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Highlights

- EO type used in the formulation of nanoemulsions had a significant impact on the droplet size of nanoemulsion-pectin system. Nanoemulsions containing lemongrass or mandarin EO presented a significantly small droplet-sized diameter and they were highly stable throughout the study period, and compared with those nanoemulsions containing oregano and thyme EOs which were highly unstable, presenting an increase in the droplet size, at the end of study.
- *E. coli* bacterial cells presented a significantly higher sensitivity to EO-pectin nanoemulsions compared to *L. innocua*.
- Among nanoemulsions obtained, the lemongrass-pectin nanoemulsion had the smallest droplet size ($11 \pm 1\text{nm}$) and it had also a higher antimicrobial activity, reaching a 5.9 log reduction of the *E. coli* population, said antimicrobial activity was corroborated by Transmission Electron Microscopy (TEM) images, where a significant damage in the *E. coli* cells was observed for both the cytoplasm and cytoplasmic membrane, leading to cell death.
- There is a positive correlation between the retention of volatile compounds and the antimicrobial activity of EO-nanoemulsions through the time period (56 days).

Antimicrobial activity of nanoemulsions containing essential oils and high methoxyl pectin during long-term storage

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ABSTRACT

The antimicrobial activity against *Escherichia coli* and *Listeria innocua* of nanoemulsions containing oregano, thyme, lemongrass or mandarin essential oils and high methoxyl pectin was assessed during a long-term storage period (56 days). On one hand, a higher antimicrobial activity was detected against *E. coli* compared to *L. innocua* regardless the EO type. Transmission Electron Microscopy (TEM) images showed a significant damage in the *E. coli* cells for both the cytoplasm and cytoplasmic membrane, led to cell death. The antimicrobial activity of the nanoemulsions was found to be strongly related to the EO type rather than to their droplet size. The lemongrass-pectin nanoemulsion had the smallest droplet size ($11 \pm 1\text{nm}$) and higher antimicrobial activity reaching 5.9 log reductions of the *E. coli* population. Nevertheless, the freshly made oregano, thyme and mandarin EO-pectin nanoemulsion led to 2.2, 2.1 or 1.9 *E. coli* log-reductions, respectively. However, the antimicrobial activity decreased significantly during storage regardless the EO type, which was related to the loss of volatile compounds over time according to our results. The current work provides valuable information in order to make progress in the use of nanoemulsions containing EOs as decontaminating agents in food products.

Keywords

Nanoemulsions, essential oils, stability, antimicrobial properties.

1. Introduction

Essential oils (EOs) are volatile substances obtained from aromatic plant materials (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) (Noorizadeh, Farmany, & Noorizadeh, 2011; Bakkali & Idaomar, 2008), usually extracted by steam vaporization and cold-press techniques (Saad, Muller, & Lobstein, 2013). EOs are commonly used as antioxidants, flavorings or colorants in a wide range of food products (Santin, Oliveira, Cristina, Ferreira, & Ueda-nakamura, 2009; Edris, 2007). Moreover, EOs have been described as strong natural antimicrobial agents for food preservation purposes (Muriel-Galet et al., 2012). The antimicrobial properties of EOs are mainly due to their volatile components, including terpenoids and phenolic compounds (Cosentino et al., 1999). The mechanism of EOs to inactivate food-borne microorganisms relies on their interaction with the microbial membrane. EOs phenolic compounds are known to penetrate through the microbial membrane and cause the leakage of ions and cytoplasmatic content thus leading to cellular breakdown (Burt, 2004; Bajpai, Baek, & Kang, 2012). Several studies have shown that EOs are effective antibacterial agents against a wide spectrum of pathogenic bacterial strains including *L. monocytogenes*, *L. innocua* (Solomakos, Govaris, Koidis, & Botsoglou, 2008), *E. coli* O157:H7, *Shigella dysenteria*, *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella typhimurium* (Saad et al., 2013). However, antimicrobial EOs are rarely used directly in food products as bulk oils since they present limitations such as intense aroma and low water solubility (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2014).

Nanotechnology is a tool used to modify nano-scale material characteristics, in this case, to improve the EOs properties, which can be incorporated as nano-sized delivery systems in order to overcome their limitations (Huang, Yu, & Ru, 2010). A wide variety of

delivery systems have been developed to encapsulate active ingredients, including colloidal dispersions, biopolymer matrices or emulsions (Weiss, Takhistov, & McClements, 2006). Emulsions containing very small oil droplet size are desirable for certain applications since they present advantages over systems containing larger particles. Nanoemulsions are defined as conventional emulsions that contain tiny particles (diameter between 100 to 300 nm) that make them kinetically stable to the particle aggregation and gravitational separation (Qian & McClements, 2011; Tadros, Izquierdo, Esquena, & Solans, 2004). Moreover, due to the reduced droplet sized and therefore weak light scattering, they may be incorporated in optically transparent beverages or certain food products without altering their optical properties (Qian & McClements, 2011). Additionally, nanoemulsions have a much higher surface area of the active ingredients compared to conventional emulsions and therefore present an enhanced functionality when interacting with biological systems (Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013). Microfluidization is a device widely used for the production of food-grade nanoemulsions (Guerra-Rosas, Morales-Castro, Ochoa-Martínez, Salvia-Trujillo, & Martín-Belloso, 2016; Salvia-Trujillo et al., 2014; Lovelyn & Attama, 2011). Salvia-Trujillo et al. (2014) reported an enhanced antimicrobial activity of nanoemulsions produced by microfluidization compared to conventional emulsions with a larger droplet size, which was attributed to the higher penetration of nano-sized droplets through the microbial membrane. Nonetheless, the fate of nanoemulsion functionality after being incorporated in food formulations has been hardly described. On the other hand, the stability of the antimicrobial activity of nanoemulsions during longer periods of time also needs to be evaluated. Therefore, the aim of the present work was to study and compare the antimicrobial activity of nanoemulsions containing EOs (oregano, thyme, mandarin and lemongrass) as oil phase and high-methoxyl pectin solution (1 % w/v) as the aqueous phase

against *E. coli* and *L. innocua* during storage. For that purpose, EO-pectin nanoemulsions were stored at room temperature for 56 days and the antimicrobial activity of such nanoemulsion was assessed at several time intervals in terms of *E. coli* and *L. innocua* population reductions. Moreover, changes in the volatile fraction of EOs during storage was studied and related to the antimicrobial capacity of nanoemulsions. Also changes in the microbial cell structure were assessed by **Transmission Electron Microscopy (TEM)**, a useful technique in biological science for the observation of cellular structures.

2. Materials and methods

2.1. Chemical compounds

Chemical compounds studied in this article: High methoxyl pectin (PubChem CID: 441476); carvacrol (PubChem CID: 10364); thymol (PubChem CID: 6989); citral (PubChem CID: 638011); limonene (PubChem CID: 440917); Tween 80 (PubChem CID: 443315).

2.2. Materials

Food-grade high methoxyl pectin (Unipeptine QC100 from citrus source) was kindly donated by Cargill Inc. (Spain). Oregano (*Origanum compactum*) and thyme (*Thymus vulgare*) EOs were purchased from Dietetica Intersa, S.A. (Spain), whereas lemongrass (*Cymbopogon citratus*) was purchased from Laboratories Dicana, S. L. (Spain), and mandarin (*Citrus reticulata*) was kindly provided by Indulleida, S.A. (Spain). The main volatile active compounds present in each EO used in this study based on bibliographic references is described in Table 1. Tween 80 (Poly oxyethylene sorbitan Monoesterate) (Lab Scharlab, S. L., Spain) was used as non-ionic food-grade surfactant. Ultrapure water obtained

from Millipore water system (Millipore S. A., Molsheim, France) (0.22 μ m) was used for the formulation and analysis of all nanoemulsions.

2.3. Essential oil-pectin nanoemulsions preparation

High methoxyl pectin powder (1 % w/v) was dissolved in hot water at 80-85°C, with continuous stirring until being fully solubilized and it was cooled down to 25 °C. Coarse oil-in-water emulsions were made by mixing pectin solutions as aqueous phase with oregano, thyme, lemongrass or mandarin EOs (2 % v/v) and Tween 80 (5 % v/v) emulsified with a high sheer laboratory mixer Ultraturrax T-25 (IKA, Staufen, Germany) for 2 minutes at 9500 rpm. The final volume was 1000 mL. Afterwards, the coarse emulsion was passed through a microfluidizer device (Microfluidics, Massachusetts, USA) at 150 MPa for 5 cycles. For the stability studies, 15 mL of each nanoemulsion were kept in capped plastic test tubes and stored in the dark at room temperature ($\sim 25 \pm 2$ °C).

Physical, chemical and antimicrobial assays were performed in duplicate immediately after nanoemulsions preparation and every 7 days up to 56 days. Every sampling day, new tubes were used in order to avoid the oxidation or volatilization of the volatile compounds of EOs after opening the plastic tubes.

2.4. Droplet size and droplet size distribution

The average droplet size of nanoemulsions was determined by dynamic-light-scattering (DLS), using a Zetasizer Nano-ZS laser diffractometer (Malvern Instruments Ltd., Worcestershire, UK), working at 633 nm, equipped with a backscatter detector (173°). The DLS measures particle diffusion moving under Brownian-motion. Nanoemulsions were diluted 100 times with milli-Q water to avoid multiple-scattering effect and stirred to ensure

sample homogeneity. The refractive indexes (RI) of the oil phases were measured with a manual refractometer (model J357, Rudolph research, New Jersey, USA) being 1.501, 1.497, 1.484 and 1.475 for the oregano, thyme, lemongrass and mandarin EOs, respectively. The absorbance of the EOs at 633 nm was measured with a spectrophotometer Cecil CE 1021 (Cambridge, England) being 0.002, 0.002, 0.024 and 0.004 for the oregano, thyme, lemongrass and mandarin EOs, respectively. Measurements were made and the droplet size was reported as the volume weighted average diameter (nm).

2.5. Antimicrobial properties of nanoemulsions

In order to evaluate the changes on the antimicrobial activity of nanoemulsions during storage, their bactericidal capacity was assessed against *Escherichia coli* 1.107 and *Listeria innocua* 1.17, as Gram-negative and Gram-positive bacteria, respectively. For that purpose, bacterial cultures were prepared to grow at their stationary phase and then put in contact with nanoemulsions containing the selected EOs.

2.5.1. Bacterial strains and growth conditions

Gram-negative bacteria *E. coli* 1.107 and Gram-positive bacteria *L. innocua* 1.17 (Laboratoire de répression des Fraudes, Montpellier, France) were provided to the culture collection of the Department of Food Technology of the University of Lleida (Spain). Those *E. coli* and *L. innocua* strains were chosen for this study as surrogates of the pathogenic *E. coli* O157:H7 and *L. monocytogenes*.

Stock cultures were kept in inclined test-tubes with Tryptone Soy Agar (TSA) (Biokar Diagnostics, Beauvais, France) at 5 °C until use. A stock culture of *E. coli* 1.107 was grown

in Tryptone Soy Broth (TSB) (Bioakar Diagnostic; Beauvais, France) and incubated overnight at 37 °C on a rotary shaker at 120 rpm for 11 h; while a stock culture of *L. innocua* was grown in TSB containing 0.6% of yeast extract (Biokar Diagnostics; Beauvais, France) at 35 °C and 180 rpm for 15 h, to obtain both cells in stationary growth phase at the moment of carrying out the inoculation. The final concentration reached for *E. coli* and *L. innocua* was between 10⁸-10⁹ colony-forming units per milliliter (CFU/mL).

2.5.2. Antimicrobial activity assay

The antimicrobial activity of EOs-pectin nanoemulsions was assessed evaluating the *in vitro* inhibition of both microorganisms. The method used was the previously described by Ferreira et al. (2010) with some modifications. A 0.5 mL-aliquot of overnight bacterial culture was added to 4.5 mL of sterile Milli-Q water and mixed with 0.5 mL of the EO-pectin nanoemulsions. To determine the inactivation of *E. coli* or *L. innocua* populations, a 100 µL-aliquot of the bacterial suspensions was taken immediately after mixing and after 30 minutes of contact time and diluted in 900 µL of saline peptone water (0.1% peptone, Biokar Diagnostics, Beauvais, France, adding 0.85% NaCl, Scharlau Chemie, S.A. Barcelona, Spain). Further serial dilutions were made and a 100 µL-aliquot was spread on MacConkey agar or Palcam agar containing Palcam Selective Supplement (Biokar Diagnostics, Beauvais, France) plates for *E. coli* or *L. innocua* counts, respectively. The assays were performed in duplicate. Controls for each bacterium were performed in the same way using sterile Milli-Q water instead of nanoemulsion. Plates were incubated for 24 h at 35 ± 2 °C and colonies were counted. Results were expressed as Log₁₀ CFU (Colony-forming unit) per milliliter of the survival fraction of *E. coli* and *L. innocua*, using Equation 1:

$$\log_{10} CFU = \frac{\log N}{\log N_0} \text{ Equation 1}$$

where N is the number of surviving bacteria/mL (after 30 minutes of reaction time) and N_0 is the initial number of bacteria/mL (0 minutes of reaction time).

2.6. Gas chromatography/mass spectrometry (GC/MS) analysis

Gas chromatography/mass spectrometry (GC/MS) analysis of EO-pectin nanoemulsions was used to assess changes in the concentration of the main volatile compounds of EOs during storage time (56 days at 25 °C). An Agilent 6890 high resolution gas chromatograph (HRGC) equipped with an Agilent ALS 7863 auto sampler and coupled to an Agilent 5890N mass spectrometer was used. Pentane (99 % purity) and diethyl ether (100 % purity) (extraction solvents), Milli-Q water, anhydrous sodium sulfate (activated 2 hours prior to analysis, drying at least 2 hours in the muffle furnace at 300 °C) and 2-propanol (instrumental quality) were used. The analysis was carried out using a Varian CP9205 VF-WAX ms column (30 m X 250 µm i.d., film thickness 0.25 µm). The oven temperature was programmed as follows: initial temperature, 60 °C, held for 1 min; 15 °C/min to 90 °C; 5 °C/min to 170 °C; then 5 °C/min raised to 250 °C and held for 4 min. Helium gas was used as carrier gas at a constant flow rate of 1 mL/min. The sample was diluted in 25% pentane and 75% diethyl ether (factor dilution 1:3) and 1 µL of this was injected in splitless mode in a ratio of 100:1. A calibration curve was prepared using standards considering the major components of the volatile fraction for each EO which were: carvacrol, thymol, citral and D-limonene (obtained from Sigma-Aldrich, S. L., Madrid, Spain) at concentrations from 1, 10, 100, 400, 700, 1000, 1400 and 1700 ppm. The quantification of the volatile compounds during storage time was determined in duplicate. Identification of the main compounds from

each EO-pectin nanoemulsion was achieved by comparing their relative retention time (RT) and mass spectra of the recorded chromatographic peaks with NIST 98.1 NIST/EPA/NIH Mass Spectral Library Mass Spectrometry Data and with spectra obtained from the standards used when feasible.

Results were reported as the percentage of the Relative Concentration (RC %) and calculated with the ratio of the concentration of each main volatile compounds after a determined storage time versus the initial concentration of each volatile compound in the freshly made nanoemulsions.

2.7. Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) was performed in order to elucidate the impact of nanoemulsions (at zero time) with different EOs on the *E. coli* and *L. innocua* microbial cells. For that purpose, bacteria suspension was in contact with the freshly prepared nanoemulsion and was let interact for 30 min, as explained in section 2.5.2 from materials and methods. As a control, the bacterial suspension was evaluated without the addition of nanoemulsions. After the antimicrobial assay, samples were fixed in 2.5% glutaraldehyde (0.1 M phosphate buffer, pH 7.4) for up to 24 h, the cells were rinsed three times for 10 minutes with phosphate buffer (0.1 M, pH 7.4) and post fixed with 1% osmium tetroxide for 2 h at 4 °C. After fixation, three washes were made with 0.1M sodium acetate (two for 2 min. and one for 30 min.), and cells were consecutively dehydrated using 30, 50, 70 and 100% acetonitrile for 30 min each. Then, cells were sequentially infiltrated with mixtures of propylene oxide Durcupan's ACM Epoxy Resin 1:1 (50% resin + 50% acetonitrile) for 90 min. and 3:1 (75% resin + 25% acetonitrile). Polymerization of the resin to form blocks of samples was conducted in an oven at 60 °C for a minimum time of 48 h. The polymerized

blocks were hand trimmed with a razor blade and sectioned with a diamond knife in a Reichert Ultracut R ultramicrotome (Leica, Wetzlar, Germany). From the cuts made, thin sections (70-80 nm) were placed on 300 mesh gold-copper grids. Sections were stained in 2% uranyl acetate for 30 min, washed 3 times with Milli-Q water for 2 min, and stained by floating in a drop of Reynold's lead citrate for 5 min. Finally, images of each sample were obtained observing the grids in a Jeol-JEM 1010 transmission electron microscope (Biodirect, Inc., Massachusetts, USA) at an acceleration voltage of 100 kV.

2.8. Statistical analysis

Data were analyzed using an analysis of variance (ANOVA) with statistical procedures of Statgraphics Plus version 5.1, Windows package (Statistical Graphics Co., Rockville, MD). Results were expressed as mean $\pm$ standard deviations and compared using the least significant difference (LSD) test to determine significant differences between each nanoemulsions containing different essential oils at 5% significance level (interval of confidence of 95%). Regression coefficients (R^2) were calculated to determine the correlation between the antimicrobial activities and the Relative Concentration (RC) of the main volatile compounds of EOs contained in the nanoemulsions.

3. Results and discussion

3.1. Droplet size and droplet size distribution

In the present work, the average droplet size of coarse emulsions and nanoemulsions was assessed during storage time. The average droplet size for coarse emulsions containing oregano, thyme, lemongrass and mandarin EO and pectin was 740 ± 268 , 82 ± 23 , 370 ± 16 and 601 ± 7 nm, respectively. After microfluidization, the average droplet diameter of the

nanoemulsion-pectin systems was reduced down to 27 ± 12 , 40 ± 8 , 11 ± 1 and 17 ± 1 nm, for oregano, thyme, lemongrass and mandarin respectively. The type of EO used in the formulation had a significant impact on the droplet size of nanoemulsion-pectin systems. Nanoemulsions containing lemongrass or mandarin presented a significantly smaller average droplet diameter compared to nanoemulsions containing oregano and thyme EOs. Also the appearance was strongly influenced by the emulsion droplet size as reported previously (Guerra-Rosas et al., 2016). Nanoemulsions containing lemongrass or mandarin with the smallest droplet size showed a transparent or translucent appearance compared to their respective emulsions, which were whiter or opaque, and they had a major influence on the formation and stability, results that are agree with Chang, McLandsborough, & McClements, (2015). The differences among the droplet size obtained in nanoemulsions containing several EOs has been attributed to different polarities of the volatile compounds forming each oil phase (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2015).

Regarding the long-term stability, the average droplet diameter of nanoemulsions containing lemongrass or mandarin EOs and high-methoxyl pectin remained practically unchanged during storage ($p > 0.05$), showing a high stability over time. Previously reported data indicate that reducing the droplet diameter to nano-scale level can significantly improve the stability of emulsions by diminishing aggregation, flocculation, and gravitational separation (Chang, McLandsborough, & McClements, 2015; Mason, Wilking, Meleson, Chang, & Graves, 2006). Also has been reported that a nanoemulsion formulated with a biopolymeric matrix appears to be the most promising approach, because of the immobilization of oil droplets provided high stability (Donsì & Ferrari, 2016). Food-grade biopolymers, as pectin, can impart to the nanoemulsions some desired features, such as

specific interfacial behavior (electrostatic forces, steric repulsion, and rheology) (Chen, Remondetto, & Subirade, 2006), due to its anionic nature (Dickinson, Semenova, Antipova, & Pelan, 1998).

Respect to oregano and thyme EO-nanoemulsions, they were highly unstable. Initially, fine droplets were produced by microfluidization as discussed before but rapidly increased up to 169.30 ± 10.69 and 105.10 ± 45.43 nm for nanoemulsions containing oregano or thyme EOs, respectively. After 7 days of storage and by the end of storage (56 days) they had values around 1017.00 ± 198.40 and 924.10 ± 183.80 nm, respectively. This droplet growth in turn led to the appearance of a cream layer on top of the samples, which has been published elsewhere (Guerra-Rosas et al., 2016). There are a number of possible reasons for the increase in the mean droplet size, one of them is the oil phase composition, which in some cases influences the instability of the oil droplets leading to a rapid growth (Donsi & Ferrari, 2016). This behavior may be attributed to Ostwald ripening or coalescence phenomena since these are the main mechanisms for destabilization in nanoemulsions (Rao & McClements, 2013). Nanoemulsions containing EOs are shown to be highly sensitive to Ostwald ripening (Ziani, Chang, Mclandsborough, & McClements, 2011) as EOs present a relatively high water solubility and therefore the molecular diffusion of oil between droplets through the continuous phase occurs, where migration of oil molecules from small to larger droplets takes place leading to an overall increase in the droplet size (Noor El-Din et al., 2013). The same behavior was observed by Chang et al., (2015), they found that nanoemulsions prepared from pure thyme oil were highly unstable due to Ostwald ripening. Only, they were able to increase antimicrobial efficacy of thyme oil nanoemulsions by adding a ripening inhibitor (corn oil) to the oil phase prior to homogenization.

3.2. Antimicrobial activity

The use of EOs as natural-antimicrobial agents is an alternative to synthetic food additives and is intensively explored in recent years (Donsì & Ferrari, 2016). Therefore, we evaluate the bactericidal action (against *E. coli* and *L. innocua*) of EO-pectin nanoemulsions immediately after being prepared and along storage. Results are shown in Figures 1A and 1B. It can be seen that *E. coli* (Gram negative) presented a higher sensitivity to the antimicrobial action of EO-pectin nanoemulsions compared to *L. innocua* (Gram positive). In our study was found that the decrease in particle size increases the biological activity of the encapsulated active compounds within nanoemulsions. For instance, just made nanoemulsions containing lemongrass EO, which had an initial droplet size of 27 nm, led to 5.9 log₁₀ reductions of *E. coli* whereas *L. innocua* population was reduced 0.2 log₁₀ units with the same nanoemulsions. This might be attributed to the differences of the cell membrane composition of these two microorganisms and their different sensitivity to EO active compounds (Abdollahzadeh, Rezaei, & Hosseini, 2014; Castilho, Savluchinske-Feio, Weinhold, & Gouveia, 2012; Solomakos et al., 2008). The effect of EOs on food spoilage and pathogenic bacteria has been investigated in the last decade. Accordingly to our results, other authors have reported a higher Gram negative sensitivity to EO compared to Gram positive bacteria, due to the differing structures of their respective cell walls (Seow, Yeo, Chung, & Yuk, 2014; Wilkinson, Hipwell, Ryan, & Cavanagh, 2003). Gram negative bacteria are complex and possess an external lipopolysaccharide wall surrounding the peptidoglycan cell wall that might restrict the diffusion of hydrophobic compounds through its lipopolysaccharide layer (Alboofetileh, Rezaei, Hosseini, & Abdollahi, 2014; Vaara, 1992). Whereas in Gram positive bacteria, hydrophobic molecules can easily penetrate

through the thick peptidoglycan layer, permitting the access to the cell membrane (Nazzaro, Fratianni, & Martino, 2013). Wu, Lin, & Zhong, (2014), evaluated physical and antimicrobial characteristics of thyme oil emulsified with soluble soybean polysaccharide. They reported that emulsions with droplets in the nano range can be efficiently transported through the porin proteins of the outer membrane of the bacterium, enabling an effective delivery of EOs, leading to a higher antimicrobial activity.

Other factors should also be considered when the antimicrobial activity of EOs is evaluated. In this sense, the type of EO, its composition and polymer type, also plays a significant role on determining their antimicrobial activity. According to this, a higher resistance to EO would be expected in Gram negative (*E. coli*) compared to Gram positive bacteria (*L. innocua*) (Muriel-Galet et al., 2012; Ruiz-Navajas, Viuda-Martos, Sendra, Perez-Alvarez, & Fernández-López, 2012). However, there are contradictory evidences regarding this matter. Several studies have shown a higher resistance of Gram positive bacteria against EOs compared to Gram negative depending on the EO type (Dorman & Deans, 2000; Tassou, Drosinos, & Nychas, 1995). The two studied microorganisms showed different response against different EO types. *L. innocua* presented a similar sensitivity to all the EO types studied whereas the antimicrobial activity against *E. coli* significantly depended on the EO type. The antimicrobial capacity of nanoemulsions against *E. coli* presented the following order: lemongrass > oregano > thyme > mandarin EO. It is known that the overall bactericidal effect of EO largely depends on their volatile composition. Lemongrass EO consists on a mixture of phenolic compounds such as citral, geranial and myrcene, that possess an antimicrobial activity against a number of bacteria (Hammer, Carson, & Riley, 1999). Oregano and thyme mainly contain carvacrol and thymol in the volatile fraction (Marino,

Bersani, & Comi, 2001), which have shown to inhibit some pathogenic bacterial strains such
 as *E. coli*, *S. enteritidis*, *S. choleraesuis* and *S. typhimurium* (Peñalver et al., 2005). Likewise
 other citrus EOs, mandarin EO is generally composed by D-limonene which has been shown
 to be effective against *S. aureus*, *L. monocytogenes*, *S. enterica* and *Saccharomyces bayanus*
 as well as other microorganisms (Chikhoun, Hazzit, Kerbouche, Baaliouamer, & Aissat,
 2013; Settanni et al., 2012). However, D-limonene is very hydrophobic and difficult to be
 incorporated in oil-in-water emulsions (Calo, Crandall, Bryan, & Ricke, 2015). In addition,
 pectin is a polysaccharide used in many food preparations as a stabilizer or thickening agent,
 and due to its anionic nature (Sila et al., 2009), might present electrostatic interactions and
 give negative charge to the oil droplets and in turn change their antimicrobial properties
 (Burapapadh, Kumpugdee-Vollrath, Chantasart, & Srimornsak, 2010). On the other hand,
 the antimicrobial potential of nanoemulsions against *E. coli* or *L. innocua* significantly
 changed during storage time (Figure 1AB). The fresh nanoemulsion containing lemongrass
 EO exhibited 5.92 ± 0.07 log reductions of the *E. coli* inoculated population, whereas it
 decreased up to 2.16 ± 0.06 log reduction after being stored 7 days, and it almost completely
 reduced their antimicrobial activity at the end of the storage period (56 days). Similarly, the
 rest of EO nanoemulsions presented a gradual decreasing on their antimicrobial activity
 against *L. innocua* during storage. In a study realized by Bhargava, Conti, da Rocha, &
 Zhang, (2015), they applied an oregano oil nanoemulsion on a food matrix to the control of
 foodborne bacteria. They evaluated the antimicrobial effect at 3 h, 24 h, and 72 h after being
 applied the nanoemulsion. They report that the inhibitory effect of bacteria decreased when
 the treatment continued for 72 h, results that are agree with us. Besides, in our study, the
 lessening in the antimicrobial activity of EOs might be related to the loss of the volatile
 compounds during storage observed (Table 2), as discussed in the following section.

3.3. Volatile composition

EOs may contain more than sixty individual components that constitute their complex aroma profile but only few of them are responsible for their antimicrobial activity (Bakkali et al., 2008; Burt, 2004; Cosentino et al., 1999). In this sense, it is reported that carvacrol (7.8-80%) and thymol (3.24-72%) are the major aroma/volatile compounds present in oregano that account for their antimicrobial activity (Martino, Feo, Formisano, Mignola, & Senatore, 2009; Giatrakou, Kykkidou, Papavergou, Kontominas, & Savvaidis, 2008; Daferera, Ziogas, & Polissiou, 2000). In the case of thyme EO, the same major volatile compounds that in oregano are found, but in different proportions (10 - 74.8% thymol and 2.2 - 11% carvacrol) (Salehi, Golparvar, & Hadipanah, 2014; Lee, Umamo, Shibamoto, & Lee, 2005; Daferera, Ziogas, & Polissiou, 2000). Citral (70-85%) is found to be the main volatile compound in lemongrass EO (Desai, Parikh, & De, 2014) and D-limonene (52.2-96.2%) in mandarin EO (Fisher & Phillips, 2008). Thus, a GC/MS analysis of the EO-nanoemulsions was carried out to evaluate the relative concentration (RC) of the main volatile compounds of each EO remaining in the nanoemulsions along the storage and results are presented in Table 2. The concentration of the main components of each EO-pectin nanoemulsion gradually decreased during storage time regardless the EO type. A comparison of the antimicrobial activity and the volatile compounds of the EO-pectin nanoemulsions evaluated in our assay showed greater inhibitory capacity of the EOs. For oregano EO, the RC of carvacrol and thymol was 88.42 and 74.44 %, respectively, whereas for thyme EO the RC was 67.42 and 92.29 %, respectively, after 7 days of storage. According to these results, there is a significant difference between the RCs of these two monoterpenes because oregano EO contains a high percentage of carvacrol and thyme contains mainly thymol. For lemongrass EO, the RC of

citral was 67.42 %; whereas for mandarin EO, the RC of D-limonene was 92.29 %, after 7 days of storage. At the end of storage (56 days), the retention of carvacrol/thymol (oregano and thyme), citral (lemongrass) and D-limonene (mandarin) was diminished up to 41.60/37.42, 21.53/27.59, 43.30 and 2.95 %, respectively. There are a number of reasons that might explain the decrease in the concentration of volatile compounds during storage: (i) volatile compounds might be able to migrate from the oil droplets through the water phase and further be volatilized or (ii) they might be oxidized to unstable forms (Mirhosseini et al., 2008). In fact, D-limonene has been found to be susceptible to oxidative degradation which lead to a loss of activity and (iii) the boiling points which are different (Li & Chiang, 2012; Sun, 2007). According to PubMed, the boiling points for carvacrol, thymol, citral and D-limonene are 236/237, 233, 229 and 178 °C at 760 mm Hg, respectively. Clearly, D-limonene boiling point value is found below among others it is related with the loss of this main volatile compound. Additionally, we found a positive correlation between the retention of volatile compounds and the antimicrobial activity of EO-nanoemulsions during storage (Figure 2 AB). The strongest correlation was found between the retention of thyme or lemongrass EO and the inactivation of *E. coli* (Figure 2A). These results are consistent with those observed for antimicrobial activity of nanoemulsions during storage time. Moreover, the volatilization of antimicrobial compounds in EO-loaded nanoemulsions might be accelerated due to their small droplet size compared to conventional emulsions.

3.4. Influence of the nanoemulsion on the microbial cell structure

The mechanism of antibacterial activity can be evaluated by measuring potassium, protein, and nucleic acid leakage from the cells, and electron microscopy. Several studies have used electron microscopy in the literature. However, there are not reports about TEM

observations of microorganisms inactivated by action of nanoemulsions containing EO. In our work, TEM was used to observe the alterations caused in the structure of *E. coli* and *L. innocua* cells by the action of EO-loaded nanoemulsions. In Figure 3 (AB), changes in the cellular structure of both microorganisms after contact with nanoemulsions containing different EOs can be observed in contrast with the fresh control cells. On one hand, it can be observed that the cells of *E. coli* and *L. innocua* which were without nanoemulsion contact, the populations presented a significantly different structure. *L. innocua* cells exhibited a remarkably thicker outer membrane compared to *E. coli* cells. This fact might be highly related to the higher resistance presented by *L. innocua* to the antimicrobial activity of EO-pectin nanoemulsions in comparison with the *E. coli* cells, which presented a significantly higher sensitivity to EO-pectin nanoemulsions. On the other hand, TEM micrographs also evidenced differences in the influence of the different EO tested on the substructure of microbial cells. Both in *E. coli* and *L. innocua* cells treated for 30 min with nanoemulsions containing oregano or lemongrass EOs, the appearance of pores in the cytoplasmatic membrane was observed and hollow compartments in the bacterial cytoplasm were detected. However, bacterial cells treated with nanoemulsions containing thyme or mandarin presented less damages in the cellular structure. These results are in accordance with the antimicrobial results discussed earlier. Bhargava et al., (2015), used three bacteria to evaluate the effect of an oregano oil nanoemulsion to the control of foodborne bacteria on a food matrix. Scanning Electron Microscopy reveals that cell surfaces were remarkably disintegrated and cell boundaries became irregular after application of nanoemulsion. Also, cellular fragments were observed.

Even though the mechanism of action of EOs against microorganisms is not completely understood, some studies indicate that might be due to the interaction of phenolic compounds of EOs with the proteins in the cytoplasmatic membrane leading to the formation of pores and the subsequent leakage of ions and other cell content causing the cell breakdown (Carson, Mee, & Riley, 2002). This theory would sustain our results, since EO nanoemulsions presented obvious damages in the *E. coli* and *L. innocua* cytoplasmatic membrane. In contrast with our results, other authors have reported a limited diffusion of phenolic compounds from EOs through the microbial membrane of Gram negative bacteria, such as *E. coli*, thus being more resistant to EOs (Vaara, 1992). However, Wilkinson et al., (2003) described a higher Gram negative sensitivity to EO compared to gram positive. These differences between studies might be attributed to different strains used or different purity of the EOs tested.

5. Conclusions

This work enables us to evaluate and compare the antimicrobial activity of the EOs-pectin nanoemulsions against *E. coli* and *L. innocua*. The results showed that lemongrass-pectin nanoemulsion, which maintained a droplet size between 11 to 13 nm, exhibited the strongest antimicrobial potential against the two bacteria studied. *E. coli* was the most sensitive strain to EOs-pectin nanoemulsions which lead to the cell death compared to *L. innocua*, as it was confirmed though TEM images. Moreover, a gradual decrease of antimicrobial activity of nanoemulsions along storage time was observed regardless the EO type, which could be attributed to the loss of their respective main volatile compounds. The results obtained in the present study, has demonstrated and evidenced the potential and effectiveness of the nanoemulsions to be used as antimicrobial delivery systems in beverages and foods.

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Figure 1 Changes through storage time at 25 °C in the survival fraction of *E. coli* (A) or *L. innocua* (B) during 30 min of reaction time with different essential oils-pectin nanoemulsion produced by microfluidization at 150 MPa and 5 cycles. Data shown are a mean $\pm$ standard deviation

Figure 2 Correlation factors (R^2) between the Relative concentration (%) of main volatile compounds of EOs and the loss of the antimicrobial activity of the nanoemulsions against *E. coli* (A) or *L. innocua* (B). R^2 values close to 1 indicate a high correlation

Figure 3 Transmission Electron Microscopy images of *E. coli* (A) or *L. innocua* (B) after 30 min of reaction time with fresh essential oils-pectin nanoemulsions containing oregano, thyme, lemongrass or mandarin. Scale bar represents 500-1000 nm (75000X)

Table 1 Main volatile components and percentages present of EOs used in the present work according to the bibliographic references.

Essential oil	Main volatile compounds	References
Oregano (<i>Origanum vulgare</i>)	Carvacrol (7.8 - 80) and thymol (3.24 -72%)	(Martino, Feo, Formisano, Mignola, & Senatore, 2009; Giatrakou, Kykkidou, Papavergou, Kontominas, & Savvaidis, 2008; Daferera, Ziogas, & Polissiou, 2000)
Thyme (<i>Thymus vulgaris</i>)	Thymol (10 - 74.8%) and carvacrol (2.2 - 11%)	(Salehi, Golparvar, & Hadipanah, 2014; Lee, Umamo, Shibamoto, & Lee, 2005; Daferera, Ziogas, & Polissiou, 2000)
Lemongrass (<i>Cymbopogon citratus</i>)	Citral (70 - 85 %)	(Desai, Parikh, & De, 2014)
Mandarin (<i>Citrus reticulata</i>)	Limonene (52.2 - 96.2 %)	(Fisher & Phillips, 2008)

Table 2 Changes in the relative concentration of the main volatile compound EO of each nanoemulsion during storage time at 25 °C.

Time (days)	Relative concentration (%)					
	Oregano		Thyme		Lemongrass	Mandarin
	Carvacrol	Thymol	Carvacrol	Thymol	Citral	Limonene
0	100 ± 0.00 ^a	100 ± 0.00 ^a	100 ± 0.00 ^a	100 ± 0.00 ^a	100 ± 0.00 ^a	100 ± 0.00 ^a
7	88.42 ± 1.50 ^b	74.44 ± 1.74 ^b	88.00 ± 0.32 ^b	81.19 ± 0.09 ^b	67.42 ± 3.37 ^b	92.29 ± 0.72 ^b
14	87.14 ± 1.88 ^b	72.80 ± 3.38 ^b	73.48 ± 2.23 ^c	52.46 ± 0.29 ^c	63.89 ± 0.94 ^b	78.98 ± 1.46 ^c
21	85.42 ± 1.44 ^b	53.75 ± 0.94 ^c	57.31 ± 1.52 ^d	45.54 ± 0.04 ^d	63.08 ± 1.81 ^b	74.83 ± 1.09 ^d
28	77.42 ± 1.26 ^c	51.20 ± 0.13 ^{cd}	55.67 ± 0.34 ^d	35.76 ± 0.38 ^e	56.01 ± 0.81 ^c	33.89 ± 1.57 ^e
35	76.21 ± 0.85 ^c	49.70 ± 0.98 ^d	44.25 ± 0.27 ^e	34.55 ± 0.28 ^e	51.83 ± 0.94 ^c	11.59 ± 0.28 ^f
42	75.50 ± 0.84 ^c	44.11 ± 2.65 ^e	42.23 ± 2.43 ^e	31.594 ± 0.69 ^f	51.79 ± 2.61 ^c	11.11 ± 0.07 ^f
49	64.65 ± 0.72 ^d	39.75 ± 0.99 ^f	30.79 ± 1.61 ^f	28.24 ± 0.79 ^g	44.86 ± 0.07 ^d	7.28 ± 0.07 ^g
56	41.60 ± 0.46 ^e	37.42 ± 0.95 ^f	27.59 ± 0.66 ^g	21.53 ± 0.60 ^h	43.30 ± 1.29 ^d	2.95 ± 0.09 ^h

Data shown are a mean ± standard deviation. Values in a column with the same superscript are not significantly different ($P < 0.05$)