**Development and characterization of geraniol-loaded polymeric nanoparticles with
antimicrobial activity against foodborne bacterial pathogens**

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Abstract

This study demonstrates the rose essential oil component (EOC) geraniol can be loaded into polymeric nanoparticles (NPs) with sustainable release profile. Geraniol-loaded NPs were prepared by flash nanoprecipitation and characterized for size, encapsulation efficiency, payload release during storage, inhibition of *Escherichia coli* O157:H7 and *Salmonella enterica* Typhimurium *in vitro* and on spinach surfaces, and NP-assisted transport of EOC into cellular membranes. Adjusting concentrations of stabilizing polymer, Pluronic® F-127, and geraniol produced NPs ranging in size from 26 to 412 nm. Antimicrobial NPs inhibited *S. Typhimurium* and *E. coli* O157:H7 growth at 0.25 and 0.2 wt.%, respectively. Geraniol-loaded NPs displayed sustained release with a time constant of 24 hr, maintaining their anti-pathogenic properties over a prolonged time period. Pathogen reductions on treated spinach surfaces ranged from 0.3 to 4.2 log₁₀ CFU/cm². Antimicrobial NPs may be useful for post-harvest decontamination of foods such as fresh produce from cross-contaminating microbial pathogens.

Keywords: Geraniol, Pluronic® F-127, antimicrobial nanoparticles, *Escherichia coli* O157:H7, *Salmonella* Typhimurium

Chemical compounds studied in this article:

Geraniol (PubChem CID: 637566); Pluronic F127 (PubChem CID: 24751).

1.0 Introduction

Foodborne microbial pathogens continue to impose significant public health and financial burden in the U.S. despite the advent of numerous food processing technologies and food safety oversight systems designed to prevent pathogen transmission to consumers. According to the U.S. Centers for Disease Control and Prevention (CDC; Atlanta, GA), approximately 48 million incident cases of human foodborne disease occur annually, resulting in an estimated cost to the U.S. economy of 77.7 billion USD (Scallan et al., 2011a; Scallan et al., 2011b; Scharff, 2012). Bacterial pathogens such as *Salmonella enterica* and the Shiga toxin-producing *Escherichia coli* (STEC), including *E. coli* O157:H7, have been transmitted to consumers by a variety of food vehicles, including fresh and minimally processed produce, dairy products, meat and poultry (Painter et al., 2013).

In recent years a great deal of research has been completed detailing the efficacy of plant-derived essential oil components (EOC) (e.g., thymol, carvacrol, allicin, geraniol, limonene, etc.) to inhibit the growth of foodborne bacterial pathogens (Davidson et al., 2013). Nevertheless, their utility in foods may be limited by their impacts on organoleptic properties of the food at levels sufficient to inhibit microbial growth or by low solubility in the aqueous phase of various foods (Burt, 2004; Weiss et al., 2009). One functional solution to these limitations is that of encapsulation within food-grade encapsulating materials (e.g., lipid, polymers) (Hill et al., 2013a; Sanguansri and Augustin, 2006). Gaysinsky et al. (2005a, b) reported entrapment of eugenol and carvacrol within surfactant micelles resulted in decreased growth in a liquid medium over 24 hr at pH 7.0 of *E. coli* O157:H7 and *Listeria monocytogenes* when compared to non-encapsulated EOCs. Gomes et al. (2011; 2008), likewise, reported inhibition of bacterial pathogens on spinach surfaces treated with β -cyclodextrin-entrapped cinnamon and clove oils,

reporting reduced minimum inhibitory concentrations (MIC) of encapsulated antimicrobial oils versus non-encapsulated EOC.

The rose oil component geraniol (*trans*-3,7-Dimethyl-2,6-octadien-1-ol) has been previously reported to exert growth-inhibiting activity against various foodborne bacterial and fungal microbes (Pattnaik et al., 1997; Singh et al., 2012). Kim et al. (1995) reported minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of the compound in 1% Tween 20 against *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* Typhimurium, and *Vibrio vulnificus* ranged between 500 and 1,000 µg/ml. Friedman et al. (2004) reported bactericidal activity of geraniol against *Salmonella* and *E. coli* O157:H7 was observed rapidly at 21°C, achieving 50% lethality of inoculated *E. coli* O157:H7 and *Salmonella* cells at levels of 0.089 and 0.031%, respectively, in clarified apple juice. An MIC for geraniol of 0.05% against *E. coli* O157:H7 grown in medium adjusted to pH 4.5 was reported, though researchers observed that at pH 7.2 the MIC of geraniol against the pathogen was >0.1% (Knight and McKellar, 2007). The objective of this study was to develop antimicrobial nanoparticles (NPs) loaded with geraniol using the triblock copolymer Pluronic® F-127 and characterize resulting physico-chemical and antimicrobial properties. Pluronic® F-127 was selected because it is inexpensive, non-toxic, bears high biocompatibility, and has been repeatedly utilized in drug delivery systems (Kabanov et al., 2002; Pham Trong et al., 2008; Shi et al., 2015; Tilley et al., 2013). Resulting NPs were characterized for size as a function of geraniol:polymer blending ratios, encapsulation efficiency, drug release profile, and antimicrobial capacity of geraniol NPs against *Salmonella* Typhimurium and *E. coli* O157:H7.

2.0 Materials and Methods

2.1 Materials

Geraniol (>96.0%; CAS# 106-24-1) (TCI America, Portland, OR), Pluronic® F-127 (PF127; CAS# 9003-11-6) (Sigma-Aldrich Co., St. Louis, MO), and tetrahydrofuran (THF; CAS# 109-99-9; Sigma-Aldrich Co.) were purchased and used as received for the preparation of EOC-loaded NPs. Nile Red (CAS# 7385-67-3) (Tokyo Kasei Kogyo Co., LTD, Tokyo, Japan) and poly-L-lysine (CAS# 25988-63-0) (Sigma-Aldrich Co.) were used for experiments determining nanoparticle adsorption to bacterial pathogen surfaces.

2.2 Preparation of Geraniol-Loaded Nanoparticles

Geraniol-loaded polymeric NPs were prepared with a rapid nano-precipitation method previously reported (Akbulut et al., 2009; Zhang and Akbulut, 2011). Briefly, geraniol and the amphiphilic triblock copolymer PF127 were dissolved in THF to differing ratios to determine impact of blending ratios on resulting geraniol-containing NP size. Then, THF solution was rapidly impinged against milli-Q water to produce polymer-encapsulated geraniol-bearing NPs. The flow rate of water was 50.0 mL/min, and the flow rate of the THF solution was 5.0 mL/min. Following impingement processing, the NP-contained solution was placed under a fume hood for 7.0 hr to remove THF (Figure 1).

2.3 Characterization of Nanoparticles

Particle size distribution of polymeric NPs was measured by dynamic light scattering (DLS) following four-fold dilution in milli-Q water using a Zetasizer ZS90 particle size and zeta potential analyzer (Malvern Instruments, Ltd., Westborough, MA). The measurements were carried out at a scattering angle of 90° at 25°C. Size and shape of individual NPs was characterized by use of transmission electron microscope (TEM; JEM-2010, Jeol USA, Inc., Peabody, MA) at the Microscopy Imaging Center at Texas A&M University (College Station,

TX). Nanoparticle-containing solution was diluted four-fold in milli-Q water prior to drop-wise application of NP solution onto a copper grid (400 mesh) with carbon film (CF400-Cu, Electron Microscopy Sciences, Hatfield, PA). The NP sample was dried at ambient temperature prior to TEM analysis. Observations were performed at 200 kV accelerating voltage, $<2.5 \times 10^{-5}$ Pa, at ambient temperature ($\sim 25^{\circ}\text{C}$).

2.4 *Release Kinetics of Geraniol From Polymeric NPs*

Geraniol-containing NPs (9.0 mL) were added into standard regenerated cellulose membranes (molecular weight cut-off 12,000-14,000 Da; approximately 2.0 nm diameter cut-off) (Spectrum Laboratories, Inc., Rancho Dominguez, CA); membranes were then placed into beakers containing 200.0 mL milli-Q water. Unencapsulated geraniol was expected to passively diffuse through dialysis membranes, while NPs were expected to be unable to diffuse through membranes, preventing entrapped geraniol from diffusion. Changes in concentration of free geraniol were tracked by spectroscopy using a UV-1800 UV/Visible scanning spectrophotometer (Shimadzu Corp., Columbia, MD), scanning from 190 to 800 nm (geraniol maximal absorption: 240 nm). UV measurements were performed at ambient temperature at 1, 2, 4, 8, 24, 48, 96, and 144 h. Four independent replications were completed for each measurement.

2.5 *Encapsulation Efficiency (EE) of Nanoparticles*

Geraniol loading and EE was determined by mass spectrometry (MS). Nanoparticles were added to hexane at 1:10 NP:hexane (calculated as weight NP:weight hexane) to degrade NPs and release geraniol into the solvent phase. Following geraniol release from NPs, hexane was used to complete MS analysis of geraniol at the Laboratory for Biological Mass Spectrometry (Department of Chemistry, Texas A&M University). A reference/standard solution of 0.08 wt. % geraniol was prepared to calibrate the concentration of geraniol obtained from degraded NPs in

mass spectra. Ten μL of 0.5 mg/ml of hexadecanol was added as internal standard to 50 μL of sample. 100 μL of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with trimethylchlorosilane (TMCS) was added into this solution. The mixture was heated at 60°C for 1 hr. The compounds of interest were detected as trimethylsilyl (TMS) derivatives using GC/MS. Ultra GC/DSQ (Thermo-Electron, Waltham, MA) was used for GC-MS. Chromatography was carried out using an Rxi-5ms column (60 m x 0.25 mm with 0.25 μm film thickness) (Restek, Bellefonte, PA). Helium was used as a carrier gas at constant flow of 1.5 mL/min. GC inlet was held at 225°C while transfer line and ion source temperatures were held at 250°C. An aliquot of 1.0 μL of sample was injected in splitless mode. The oven temperature was maintained at 50°C for 5 min, then raised to 320°C at 20°C/min. Electron impact ionization at 70 eV was used for ionization and mass spectra were acquired in full scan mode in the range of 30-500 m/z. Three replications were completed for MS analysis of NP EE; EE was determined as:

$$EE (\%) = (\text{Weight of drug in NPs} / \text{Initial weight of drug}) \times 100\% \quad (\text{Eq. 1})$$

2.6 Microorganisms and Inoculum Preparation

Salmonella Typhimurium LT2 (American Type Culture Collection [ATCC] No. 700720) (Manassas, VA) and *E. coli* O157:H7 (ATCC No. 700728) were obtained from the Food Microbiology Laboratory (Department of Animal Science, Texas A&M University) culture collection and revived according to previously published methods (Perez et al., 2012). Working cultures were obtained by aseptically scraping a loopfull (10 μL) of culture from tryptic soy agar (TSA; Becton, Dickinson and Co., Sparks, MD) slants into 9.0 mL sterile tryptic soy broth (TSB; Becton, Dickinson and Co.), followed by incubation at 35°C for 24 hr. A second passage was completed in identical fashion, with subsequent incubation at 35°C for 24 hr prior to antimicrobial assay completion.

2.7 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration Assays

Salmonella Typhimurium and *E. coli* O157:H7 cultures were diluted in 9.9 mL double strength TSB (2xTSB) to 5.0 log₁₀ CFU/mL. To quantify the initial numbers of each pathogen inoculated into reaction plates, decimal dilutions were prepared in 0.1% (w/v) peptone diluent (Becton, Dickinson and Co.). Diluted cells were spread onto surfaces of TSA-containing Petri dishes; bacterial cells were enumerated following incubation of Petri dishes at 35°C for 24 hr.

A micro-broth dilution assay was utilized to determine MIC and MBC of free and NP-loaded geraniol against both bacterial organisms (Brandt et al., 2010). Wells of a 96-well sterile microplate (Falcon®, Corning, Inc., Tewksbury, MA) were loaded with 200.0 µL free or NP-encapsulated geraniol. Following loading, EOC (free, NP-entrapped) was diluted via addition of 100.0 µL sterile phosphate-buffered saline (0.1%) (PBS; Calbiochem, EMD Biosciences, Inc., La Jolla, CA), mixed, and then 100.0 µL of mixture was removed and loaded into adjacent wells. This process of dilution with PBS and transfer of 100 µL was repeated to produce two-fold dilutions of free or NP-loaded geraniol. Following loading of EOC in wells, prepared cultures were aseptically loaded (100.0 µL) into reaction wells. Negative controls were prepared consisting of only sterile 2xTSB (100.0 µL) and sterile PBS (100.0 µL) to confirm no cross-contamination of NPs. Positive controls consisted of 2x TSB (100.0 µL) and *S. Typhimurium* or *E. coli* O157:H7 in PBS (100.0 µL) to confirm pathogen ability to grow under experimental conditions. Additionally, geraniol-containing (free, encapsulated) non-inoculated controls were prepared to allow for baseline correction during determination of bacterial growth via observation of changes in optical density at 630 nm (OD₆₃₀). A PF127 control was not included based on review of previously published research showing no inherent antimicrobial activity of the polymer against various Gram-positive and Gram-negative bacterial organisms (Chudasama

et al., 2010; Veyries et al., 2000). Immediately following preparation (0 hr), and again at 24 hr incubation at 35°C, microplates were loaded into an Epoch UV/Visible scanning spectrophotometer (Bio-Tek® Instruments, Inc., Winooski, VT) and sample OD₆₃₀ was read. Free or encapsulated EOC producing a change in OD₆₃₀ < 0.05 were deemed inhibitory to pathogen growth; the lowest concentration of free or NP-loaded geraniol producing pathogen inhibition was identified as the MIC (Branen and Davidson, 2004). Duplicate identical wells were constructed for all combinations of antimicrobial EOC and microorganism, as well as required controls. The assay was replicated independently two times (n=4); MICs were identified as the lowest concentrations of free or entrapped geraniol inhibiting the pathogen.

Bactericidal activity of free or encapsulated geraniol was assayed following completion of MIC determination. Numbers of surviving pathogens from wells containing inhibitory concentrations of free or encapsulated EOC were determined by spreading 0.1 mL of culture fluid directly from a sample well onto the surface of a TSA-containing Petri dish. Inoculated Petri dishes were then incubated for 24 hr at 35°C prior to enumeration of colonies. The lowest concentration of free or NP-loaded geraniol producing a ≥ 3.0 log₁₀-cycle decline in numbers of pathogen (log₁₀-transformed plate count of bacterial cells prior to microplate inoculation – log₁₀-transformed plate count of bacterial cells following incubation of inoculated Petri dish from pathogen-inhibiting microplate well) was identified as the MBC for free or NP-encapsulated EOC for each pathogen.

2.8 *Interaction of Fluorescent NPs with Bacterial Pathogen Cells by Confocal Microscopy*

For confocal microscopy analysis of NP/bacterial cell interaction, Nile Red (0.001 wt. %) was co-entrapped with geraniol to produce fluorescing NPs. Poly-L-lysine was used to provide cell immobilization on glass cover slides while taking confocal images. Glass cover slides were

coated by dipping into poly-L-lysine solution (0.1 mg/mL) for 5 min. Excess poly-L-lysine was removed and slides were dried completely under a fume hood. A 1.0 mL suspension of *E. coli* O157:H7 cells, prepared as described in Section 2.6, was mixed with 1.0 ml Nile Red-containing NPs and incubated 2 hr; samples were then washed once in 0.1% peptone water. For the control, Nile Red was prepared to 0.001%; a 1.0 mL suspension of *E. coli* O157:H7 was similarly mixed with 1.0 ml Nile Red, incubated 2 hr, and then samples were washed once in 0.1% peptone water. One drop of sample was then placed in the middle of the poly-L-lysine coated glass cover slides. The images were obtained using a confocal microscope (Zeiss LSM 780 NLO Multiphoton Microscope) using an excitation wavelength of 561 nm to determine the distribution of nanoparticles; fluorescence emission was detected at 579-624 nm. Confocal microscopy was conducted at the Image Analysis Laboratory, College of Veterinary Medicine & Biomedical Sciences at Texas A&M University (College Station, TX).

2.9 Antimicrobial NP Inhibition of Pathogens Inoculated on Spinach Surfaces

As a follow-up to the completion of MIC and MBC experiments, researchers hypothesized geraniol NPs would exert antimicrobial effects against pathogens inoculated onto fresh produce commodity surfaces. A small experiment was designed to: i) gather preliminary understanding of the antimicrobial utility of geraniol NPs on a produce surface, and ii) guide the development of subsequent experimentation designed to comprehensively determine optimal NP application methods for produce decontamination for industry application. Working cultures of *E. coli* O157:H7 and *S. Typhimurium* were prepared as previously described (Section 2.6). The cells were washed by centrifugation at 2191 x g in a Jouan B4i centrifuge (Thermo-Fisher Scientific, Inc., Waltham, MA) for 15 min at 22°C. Resulting pellets were suspended in 10 mL PBS and again washed by centrifugation for 15 min at 22°C; centrifugation and washing procedures were

repeated identically twice. After the final cycle, pellets were suspended in 10 mL PBS; both microbes were mixed and serially diluted in 0.1% peptone to achieve an inoculum of $8.0 \log_{10}$ CFU/mL. The concentration of the inoculum was confirmed via selective/differential plating on lactose-sulfite-phenol red-rifampicin (LSPR) agar (Castillo et al., 1998). Bunched, non-waxed spinach was locally purchased and immediately returned to the Food Microbiology Laboratory. Spinach leaves were washed in sterile water and surface disinfected with 70% ethanol (Alegre et al., 2013). After 1 hr of drying at 25°C, 10 cm² samples were aseptically excised from the spinach leaves with flame-sterilized implements. Samples were spotted with ten 10.0 µL spots of microbial cocktail on to the adaxial surfaces of the leaf. Samples were then stored at 25°C for 1 hr to allow pathogen attachment to spinach surface. **Nanoparticle treatments were applied to spinach samples to determine decontamination capacity of antimicrobial NPs as a function of application method. In the case of spraying, the impact of the number of spray applications was also tested, and in the case of immersion, the immersion period (2 and 5 min).** The treatments were spray application of NPs via one, two or three sprays (~1.0 mL/spray), or spinach sample immersion in 20 mL of NP-containing buffer for 2 or 5 min. A negative control sample consisting of no added inoculum and no NPs was plated along with a positive control sample consisting of the inoculum without NP treatment. All samples were serially diluted in 0.1% peptone and surviving microbial cells spread on LSPR; Petri dishes plates were incubated aerobically at 35°C for 24 hour prior to pathogen enumeration. The procedure was replicated identically four times (n=4).

2.10 Statistical Analysis of Data

Data analysis was completed using ORIGIN® v.8 software (OriginLab Corp., Northampton, MA). One-way analysis of variance (ANOVA) was used to test the differences between

treatments and Tukey's Honestly Significant Differences (HSD) test was performed to separate means differing at $p < 0.05$.

3.0 Results and Discussion

3.1 Characterization of Nanoparticles and Encapsulation Efficiency

Figure 2A depicts the intensity-weighted size distribution for one of such NPs as obtained by DLS analysis. Hydrodynamic NP size ranged from 200 to 1000 nm, with an average diameter of 412 ± 25 nm for particles constructed from a 1:1 ratio geraniol:PF127 (Table 1); mean particle size differed ($p < 0.05$) as a function of formulation, except for the 1.0:0.25 and 0.6:1.0 (geraniol:PF127) formulations. Zhang and Akbulut (2011) reported similar trends in polymeric NP size with systematic variation in particle composition by the addition of increasing amounts of polystyrene to poly(ethyleneglycol-*b*- ϵ -caprolactone) for drug loading. Polydispersity index (PDI) values approximated 0.19 for freshly prepared NPs at ambient temperature. Transmission electron microscopy analysis revealed NPs were spherically shaped, as well as agreeing with particle size estimates obtained by DLS (Figure 2B). It was also possible to obtain EOC-loaded NPs of differing mean hydrodynamic sizes by altering geraniol:PF127 mixing ratio (Table 1). Encapsulation efficiency analysis can indicate the percent of drug entrapped within a polymeric matrix from the amount of drug applied during NP formulation and manufacture. The mean EE for geraniol in PF127 NPs was 57.5 ± 5.5 %; non-entrapped geraniol was removed during THF evaporation. The EOC loading ratio (wt. of drug divided by wt. of whole particles) was 0.365. Manaspon et al. (2012) reported Pluronic® F127/chitosan NPs containing doxorubicin entrapped between 58.1 and 51.2% when PF127 was loaded at 5 and 10% (w/v), respectively, similar to the EE obtained in the current study.

3.2 Release of Geraniol from PF127 Nanoparticles

The release of geraniol from NPs into water was observed by spectroscopic determination of geraniol concentration as a function of incubation at 25°C over 144 h (Figure 3). Early in the experiment, the concentration of geraniol diffusing across the dialysis membrane increased significantly from 0 to 8 h, as well as from 8 to 24 h ($p < 0.05$). The concentration of geraniol diffusing across the membrane appeared to be maximal at 24 h, with a plateau observed in geraniol content in dialysis water from 24 until 144 h of storage. The resulting diffusion curve was fitted with an exponential regression curve ($R^2 = 0.9994$), with a half-release occurring within 7.25 h. Drug release properties from nanoparticles in the current study are similar to those reported previously for PF127 micelles constructed for pharmaceutical and antimicrobial drug delivery applications (Kelishady et al., 2014; Taha et al., 2014). Nonetheless, NPs in the current study outperformed PF127 micelles encapsulating the plant oil curcumin with respect to release profile where 80% drug release was observed to occur within 6 hr at 37°C (Peng et al., 2014).

3.3 Antimicrobial Activity of Geraniol NPs against Foodborne Bacterial Pathogens

The MICs and MBCs of nano-encapsulated and unencapsulated geraniol applied to *S. Typhimurium* and *E. coli* O157:H7 are provided in Table 2. The MICs for nano-encapsulated geraniol were lower than those obtained for unencapsulated geraniol for both *S. Typhimurium* LT2 and *E. coli* O157:H7 ($p < 0.05$). The MIC of nano-encapsulated geraniol was slightly higher (0.25 wt.%) for *S. Typhimurium* than for *E. coli* O157:H7 (0.2 wt.%). Similar results were observed with respect to difference in pathogen-specific MICs for cells exposed to unencapsulated geraniol, where MICs of free EOC were 0.8 and 0.4 wt.% against *S. Typhimurium* LT2 and *E. coli* O157:H7, respectively. The MBC for unencapsulated geraniol did

not differ between pathogens, though the MBC for the nano-encapsulated geraniol was lower for *E. coli* O157:H7 (0.4 wt.%) as compared to *S. Typhimurium* (0.8 wt.%) ($p<0.05$) (Table 2).

Antimicrobial EOC in NPs inhibited pathogen growth at lower geraniol concentrations as compared to unencapsulated geraniol. Similar decreases in MICs of plant-derived antimicrobials following nano-encapsulation were reported for cinnamon bark extract-containing poly-D,L-lactide co-glycolide (PLGA) nanoparticles tested against *S. Typhimurium* and *L. monocytogenes*, as well as for cinnamon bark extract, clove bud extract, *trans*-cinnamaldehyde, and eugenol entrapped within β -cyclodextrin inclusion complexes (Hill et al., 2013a; Hill et al., 2013b). Our findings are also in accord with others reporting greater susceptibility of *E. coli* O157:H7 to free and nano-encapsulated plant phenolic acids as compared to *Salmonella* (Gutierrez et al., 2008; Ravichandran et al., 2011). Other authors have previously theorized that nano-entrapment within polymeric nanocapsules of plant-derived antimicrobials (including geraniol) enhances their interaction with pathogenic cells through allowing greater suspension of active compound(s) in aqueous medium as well as limiting interactions with medium components that would degrade antimicrobial activity (e.g., partitioning within fat phases in emulsified foods, oxidation, etc.) (Liolios et al., 2009; Mourtzinis et al., 2008). Although we did not complete studies determining the impact of NP size on resulting antimicrobial activity, previous research has demonstrated that surfactant-based micro-emulsions with larger particle size have resulted in enhanced microbe inhibition (Terjung et al., 2012). These and others have reported that increased NP diameter provides for increased EOC loading and greater antimicrobial effect, observed as reduced MIC or lower counts of surviving microbial cells (Shakeri et al., 2014). Given these results, it is likely that use of NPs described here in antimicrobial assays (Sections 2.7-2.9) would have produced

lesser observed antimicrobial activity versus those reported here utilizing 1.0:1.0 (geraniol:PF127)-made NPs.

Nano-encapsulated geraniol reduced populations of both pathogens on the surfaces of spinach leaves, though reductions achieved were variable (Figure 4). The more effective method of NP delivery to pathogens inoculated onto spinach was through immersion. For *S. Typhimurium*, 2 and 5 min immersion produced reductions in pathogen numbers of 3.2 ± 1.7 and $4.2 \pm 1.2 \log_{10} \text{CFU/cm}^2$, respectively. For *E. coli* O157:H7, immersion for 5 min in NP-containing fluid resulted in a reduction in pathogen numbers of $4.2 \pm 1.5 \log_{10} \text{CFU/cm}^2$, while 2 min immersion produced only a $3.0 \pm 2.0 \log_{10} \text{CFU/cm}^2$ reduction in the pathogen (Figure 5). Application of EOC-containing NPs via spraying produced pathogen reductions not exceeding $1.5 \log_{10} \text{CFU/cm}^2$, indicating reduced antimicrobial efficacy of NPs or reduced contact between pathogen cells attached to spinach surfaces and antimicrobial EOC. Data indicate that while some pathogen decontamination may have occurred by a simple washing action via water used for NP application, previous research has reported that pathogen reductions on produce commodity surfaces via water washing produce $\log_{10}$ -cycle reductions of 0.7 to 1.5 (Alvarado-Casillas et al., 2007; Burnett and Beuchat, 2001; Gragg and Brashears, 2010; Martínez-González et al., 2011; Parish et al., 2003; Van Haute et al., 2015).

Reductions in pathogen numbers observed in the current study are similar to findings from previous research exploring the capacity of plant-derived antimicrobials to decontaminate inoculated leafy green vegetables. Viazis et al. (2011) reported that application of 0.5% *trans*-cinnamaldehyde dispersed in TSB produced a $3.3 \log_{10}$ -cycle reduction in *E. coli* O157:H7 numbers on baby spinach leaves following 10 min exposure at 23°C. Application of 10% emulsified clove oil, as well as 5% and 10% zataria oil extract, produced reductions of 2.5-3.5

log₁₀-cycles in *E. coli* O157:H7 on surfaces of baby-leaf salad vegetables after 5 days of storage at 7°C (Azizkhani et al., 2013). Orue et al. (2013) reported reductions in numbers of *Salmonella*, *Shigella sonnei*, and *E. coli* on spinach following 20 min exposure to various essential oil mixtures ranged from 2.0 to 3.0 log₁₀ CFU/g. These researchers also reported that *E. coli* O157:H7 reductions were greater than those of *Salmonella*, similar to findings presented in the current study (Table 2; Figure 4).

E. coli O157:H7 cells were exposed to fluorescent geraniol-loaded NPs to characterize nanoparticle absorption and interaction with bacterial membrane lipids by confocal microscopy. Fluorescence was detected in cells incubated with NPs, while no fluorescent signal was detected from cells incubated with free Nile Red (Figure 5). Non-entrapped Nile Red was not absorbed into cell membranes and showed aggregation (Fig. 5A). However, NP-encapsulated Nile Red and geraniol was taken up into the internal compartments of *E. coli* O157:H7 cells (Fig. 5B). These results suggest that encapsulation of plant-derived EOC such as geraniol can indeed enhance their bio-availability and the transport of EOC, which may explain observed reductions in pathogen-specific MICs for encapsulated versus free geraniol (Table 2).

A rapid nano-precipitation method for encapsulating the plant-derived terpene geraniol in the polymer Pluronic® F-127 was demonstrated to produce a unimodal population of NPs, with variable particle hydrodynamic size that differed according to PF127:geraniol mixing ratio. Geraniol release against dialysis water followed an exponential release kinetic, with 50% of drug being released within the first 7.25 hr of storage at 25°C. Nano-encapsulation of geraniol enhanced antimicrobial activity against the enteric pathogens *S. Typhimurium* and *E. coli* O157:H7 by lowering the required amount of EOC necessary for inhibition through improved transport of EOC to pathogen membranes.. Further, immersion application of antimicrobial NPs

to spinach surfaces produced greater reductions in pathogen numbers compared to spraying of NPs onto spinach. Geraniol-loaded NPs may serve as useful delivery vehicles for the post-harvest decontamination of fresh produce from cross-contaminating bacterial pathogens.

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6.0 Figure Captions

Figure 1: Process for preparation of geraniol-loaded Pluronic® F-127 polymeric nanoparticles.

Figure 2: Size distribution for geraniol-loaded nanoparticles obtained by dynamic light scattering (DLS) analysis (a), and TEM micrograph of typical geraniol-loaded nanoparticles (b). DLS analysis was carried out at 25°C with 4X-diluted nanoparticles in milli-Q water; a 90° incident angle was used for application of light.

Figure 3: Geraniol release from nanoparticles stored at 25°C as a function of time. Symbols depict means from four independent replications while error bars depict one sample standard deviation (n=4).

Figure 4: Least squares of mean reductions of *Salmonella* Typhimurium (open bars) and *Escherichia coli* O157:H7 (filled bars) on spinach by application of nano-encapsulated geraniol. Error bars indicate standard error about means (SEM). Mean populations of *E. coli* O157:H7 and *S. Typhimurium* LT2 on spinach prior to antimicrobial exposure were 6.2 ± 0.7 and 6.0 ± 0.5 $\log_{10}$ CFU/cm², respectively. Means for sharing same superscripted letters do not differ at p=0.05 (n=4). Limit of detection was 1.0 $\log_{10}$ CFU/cm². **1 Spray: application of 1.0 ml antimicrobial nanoparticle via spray; 2 spray: application of 2.0 ml antimicrobial nanoparticle via two sequential 1.0 ml sprays; 3 spray: application of 3.0 ml antimicrobial nanoparticle via three sequential 1.0 ml sprays. 2, 5 min immerse: immersion of pathogen-inoculated spinach for 2 or 5 min in geraniol-loaded nanoparticle buffer (20.0 ml).**

Figure 5: Exposure of *E. coli* O157:H7 cells to 0.001% free (a) or nanoparticle-loaded Nile Red (b). Fluorophore excitation was at 561 nm, with emission scanned from 579-624 nm.

567 *Table 1: Impact of nanoparticle formulation on resulting particle size.*

Geraniol:PF127 ratio	Particle Size ^a (nm)
0.25:1.0	25 ± 2A
2.0:0.5	155 ± 3B
1.0:0.25	290 ± 6CD
0.6:1.0	315 ± 6D
1.0:1.0	412 ± 25E

568 ^a Values are means from three identically completed replicates (n=3) ± one standard deviation
569 from mean. Means not sharing letters (A, B, C) differ, determined by analysis of variance
570 (ANOVA) and Tukey's Honestly Significant Differences (HSD) test at p=0.05.

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Table 2: Least squares means of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of free and polymeric NP-encapsulated geraniol against foodborne bacterial microbes.

	<i>E. coli</i> O157:H7		<i>Salmonella</i>	
	Encapsulated	Unencapsulated	Encapsulated	Unencapsulated
MIC ^a (wt. %)	0.20A ^d	0.40B	0.25A	0.70B
MBC ^b (wt. %)	0.40A	0.8B	0.8A	0.8A
Reduction ^c (log ₁₀ CFU/mL)	4.5±0.1A	4.5±0.1A	4.4±0.1A	4.6±0.2A

^aMICs are defined as the lowest concentration of free or encapsulated geraniol producing <0.05 change in baseline-corrected optical density at 630 nm after 24 h incubation at 35°C from two independent replications with duplicate identical samples per replicate (n=4).

^bMBCs are the lowest concentration of free or encapsulated geraniol producing ≥3.0 log₁₀-cycle decrease in numbers of bacterial organisms (calculated as log₁₀-transformed plate count of bacterial cells prior to microplate inoculation – log₁₀-transformed plate count of bacterial cells following incubation of inoculated Petri dish from pathogen-inhibiting microplate well) across two independent replications with duplicate identical samples completed per replicate (n=4).

^cValues depict mean log₁₀ reductions in pathogen numbers from two independent replications, with duplicate identical samples per replicate (n=4), ± one standard deviation. Log₁₀ reductions were determined as the difference in pathogen-specific log₁₀-transformed plate counts of inoculated cells – surviving cells following completion of bactericidal activity assay.

^dValues are means of duplicate identical replicates, with two identical samples per replicate (n=4). MIC and MBC means for encapsulated versus unencapsulated geraniol within a row not

601 sharing common letters (A, B) after the mean MIC or MBC differ for each pathogen (*E. coli*
602 O157:H7, *Salmonella*) at $p < 0.05$.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5