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Liposome Containing Nutmeg Oil as Targeted Preservative against *Listeria monocytogenes* in Dumplings

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ABSTRACT: In this work, antibacterial activities of nutmeg oil and nutmeg oil encapsulated in liposome were evaluated. Firstly, nutmeg oil exhibited significant antibacterial effect on *L. monocytogenes* by damaging integrity of cell membrane and leading to leakage of ATP and nucleic acid. Subsequently, nutmeg oil was encapsulated in liposome to enhance its chemical stability by thin film dispersion method. The optimal Zeta potential (-49.2 mV) and entrapment efficiency (26.90%) of liposome were achieved at 5.0 mg/mL nutmeg oil encapsulation. Finally, selective antibacterial activity for *L. monocytogenes* by utilizing pore-forming toxins (PFTs) to trigger nutmeg oil release from liposome was testified via Time-kill analysis and Gas chromatography (GC) assay. Besides, long-term antibacterial activity test illustrated that nutmeg oil encapsulated in liposome could extend treatment time and improve antibacterial effect on *L. monocytogenes* in dumplings.

KEYWORDS: Pore-forming toxins, controlled release, liposome, nutmeg oil, *L. monocytogenes*, dumpling

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1. Introduction

Ready-to-eat (RTE) foods have increased considerably in the world because it is delicious and convenient. Dumplings, a traditional Chinese food consisting mainly of meat, vegetables, wheat flour and spices, contribute to an important part of RTE foods in the East Asian countries,[1,2] and become more and more popular in the western countries. However, as the main ingredient of dumplings, meat is easy to be contaminated with *Listeria monocytogenes* (*L. monocytogenes*). It increases the microbial contamination risk of dumpling.

L. monocytogenes is one of these major food-borne pathogens in RTE that has the capacity to survive and adapt to various environments, such as a wide range of temperature, pH and water activity.[3, 4] According to statistics, approximately 2500 cases of human illness and 500 deaths result from this pathogen every year in the United States.[5] In order to eliminate *L. monocytogenes* or delay its growth in dumplings, new food preservatives shall be introduced. Essential oils, one of the most widely used natural antibacterial agents, are extracted from plants or spices used in food and beverages to enhance its preservation and taste quality.[6]

Nutmeg is a widely used spice and aromatic component in food industry, and has been widely applied in fragrance and flavor industries.[7] Some previous studies have demonstrated that nutmeg oil exhibits antioxidant,[8-10]anti-inflammatory,[7] and antibacterial activities.[11,12] Moreover, nutmeg oil has been classified as a kind of substances generally recognized as safe (GRAS) for food preservation by the Food and Drug Administration (FDA).[13]However, it is well known that volatility of

essential oil and sensibility toward oxygen, light, water and high temperatures reduce its stability in processing and storage ultimately decrease its bioavailability.[14,15] In order to enhance the stability and bioavailability of essential oil during its application, some molecules embedding processes have been developed, among which is use of liposomal encapsulation.[16-18]

In food industry, liposome is often used to encapsulate flavors and food antiseptic, and it primarily depends on slow release to work.[19] Nowadays, stimuli-responsive liposome, a new kind of liposomes, can respond to different stimulating agents (such as pH, temperature, redox potential, light, and ultrasound) to release bioactive molecules.[20,21]However, most of these stimulating agents rely on the external environment, and their biological safety need to be assessed when they are applied in food industry. Thus, if the stimuli-responsive liposome is utilized in food preservation, the endogenous and safe stimulating agents should be requested in advance. Based on this, to seek a new stimulating agent derived from target pathogens will be the best option.

Listeriolysin O (LLO) of *L. monocytogenes* is a kind of pore-forming toxins (PFTs) of the cholesterol-dependent cytolysins (CDCs) family, and a primary virulence factor of *L. monocytogenes*.[22, 23] Simultaneously, liposome, a spherical vesicle, is formed by amphipathic phospholipid bilayer membrane, which is similar as a biological membrane.[19, 24] Therefore, a new approach of antibacterial agent controlled release could be designed by combining the characteristics of PFTs and liposome. It is that LLO secreted by *L. monocytogenes* can form pores on the

liposome and trigger the release of antibacterial agent from liposome.

In this context, the objective of this study was to prepare the liposome containing nutmeg oil. As a proof-of-concept, we proved that nutmeg oil can be released from the liposome in the presence of LLO secreted by *L. monocytogenes*and then inhibited the growth of *L. monocytogenes*. As a specific preservative, liposome containing nutmeg oil showed satisfactory antibacterial activity for *L. monocytogenes* in dumpling model.

2. Experiments

2.1 Materials and Culture

Nutmeg oil was purchased from J.E International (Caussols plateau, France). The conventional fillings of dumpling (pork and cabbage) were purchased from the local supermarket. Two food-related microorganisms were used to the antibacterial activities, including Gram-positive *L. monocytogenes* ATCC 19115 and Gram-negative *Escherichia coli* ATCC 25922. These strains were provided by China General Microbiological Culture Collection Center (Beijing, China), and stored with liquid paraffin wax at 4 °C. *L. monocytogenes* was cultured at 37 °C on peptone yeast glucose broth (PYG), and *E. coli* on nutrient broth (NB).

2.2 The Antibacterial Activity of Nutmeg Oil

2.2.1 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). MIC of nutmeg oil was tested by two-fold serial dilution method.[25]Nutmeg oil was diluted into tubes containing sterile

PYG ranging from 0.125 to 4.0 mg/mL. Subsequently, cell suspensions were transferred to each tube to maintain a bacterial concentration of 10^{5-6}CFU/mL. The tubes were then incubated under agitation at 37 °C and examined for evidence of the growth. The lowest concentration of nutmeg oil, which did not show any visible growth of tested bacteria after macroscopic evaluation, was regarded as the MIC. Cell suspensions from each of the tubes showing no visible growth was sub-cultured on peptone yeast glucose agar. MBC was determined as the lowest concentration at which no growth of bacterial colonies on agar surface.[26]

2.2.2 Time-Kill Analysis of Nutmeg Oil. Time-kill curve assay was used to detect the bactericidal effects of nutmeg oil according to the plate colony-counting method. Nutmeg oil was diluted into tubes containing *L. monocytogenes*or *E. coli* (approximately 10^{5-6} CFU/mL) to obtain a concentration of 0.5 mg/mL. As control, bacterial suspensions in sterile phosphate buffer solution (PBS, pH 7.2) without nutmeg oil were also tested. All tubes were then incubated under agitation at 150 rpm and 37 °C. Finally, numbers of residual bacteria were observed at 0 h, 0.5 h, 1 h, 2 h, 4 h, 8 h and 16 h. [27, 28]

2.2.3 Transmission Electron Microscopy (TEM) Analysis. Morphological changes of *L. monocytogenes* were identical with those reported in the previous literauture.[29] Nutmeg oil was added to test tubes to obtain a concentration of 2.0 mg/mL. An inoculated growth medium without essential oil was used as a control. The bacterial samples (approximately 10^8 CFU/mL) were collected by centrifugation at 8000 rpm for 10 min, washed thrice by 0.03 M PBS (pH 7.2). Bacterial suspension

was dripped on a sterile slab. Copper screen was in touch with bacterial suspension for 3 min and dried. Afterwards, copper screen was dyed with 3% (w/w) phosphotungstic acid for 3 min and dried, followed by microscopic examinations (JEM-2100, JEOL, Japan).

2.2.4 Measurement of Cellular ATP Concentrations. Cellular ATP concentrations were measured using the method modified from Turgis et al.[30]Cell suspensions (approximately 10^8 CFU/mL) were centrifuged at 8000 rpm for 10 min, washed 3 times, and suspended in 0.03 M PBS (pH 7.2). Then, the different concentration (0 (control), MIC, 2MIC) of nutmeg oil was added to cell suspensions. All samples were reacted at 37 °C for 1 h, centrifuged at 8000 rpm for 10 min, and then incubated in ice to prevent ATP loss. Finally, cellular ATP concentrations of samples were tested using Clean Sense™ Surface Hygiene Test Kit (i-Genie, LEYU Biotechnology, Shanghai, China).

2.2.5 Observed Nucleic Acid with Fluorescent Staining Method. The nucleic acids were detected using the DAPI staining method.[31]Cell suspensions (approximately 10^8 CFU/mL) were centrifuged at 8000 rpm for 10 min, washed 3 times, and suspended in 0.03 M PBS (pH 7.2). Nutmeg oil was added to cell suspension to obtain a concentration of 2.0 mg/mL, and then incubated under agitation at 37 °C for 24 h. The equal volume of diluted 4'6-diamidino-2-phenylindole (DAPI) (10 μg/mL, Roche Diagnostics GmbH, Germany) and cell suspension were mixed, and then dripped on a glass slide and kept in dark for 10 min. Sample was observed by laser scanning confocal microscopy (LSCM, Leica TCS SP5 II, Leica,

Germany). The bacterial suspension in sterile PBS without nutmeg oil was observed as a control.

2.3 Preparation and Characterization of Liposome

2.3.1 Preparation of Liposome-Encapsulated Nutmeg Oil. Liposome-encapsulated nutmeg oil was prepared according to thin film dispersion method.[24, 32] A weight ratio at 1:1 of Soy lecithin and cholesterol were dissolved in chloroform, and the different concentration (2.0 mg/mL, 3.0 mg/mL, 4.0 mg/mL, 5.0 mg/mL, and 6.0 mg/mL) of nutmeg oil was added. The solution was transferred into a round bottom flask and was evaporated on a rotary evaporator (RE-2000B, Ya Rong Biochemistry Instrument, Shanghai, China) until a thin film was formed on walls. Subsequently, round bottom flask was dried in vacuum oven at 30 ℃ for 24 h to remove chloroform cleanly. Film was suspended in 0.03 M PBS (pH 7.2) which contained polyvinylpyrrolidone (1.0 mg/mL), then homogenized in cell ultra-fine grinding instrument (scientz-IID, Scientz Biotechnology, Ningbo, China) at 360 W for 30 min. Solution was centrifuged at 4000 rpm for 15 min, and the precipitation was removed. Finally, liposomes were passed through 0.22 μm pore size filter and then stored at 4 ℃.

2.3.2 Characterization of Liposome-Encapsulated Nutmeg Oil

2.3.2.1 Determination of Particle Size and Zeta Potential. The particle size and Zeta potential of the liposome-encapsulated nutmeg oil were determined using a dynamic light scattering zetasizer (Nano ZS90, Malvern Instruments, Malvern, UK). Breadth of particle size distribution was expressed according to the polydispersity

index (PDI).[33]

2.3.2.2 Determination of Encapsulation Efficiency. Preparation of standard curve: nutmeg oil was dissolved in ethanol to obtain 0.1, 0.2, 0.4, 0.6, 0.8 mg/mL standard solution, and determined by Gas chromatograph-mass spectrometer (GC-MS) (Agilent 6890N/5973N, Agilent Technologies, USA). The linear regression equation between peak areas of sabinene and concentration of nutmeg oil was made. Then, liposomes were centrifuged at 15000 rpm at 4 °C for 3 h. Supernatant was removed immediately, and vesicles were dissolved in ethanol. After being treated by ultrasonic wave for 3 h, mixture was centrifuged at 6000 rpm for 20 min. Supernatant was determined through GC-MS. The regression equation between peak areas of sabinene and concentration of nutmeg oil was used to calculate concentration of nutmeg oil.[34, 35]

2.3.2.3 Atomic Force Microscopy (AFM) Analysis. AFM analysis was used to observe morphology of liposome-encapsulated nutmeg oil.[29]A drop of liposome was applied evenly on a mica sheet for 3 min, and the excess liquid was removed. Subsequently, the equal volume of tri-distilled water was applied on mica sheet to disperse liposome. After air-dried at room temperature, sample was observed using the Atomic Force Microscope (MFP-3D-SA, Asylum Research, USA).

2.4 Antibacterial Activity of Liposome-Encapsulated Nutmeg Oil in Dumplings

2.4.1 Time-Kill Analysis of Liposome-Encapsulated Nutmeg Oil. Time-kill analysis of liposome-encapsulated nutmeg oil was detected according to plate colony-counting method.[27, 28] The traditional fillings of dumpling (pork: cabbage, 4:1,

w/w) and water (1:9, w/w) were weighed and blended into slurry, and then was put into bottles and sterilized.[36,37] Liposome-encapsulated nutmeg oil was diluted into bottle containing *L. monocytogenes* or *E. coli* (approximately $10^{5\text{-}6}$ CFU/mL) to obtain a concentration of 300 mg/mL. As control, bacterial suspensions without liposome were also tested. All the bottles were incubated under agitation at 25 °C and at 150 rpm. Then, numbers of residual bacteria were observed at 0 d, 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, and 7 d.

2.4.2 Controlled Release of Liposome-Encapsulated Nutmeg Oil. GC analyses of nutmeg oil, liposome, and liposome incubated with *L. monocytogenes* or *E. coli* for 7 d were carried out on an Agilent 7890A gas chromatograph, with a HP-5(30 m × 0.32 mm × 0.25 μm) capillary column. Nitrogen was used as carrier gas. Temperature of column was programmed from 60 to 250 °C at 10 °C/min, and held for 3 min. Injection temperature was programmed at 250 °C and detector temperature at 280 °C.[16]

2.4.3 Scanning Electron Microscopy (SEM) Analysis. Liposome-encapsulated nutmeg oil was added to test bottles to obtain a concentration of 300 mg/mL. The cell suspension without liposome was used as a control. Further, to observe the morphological changes, the method of SEM was modified from Diao et al. method.[38] Cell suspensions were collected by centrifugation at 8000 rpm for 10 min, washed thrice by 0.03 M PBS (pH 7.2), and fixed with 2.5% (v/v) glutaraldehyde and osmic acid solution at 4 °C. After centrifuging, cell was dehydrated using various ethanol concentrations (30%, 50%, 70%, 80%, 90% and 100% (v/v)) for 10 min. Then, cell

was dried. Finally, cells were gold-covered by cathodic spraying, followed by microscopic examinations using a scanning electron microscope (JSM-7001F, JEOL, Japan).

2.4.4 Long-Term Antibacterial Activity of Liposome-Encapsulated Nutmeg Oil in Dumplings. Long-term antibacterial activities of nutmeg oil and liposome-encapsulated nutmeg oil were tested by plate colony-counting method.[27, 28] Nutmeg oil and liposome-encapsulated nutmeg oil were diluted into slurry of fillings containing *L. monocytogenes* (approximately 10^5 CFU/mL) to obtain the concentration of 0.5 and 400 mg/mL, respectively. Bacterial suspensions without liposome were also tested as a control. All the samples were incubated in bottles equipped with breathable sealing films instead of caps at 25 ℃ and at 150 rpm to simulate the situation that preservatives were added to food directly. Subsequently, numbers of residual bacteria were observed at 0 d, 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, and 7 d.

2.5 Statistical analysis

All experiments were conducted in triplicate, and the results were analyzed with SPSS software (version 22.0 for Windows). The one-way ANOVA and Bonferroni post hoc tests were used to determine the level of significance and $p< 0.05$ was considered to be significant.

3. Results and Discussion

3.1 Antibacterial Activity of Nutmeg Oil. To date, some studies have reported that terpenoids, the main components of some essential oils, have significant

antibacterial activities.[39] Based on our earlier research, sabinene and α-pinene were the major components of nutmeg oil tested in this study.[40]They were all the terpenoids, and these results strongly supported that nutmeg oil could exhibited efficient antibacterial effects as a natural antiseptic.

First of all, antibacterial activity of nutmeg oil was evaluated. For *L. monocytogenes*, MIC value of nutmeg oil was 1.0 mg/mL, and MBC was 2.0 mg/mL. Meanwhile, MIC was 0.5 mg/mL, and MBC was 1.0 mg/mL for *E. coli*.[40]Results showed that nutmeg oil had obvious antibacterial activities for all tested bacterial strains, including both Gram-positive and Gram-negative bacteria. We previously had demonstrated that nutmeg oil also exhibited certain antibacterial activities against *Staphylococcus aureus, Bacillus subtilis, Salmonella typhi, Klebsiella pneumonia, Pseudomonas aeruginosa, and Bacillus pumilus*.[40] These all showed that nutmeg oil was a potent bacterial inhibitor and bactericide with a broad antibacterial spectrum.

Subsequently, effects of nutmeg oil on growth of bacteria were determined by time-kill analysis. As observed in Figure 1a, nutmeg oil has strong antibacterial activity against *L. monocytogenes.* Compare to control, almost 99.968% reduction in population was observed in *L. monocytogenes*, after 8 h of nutmeg oil treatment. And about 99.999% reduction in population was achieved at incubated time of 16 h. It indicated that incubated time of essential oil had a great influence on antibacterial activity, and nutmeg oil could be a natural and efficient food preservative, achieving complete bacterial elimination at a short time. According to former research, about 99.999% reduction was achieved for *E. coli* at 8 h treatment.[40] Nutmeg oil was more

effective against Gram-negative strains than Gram-positive strains, which was in agreement with a report on the sensitivity of bacteria to *Saturejahorvatii* essential oil.[41]

Previous findings showed that essential oil principally performed against cell membrane of bacteria.[2] Hence, TEM analysis was carried out to visualize efficacy of nutmeg oil on morphological and physical changes of *L. monocytogenes*. In the case of TEM, untreated bacteria had a normal and intact cell structure (Figure 1b), and *L. monocytogenes* treated with nutmeg oil showed extensive damage to cell membrane and cellular disintegration (Figure 1c), all of which were irreversible changes. These all illustrated that cell membrane was the first target of essential oil. And integrity of cell membrane was crucial to survival of bacteria because it was a key element for the basic physiological activities taking place within cells.[39]

Furthermore, to observe the level of *L. monocytogenes* membrane damage caused by nutmeg oil, the amount of intracellular ATP was measured using an ATP bioluminescence assay and nucleic acid was analyzed qualitatively by fluorescent staining method. Result of ATP bioluminescence assay showed that 91.95% reduction of intracellular ATP content was detected in suspension treated with MIC of nutmeg oil, and 98.76% reduction was observed in suspension treated with 2 MIC (Figure 2a). ATP release was steadily increased with increase of nutmeg oil concentration. This indicated that at concentration of 2 MIC and higher, antibacterial activity of nutmeg oil might be due to bactericidal damage to cell membrane rather than bacteriostasis. Simultaneously, outcome of LSCM revealed that fluorescence intensities of bacteria

266 treated with nutmeg oil were significantly lower compared to the control groups
267 (Figure 2b and c). Marked leakage of intracellular material was demonstrated that
268 obvious and irreversible damage to cell membrane was occurred by nutmeg oil, which
269 was consistent with observations of TEM analysis. Therefore, nutmeg oil could
270 damage cell membrane of *L. monocytogenes*, lead to extensive leakage of intracellular
271 material and cause the death of bacteria.

272 **3.2 Characterization of Liposome-Encapsulated Nutmeg Oil.** Essential oils
273 had a wide spectrum of biological activity and had natural antibacterial properties
274 with potential to extend shelf life of foods.[15] Due to volatility and chemically
275 instability of most of essential oils, new methods have been developed to improve
276 stability, and among these is encapsulation of the essential oils in liposomes.[16]

277 The primary physical characteristics of the liposomes indicated their potential
278 applications in food industries were analyzed. Properties of liposomes with different
279 nutmeg oil envelopment quantum were shown in Figure 3. Particle size of liposomes
280 is an important parameter that determines their physicochemical properties and
281 biological functionalities. As seen, average particle sizes of liposome varied from
282 138.5 ± 1.68 to 172.1 ± 2.41 nm, and were significantly bigger than those of blank
283 liposome (approximately 70 nm).Average particle sizes of the liposome gradually
284 increased with the increasing of nutmeg oil content. It is proposed that as the nutmeg
285 oil content was increased, more essential oil were incorporated inside each liposome,
286 thereby increasing size of the liposome. It was reported that excessive small particle
287 sizes (< 50 nm) had high surface tension, thus they were very unstable and easy to

fuse with others and biological membranes.[42]Therefore, liposome prepared in this study was stable with a small size.

PDI is a measure of particle size distribution, which was calculated from cumulant analysis of dynamics light scattering measurements. PDI values of liposome varied from 0.218 ± 0.018 to 0.229 ± 0.026, which suggested a narrow size distribution. Then, entrapment efficiency of liposome-encapsulated nutmeg oil was investigated. Entrapment efficiencies of liposome varied from 22.81 ± 0.99 to 26.90 ± 1.22%.As for nutmeg oil entrapment efficiency, encapsulation of 5 mg/mL nutmeg oil led to saturation of liposome and attained a highest entrapment efficiency of 26.90 ± 1.22%, whilst higher nutmeg oil encapsulating concentration of 6 mg/mL just resulted in a decreased level of essential oil encapsulation inside liposome. To sum up, optimal PDI and entrapment efficiency were obtained at the concentration of nutmeg oil to 5.0 mg/mL.

Moreover, the other physical and chemical characteristics of optimal liposome were determined. Size distribution of liposome was shown in Figure 4a. Average particle size was 165.5 ± 1.46 nm with a PDI of 0.218 ±0.018. As a carrier system, small particle sizes (< 200 nm) had been reported to ensure to increase vascular permeability and enhance utilization ratio of entrapped compound.[19] Hence, liposome prepared in this study could have high efficacy in application. PDI value was low (< 0.220) and showing a relatively narrow a narrow size and stable distribution.[43]Zeta potential is used as an important parameter for particle stability, and it is a measure of liposomal surface charges.[44]A relatively high Zeta potential (positive or negative)

could increase the repulsive interactions, reduce frequency of liposome aggregation and precipitation, thus enhancing the stability of solution.[45,46] Generally, <-30 mV and > +30 mV would both be considered as high Zeta potentials. Meanwhile, Manosroi et al.[47] had reported that leakage rate in negative liposome was lower than this in positive liposome. From the date obtained, liposome-encapsulated nutmeg oil had a high negative Zeta potential of −49.2 ± 2.72 mV. It was reasonable to conclude that liposome we prepared might have high repelling force, prevent aggregation or precipitation happening, and result in a good stability.

With respect to entrapment efficiency of liposome, the linear regression equation between peak areas of sabinene and concentration of nutmeg oil was y = 8646233.75x + 444988.15, R^2 = 0.996. In the case of 5 mg/mL nutmeg oil encapsulation, value of entrapment efficiency was 26.90 ± 1.22%. Finally, AFM analysis was conducted to observe morphology of liposomes. As seen from Figure 4d, liposome was vesicles with near spherical shape, and exhibited a well-distributed particle. Besides, AFM image revealed that liposome was nanometric size, and ranged in size from 100 to 800 nm, which was well consistent with size distribution (Figure 4a). From these results, it could be deduced that liposome-encapsulated nutmeg oil with small particle size and high Zeta potential was a stable dispersion, and had potential applications in food industries.

3.3 Antibacterial Activity of Liposome-Encapsulated Nutmeg Oil in Dumplings. In order to prove our hypothesis that PFTs could form pores on liposome and trigger release of bioactive molecules from liposome, two kinds of bacteria were

selected to explain this theory. *L. monocytogenes*, which could secrete LLO, acted as a bacteria model that secreted PFTs while *E. coli*, which didn't secrete PFTs, acted as a control. Meanwhile, nutmeg oil acted as an antibacterial model that could have strong inhibitory effects against these bacteria.

Initially, antibacterial activities of liposome-encapsulated nutmeg oil against these tested bacteria were detected, and results were shown in Figure 5. As expected, population of *E. coli* treated with liposome was gradual and steady increased and sustained in approximately 10^8 CFU/mL (Figure 5a), which was accorded with control bacteria. In contrast, almost 99.46% reduction in population was observed in liposome-treated *L. monocytogenes*at cultivation time of 1 d, compared to control. Afterwards, around 99.86% reduction in population was detected, after 7 d of liposome treatment (Figure 5b). These indicated liposome-encapsulated nutmeg oil could release encapsulated nutmeg oil in the presence of *L. monocytogenes* and lead to inhibition of *L. monocytogenes* growth. Conversely, no antibacterial effect on *E. coli* was observed because nutmeg oil had been encapsulated in liposome.

Subsequently, GC assay was used to further prove the release of nutmeg oil from liposome. As showing in Figure 5c, the characteristic peak of sabinene, which is the main component of nutmeg oil, didn't be observed in pure liposome. It illustrated that 5 mg/mL nutmeg oil had been completely encapsulated in liposome and almost no free essential oil in suspension due to wastage of free oil in preparation or the low sensitivity of equipment. In the same, the characteristic peak didn't be viewed in liposome cultivate with *E. coli* that didn't secrete PFTs. Inversely, the

354 characteristic peak was detected in liposome incubated with *L. monocytogenes*. It
355 demonstrated that LLO enabled nutmeg oil release from liposome, which was
356 concordant with our hypothesis. From the above, released nutmeg oil from liposome
357 in the presence of *L. monocytogenes* was efficient to inhibit growth of bacteria. New
358 stimulating agent we looked for was LLO that secreted by *L. monocytogenes*, thus
359 stimulating agent had an excellent biological safety.

360 Besides, applications of free nutmeg oil and nutmeg oil encapsulated in liposome
361 in dumplings were compared. All the samples were incubated in bottles equipped with
362 breathable sealing films instead of caps to simulate the situation that preservatives
363 were added to food directly. After 1 d treatment of nutmeg oil, almost 99.94%
364 reduction in population was achieved in *L. monocytogenes.* However, nutmeg oil
365 could only inhibit increase in bacterial population up to 2 d and then steady growth of
366 *L. monocytogenes* was observed (Figure 6a). Increase in bacteria growth was due to
367 volatilization of essential oil from breathing hole. It could assume that when essential
368 oil was applied in food industrial directly in air, its volatilization would become more
369 efficient, and antibacterial effect was going to be much worse along with it. In
370 contrast, around 99.79% reduction in population was observed after 1 d of liposome
371 treatment. Moreover, about 99.96% reduction was detected for *L. monocytogenes*, at
372 treatment time of 7 d (Figure 6b). Thus it could be seen that nutmeg oil encapsulated
373 in liposome could prolong treatment time and improve antibacterial effect, which is
374 because release dosage of nutmeg oilcan timely adjust based on magnitude of
375 bacterial infections, leading to the minimal amounts of nutmeg oil release and higher

antibacterial efficiency.

Further, SEM analysis was utilized to observe morphological changes of *L. monocytogenes* treated by liposome. It could be found that untreated bacteria were rod shaped, regular, smooth (Figure 6c), while some bacteria treated with liposome became deformed, destroyed and stuck together (Figure 6d). Schematic of LLO triggered nutmeg oil release from liposome was shown in Figure 6e. The first step is interaction of the soluble LLO monomers with membrane. After membrane binding, LLO monomers diffused laterally to initiate formation of membrane oligomer. Then, oligomerization of membrane-bound monomers transformed to pre-pore complex. Once pre-pore complex made transition to pore complex, large transmembrane β-barrel pore was formed in liposome.[48]Through these pores, nutmeg oil could be released from liposome, which exhibited obvious inhibiting effects on *L. monocytogenes*. Therefore, liposome-encapsulated nutmeg oil would be a promising food preservative in dumplings.

4. Conclusions

Utilization of nanotechnology to enhance control of bacteria in food industry is quite novelty. As a kind of natural and safe spice, nutmeg oil possesses excellent antibacterial activity against pathogenic bacteria. Encapsulation could preserve biological activities of nutmeg oil, and can be an alternative to improve stability of these compounds when they are applied in food industry. In addition, PFTs could trigger nutmeg oil release from liposome and inhibit bacteria growth steadily. Besides *L. monocytogenes*, some other bacteria also could secrete PFTs. Therefore, this

technique has potential application as a targeted food preservative to treat various food-borne pathogens that can secrete PFTs.

ABBREVIATIONS USED

Listeria monocytogenes	*L. monocytogenes*
Escherichia coli	*E. coli*
Pore-forming toxins	PFTs
Gas chromatography	GC
Ready-to-eat	RTE
Generally recognized as safe	GRAS
Listeriolysin O	LLO
Cholesterol-dependent cytolysins	CDCs
Peptone yeast glucose broth	PYG
Nutrient broth.	NB
Minimum inhibitory concentration	MIC
Minimum bactericidal concentration	MBC
Phosphate buffer solution	PBS
Scanning electron microscopy	SEM
Transmission electron microscopy	TEM
4'6-diamidino-2-phenylindole	DAPI
Laser scanning confocal microscopy	LSCM
Polydispersity index	PDI
Gas chromatograph-mass spectrometer	GC-MS
Atomic Force Microscopy	AFM

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FIGURE CAPTIONS

Figure 1. Antibacterial activity of nutmeg oil against *L. monocytogenes*

(a) Time-kill curve of nutmeg oil against *L. monocytogenes*, TEM analysis of (b) *L. monocytogenes*, (c) *L. monocytogenes*treated with nutmeg oil. *p< 0.05 when compared to control

Figure 2. Intracellular material changes of *L. monocytogenes*

(a) Intracellular ATP content, (b) LSCM of untreated cell, (c) LSCM of cells after treatment withMBC.*p< 0.05 when compared to control

Figure 3. Properties of liposomes with different nutmeg oilenvelopment quantum

Figure 4. Properties of liposome with 5mg/mL nutmeg oilencapsulation.

(a) Size distribution, (b) Zeta potential distribution, (c) the linear regression equation of entrapment efficiency, (d) AFM analysis.

Figure 5. Time-kill analysis of liposome in dumplings

(a) *E. coli*, (b) *L. monocytogenes*, (c) controlled release of liposome. *p< 0.05 when compared to control

Figure 6. Long-term antibacterial activity of liposome in dumplings

(a) Nutmeg oil, (b) liposome, (c) SEM analysis of untreated cell, (d) SEM analysis of cells after treatment withliposome, (e) schematic of LLO triggered nutmeg oil release from liposome.*p< 0.05 when compared to control

FIGURE GRAPHICS

Figure 1

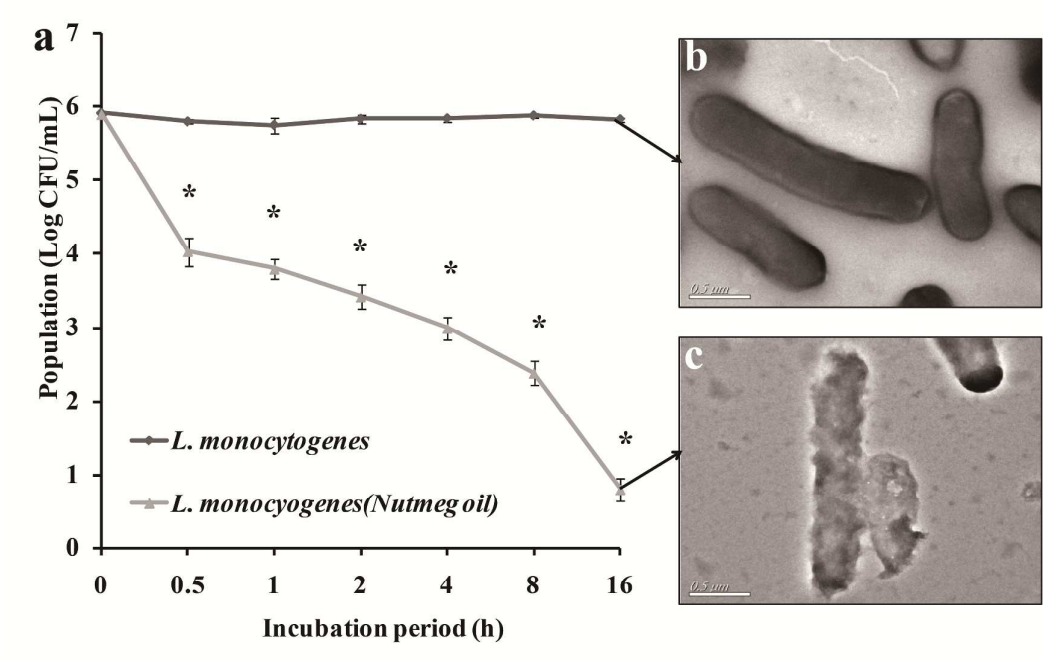
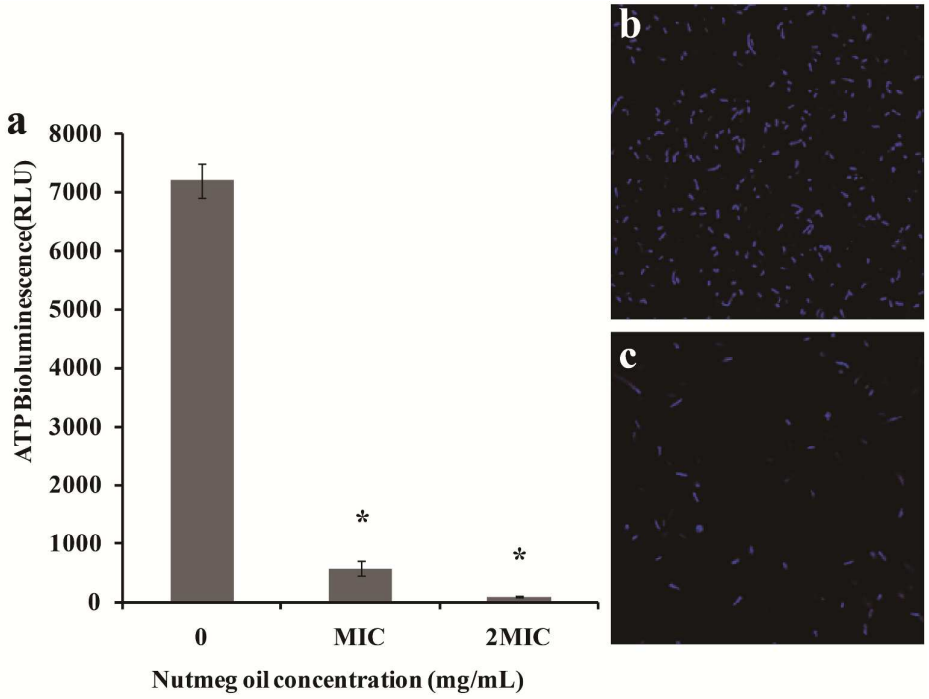
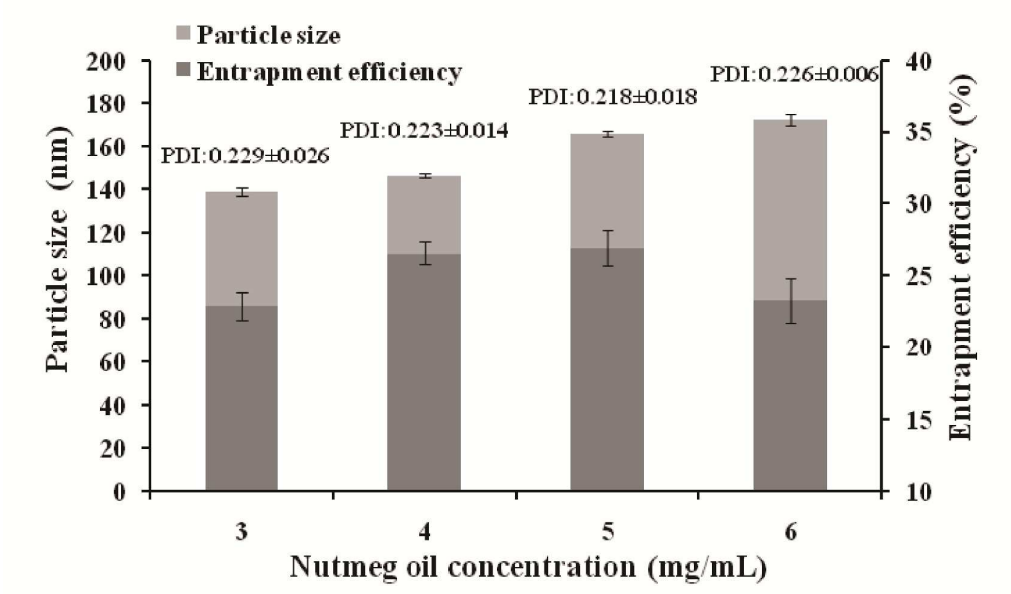


Figure 2



544 **Figure 3**



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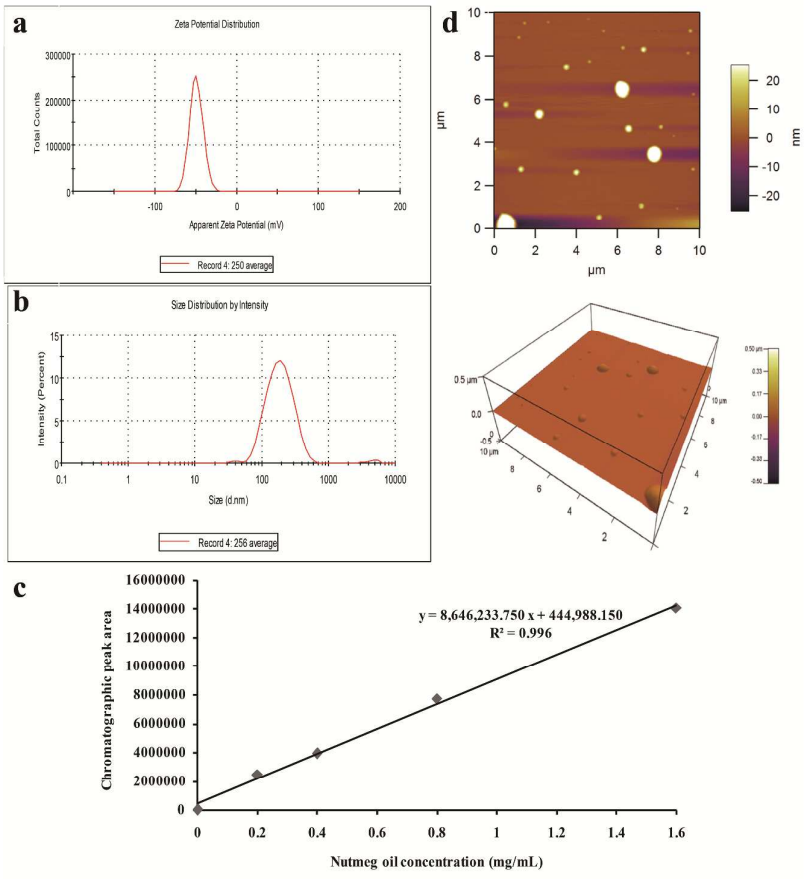
546 **Figure 4**



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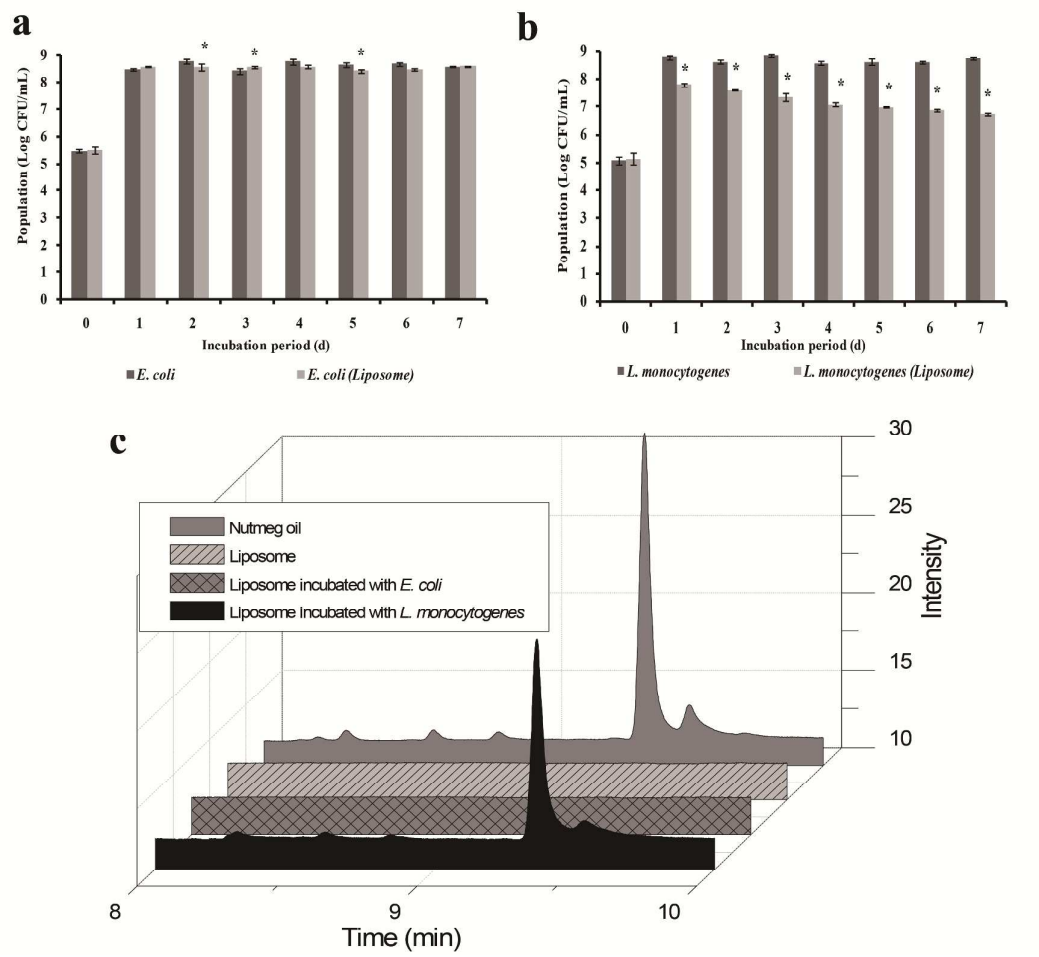
549 **Figure 5**



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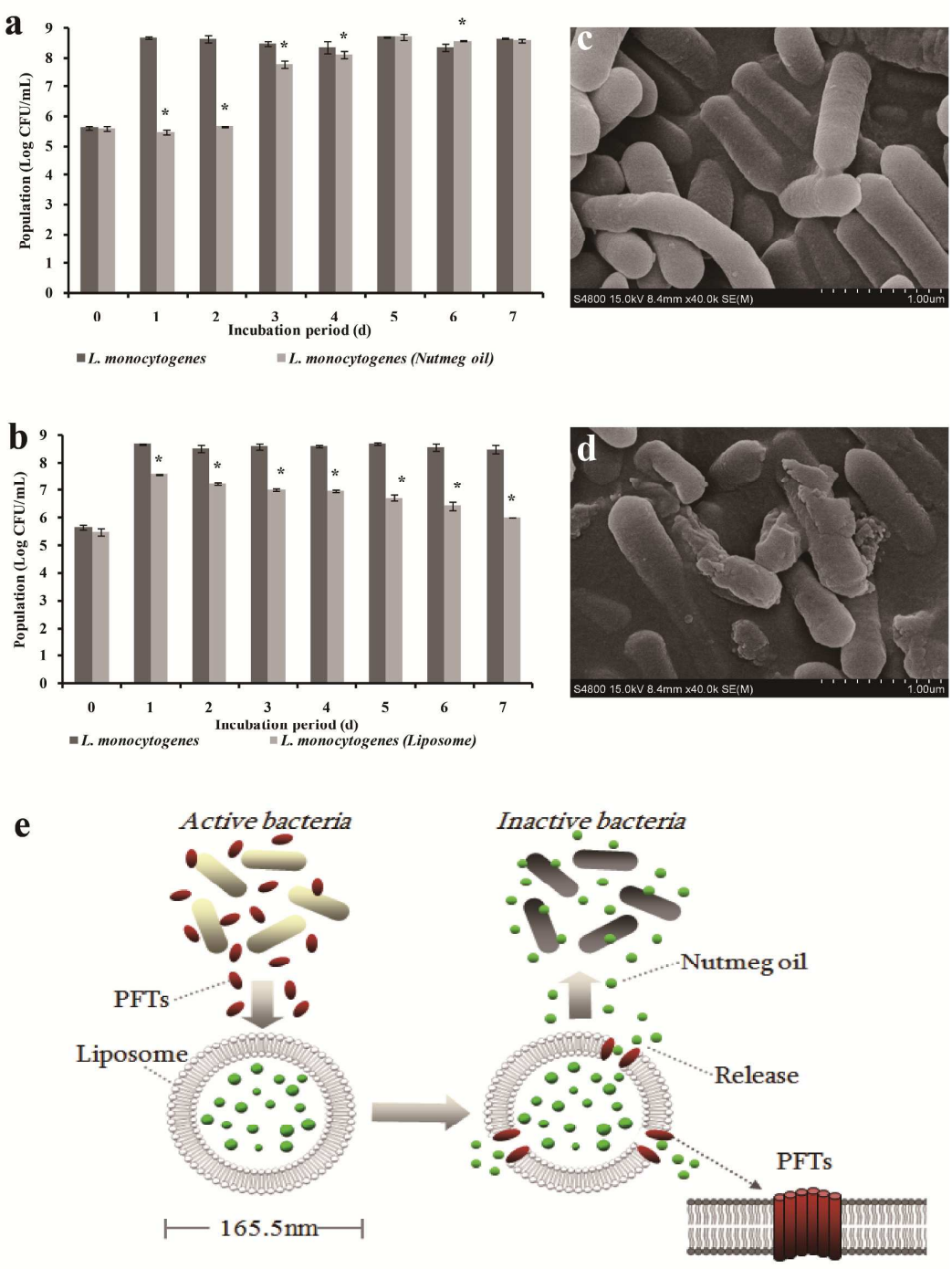
560 **Figure 6**



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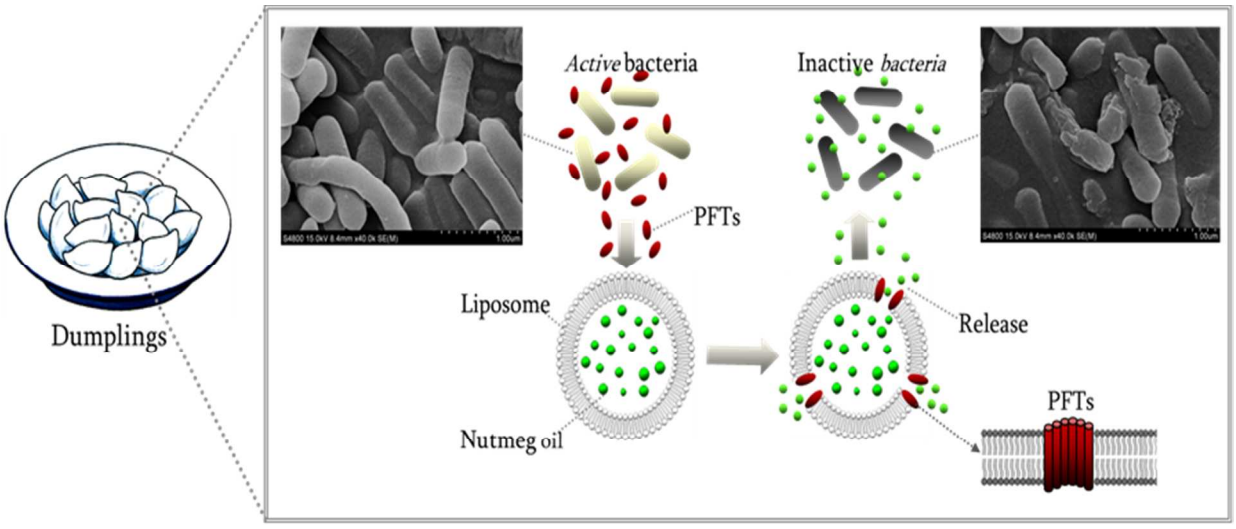
TOC Graphic：


Dumplings
Active bacteria
Inactive bacteria
PFTs
Liposome
Nutmeg oil
Release
PFTs