



Enhancing the antimicrobial activity of D-limonene nanoemulsion with the inclusion of ϵ -polylysine



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ABSTRACT

The objective of this research was to investigate the synergism between ϵ -polylysine and D-limonene and develop a novel nanoemulsion system by merging the positive effect of these two antimicrobial agents. Results from the checkerboard method showed that ϵ -polylysine and D-limonene exhibit strong synergistic and useful additive effects against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Saccharomyces cerevisiae*. In addition, D-limonene nanoemulsion with the inclusion of ϵ -polylysine was successfully prepared by high pressure homogenizer technology. Its antimicrobial efficiency was compared with pure D-limonene nanoemulsion by measuring the minimal inhibitory concentration, electronic microscope observation and the leakage of the intercellular constituents. The results demonstrated a wide improvement of the antimicrobial activity of D-limonene nanoemulsion following the inclusion of ϵ -polylysine. Overall, the current study may have a valuable contribution to make in developing a more efficient antimicrobial system in the food industry.

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1. Introduction

The survival of microorganisms in food has for decades posed a major public health concern worldwide (Settanni et al., 2012). There are persistent concerns about food safety because of the occurrences of new food-borne disease caused by pathogenic microorganisms (Powella, Jacoba, & Chapmanb, 2011). The food industry has continuously used a wide range of synthetic antimicrobial agents to inhibit the growth of these pathogens (Bajpai, Baek, & Kang, 2012). However, their use has been recognized to cause several hazards to human being health, including respiratory allergies, and a rise in carcinogens and toxic substances (Gutierrez, Barry-Ryan, & Bourke, 2009; Ho, Ishizaki, & Tanaka, 2000). Thus, natural antimicrobial agents (such as: ϵ -polylysine and D-limonene) have become a priority in the food industry in terms of improving the safety of food products by acting against food-borne pathogens (Najjar, Kashtanov, & Chikindas, 2007).

ϵ -Polylysine (ϵ -PL) is a cationic homo-polyamide of 25–30 lysine residues with an amide linkage between 3-amino and a carboxyl function (Shih, Shen, & Van, 2006; Szókan et al., 1997). This peptide was initially isolated from a *Streptomyces albulus* sp. *Lysinopolymerus* strain (Shih et al., 2006). ϵ -PL displayed a high

water solubility, high thermal stability (even when it was autoclaved for 20 min at 120 °C) and low toxicity (Hiraki et al., 2003; Yoshida & Nagasawa, 2003). It is usually used as an antimicrobial food additive due to its broad spectrum of antimicrobial activity against Gram-positive and negative bacteria, yeasts and moulds (Geornaras, Yoon, Belk, Smith, & Sofos, 2007; Najjar et al., 2007). In 2003, ϵ -PL was approved by the food and drug administration (FDA, USA) as a safe food additive and has since been widely used in Japan (Hiraki et al., 2003).

D-Limonene is the main compound contained in all citrus-derived essential oils (EOs). It is widely used as a flavouring agent in commercial applications such as the food and beverage industry due to its transparency and its pleasant citrus fragrance. It is tabulated in the code of federal regulations as generally considered safe (GRAS) for food preservation (Sun, 2007). In addition, D-limonene has aroused the interest of many researchers due to its wide spectrum of antimicrobial activities, making it a promising antimicrobial agent (Chikhounne, Hazzit, Kerbouche, Baaliouamer, & Aissat 2013; Van Vuuren & Viljoen, 2007), and as a green solvent for the extraction of natural products (Inamuddin & Mohammad, 2012). However, this compound undergoes oxidation under standard storage conditions, resulting in the loss of its lemon-like taste (Li & Chiang, 2012). Its oxidative degradation begins by the formation of D-limonene hydroperoxide and then progresses to scission reactions that produce epoxides, ketones and alcohols (Nguyen, Campi, Roy Jackson, & Patti, 2009). Furthermore, its hydrophobicity

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is another limitation of its application in the field of water-rich surfaces or liquid-solid interfaces (Sootitawatt, Yoshii, Furuta, Ohkawara, & Linko, 2003). Due to these two drawbacks, the utilization of α -limonene in food requires the application of an elevated concentration in order to obtain an adequate antimicrobial activity.

In order to minimize the oxidation degradation and hydrophobicity of α -limonene, nanoemulsion technology has been widely used for its encapsulation (Donsì, Annunziata, Sessa, & Ferrari, 2011; Li & Chiang, 2012). Nanoemulsions are very efficient in terms of improving the stability and increasing the disruption of encapsulated compounds (Weiss, Gaysinsky, Davidson, & McClements, 2009). Furthermore, it is believed that the same encapsulated component displays higher antimicrobial activity compared to the bulk form. This might be due to tiny droplets of the formulation that can easily cross the bacterial cells and destabilize the lipid envelope of the treated microorganisms (Baker, Hamouda, Shih, & Myc, 2003; Zahi, Liang, & Yuan, 2015). On the other hand, novel combination of antimicrobial agents has also been extensively used in order to improve their antimicrobial activity. It has been reported that combinations of EOs with other EOs or with natural antimicrobial agents could achieve a strong inhibitory effect at very low dosages and efficiently reduce the negative sensory impact on food (Govaris, Solomakos, Pexara, & Chatzopoulou, 2010; Moosavy et al., 2008).

To the best of our knowledge, the combination of ϵ -PL with α -limonene and the formation of a α -limonene with the inclusion of ϵ -PL have not been investigated until now. Thus, the purpose of this study was to evaluate the antimicrobial efficacy of ϵ -PL and α -limonene combination against four food-borne pathogens, and, further, to develop a novel nanoemulsion with a high inhibitory effect by combining the positive effect of these two compounds.

2. Materials and methods

2.1. Chemicals

ϵ -PL ($C_6H_{12}N_2O_n$) was obtained from Lanzhou Weiri Bio-Engineering Co., Ltd., (Lanzhou, China). α -Limonene was purchased from Floride Worldwide Citrus Products Group Inc. (Bradenton, Florida, USA). Nonionic surfactant (Tween 80) and kanamycin sulfate were obtained from the Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). The microbial strains were purchased from the China General Microbiological Culture Collection Center (Beijing, China). Slants of nutrient agar for bacteria and Yeast Peptone Dextrose for yeast were obtained from (NA, Abxing, Beijing, China).

2.2. Microorganisms and growth conditions

The Gram-negative bacteria *Escherichia coli* ATCC 8739, the Gram-positive bacteria *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, and the yeast *Saccharomyces cerevisiae* ATCC 9763 were used to evaluate the antimicrobial properties. All of the strains were kept at 4 °C on their appropriate slants. The active microbial cultures were prepared by transferring a single loop of cells from the agar slant to a test tube containing 5 ml of Nutrient Agar and Yeast Peptone Dextrose for bacteria and yeast, respectively. The bacterial and yeast cultures were then incubated overnight at 37 °C for bacteria, and 30 °C for the yeast. The turbidity of the strains' suspension was measured at 600 nm using a UV-visible spectrophotometer (Ultraspex 2450, SHIMADZU Ltd., Japan) and adjusted to the optimal concentration (1×10^8 CFU/ml) (Firuzi, Asadollahi, Gholami, & Javidnia, 2010).

2.3. Nanoemulsions preparation

The preparation of α -limonene nanoemulsions with or without ϵ -PL was assessed by high pressure homogenization technology. The emulsions were prepared by weighing α -limonene (4%, w/w), Tween 80 (10%, w/w) and Milli-Q water (86%, w/w) into a container and mixing them together using a high blending homogenizer for 5 min at 24,000 rpm. The obtained coarse emulsions were then passed through a high pressure homogenizer (AH-BASIC, Shanghai, China) for 10 passes at 100 MPa to form the nanoemulsions. Using the above method, distinct nanoemulsions were prepared by varying the content of ϵ -PL in the water to 0.5%, 1%, 2% (w/w).

2.4. Particle size measurements

The mean droplet diameters and particle size distributions of the formulations were measured using dynamic light scattering. The analyses were performed on an average of 10 scans at a scattering angle of 90° and every sample was diluted approximately 1000 fold with Milli-Q water. The measurements were repeated in triplicate for each sample.

2.5. Antimicrobial activity experiments

2.5.1. Determination of the minimal inhibitory concentration (MIC)

The minimal inhibitory concentration of the two antimicrobial agents and nanoemulsions was assessed as described by Weerakkody, Caffin, Turner, and Dykes (2010) with minor modifications. Briefly, a series of the tested samples were prepared in 10 ml sterile test tubes by two-fold dilution with Nutrient Agar, and Yeast Peptone Dextrose, for bacteria and yeast, respectively. After that, microbial suspensions ($400 \mu\text{l}$, 1×10^8 CFU/ml) were added to each test tube. In addition, a positive control containing 50 $\mu\text{g/ml}$ of Kanamycin sulfate and a negative control containing the microbial suspensions alone were also prepared. The MIC was ascribed as the lowest concentration in the serial dilution of the samples resulting in the lack of visible microorganism growth after 24 h and 48 h for the bacteria and the yeast, respectively (Lv, Liang, Yuan, & Li, 2011; Zahi et al., 2015).

2.5.2. Synergism testing: checkerboard method

The checkerboard method is commonly used to determine the *in vitro* interactive inhibition of the bioactive compounds (Hemaiswarya, Kruthiventi, & Doble, 2008). In the present study, this method was used to evaluate the antimicrobial efficiency of ϵ -PL and α -limonene combinations. In brief, ϵ -PL was diluted two-fold in the vertical orientation, whereas α -limonene was diluted in the horizontal one. Their respective concentrations were 1/2, 1/4, and 1/8 in terms of MIC values. Next, 400 μl of freshly prepared microbial suspensions were added to each tube. The tubes were then incubated overnight at 37 °C and 30 °C for the bacteria and yeast, respectively, and their microbial growth was evaluated. In order to examine the antimicrobial effect of ϵ -PL and α -limonene combinations, the checkerboard method was associated with calculation of the minimal inhibitory concentrations of ϵ -PL and α -limonene (alone and in combination) and the fractional inhibitory concentration index (FICI). This last was calculated as (FICA + FICB), where (FICA = MICA of the combination/MICA alone) for ϵ -PL, and (FICB = MICB of the combination/MICB alone) for α -limonene. The data was interpreted as: synergy (FICI ≤ 0.5), addition ($0.5 < \text{FICI} \leq 1$), indifference ($1 < \text{FICI} \leq 4$), and antagonism (FICI > 4).

2.6. Mechanism of the antimicrobial action against the tested cells

2.6.1. Scanning electron microscopy (SEM)

SEM studies were established as reported by Moosavy et al. (2008) with some modifications. Briefly, microbial suspensions (1×10^8 CFU/ml) were prepared in test tubes and mixed with α -limonene nanoemulsions, with or without ϵ -PL, to the required MIC values. A negative control without the nanoemulsions was also prepared. The tubes were then incubated at 25 °C for 3 h. After that, microbial cells were collected by centrifugation at 5000g for 10 min. They were then rinsed twice with 0.1 M (PBS, PH = 7.0) buffer solution and re-suspended in PBS solution containing glutaraldehyde (2.5%, w/w) and stored at –4 °C for 4 h to fix the cells. Later, the fixed cells were harvested by centrifugation and dehydrated in water/ ethanol solutions (30–100%, w/w) for 15 min each. Finally, the cells were placed onto a support and analyzed by a scanning electron microscope.

2.6.2. Leakage of the intercellular constituents

The leakage of cell constituents into their supernatants was evaluated according to the procedure of Rhayour, Bouchikhi, Tantaoui-Elaraki, Sendide, and Remmal (2003) with minor modifications. Microbial suspensions of the four tested microorganisms (100 ml, 1×10^8 CFU/ml) were prepared and the cells were harvested by centrifugation at 5000 g for 10 min. The cells were then rinsed three times with 0.1 M (PBS, PH = 7.0) buffer solution, and re-suspended in 100 ml of the PBS solution. Next, the cells solutions were treated with α -limonene nanoemulsion with the inclusion of ϵ -PL at three distinct concentrations (control, MIC, and $2 \times$ MICs) and incubated at 37 °C and 30 °C for the bacteria and the yeast respectively, for 1 h. After incubation, 2 ml of each suspension were centrifuged at 12,000 g for 2 min and 1 ml of the supernatant was collected and analyzed by UV at 260 nm to measure the content of the cell constituents' leakage.

2.7. Statistical analysis

All of the experiments were carried out in triplicate on freshly prepared samples and are recorded as mean \pm standard deviation for the measurements.

A statistical package (SPSS, version 12.0 for windows, SPSS Inc., Chicago IL) was used for the data analysis.

3. Results and discussions

3.1. Antimicrobial efficiency of ϵ -PL and α -limonene

The MIC values of ϵ -PL and α -limonene against the tested microorganisms are presented in Table 1. ϵ -PL displayed a good inhibitory effect with respect to the growth of all microorganisms with an MIC value of 50 μ g/ml against *E. coli*, *S. aureus*, and *S. cerevisiae*. However, this compound was more efficient with respect to

the growth of *B. subtilis* with a MIC score of 25 μ g/ml. These results are in agreement with the findings of Hiraki (2000) who reported that MIC values of ϵ -PL against a wide range of bacteria were less than 100 μ g/ml. Similarly, Zhou et al. (2011) reported that the MICs values of ϵ -PL against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Serratia marcescens* and *Fusarium solani* were less than 100 μ g/ml, and that the fungus was more resistant than bacteria. On the other hand, α -limonene exhibited a higher inhibitory effect compared to ϵ -PL, which is evident from the low MIC value (1 μ g/ml) against *E. coli*, *S. aureus* and *B. subtilis*. In addition, the most effective antimicrobial activity was shown against *S. cerevisiae* with an MIC score of 0.5 μ g/ml.

3.2. Synergism testing between ϵ -PL and α -limonene

ϵ -PL has been synergistically tested with a wide range of antimicrobial agents, including carvacrol, nisin, lauric arginate ester, acidic calcium sulfate, and caprylic acid, to investigate any possibility of reducing undesirable effects on the organoleptic properties of food. The results showed that ϵ -PL had a good synergistic effect in combination with the majority of the tested components (Brandt et al., 2010; Moschonas et al., 2012). In the current study, the synergistic effect between ϵ -PL and α -limonene against the four food-borne pathogens was investigated by the checkerboard test, and the FICI results are presented in Table 2. According to the FICI values, ϵ -PL and α -limonene exhibited a useful additive effect with an FIC of 0.625, and 0.5, for *S. aureus* and *S. cerevisiae*, respectively. In addition, a synergistic effect (FICI = 0.375) was found with regards to both *E. coli* and *B. subtilis*. In contrast, neither non-antagonism nor indifference effects were found in this experiment, which may be due to the good synergism between these two antimicrobial agents.

3.3. The effect of ϵ -PL concentration on the α -limonene nanoemulsions formation

The impact of ϵ -PL concentration on the formation and stability of a α -limonene nanoemulsion was investigated. The nano-dispersions were prepared using a high pressure homogenization method. Their respective mean droplet diameters are shown in Fig. 1A. The droplet diameters of the four nanoemulsions were 12.21 nm, 12.40 nm, 13.29, and 15.65 nm for 0% (control), 0.5%, 1% and 2% ϵ -PL respectively. The inclusion of ϵ -PL at different concentrations contributed toward a slight increase of droplets size. This increase is may be attributed to the high molecular weight of ϵ -PL, but it does not greatly affect the formation of the nanoemulsion. In addition, the obtained droplets are very small, and could meet with the requirement of good nanoemulsions. α -Limonene nanoemulsion prepared with the inclusion ϵ -PL (2% ϵ -PL, 4% α -limonene w/w) exhibited good stability. Samples stored at room temperature for about three months did not show any phase separation (Fig. 1B).

3.4. A comparative study between the antimicrobial activity of α -limonene nanoemulsion with or without ϵ -PL

To evaluate the effect of ϵ -PL on the antimicrobial activity of the α -limonene nanoemulsion, the experimental work was extended to measure the MIC values of the α -limonene nanoemulsion with, or without, ϵ -PL against the test microorganisms, and the results are presented in the Table 3. As shown in the table, the MIC values of the α -limonene nanoemulsion were all lower than those of the bulk α -limonene. These results are in agreement with Karthikeyan, Amaechi, Rawls, and Lee (2012) and Sugumar, Nirmala, Anjali, Mukherjee, and Chandrasekaran (2012), which

Table 1
The minimal inhibitory concentration of ϵ -PL and α -limonene against the four target microorganisms' tested.^a

Microorganisms	MIC ^b		
	ϵ -Polylysine	α -limonene	KS ^c
<i>E. coli</i>	50	1	0.156
<i>S. aureus</i>	50	1	0.3125
<i>B. subtilis</i>	25	1	0.3125
<i>S. cerevisiae</i>	50	0.5	0.3125

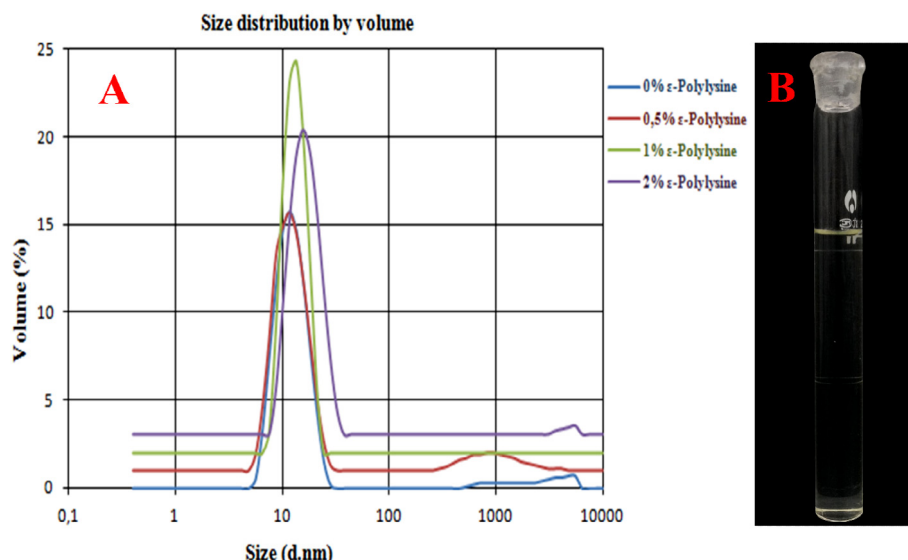
^a Results are means of three replicates.

^b MIC: minimal inhibitory concentration (μ g/ml).

^c KS: Kanamycin sulfates (50 μ g/ml).

Table 2FIC (FICI) of ϵ -PL and D-limonene combination against the microorganisms' tested.^a

Combination	<i>E. coli</i>		<i>S. aureus</i>		<i>B. subtilis</i>		<i>S. cerevisiae</i>	
	FIC	FICI	FIC	FICI	FIC	FICI	FIC	FICI
ϵ -Polylysine-D-limonene	0.25 0.125	0.375(S) ^b	0.5 0.125	0.625(A)	0.25 0.125	0.375(S)	0.25 0.25	0.5(A)

^a Results are the mean of three different experiments.^b The results were interpreted as: synergy (S, FICI <0.5), addition (A, 0.5 ≤ FICI ≤ 1), indifference (I, 1 ≤ FICI ≤ 4), and antagonism (AN, FICI ≥ 4).**Fig. 1.** Particle size distribution of the D-limonene nanoemulsions prepared with different amounts of ϵ -PL (0%, 0.5%, 1%, and 2%) (A), and the typical picture of the D-limonene nanoemulsion with ϵ -PL (2% ϵ -PL, 4% D-limonene w/w) after 3 months of storage at 28 °C (B).**Table 3**Impact of ϵ -PL on the MIC values of D-limonene nanoemulsion against the four microorganisms tested.

Microorganisms	MIC ^a			
	ϵ -Polylysine	D-limonene	D-limonene nanoemulsion	(ϵ -Polylysine, D-limonene) nanoemulsion
<i>E. coli</i>	50	1	15 (0.6) ^b	1.69 ^c (0.067 ^d , 0.033 ^e)
<i>S. aureus</i>	25	1	7.5 (0.3)	3.39 (0.135, 0.067)
<i>B. subtilis</i>	50	1	15 (0.6)	1.69 (0.067, 0.033)
<i>S. cerevisiae</i>	50	0.5	7.5 (0.3)	3.39 (0.135, 0.067)

^a MIC of D-limonene nanoemulsion against the bacteria.^b The real content of the D-limonene on the nanoemulsion.^c MIC of the D-limonene nanoemulsion with the inclusion of ϵ -PL.^d The real content of ϵ -PL in the D-limonene nanoemulsion with the inclusion of ϵ -PL.^e The real content of D-limonene in the D-limonene nanoemulsion with the inclusion of ϵ -PL.

confirms that the antimicrobial efficiency can be increased using nanoemulsion technology. On the other hand, D-limonene nanoemulsion with ϵ -PL (2% ϵ -PL, 4% D-limonene w/w) exhibited a higher antimicrobial activity than the nanoemulsion without ϵ -PL, with MIC values of 0.067 μ g/ml against both *E. coli* and *B. subtilis*, and 0.135 μ g/ml against both *S. aureus* and *S. cerevisiae*. The most efficient inhibitory effect was mainly shown with respect to *E. coli* and *B. subtilis*, which is evident from the good synergism between these two compounds. Moreover, not only the MIC scores of D-limonene were lower following the inclusion of ϵ -PL into the D-limonene nanoemulsion, but the MIC scores of ϵ -PL were also inferior compared to the unprocessed one (Table 3). The combination of ϵ -PL and D-limonene was more efficient in terms of enhancing the antimicrobial proprieties against the tested microorganisms. Our results might have an appreciable application in terms of minimizing the amount of the antimicrobial agents in food matrices.

3.5. Mechanism of D-limonene with ϵ -PL nanoemulsion against the cells tested

The investigations on the chemical composition of EOs have shown that terpenes, phenols, ketones and aldehydes are responsible for their antimicrobial capability (Ceylan & Fung, 2004). It is also believed that these active components might bind the cell cytoplasm membranes, and then penetrate to the phospholipid bilayer (Gill & Holley, 2006). This can lead to the disruption of the proteins, DNA, RNA or polysaccharides, resulting in the death of the target microorganisms (Rhayour et al., 2003; Wu, Qiu, De Los Reyes, Lin, & Pan, 2009). In addition, some researchers have suggested that the leakage of vital intracellular constituents is directly related to the distortion of the cell walls and their cytoplasmic membranes leading to a colossal destabilization of the membrane and causing cell death (Kim, Marshall, Cornell, Preston, & Wei, 1995). The main mechanisms of action of ϵ -PL

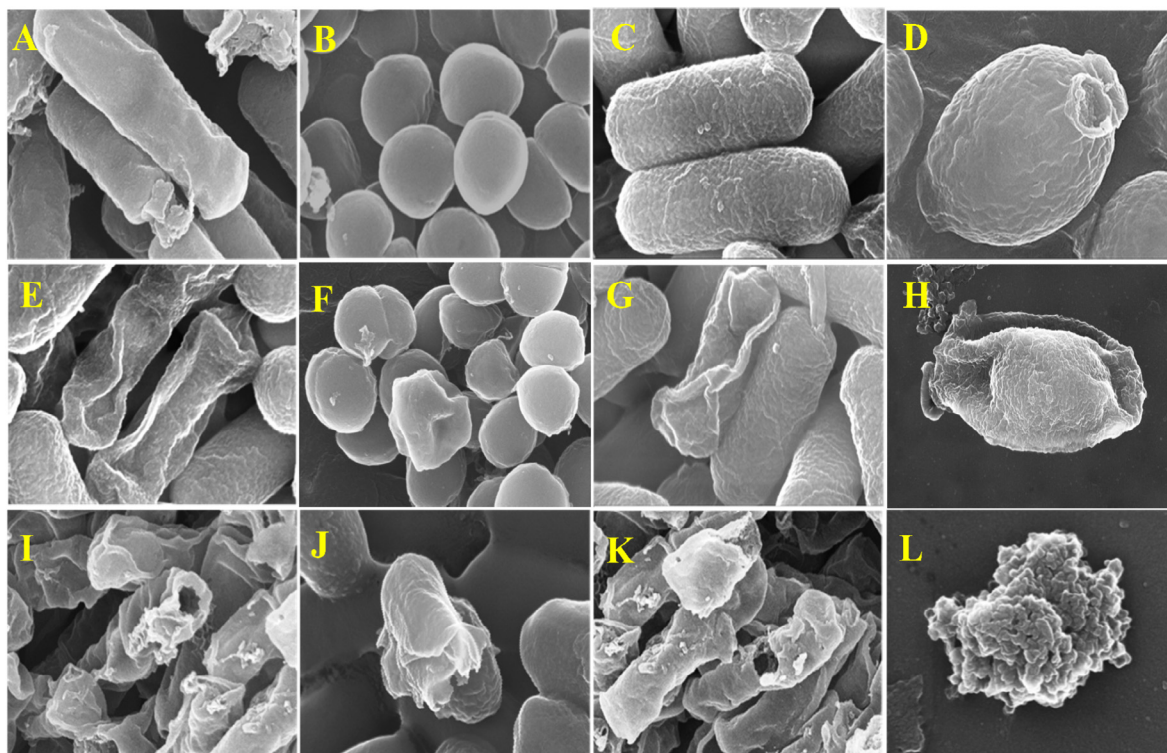


Fig. 2. Scanning electron micrographs of *E. coli* (A, E, and I), *S. aureus* (B, F, and J), *B. subtilis* (C, G, and K) and *S. cerevisiae* (D, H, and L) cells, (A, B, C, and D) Control, (E, F, G, and H) treated with *D*-limonene nanoemulsion, (I, J, K, and L) treated with *D*-limonene nanoemulsion with the inclusion of ϵ -PL (2% ϵ -PL, 4% *D*-limonene w/w) at MIC values for 3 h, (magnification $\times 15,000$, $20,000$ or $50,000$).

and *D*-limonene are against the cytoplasmic membranes of microorganisms, causing a disturbance in membrane integrity, thereby changing its permeability and resulting to the leakage of some cellular components such as ions and proteins; dissipation of the protons' motive-force; and the inhibition of respiratory enzymes (Li, Han, Feng, Tian, & Mo, 2014; Sun, 2007). With the aim of observing the effect of *D*-limonene nanoemulsion with or without ϵ -PL on the structural morphology of the tested microorganisms, SEM of the cells treated with the *D*-limonene nanoemulsion (4% *D*-limonene) or *D*-limonene nanoemulsion with ϵ -PL (2% ϵ -PL, 4% *D*-limonene w/w) at their respective MIC values was employed, and the results are presented in Fig. 2. As is clearly shown in the figure, the untreated cells (control) had a spherical structure for *S. aureus* and *S. cerevisiae* (Fig. 2B and D) and a bacilliform structure for *E. coli* and *B. subtilis* (Fig. 2A and C). However, the photomicrographs of the cells treated with *D*-limonene nanoemulsion (without ϵ -PL) show different forms of distortion and deformation (Fig. 2E–H). In contrast, the four microorganisms treated by *D*-limonene nanoemulsion with ϵ -PL endured a complete collapse of the cells' morphology accompanied by an intercellular leakage (Fig. 2I–L). These results provide evidence that the *D*-limonene nanoemulsion with ϵ -PL is endowed with a stronger antimicrobial activity against the tested microorganisms compared to *D*-limonene nanoemulsion.

In addition, it is worth noting that a measurement of cell leakage markers, such as proteins or the cell constituent leakage at 260 nm, could be a good indicator of membrane integrity to specific antimicrobial agents in relation to unprocessed cells (Bajpai et al., 2012). The cell constituents' leakage following the application of *D*-limonene nanoemulsion with ϵ -PL was estimated by the measurement of the absorbance of the supernatants of the tested microorganisms.

The results indicated that following the addition of *D*-limonene nanoemulsion with ϵ -PL (at MIC, and $2 \times$ MIC) to the

Table 4

The effect of *D*-limonene nanoemulsion with the inclusion of ϵ -PL on the cell constituents' release of the four target microorganisms.

Microorganisms	(ϵ -Polylysine, <i>D</i> -limonene) nanoemulsion concentration	Cells constituents release (OD 260 nm) ^a
<i>E. coli</i>	0	0.064 \pm 0.003 ^b
	MIC	0.224 \pm 0.006
	$2 \times$ MIC	0.620 \pm 0.012
<i>S. aureus</i>	0	0.055 \pm 0.007
	MIC	0.251 \pm 0.014
	$2 \times$ MIC	0.337 \pm 0.019
<i>B. subtilis</i>	0	0.096 \pm 0.002
	MIC	0.191 \pm 0.009
	$2 \times$ MIC	0.732 \pm 0.021
<i>S. cerevisiae</i>	0	0.082 \pm 0.003
	MIC	0.147 \pm 0.005
	$2 \times$ MIC	0.297 \pm 0.017

^a Results are presented as mean \pm standard deviation from the experiments in triplicates. Optical density at 260 nm.

^b Means within the same strain, those which aren't followed by a common letter, they are significantly different ($p < 0.05$).

microorganisms, a rapid loss of the cell constituents occurred and was increased by raising the concentration of the formulation (Table 4). This provides evidence that an increase in the permeability of the cell membranes has been occurred, which is supported by the results of SEM. In addition, the most effective cell constituents' leakage took place following the $2 \times$ MIC treatment of *E. coli* and *B. subtilis*, which gave absorbance readings of 0.620 and 0.732 respectively. These results (Table 4) appear to match well with the damaged cells observed in SEM experiments (Fig. 2I, 2K). These results are in agreement with those previously reported by Lv et al. (2011). The authors have determined that a high leakage of cell constituents indicates that irreversible damage

to the cytoplasmic membranes has occurred. Thus, the results suggest that application of a α -limonene nanoemulsion with the inclusion of ϵ -PL could cause irreversible damage to the cytoplasmic membranes, which might be in accordance with the deteriorated morphology of the treated cells.

4. Conclusion

A novel antimicrobial nano-dispersion system with good stability and high antimicrobial efficiency was successfully developed by the incorporation of ϵ -PL into a α -limonene nanoemulsion. This formulation has combined the advantages of the synergistic effect of two strong antimicrobial agents and nanoemulsion technology into one system. The use of this delivery system has contributed to the improvement of the antimicrobial efficiency of the α -limonene nanoemulsion, and to the overall reduction in the amount of ϵ -PL and α -limonene required to impose a strong inhibitory effect. These results indicate that a α -limonene nanoemulsion and ϵ -PL could be combined into a food-grade delivery system that can be used for the preservation of food and beverage products.

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References

- Bajpai, V. K., Baek, K. H., & Kang, S. C. (2012). Control of *Salmonella* in foods by using essential oils: A review. *Food Research International*, 45(2), 722–734.
- Baker, J. R., Hamouda, T., Shih, A., & Myc, A. (2003). U.S. Patent No. 6,559,189. Washington, DC: U.S. Patent and Trademark Office.
- Brandt, A. L., Castillo, A., Harris, K. B., Keeton, J. T., Hardin, M. D., & Taylor, T. M. (2010). Inhibition of *Listeria monocytogenes* by food antimicrobials applied singly and in combination. *Journal of Food Science*, 75(9), M557–M563.
- Ceylan, E., & Fung, D. Y. C. (2004). Antimicrobial activity of spices. *Journal of Rapid Methods and Automation in Microbiology*, 12, 1–55.
- Chikhoun, A., Hazzit, M., Kerbouche, L., Baaliouamer, A., & Aissat, K. (2013). *Tetraclinis articulata* (Vahl) Masters essential oils: Chemical composition and biological activities. *Journal of Essential Oil Research*, 25(4), 300–307.
- Donsi, F., Annunziata, M., Sessa, M., & Ferrari, G. (2011). Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *LWT Food Science and Technology*, 44(9), 1908–1914.
- Firuzi, O., Asadollahi, M., Gholami, M., & Javidnia, K. (2010). Composition and biological activities of essential oils from four *Heracleum* species. *Food Chemistry*, 122(1), 117–122.
- Geornaras, I., Yoon, Y., Belk, K. E., Smith, G. C., & Sofos, J. N. (2007). Antimicrobial activity of ϵ -polylysine against *Escherichia coli* O157: H7, *Salmonella Typhimurium*, and *Listeria monocytogenes* in various food extracts. *Journal of Food Science*, 72(8), M330–M334.
- Gill, A. O., & Holley, R. A. (2006). Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. *International Journal of Food Microbiology*, 108, 1–9.
- Govaris, A., Solomakos, N., Pexara, A., & Chatzopoulou, P. S. (2010). The antimicrobial effect of oregano essential oil, nisin and their combination against *Salmonella Enteritidis* in minced sheep meat during refrigerated storage. *International Journal of Food Microbiology*, 137(2), 175–180.
- Gutierrez, J., Barry-Ryan, C., & Bourke, P. (2009). Antimicrobial activity of plant essential oils using food model media: Efficacy, synergistic potential and interactions with food components. *Food Microbiology*, 26(2), 142–150.
- Hemaiswarya, S., Kruthiventi, A. K., & Doble, M. (2008). Synergism between natural products and antibiotics against infectious diseases. *Phytomedicine*, 15(8), 639–652.
- Hiraki, J. (2000). ϵ -Polylysine, its development and utilization. *Fine Chemistry*, 29, 18–25.
- Hiraki, J., Ichikawa, T., Ninomiya, S. I., Seki, H., Uohama, K., Seki, H., & Barnett, J. W. Jr., (2003). Use of ADME studies to confirm the safety of ϵ -polylysine as a preservative in food. *Regulatory Toxicology and Pharmacology*, 37(2), 328–340.
- Ho, Y. T., Ishizaki, S., & Tanaka, M. (2000). Improving emulsifying activity of ϵ -polylysine by conjugation with dextran through the Maillard reaction. *Food Chemistry*, 68, 449–455.
- Inamuddin, M., & Mohammad, A. (2012). *Green solvents I: Properties and applications in chemistry*. Springer.
- Karthikeyan, R., Amaechi, B. T., Rawls, H. R., & Lee, V. A. (2012). Antimicrobial activity of nanoemulsion on cariogenic *Streptococcus mutans*. *Archives of Oral Biology*, 56(5), 437–445.
- Kim, J. M., Marshall, M. R., Cornell, J. A., Preston, J. F., & Wei, C. I. (1995). Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella typhimurium* culture medium and on fish cubes. *Journal of Food Science*, 60, 1364–1368.
- Li, P. H., & Chiang, B. H. (2012). Process optimization and stability of α -limonene-in-water nanoemulsions prepared by ultrasonic emulsification using response surface methodology. *Ultrasonics Sonochemistry*, 19(1), 192–197.
- Li, Y. Q., Han, Q., Feng, J. L., Tian, W. L., & Mo, H. Z. (2014). Antibacterial characteristics and mechanisms of ϵ -poly-L-lysine against *Escherichia coli* and *Staphylococcus aureus*. *Food Control*, 43, 22–27.
- Lv, F., Liang, H., Yuan, Q., & Li, C. (2011). In vitro antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms. *Food Research International*, 44(9), 3057–3064.
- Moosavy, M. B., Basti, A. A., Misaghi, A., Salehi, T. Z., Abbasifar, R., Mousavi, H. A. E., & Noori, N. (2008). Effect of *Zataria multiflora* Boiss. Essential oil and nisin on *Salmonella typhimurium* and *Staphylococcus aureus* in a food model system and on the bacterial cell membranes. *Food Research International*, 41(10), 1050–1057.
- Moschonas, G., Geornaras, I., Stopforth, J. D., Wach, D., Woerner, D. R., Belk, K. E., & Sofos, J. N. (2012). Activity of caprylic acid, carvacrol, ϵ -polylysine and their combinations against salmonella in not-ready-to-eat surface-browned, frozen, breaded chicken products. *Journal of Food Science*, 77(7), M405–M411.
- Najjar, M. B., Kashtanov, D., & Chikindas, M. L. (2007). ϵ -poly-L-lysine and nisin A act synergistically against gram-positive food-borne pathogens *Bacillus cereus* and *Listeria monocytogenes*. *Letters in Applied Microbiology*, 45, 13–18.
- Nguyen, H., Campi, E. M., Roy Jackson, W., & Patti, A. F. (2009). Effect of oxidative deterioration on flavour and aroma components of lemon oil. *Food Chemistry*, 112(2), 388–393.
- Powella, D. A., Jacoba, C. J., & Chapman, B. J. (2011). Enhancing food safety culture to reduce rates of foodborne illness. *Food Control*, 22, 817–822.
- Rhayour, K., Bouchikhi, T., Tantaoui-Elaraki, A., Sendide, K., & Remmal, A. (2003). The mechanism of bactericidal action of oregano and clove essential oils and of their phenolic major components on *Escherichia coli* and *Bacillus subtilis*. *Journal of Essential Oil Research*, 15(4), 286–292.
- Settanni, L., Palazzolo, E., Guarrasi, V., Aleo, A., Mammina, C., Moschetti, G., & Germanà, M. A. (2012). Inhibition of foodborne pathogen bacteria by essential oils extracted from citrus fruits cultivated in Sicily. *Food Control*, 26(2), 326–330.
- Shih, I. L., Shen, M. H., & Van, Y. T. (2006). Microbial synthesis of poly (3-lysine) and its various applications. *Bioresource Technology*, 97, 1148–1159.
- Sootitnantawat, A., Yoshii, H., Furuta, T., Ohkawara, M., & Linko, P. (2003). Microencapsulation by spray drying: Influence of emulsion size on the retention of volatile compounds. *Journal of Food Science*, 68(7), 2256–2262.
- Sugumar, S., Nirmala, J., Anjali, H., Mukherjee, A., & Chandrasekaran, N. (2012). Bio-based nanoemulsion formulation, characterization and antibacterial activity against food-borne pathogens. *Journal of Basic Microbiology*, 52, 1–10.
- Sun, J. (2007). α -limonene: Safety and clinical applications. *Alternative Medicine Review*, 12(3), 259.
- Szókán, G., Almas, M., Krizsan, K., Khlafula, A. R., Tyihak, E., & Szende, B. (1997). Structure determination and synthesis of lysine isopeptides influencing on cell proliferation. *Biopolymers*, 42, 305–318.
- Van Vuuren, S. F., & Viljoen, A. M. (2007). Antimicrobial activity of limonene enantiomers and 1, 8-cineole alone and in combination. *Flavour and Fragrance Journal*, 22(6), 540–544.
- Weerakkody, N. S., Caffin, N., Turner, M. S., & Dykes, G. A. (2010). In vitro antimicrobial activity of less-utilized spice and herb extracts against selected food-borne bacteria. *Food Control*, 21(10), 1408–1414.
- Weiss, J., Gaysinsky, S., Davidson, M., & McClements, J. (2009). Nanostructured encapsulation systems: Food antimicrobials. In *IUFoST world congress book: Global issues in food science and technology* (pp. 425–479).
- Wu, V. C. H., Qiu, X., De Los Reyes, B. G., Lin, C. S., & Pan, Y. (2009). Application of cranberry concentrate (*Vaccinium macrocarpon*) to control *Escherichia coli* O157: H7 in ground beef and its antimicrobial mechanism related to the down regulated *slp*, *hdeA* and *cfa*. *Food Microbiology*, 26, 32–38.
- Yoshida, T., & Nagasawa, T. (2003). ϵ -Poly-L-lysine: Microbial production, biodegradation and application potential. *Applied Microbiology and Biotechnology*, 62(1), 21–26.
- Zahi, M. R., Liang, H., & Yuan, Q. (2015). Improving the antimicrobial activity of α -limonene using a novel organogel-based nanoemulsion. *Food Control*, 50, 554–559.
- Zhou, C., Li, P., Qi, X., Sharif, A. R. M., Poon, Y. F., Cao, Y., et al. (2011). A photopolymerized antimicrobial hydrogel coating derived from epsilon-poly L-lysine. *Biomaterials*, 32, 2704–2712.