunocompromised.[10] There are about 500 deaths and 12 500 infected persons annually in the U.S. caused by *Lm* infection.[11] Several outbreaks of listeriosis have been linked to contaminated milk,[12,13] including one outbreak in Massachusetts in 2007 that was linked to pasteurized milk.[14]

Antimicrobial activity of nisin in food matrices was observed in several literature studies to be much lower than that in a growth medium or buffer.[7,15] Nonspecific binding of nisin with lipids and proteins has been reported.[4−6] Incorporation of nisin within capsules of edible polymers may reduce the binding between nisin and food components and improve its efficacy in foods.[16−18] For example, Salmoso et al.[19] 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 (free) nisin. However, the authors used an expensive process based on precipitation in antisolvent supercritical carbon dioxide, and the carrier polymer poly(L-lactide) is not GRAS. In a recent study,[20] nisin was encapsulated in liposomes. Although nisin capsules showed antilisterial properties similar to those of free nisin at a level of 500 IU/mL in a microbial medium and skim milk during incubation at 6−8 °C, the antilisterial properties of capsules were less effective than free nisin when incubated at 30 °C.

Much work is still needed to develop food antimicrobial delivery systems based on GRAS, sustainable, and inexpensive ingredients and low-cost and scalable processes. In our previous study, sustained release of hen egg white lysozyme from spray-dried capsules of zein (alcohol-soluble storage protein, prolamins, extracted from maize kernels) was observed at an appropriate formulation.[21] Spray drying is a technology extensively used to prepare powdered products in the food industry, while zein is available in large quantity. Thymol, a naturally occurring antimicrobial extracted from the thyme plant, was needed in the spray drying formulation to achieve sustained release of lysozyme from subsequently spray-dried capsules. Although we did not test antilisterial properties of capsules, our study demonstrated an approach to produce low-cost GRAS antimicrobial delivery systems.

Nisin and lysozyme differ in primary structures and physicochemical properties, and the conditions previously established for lysozyme[21] may not be applicable to nisin. The primary objective of this work was thus to establish formulations that enabled sustained release of nisin from spray-dried zein capsules,

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Table 1. Encapsulation Performance of Samples Spray-Dried from Nisin Extracts Added with 2% w/v Zein but Different Amounts of Thymol and Glycerol[a]

sample	thymol % (w/v)	glycerol % (w/v)	mass yield[b] (%)	nisin EE[c] (%)	total solids (%)	nisin SA ratio[d] (%)	thymol EE[e] (%)	thymol content change[f] (%)
A			66.71	56.77^{C}	98.31^{A}	123.06		
B	0.02		79.21	84.16^{A}	98.44^{A}	107.94		
C	0.10		70.95	78.51^{AB}	97.96^{A}	112.96		
D	0.40		67.50	71.6^{A}	93.44^{B}	135.22		
E	1.00		31.67	35.96^{DE}	93.33^{B}	121.67	7.56^{A}	25.14^{A}
F	1.00	0.05	30.00	31.03^{E}	93.91^{B}	110.12	5.22^{BC}	19.36^{A}
G	1.00	0.10	33.87	35.64^{DE}	89.10^{C}	118.10	7.02^{AB}	20.42^{A}
H	1.00	0.50	38.29	45.87^{CD}	84.48^{D}	141.81	4.57^{C}	10.40^{B}

[a] Values in a column sharing same superscript letters are not statistically different. [b] Defined in eq 1. [c] Encapsulation efficiency (EE) as defined in eq 2. [d] Specific activity (SA) ratio as defined in eq 3. [e] Encapsulation efficiency (EE) as defined in eq 4. [f] Defined in eq 5.

demonstrated for capsules with a right amount of glycerol. The second objective was to compare antilisterial properties of free and encapsulated antimicrobials in the growth medium and in 2% reduced fat milk. This class of low-cost GRAS antimicrobial systems may be practically used in food matrices to enhance microbiological food safety and quality.

MATERIALS AND METHODS

Materials. The 2.5% nisin preparation was a powdered product from MP Biomedicals, LLC (Solon, Ohio). The product was labeled with a nisin concentration of 1000 IU/mg solids. Ethanol (200 proof) and zein were purchased from Acros Organics (Morris Plains, NJ). Tryptic soy broth (TSB), yeast extract (YE), peptone, and agar (chemical grade) were products of Becton, Dickinson and Company (Sparks, MD). Ultrahigh-temperature-processed milk used in antilisterial tests was a 2% reduced fat product from Farmland Dairies, LLC (Wallington, NJ). Other chemicals were obtained from Sigma-Aldrich Corp. (St. Louis, MO).

Encapsulation by Spray Drying. The 2.5% nisin preparation was partially purified by extraction using aqueous ethanol at previously optimized conditions.[22] Briefly, the nisin solids were suspended at a concentration of 6 mg mass per mL in 50% v/v aqueous ethanol, and the slurry was vigorously agitated using a stirring plate to fully suspend particulates. After extraction for 6 h at room temperature, the suspension was centrifuged at 1520 × *g* for 5 min (model Tabletop Centrifuge E9, Beckman, Palo Alto, CA). The supernatant (extract) was then transferred and constituted to 70% ethanol (v/v) to dissolve 2% w/v zein and different concentrations of thymol and glycerol according to formulations in Table 1. Thymol was previously observed to have an impact on release kinetics of lysozyme from spray-dried zein capsules,[21] while glycerol was used because it is a common plasticizer and is effective in modulating microstructure and physical properties of protein and starch films.[23,24] The solution was then spray-dried using a benchtop spray dryer (mini spray dryer B-290, BÜCHI Corporation, Flawil, St. Gallen, Switzerland) with the following parameters: 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.

Evaluation of Encapsulation Performance. The total solids content of capsules was determined using the AOAC Official Method 925.09.[25] Vacuum drying was performed at 100 °C and 500 mmHg under-pressure until a constant weight (in about 5 h). The weight difference of each sample before and after drying was used to calculate the total solids content.

Parameters in evaluating encapsulation performance of lysozme in our previous work were adopted here,[20] with modifications. The mass yield of spray drying was determined as the percentage of a collected product mass normalized to the corresponding nonsolvent mass in the solution used in spray drying:

$$\text{mass yield}\% = \frac{\text{mass of collected product}}{\text{nonsolvent mass in the feed}} \times 100\% \quad (1)$$

Encapsulation efficiency of nisin was defined as in eq 2 by comparing the total nisin units in a spray-dried product and that used in spray drying. To determine nisin concentration in spray-dried capsules, a powdered sample was dissolved in 70% ethanol for the activity assay detailed below.

$$\text{encapsulation efficiency}\% = \frac{\text{total nisin units in a collected product}}{\text{total nisin units in the feed}} \times 100\% \quad (2)$$

The total nisin units in a collected product were estimated by multiplying the nisin units in unit mass and the total mass of a collected product. Because of significant errors in collecting powders from spray dryer chambers and a small amount of material (~3 g solids) in each batch, encapsulation efficiency as defined in eq 2 may not reflect the encapsulation process accurately. An additional parameter was calculated by comparing the specific activity (SA) of nisin before and after spray drying, where SA of nisin (IU/mg) was defined as nisin activity per unit nonsolvent mass:

$$\text{SA ratio}\% = \frac{\text{SA in spray-dried capsules}}{\text{SA in the feed}} \times 100\% \quad (3)$$

Encapsulation efficiency of thymol was similarly calculated according to eq 4 by comparing the total thymol mass in a spray-dried product and that used in spray drying. Powders were dissolved in a methanol−water−acetic acid ternary mixture (v/v/v = 60/40/2) for quantification of thymol using the following HPLC method. The thymol % in unit mass of spray-dried capsules and that of nonsolvent mass before spray drying were used to calculate percentages of thymol content changes as in eq 5, similar to SA ratio % of nisin.

$$\text{encapsulation efficiency}\% = \frac{\text{total thymol mass in collected products}}{\text{total thymol mass in the feed}} \times 100\% \quad (4)$$

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

In Vitro Release Kinetics of Nisin and Thymol. According to the U.S. FDA Center for Food Safety and Applied Nutrition,[26] the pH of most food products is from 2.0 (for lemon juice) to 7.96 (for egg white). For release studies, 1.0 mL of a 20 mM sodium phosphate buffer, preadjusted to pH 2.0, 6.0, and 8.0 using 1 N NaOH or HCl, was used to suspend 4 mg of spray dried particles in a 1.5 mL microcentrifuge tube. An end-to-end shaker (Lab Industries Inc., Berkeley, CA) was used to continuously mix the suspensions at room temperature (21 °C). At a designated incubation time, 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. A 700 μL portion of the corresponding fresh phosphate buffer was supplemented to the remaining suspension, and the capsules were resuspended for longer release time points. The cumulative release of nisin was calculated by the following equation:

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

where R_{ti} (%) is the cumulatively released nisin at time t_i, the *i*th 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.

In vitro release of thymol in the above phosphate buffers at pH 2.0, 6.0, and 8.0 was studied similarly to nisin samples. Capsules (40 mg) were used for each sample, and release time points were 0.5−144 h. After centrifugation, 700 μL of the supernatant was withdrawn, and 700 μL of corresponding phosphate buffer was supplemented for continued release tests. A 400 μL portion of the withdrawn supernatant was transferred and mixed with 600 μL of methanol and 20 μL of acetic acid, which was used as a running solvent mixture to quantify thymol concentration using the HPLC protocol below.[41] To test total thymol content in capsules, 40 mg of a powdered sample was dissolved in a mixture of 1 mL 60% aqueous methanol and 20 μL acetic acid, and the solution was assayed by HPLC. Cumulative release of thymol was calculated similarly to that of nisin (eq 6).

Determination of Nisin Activity. Nisin activity of samples was determined by the standard agar diffusion assay[27] using *Micrococcus luteus* ATCC 10240 as a test microorganism. The total nisin activity in zein capsules was determined after dissolving capsules in 70% v/v aqueous ethanol, while nisin extract and samples from release studies (in buffers) were used directly. Two sample replicates were tested, and each sample was loaded in 4 well replicates in an agar gel. An average of 8 inhibition zone diameters from each sample was used to estimate nisin activity using an appropriate standard curve corresponding to standard solutions with a same solvent (water or 70% aqueous ethanol). Standard curves were prepared separately for solvents of deionized water and 70% v/v aqueous ethanol because of the synergism between nisin and ethanol in the assay, as detailed in our previous work.[22] After linear regression, the correlation between inhibition zone diameter and nisin activity was expressed in the following equation:

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

Here, *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.

HPLC Quantification of Thymol. A literature HPLC protocol[28] was adopted to quantify thymol using an Agilent Technologies (Waldbronn, Germany) 1200 series chromatography system. The system included a quaternary pump module, a degasser, an autosampler, a temperature-controlled column chamber, and an Agilent diode array and multiple wavelength detector. The Chemstation software was used for signal acquisition and analysis. Chromatography separation was achieved using an Agilent ZORBAX Eclipse Plus C-18 (4.6 mm × 150 mm, 5 μm stationary phase) column that was incubated at 25 °C. The mobile phase consisted of a methanol−water−acetic acid ternary mixture at a volume ratio of 60/40/2. The sample injection volume was 25 μL. The flow rate was 0.5 mL/min, and the detection wavelength was 274 nm.

Bacterial Inhibition Assay in TSB-YE and in Milk. The *Lm* Scott A culture was incubated at 30 °C in TSB-YE for 24 h, followed by transferring to fresh TSB-YE and incubating for another 24 h at 30 °C. The pathogen population after two consecutive transfers was 9.31 log CFU/mL based on plate count. For tests in medium, antimicrobials (the 2.5% nisin preparation, pure thymol, spray-dried capsules) were added to 18 mL TSB + 0.6% YE (TSB-YE) medium preadjusted to pH 6.0 using 1 N HCl that was then mixed with 2 mL of culture previously diluted to 7.0 log CFU/mL *Lm* using TSB-YE. The overall nisin concentration was used at 100 IU/mL for *in vitro* tests using capsule samples A, E, F, G, and H (Table 1). After incubation at 30 °C for 0, 4, 8, 12, 24, 48, 72, 96, 120, and 144 h, the surviving bacteria population was determined by the plate count on tryptic soy agar (TSA) plates. Four antimicrobial controls were used to compare with capsules with coencapsulated nisin and thymol: (1) zein capsules prepared as sample E (with 1% thymol in the spray drying solution, Table 1) but without nisin, (2) free thymol applied at 0.02 mg/mL in the growth medium, (3) free nisin (the 2.5% preparation as received) used at 100 IU/mL, and (4) free thymol + free nisin at concentrations identical to controls 2 and 3. The medium with no supplements added was used as a negative control in all treatments.

The UHT milk was confirmed for sterility. Tests of antilisterial properties of free and encapsulated antimicrobials in milk were conducted as above by substituting the TSB-YE with 2% reduced fat milk. Treatments containing nisin were used at an overall nisin concentration of 400 IU/mL because our preliminary tests showed a level of 100 IU/mL for both free and encapsulated nisin was ineffective in inhibiting *Lm*. Free thymol was used at 0.08 mg/mL. The *Lm* population in milk was enumerated by serial dilution and the plate count method after incubation at 25 °C for 0, 4, 8, 12, 24, and 48 h.

Scanning Electron Microscopy (SEM). The powdered sample was glued directly onto an adhesive tape mounted on the specimen stub and sputter-coated with a gold layer of ca. 5 nm thickness before imaging using a LEO 1525 SEM microscope (LEO Electron Microscopy, Oberkochen, Germany). In addition, the internal particle structure was imaged after fracturing particles using a sharp blade (Accu-Knife, Fisher Scientific Inc.).[21]

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 conduct the analysis.

RESULTS AND DISCUSSION

Encapsulation Performance. The nisin extract had 684 μg/mL total protein and 5760 IU/mL, a partial purification in comparison to 1260 μg/mL total protein and 6000 IU/mL for the 2.5% preparation. After adding 2.0% zein and thymol and/or glycerol as in Table 1, encapsulation performances of spray-drying experiments are listed in Table 1. The mass yield was generally lower for samples prepared with a higher content of thymol in the aqueous ethanol solution, which coincided with difficulty of collecting powder sticking to spray dryer chambers. Nisin encapsulation efficiencies of treatments followed the same trend as mass yields. This is expected because the mass yield impacts the numerator in eq 1. Nisin encapsulation efficiency of the treatment without

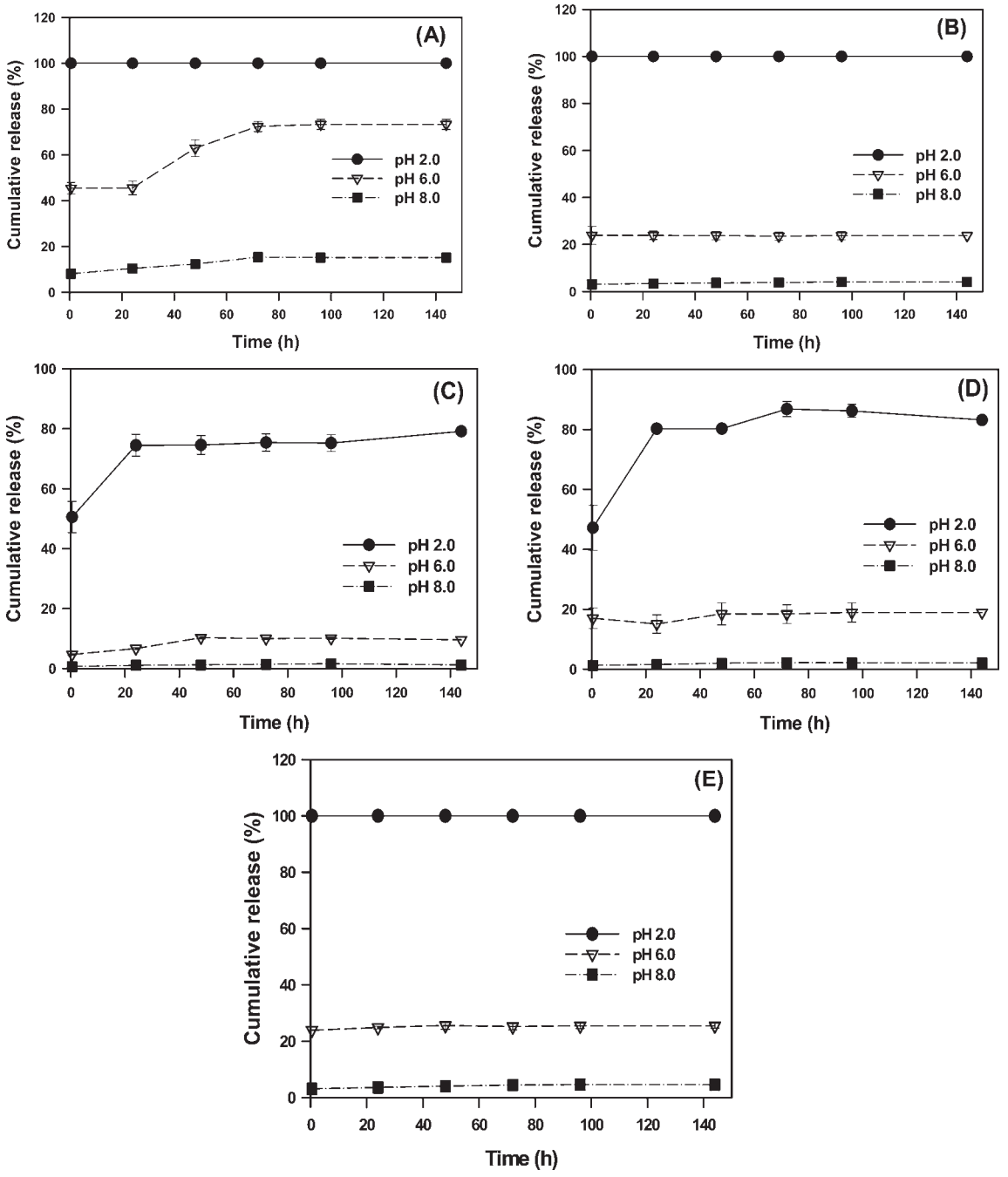

Figure 1. Release kinetics of nisin from zein capsules produced by spray drying a nisin extract (adjusted to 70% aqueous ethanol) with 2% zein and (A) 0%, (B) 0.02%, (C) 0.1%, (D) 0.4%, or (E) 1% (w/v) thymol. Error bars are standard deviations from 8 readings in nisin assay, 4 each for 2 sample replicates.

thymol (sample A) was statistically lower ($P < 0.05$) from treatments with thymol at 0.02%, 0.1%, and 0.4% w/v in the solution spray-dried (samples B−D). There was no significant difference among treatments when thymol was used at 0.02%, 0.1%, and 0.4% w/v in the formulation.

Samples prepared with 1% w/v of thymol in formulation (samples E−H) had a significantly lower encapsulation efficiency of nisin than those with lower thymol usage levels. Nisin encapsulation efficiency of the treatment with the highest glycerol level (sample H) was significantly higher ($P < 0.05$) than other treatments with lower glycerol usage levels (samples E, F, and G). As discussed above, because of errors in collecting spray-dried powder during experiments and thus estimation of encapsulation efficiency, it is difficult to interpret how nisin encapsulation is impacted by the experimental conditions in Table 1.

For samples without glycerol, the total solids content of samples A, B, and C was 98% or greater, indicating good drying conditions. Samples D and E had a solids content of ~93% that was lower than those of samples A, B, and C, possibly due to more significant impact of thymol evaporation during 5-h vacuum drying at 100 °C, as previously observed and discussed.[21]

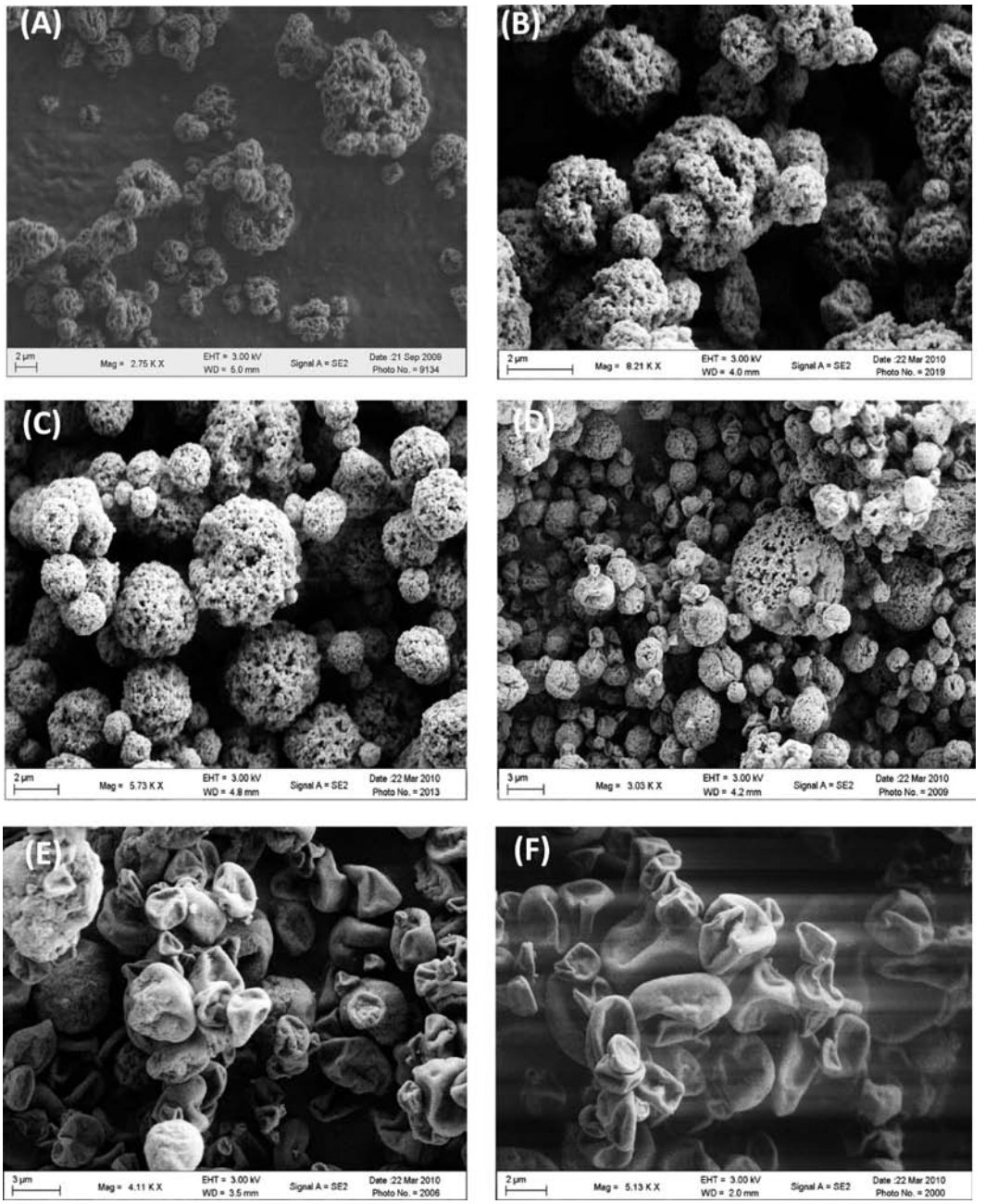

Figure 2. SEM images of nisin-loaded zein capsules produced by spray drying a nisin extract (adjusted to 70% aqueous ethanol) with 2% zein and (A) 0%, (B) 0.02%, (C) 0.1%, (D) 0.4%, or (E) 1% (w/v) thymol. Image F is a thymol capsule sample spray-dried from a solution with 2% w/v zein and 1% w/v thymol in 70% aqueous ethanol.

For samples with glycerol, there was no difference ($P > 0.05$) between samples E (without glycerol) and F (with the lowest glycerol content), while a lower total solids content was observed when glycerol was used at two larger amounts (samples G and H). Glycerol is a well-known hydrophilic plasticizer that increases the desorption time of water from films.[29] As a result, more solvent may have been retained in spray-dried capsules containing a larger amount of glycerol, which in turn resulted in a lower total solids content.

SA of nisin in spray-dried products was higher than that before spray drying (SA ratio >100%, Table 1). Assuming nonsolvent compounds precipitate proportionally during spray drying, i.e., same denominator in the definition of specific activity (IU/mg mass), no decrease of specific activity after spray drying indicates no loss of nisin activity. Nisin has a good thermal stability because 50−75% of residual nisin activity was observed when heated in low-acid foods (pH 4.6−6.9) at 121 °C for 3 min.[30] The spray drying in this work was performed at an inlet temperature of 105 °C, and the high temperature−short time nature of this drying process may have caused insignificant inactivation of nisin.

Encapsulation efficiency of thymol was quantified for samples prepared from solutions containing 1% thymol (samples E−H in Table 1). These samples had low mass yields, but thymol encapsulation efficiency was much lower than the corresponding

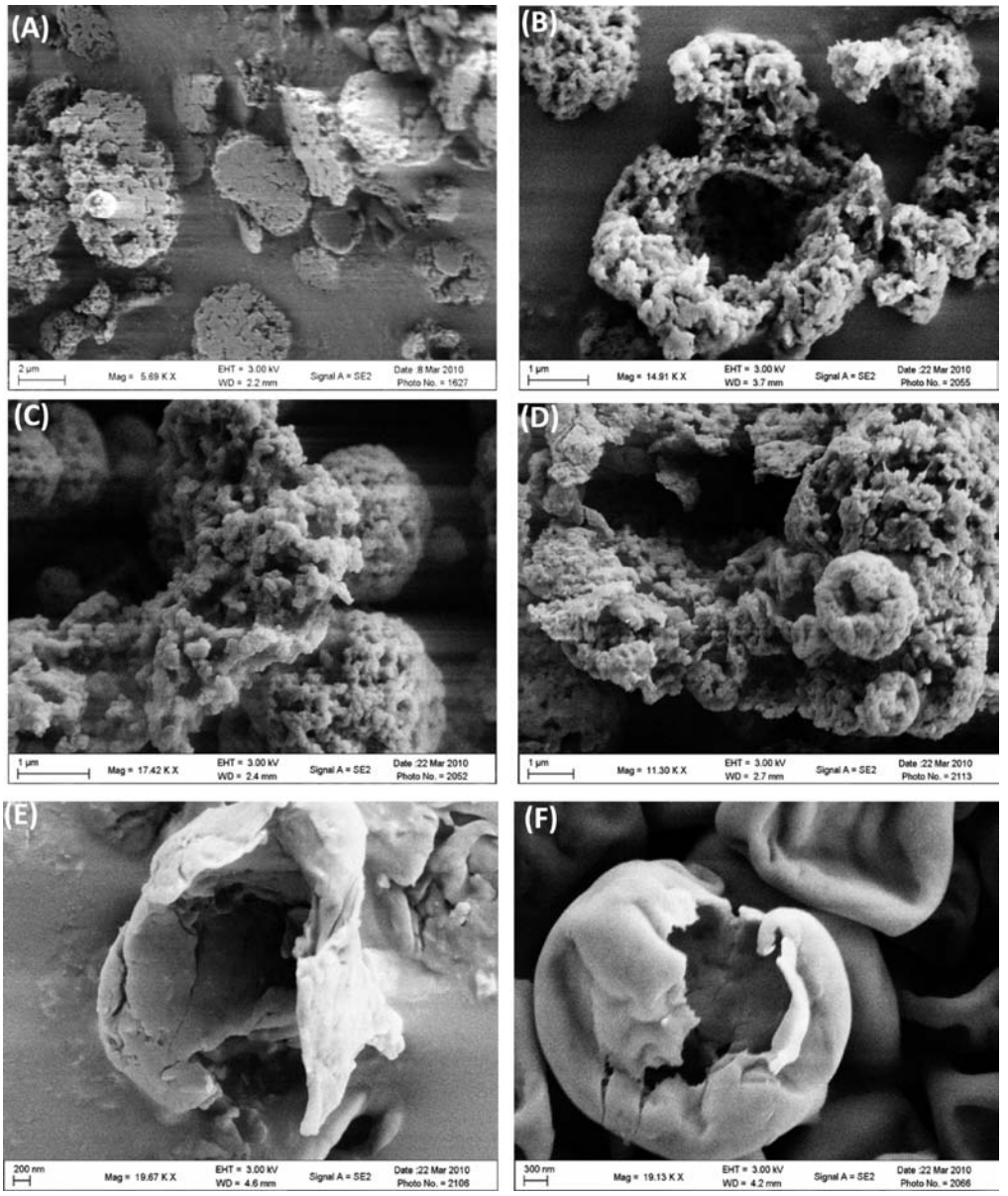

Figure 3. SEM images of the samples in Figure 2 after fracturing capsules using a blade.

mass yield, indicating thymol did not precipitate proportionally with other nonsolvent compounds during spray drying. This was confirmed by comparing thymol% in spray-dried particles and that in the stock solution before spray drying, shown in the last column of Table 1: thymol content change% that indicates more than 3 quarters of thymol was lost. The sample with the highest level of glycerol had a significantly higher loss of thymol ($P < 0.05$).

Release Properties and Structures of Capsules Produced Using Different Amounts of Thymol. Release kinetics of nisin from samples A−E, prepared with different levels of thymol, are presented in Figure 1. Similar to our previous study based on lysozyme encapsulated in zein by spray drying,[21] more complete release of nisin was observed at a lower pH. Nisin has an isoelectric point (pI) of 8.8.[31] Therefore, nisin is overall more positively charged at a lower pH between 2.0 and 8.0. On the other hand, zein has a pI of 6.8 [32] and thus is overall positively charged at pH 2.0, slightly positively charged at pH 6.0, and negatively charged at pH 8.0. Electrostatic interactions between nisin and carrier zein are thus repulsive at pH 2.0, slightly repulsive at pH 6.0, and attractive at pH 8.0, which may have partially contributed to more complete release of nisin at a lower pH. Because nisin is less soluble at a higher pH,[8] release profiles may also be impacted by its solubility. According to the estimation of Wei and Hansen,[33] the solubility of nisin at pH 6.0 and 8.0 is 1.5 and 0.25 mg/mL, respectively, which is much higher than the 0.018 mg/mL (equivalent to 714 IU/mL) in our release experiments. Therefore, less than 100% release of nisin in our study was not mainly caused by the solubility of nisin.

In our previous study, zein solutions containing lysozyme (at 10% mass of zein) and thymol (at 0−25% mass of zein) were spray-dried, and the capsule sample prepared from the solution with thymol equaling to 2% mass of zein demonstrated sustained release of lysozyme over 49 d at pH 6.0.[21] In this work, the addition of thymol not only reduced the completeness of nisin release but did not result in sustained release (Figure 1).

The sample without thymol had the most sustained release at pH 6.0, increasing from 45% to 74% in 96 h (4 d). At pH 6.0, zein is more hydrophobic than at pH 2.0 and 8.0, and hydrophobic attraction between nisin and zein may become stronger when thymol was present in capsules. There are three factors that may have contributed to differences in release profiles of lysozyme and nisin when both are encapsulated in zein by spray drying. First, an inlet temperature of 90 °C was used in our previous lysozyme study,[21] in comparison to 105 °C in this study. An inlet temperature of 105 °C was selected because the most sustained release of nisin was observed in our preliminary experiments for capsules spray-dried at 90−120 °C. Second, the lysozyme sample used was a purified product, while nisin in this work was extracted from a 2.5% preparation and the extract had impurity dairy proteins[22] that likely change microstructures of capsules and release properties. A similar observation was noted for no sustained release of lysozyme at pH 6.0 when lysozyme directly extracted from hen egg white was used in spray drying.[34] Lastly, nisin is more hydrophobic than lysozyme: the former has a grand average of hydropathy of 0.415 and the latter −0.477.[31] The exact mechanisms are to be studied in the future.

SEM images for sample surface morphology are shown in Figure 2. With the increase of thymol usage level in spray drying, particles gradually changed from mostly spherical, porous structures to a collapsed, red blood cell shape with smooth surfaces. After manually fracturing capsules, representative images showing exposed internal structures are presented in Figure 3. When thymol was not used, capsules had a continuous matrix, while the addition of thymol resulted in hollow particles with wall structures that became less heterogeneous at a higher usage level of thymol. Possibly, thymol acts as a plasticizer that impacts precipitation of biopolymers during spray drying, as previously discussed.[21]

Release Properties and Structures of Capsules Produced Using Different Amounts of Glycerol. This group of treatments (samples F, G, and H) were studied for capsules spray-dried from solutions containing 1% w/v thymol because of known synergism of nisin and thymol when the two antimicrobials are applied simultaneously to inhibit the growth of *Lm*.[35−37] Since glycerol has a very low vapor pressure at the studied conditions[38] and is thus expected in spray-dried capsules, samples F, G, and H are referred to samples with low, medium, and high glycerol levels, respectively, hereafter to simplify discussion. Release properties of both nisin and thymol were characterized and discussed below.

As shown in Figure 4, the addition of glycerol did not change nisin release properties at pH 2.0 as all samples reached 100% release of nisin shortly. At pH 6.0, the low glycerol sample showed only 8% release at the first time point (30 min) and sustained release to 87% in 96 h, with no apparent further increase afterward (Figure 4A). For the sample with a medium glycerol level at pH 6.0, about 10% nisin was released in 30 min, and sustained release up to 100% was observed when tested after 72 h, before reaching release equilibrium (Figure 4B). For the sample with the high glycerol level at pH 6.0, nisin was released quicker than the other two samples: 40% of nisin after 30 min and 100% release detected after 24 h (Figure 4C). When compared to the treatment without glycerol that showed ~25% release throughout 144 h incubation at pH 6.0 (Figure 1E), the addition of glycerol in zein capsules significantly improved release profiles of nisin.

Similar improvements of nisin release properties were also observed at pH 8.0. When glycerol was absent, less than 10% nisin was released in 144 h (Figure 1E). Conversely, gradual

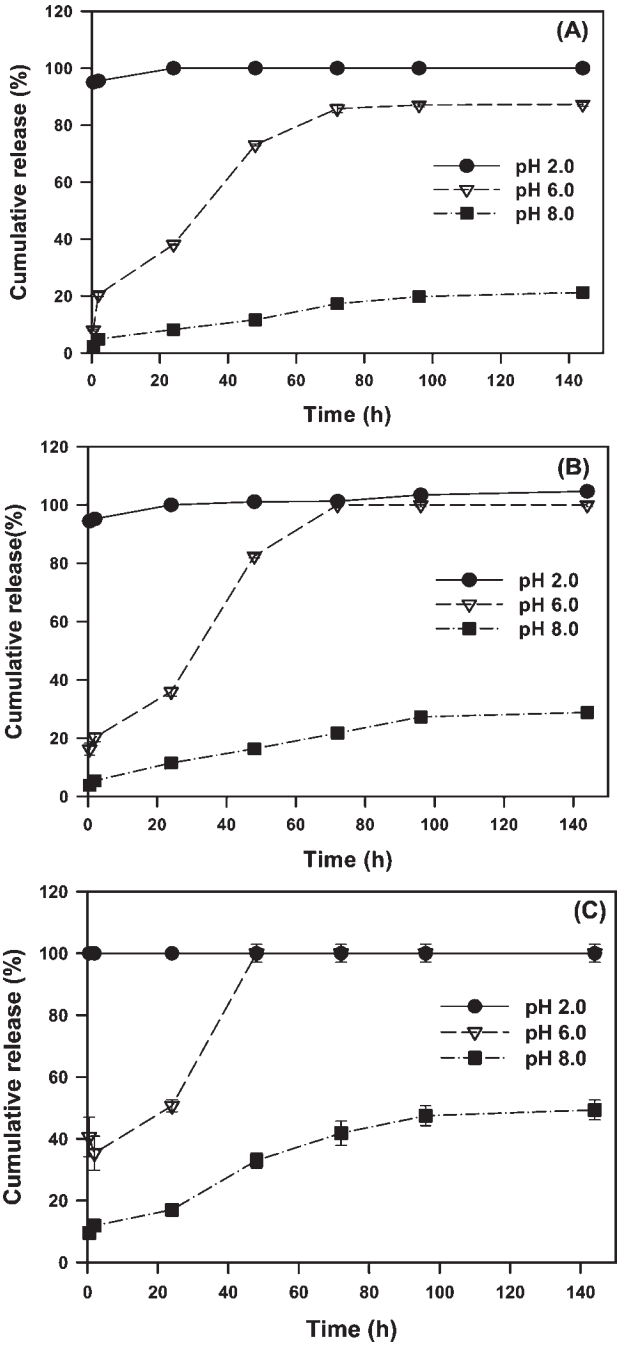

Figure 4. Release kinetics of nisin from zein capsules produced by spray drying a nisin extract (adjusted to 70% aqueous ethanol) with 2% w/v zein, 1% w/v thymol, and different concentrations of glycerol: (A) 0.05%, (B) 0.1%, and (C) 0.5% w/v. Error bars are standard deviations from 8 readings in nisin assay, 4 each for 2 sample replicates.

release of nisin was observed in 144 h for all glycerol treatments that showed 2−21%, 3−28%, and 9−49% cumulative release for samples with low, medium, and high glycerol levels, respectively.

Release kinetics of thymol is presented in Figure 5 for samples E−H where 1% thymol was incorporated in the solution used for spray drying. All samples showed gradual release of thymol, and the release was less complete for the sample with a higher level of glycerol. There was no apparent trend as for how pH impacted release characteristics of thymol.

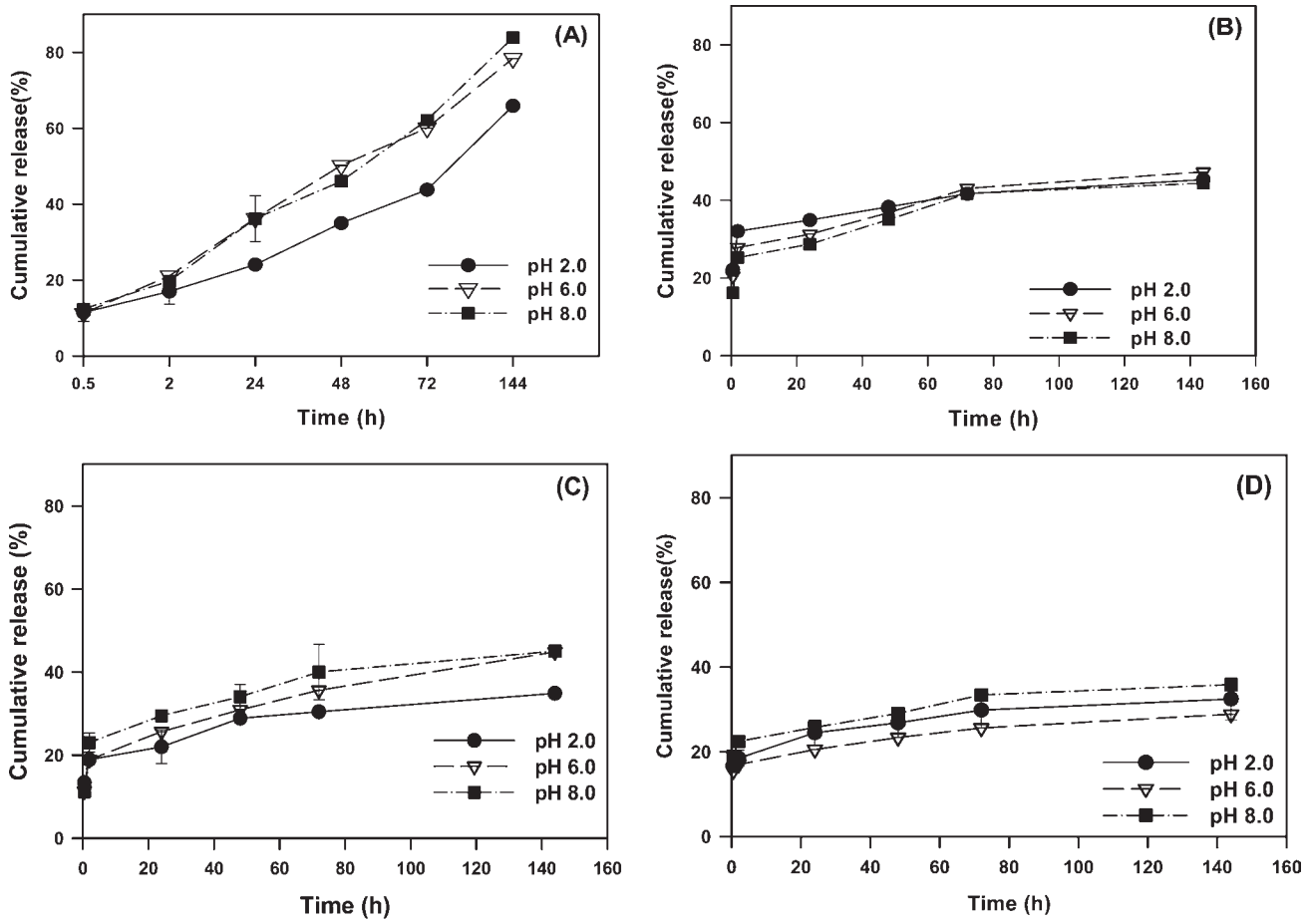

Figure 5. Release kinetics of thymol from zein capsules produced by spray drying a nisin extract (adjusted to 70% aqueous ethanol) with 2% w/v zein, 1% w/v thymol, and different concentrations of glycerol: (A) 0%, (B) 0.05%, (C) 0.1%, and (D) 0.5% w/v. Error bars are standard deviations from 2 replicates.

SEM images of samples G and H are shown in Figure 6. When compared to the sample without glycerol (Figure 2E), fewer percentages of collapsed capsules were observed at a higher level of glycerol (Figure 6), but hollow particles were observed in all cases. Because glycerol is a known plasticizer, precipitation of biopolymers in atomized droplets during spray drying may be slower at a higher level of glycerol,[39] resulting in gradual accumulation into spherical shells.

At least two factors are to be considered to interpret the exact mechanisms of nisin and thymol release from zein capsules. The first one is internal structures of capsules since an encapsulated compound needs to overcome diffusion resistance posed by capsule matrices. When capsules are suspended in a buffer, diffusion of water-soluble compounds out of capsules and inward diffusion of water are both expected to change capsule structures. Zein is the most abundant component of capsules and is practically insoluble in the buffers used. However, water is known to have plasticization functions in prolamin-based materials,[23,40] and this may result in rearrangement of internal capsule structures. On the other hand, glycerol is fully miscible with water, and the depletion of glycerol from capsules theoretically would increase the capsule porosity, which may have been responsible for faster release of nisin for samples with more glycerol (Figure 4). The second factor is molecular interactions between capsule components. The impact of electrostatic interactions between nisin and zein has been discussed above regarding more complete release of nisin at a lower pH. As for thymol, its solubility in water at 20 °C is about 1 g/L.[41] A summary of thymol release from samples E−H is given in Table 2 indicating incomplete thymol release in the studied time frame. The cumulative release of thymol from samples E−G was greater than the solubility limit of thymol because fresh buffer was supplemented during sampling, while that from sample H was below the solubility limit of thymol. Because thymol is not charged, interactions between thymol and zein should be mostly hydrophobic in nature. Capsules with a higher content of hydrophilic glycerol are overall less hydrophobic, and thymol is expected to release faster and more complete from capsules. The outward diffusion of glycerol increases the overall hydrophobicity of capsules, and this strengthens hydrophobic interactions between thymol and zein. However, release profiles of thymol showed faster and more complete release for capsules with less glycerol (Figure 5), opposite from expectations based on molecular interaction theory. The internal microstructural changes of capsules upon suspension in buffers, however, were not examined in this work, and the exact mechanisms are to be studied in the future to explain release properties of nisin and thymol.

Antilisterial Properties in TSB-YE. Antilisterial properties in the TSB-YE medium adjusted to pH 6.0 were performed for

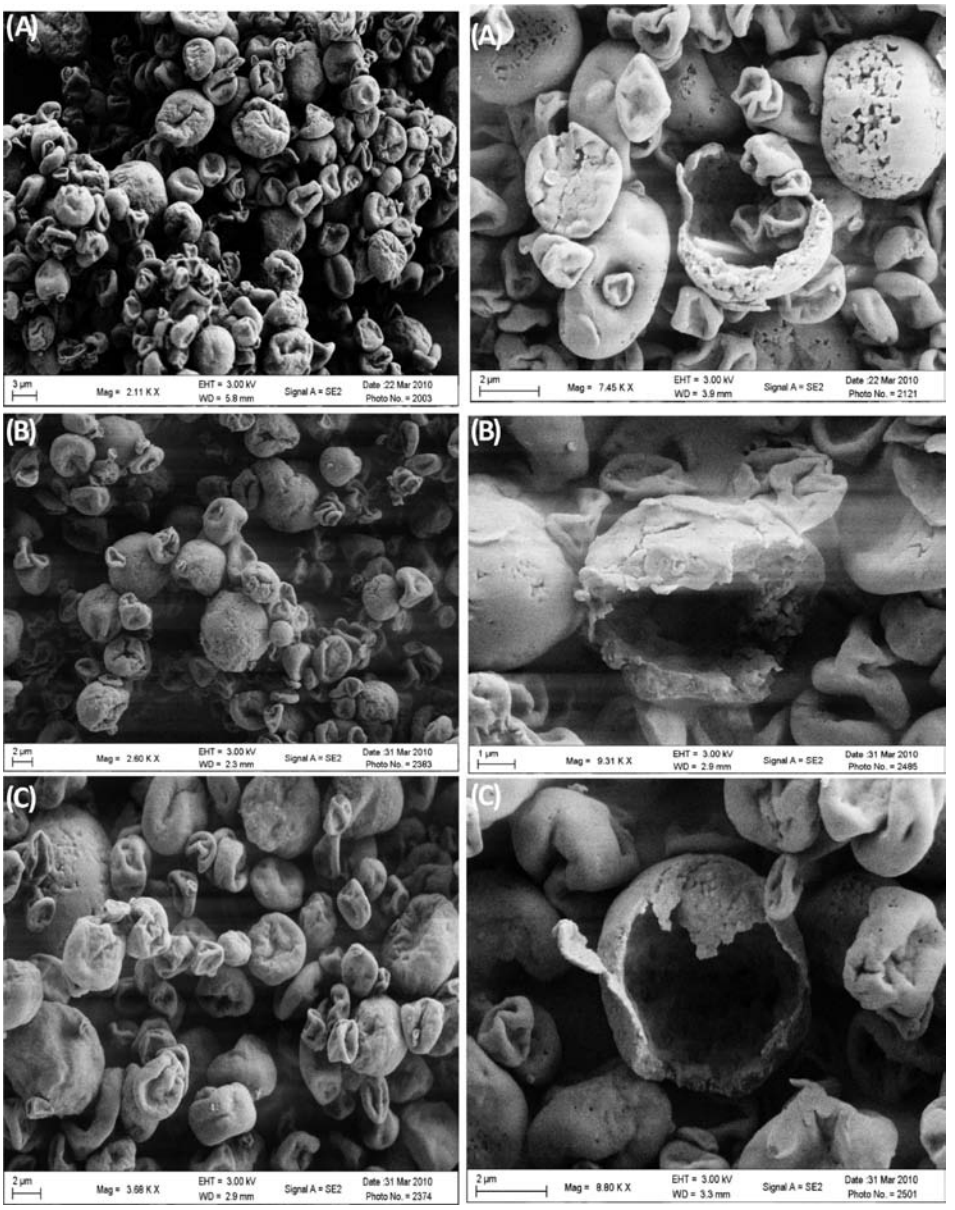

Figure 6. SEM images showing surface morphology (left images) and fractured structures (right images) of nisin-loaded zein capsules produced by spray drying a nisin extract (adjusted to 70% aqueous ethanol) with 2% w/v zein, 1% w/v thymol, and different concentrations of glycerol: (A) 0.05%, (B) 0.1%, and (C) 0.5% w/v.

Table 2. Summary of Thymol Release Properties

sample in Table *1*	thymol % (w/w) in spray-dried particles	cumulative release[a] after 144 h (mg/mL)	thymol % released after 144 h
E	7.70 ± 0.74	2.40 ± 0.29	75.00 ± 0.05
F	5.00 ± 1.05	1.16 ± 0.42	47.00 ± 0.04
G	6.00 ± 0.34	1.16 ± 0.15	45.00 ± 0.02
H	3.00 ± 0.69	0.39 ± 0.30	32.00 ± 0.01

[a] Capsules were suspended at 40 mg/mL in a pH 6.0 phosphate buffer.

treatments with a nisin amount equivalent to 100 IU/mL and/or 0.02 mg/mL thymol. The thymol level was equivalent to cumulatively released thymol from the low glycerol sample after 144 h in the pH 6.0 buffer (Table 2). Figure 7A shows results from free antimicrobial treatments and capsule samples produced without glycerol. Free nisin initially deactivated *Lm* to an undetectable level in ~<2 h, and the effectiveness against the growth of *Lm* was observed for 8 h (Figure 7A). After 12 h, however, *Lm* appeared to have recovered from the antimicrobial action and grew to a population no different from the negative control (without antimicrobial treatment) after 72 h. The initial bactericidal activity of free thymol used at 0.02 mg/mL was lower

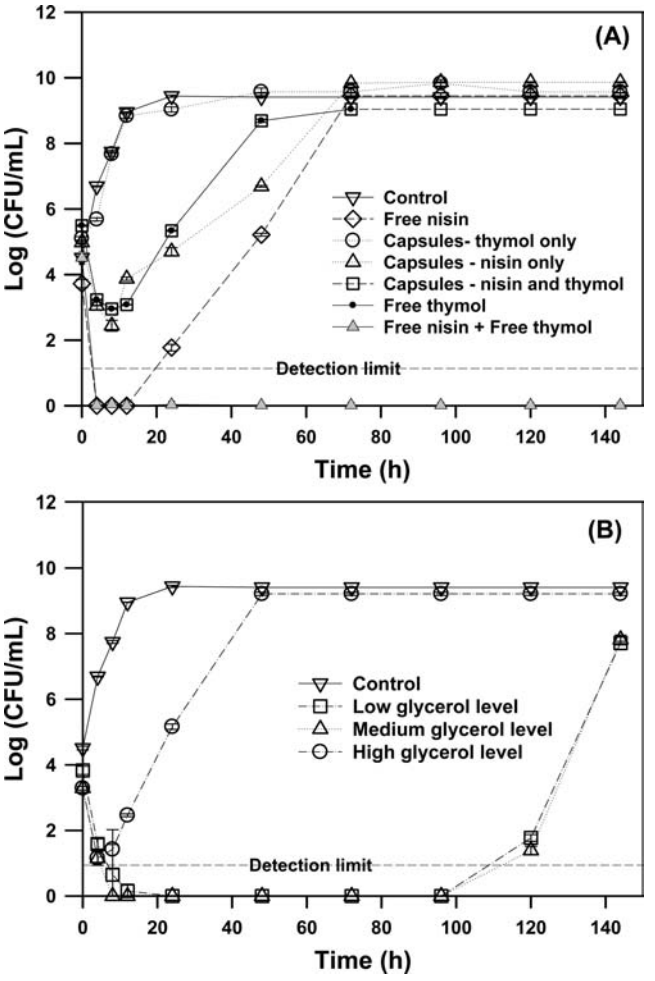

Figure 7. Growth of *Listeria monocytogenes* in a growth medium adjusted to pH 6.0 during incubation at 30 °C after treatment by (A) free antimicrobials and capsules prepared without glycerol, and (B) capsules containing both nisin and thymol and prepared with additional amounts of glycerol. "Capsules with thymol only", "capsules with nisin only", and "capsules with nisin and thymol" in part A were produced by spray drying a 70% aqueous ethanol solution with 2% w/v zein and 1% w/v thymol, a nisin extract (adjusted to 70% aqueous ethanol) with 2% w/v zein, and a nisin extract (adjusted to 70% aqueous ethanol) with 2% w/v zein and 1% w/v thymol, respectively. Capsule samples in part B were prepared spray drying a nisin extract (adjusted to 70% aqueous ethanol) with 2% w/v zein; 1% w/v thymol; and 0.05% (low level), 0.1% (medium level), or 0.5% (high level) w/v of glycerol. All nisin treatments contained 100 IU/mL, while free thymol was used at 0.02 mg/mL. Error bars are standard deviations from 2 replicates.

than that of 100 IU/mL nisin, and *Lm* grew again after 24 h, reaching a similar population as the negative control after 72 h. The best antilisterial efficacy was observed when the two antimicrobials were used in combination: no detectable *Lm* was observed throughout incubation. Synergistic antilisterial properties of nisin and thymol have been reported in many studies.[36,42−44] Antilisterial properties of capsules produced with thymol only, nisin only, or both but without glycerol were less effective than free nisin and the combination of free nisin and free thymol (Figure 7A), possibly due to limited release of nisin at pH 6.0 (Figure 1).

Antilisterial functions of capsules prepared from same amounts of nisin and thymol but different glycerol usage levels are shown in Figure 7B. For the low and medium glycerol level treatments, no *Lm* was detected after 96 h, compared to 12 h of the free nisin treatment. At the 120 h time point, *Lm* became detectable again. However, for the treatment with the high level of glycerol, nisin capsules with coencapsulated thymol did not work as effectively as free nisin. When compared to release profiles of nisin and thymol, capsules with the high glycerol level showed 100% release of nisin after 48 h at pH 6.0, but only 25% of thymol was released after 144 h at pH 6.0, corresponding to cumulative release of 0.39 mg/mL in the buffer (Table 2). Capsules with low and medium levels of glycerol both demonstrated sustained release of nisin over 72 h and ∼45% release of thymol after 144 h or a cumulative release of 1.16 mg/mL in the buffer (Table 2). Figure 7B indicates that sustained release of both antimicrobials to a sufficient concentration is needed to receive long-term efficacy to inhibit the growth of pathogenic bacteria. Chi-Zhang et al.[45] compared three modes of nisin use in inhibition of growth of *Lm* Scott A in BHI broth at 10 °C: one-time application, slow-addition, and combination of one-time and slow additions. For the one-time application treatment, *Lm* Scott A was inactivated effectively but started to recover after 24 h. Slow-addition of nisin inhibited the growth of *Lm* Scott A modestly during incubation. Combination of both application modes was effective in keeping population at low levels (>4 log lower the starting population) during incubation for 100 h. Further, the *in vitro* tests in Figure 7 show that, in simple systems like the growth medium, antilisterial properties can be improved by appropriate compounds that enable enhanced antimicrobial activity. For delivery systems, Figure 7B shows that sustained release of both antimicrobials is needed to improve antilisterial properties of free nisin, which may be important for applications in realistic food systems where binding between antimicrobials and food components is a concern, demonstrated below.

Antilisterial Properties in 2% Reduced Fat Milk. Figure 8 compares antilisterial properties of free and encapsulated nisin in 2% reduced fat milk. Antilisterial properties of all treatments in milk (Figure 8), although with 400 IU/mL nisin, were much lower than those in TSB-YE where nisin was used at 100 IU/mL (Figure 7). There was no difference between the negative control and free thymol treatment used at 0.04 mg/mL, showing the ineffectiveness of this thymol level in 2% reduced fat milk. For two free nisin treatments, i.e., with and without thymol, the *Lm* population was reduced more significantly by the treatment with thymol after 4 h, before recovering to a similar population greater than the initial population after 12 h or longer. Because the kinetics of antimicrobials binding with milk components may be fast, the enhanced antilisterial properties of combined antimicrobials in Figure 7A had apparently lost when the environment was switched from a simple system of TSB-YE to milk where both electrostatic and hydrophobic interactions are expected to be responsible for binding.

Capsules containing nisin showed improvements from free nisin treatments with and without thymol (Figure 8B). About 1 log CFU/mL better reduction than free antimicrobials was observed at the 4 h time point for two capsule samples E and H. In contrast to no improvement of nanovesicle-encapsulated nisin,[20] our results in Figure 8 showed better antilisterial properties of nisin-containing capsules than free nisin in 2% milk at 25 °C. Although much work is needed to further improve antilisterial properties of nisin in milk during long-time incubation, our work demonstrated the promise of studying low-cost, GRAS antimicrobial delivery systems to enhance microbiological food safety and quality.

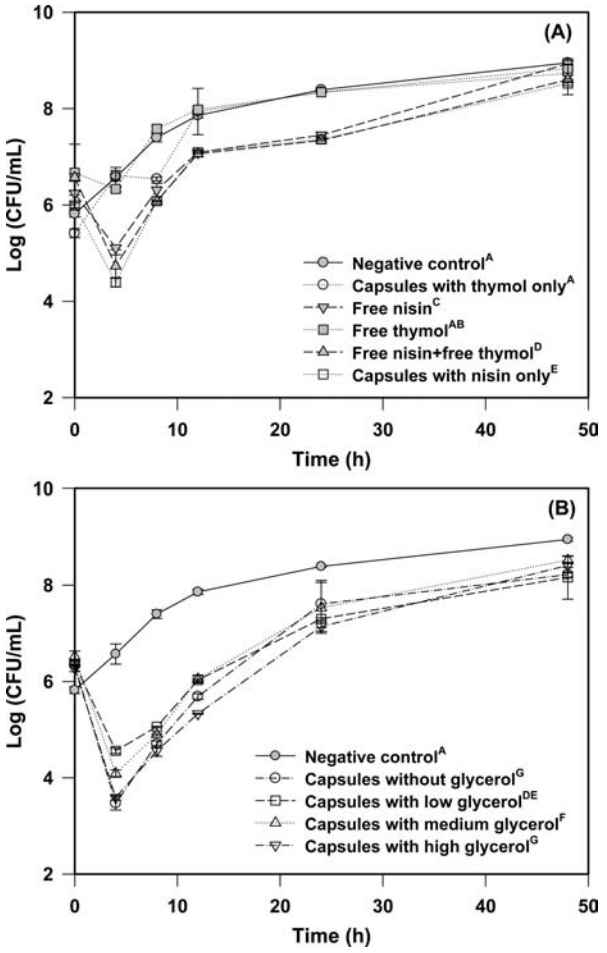

Figure 8. Growth of *Listeria monocytogenes* in 2% reduced fat milk incubated at 25 °C when treated by free antimicrobials and their capsules. A level of 400 IU/mL was used for all nisin treatments. Free thymol was used at 0.04 mg/mL. "Capsules with thymol only" and "capsules with nisin only" in part A were produced by spray drying a 70% aqueous ethanol solution with 2% w/v zein and 1% w/v thymol and a nisin extract (adjusted to 70% aqueous ethanol) with 2% w/v zein, respectively. Capsule samples in part B were prepared by spray drying a nisin extract (adjusted to 70% aqueous ethanol) with 2% w/v zein; 1% w/v thymol; and 0% (without glycerol), 0.05% (low level), 0.1% (medium level), or 0.5% (high level) w/v of glycerol. Different superscripts in the legend represent statistical differences ($P < 0.05$) for 4-h data points. Error bars are standard deviations from 2 replicates.

In summary, our work demonstrated a novel antimicrobial delivery system that had improved capability of inhibiting the growth of *Lm* in milk. Characterization of release profiles of antimicrobials unveiled that sustained release of both nisin and thymol was required to improve long-term efficacy of encapsulated antimicrobials against the potent pathogen. Our delivery system, based on GRAS ingredients produced using commercially feasible processes, may be developed into practical intervention systems to enhance microbial safety of various food products.

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