Phytochemical-loaded electrospun nanofibers as novel active edible films: characterization and antibacterial efficiency in cheese slices.

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Abstract

The aim of this work was to develop novel active edible electrospun coatings with antimicrobial properties through the encapsulation of essential oils (EOs) from two widely used spices (*Laurus nobilis* and *Rosmarinus officinalis*) for inactivating or reducing spoilage and pathogenic microorganisms. Firstly, EOs from dried leaves of both plants were obtained by hydrodistillation and their composition was analysed by Gas Chromatography – Mass Spectrometry (GC – MS). Interestingly, the main component in both extracts, probably as a consequence of the extraction conditions, was 1,8 cineole. Both EOs showed highest antimicrobial activity against Gram-positive than Gram-negative bacteria.

Three different concentrations of the 1,8 cineole-rich EOs were incorporated within a zein matrix through electrospinning and solution properties, encapsulation yield, morphological and thermal stability of the edible coatings developed were evaluated. Then, the antibacterial efficiency of EOs-zein nanofibers (ZNF) films against *Listeria monocytogenes*, *S. aureus* and mesophilic bacteria counts (MBC) was compared with their counterparts prepared by casting (ZF) when applied directly on an inoculated side of cheese slices. EO obtained from *Laurus nobilis* showed higher antibacterial activity than *Rosmarinus officinalis*. The antibacterial effectiveness of the active films increased through storage time, showing a significant reduction of ~ 2 logarithm units of *L. monocytogenes* and *S. aureus* as compared with the control samples, after 28 days at 4 °C, using the highest content of 1,8 cineole-rich EOs and in films prepared by means of the electrospinning process. Interestingly, the growth of aerobic mesophilic bacteria was also inhibited in cheese slices coated with EOs-loaded ZNF based films.

1. Introduction

Current trends in the food area, driven by consumer's concerns towards the use of synthetic food additives, have boosted research in natural compounds able to keep the quality and safety of food products. Amongst the substances with antimicrobial properties, which can be used as natural preservatives in food products, plant secondary metabolites, like essential oils (EOs), are highly promising given their other functional attributes, like antioxidant properties, antiviral, or even anti-inflammatory. However, their use is restricted mainly because of their water insolubility, high volatility, rapid oxidation and high sensitivity to a number of environmental factors such as heat, pressure, light and oxygen (Asbahani et al., 2015; Fernandes, Marques, Borges, & Botrel, 2014). An additional drawback is related to the flavour or smell that they can impart to the products where they are applied, being thus restricted to very low amounts, fact that could limit their practical functionality. A strategy that has been widely explored and that can broaden EOs applications is their encapsulation in suitable matrices, which apart from increasing their stability against environmental factors, could also result in avoiding unwanted sensorial changes in the applied products. Numerous techniques have been used for EO encapsulation like spray drying (Fernandes et al., 2014), fluid bed coating, spray chilling (Vishwakarma, Gautam, Babu, Mittal, & Jaitak, 2016), melt injection, coacervation and electrohydrodynamic processing (Rezaei, Fathi, & Jafari, 2019). The latter has received increased attention in recent years due to its advantages, such as mild processing conditions (not requiring high temperatures for processing), versatility and possibility of using all food-grade materials. Electrohydrodynamic processing is a simple, cost-effective, efficient, versatile, and scalable technology to manufacture dry nano- and microencapsulation structures in the form of fiber mats (electrospinning) or particulate powders (electrospraying), by subjecting a polymeric fluid to an external high-voltage electric field (Alehosseini, Gómez-Mascaraque, Martínez-Sanz, & López-Rubio, 2019). Some of the advantages of the nanofiber mats produced using electrospinning are that they possess a large

surface to volume ratio, and the processing conditions can be tuned to modify the size and morphology of the structures obtained, giving rise to an intertwined porous fibrous structure. Encapsulation of substances using these techniques is rather straightforward, with generally high encapsulation yields and allowing a sustained release of the encapsulated compounds (Anu Bhushani & Anandharamakrishnan, 2014; Fabra, Lopez-Rubio, & Lagaron, 2016). Several EOs have been already loaded into nanofibers through electrospinning including cinnamon (Wen et al., 2016), thyme (Lin, Zhu, & Cui, 2018), peppermint and chamomile (Huang et al., 2019), clove (Cui, Bai, Rashed, & Lin, 2018), eucalyptus (Antunes et al., 2017), ginger (Silva et al., 2018), chrysanthemum (Lin, Mao, Sun, Rajivgandhi, & Cui, 2019) and orange (Tavassoli-Kafrani, Goli, & Fathi, 2018). Concretely, because of their great abundance and excellent potential, essential oils from bay (*Laurus nobilis*) and rosemary (*Rosmarinus officinalis*) have attracted great interest, having major volatile compounds (terpenes, terpenoids, phenols), which can modify cell membrane permeability and integrity of bacteria, thus acting as strong antimicrobial natural cocktails (Okoh, Sadimenko, & Afolayan, 2010).

The aim of this study was to develop all-natural edible structures through electrospinning containing *Laurus nobilis* essential oil (LEO) and *Rosmarinus officinalis* essential oil (REO) for active food packaging applications. The matrix chosen for EOs encapsulation was zein, as it is a biodegradable hydrophobic plant protein that has demonstrated to be an excellent excipient in numerous studies for controlled drug delivery (Tran, Duan, Lee, & Tran, 2019). Moreover, it also has excellent film forming and gas barrier properties, which make it an ideal matrix for edible active food packaging applications (Gaona-Sánchez et al., 2015). Firstly, the chemical composition of LEO and REO extracted via hydrodistillation was analysed by GC-MS and the antimicrobial activity of both EOs against Gram-positive and Gram-negative bacteria was evaluated. Then, the encapsulation efficiency, morphology, molecular organisation and thermal stability of the obtained structures were studied as a function of EO concentration (1, 5 and 10%

w/v). Finally, the effectiveness of the EOs and EOs-loaded zein edible films for inactivating or reducing spoilage and pathogenic microorganisms on cheese surfaces, including *Listeria monocytogenes*, *Staphylococcus aureus* and aerobic mesophilic bacteria, was assessed.

2. Materials and Methods

2.1. Materials

The leaves of laurel (*Laurus nobilis* L.) and rosemary (*Rosmarinus officinalis* L.) were harvested in mid-October 2015 from naturally occurring grown plants in Tarsus/Mersin, Turkey. Zein from maize (Z3625) and phosphate-buffered saline (PBS) were obtained from Sigma-Aldrich (St. Louis, Mo., U.S.A). Ethanol (96% v/v purity), glacial acetic acid (100% purity), hexane (95%, anhydrous) and sodium sulphate (anhydrous) were purchased by Merck (Darmstadt, Germany). The strain of *Listeria monocytogenes* CECT 4032 (NCTC 11994), and *Staphylococcus aureus* CECT 435 (ATCC 25923) were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). *Escherichia coli* O157:H7 (ATCC 35150), *Salmonella typhimurium* (ATCC 14028), and *Salmonella enteritidis* (ATCC 13076) were supplied from KWIK STIK™. Tryptone Soy Broth (TSB), Tryptone Soy Agar (TSA) and Mueller Hinton agar (MHA), Oxford-Listeria-selective agar base and Baird-parker agar base were purchased from Thermo Scientific-Oxoid (Basingstoke, UK). A commercial semi-hard cheese (Queso Guoda cheese; protein 23.5%, fat 28%, salt content 1.9%) was purchased from a local supermarket in Valencia, Spain.

2.2. Extraction of essential oils (EOs) from *Laurus nobilis* and *Rosmarinus officinalis*

The dried leaves of *Laurus nobilis* and *Rosmarinus officinalis* (air-dried at a room temperature of 20-25°C) were separated from the other parts of plants. 250 g of samples and 2500 mL of distilled water were added to a 5-L flask and subjected to hydrodistillation for 3 h using a glass Clevenger

type apparatus. The LEO and REO were dried with anhydrous sodium sulphate and stored at 4 °C in sealed amber glass bottles until analysis. The yield was expressed in % v/w.

2.3. Composition of essential oils (EOs) by gas chromatography-mass spectrometry (GC-MS)

Essential oil constituents were determined by GC–MS analysis. GC–MS analyses were carried out on an Agilent 7890A GC, coupled to Agilent 5975C MS system equipped with HP-5MS capillary column (30 m× 0.25 mm × 0.25 µm). Oven temperature was held at 50 °C for 5 min and then programmed to 150 °C at a rate of 3 °C/min, kept constant at 150 °C for 10 min and then increased to 250 °C at a rate of 3 °C/min. Injector and detector temperatures were adjusted to 250 and 280°C respectively. Injection Split ratio was 1/50. Helium was used as carrier gas with a linear velocity of 1.0 mL/min. Mass spectra (MS) were recorded in electron ionization mode at 70 eV and with a mass range of m/z 30–550. The components were identified by comparing linear Kovats indices, their retention times and mass spectra with those obtained from the MS library. The library search was carried out using Wiley GC-MS and NIST Library of Essential Oil Constituents. The LEO and REO were diluted in hexane: 1/30.

2.4. Preparation and characterization of zein electrospun fibers

2.4.1. Preparation of EOs-containing electrospun zein structures

Zein solutions for electrospinning were prepared by dissolving 25 % (w/v) zein powder in a mixture of glacial acetic acid and ethanol using a volume ratio of 30:70. Then, various amounts of the LEO and REO (1, 5 and 10% (w/w) with respect to the zein powder weight) were added to the zein solution and stirred for 12 h at room temperature under magnetic stirring to get homogeneous solutions. Nanofibers loaded with different concentrations of EOs were designated as: Neat zein nanofibers (ZNF), 1% LEO loaded zein nanofibers (LEO1-ZNF), 5% LEO loaded zein nanofibers (LEO5-ZNF), 10% LEO loaded zein nanofibers (LEO10-ZNF), 1% REO loaded zein nanofibers

(REO1-ZNF), 5% REO loaded zein nanofibers (REO5-ZNF) and 10% REO loaded zein nanofibers (REO10-ZNF).

2.4.2. Characterization of solutions

The apparent viscosity and electrical conductivity of zein solutions with different concentrations of LEO and REO were measured. Solution viscosity was measured using a viscometer (Brookfield DV-II+ProEXTRA, USA). Approximately 7 mL of each solution sample were placed into the stainless steel cup of the viscometer and then the apparent viscosity value was obtained at 25 °C using a S18 spindle. Spindle selection was adjusted to be within 10 to 90% torque values.

The electrical conductivity of the solutions was determined, in triplicate, using a conductivity meter (Orion5Star, Thermo Fisher Scientific Inc., USA) and expressed as $\mu\text{S}/\text{cm}$. All measurements were recorded at room temperature (23 ± 2 °C).

2.4.3. Electrospinning process

Electrospinning was performed at room temperature using an electrospinning apparatus (Nanodev Scientific, Ankara, Turkey) equipped with a syringe pump (New Era Pump Systems Inc., NE-300, Hauppauge, NY), a high voltage power supply (Spellman, CZE 1000R, High Voltage Electronics Corporation, Hauppauge, NY) and a collector plate. The solutions were introduced in 5 mL plastic syringes horizontally placed on a syringe pump and the electrospun nanofibers were collected on aluminum foil. The needle tip to collector distance was adjusted to 11 cm and the applied voltage was set to 18 kV. The solution flow rate was kept constant at 0.04 mL/h. These values were set as optimum according to preliminary trials.

2.4.4. Scanning electron microscopy (SEM)

The morphology of the electrospun nanofibers was characterized using a scanning electron microscope (SEM) (Supra 55, Carl Zeiss, Germany) at an accelerating voltage of 5 kV and a magnification of 25,000×. The samples were sputter-coated with 2 nm platinum. In order to determine average fiber diameter, 100 randomly selected fibers for each sample were analysed using ImageJ software (Image J, NIH, Maryland, USA).

2.4.5. Fourier transform infrared spectroscopy (FTIR)

The infrared spectra of the electrospun nanofiber mats and EOs were obtained using FTIR spectroscopy (Bruker Rheinstetten, Germany). The FTIR spectra were recorded in a range of 650 to 4000 cm⁻¹ from an average of 16 scans and with a resolution of 4 cm⁻¹.

2.4.6 Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) was performed with a TG-STDA Mettler Toledo model TGA/STDA851e/LF/1600 analyser. The samples (ca. 10 mg) were heated from 25 °C to 600 °C at a heating rate of 10 °C/min under dynamic nitrogen flow. Thermogravimetric curves express the weight of the sample as a function of temperature.

2.4.7. Determination of encapsulation efficiency (EE) and loading capacity (LC)

The encapsulation efficiency was determined as previously reported by (Yao, Chang, Ahmad, & Li, 2016) with some modifications and calculated using Equation (1):

$$EE(\%) = \frac{\text{Mass of essential oil in the fibers (experimental)}}{\text{Total mass of essential oil added (theoretical)}} \times 100 \quad \text{Eq. (1)}$$

First, LEO and REO were dissolved in hexane to prepare a calibration curve covering a broad range of EOs concentration (2,5–50 µg/ml), followed by examining the solution's absorbance at 262 nm and 294 nm respectively. The LEO and REO calibration curve were obtained using linear regression ($R^2 = 0.998$ and $R^2 = 0.999$ respectively). The LEO and REO content of the various electrospun samples was calculated according to this calibration curve. 10 mg each sample were

dissolved in 5 mL ethanol solution under magnetic stirring at 100 rpm for 30 min and, the supernatant of samples was extracted with 5 mL hexane and then filtered through a syringe filter (0.45-µm pore size). Then, the absorbance was measured in triplicate using a Cary60 UV-Vis spectrophotometer (Agilent Technologies, Malaysia).

The loading capacity of the electrospun fibers was also determined following Equation (2):

$$LC (\%) = \frac{\text{Mass of entrapped essential oil (experimental)}}{\text{Total mass of fibers (experimental)}} \times 100 \quad \text{Eq. (2)}$$

2.5. Preparation of zein films through casting

For comparative purposes, casting films were also prepared for the antimicrobial assay. To these end, film-forming solutions were prepared by dissolving 13 g of zein powder in glacial acetic acid and ethanol 70:30% (w/w). EO at 5 or 10% (g essential oil/g dry zein powder) was incorporated and then stirred for 12 h at room temperature under magnetic stirring to get homogeneous solutions. Neat zein film solutions were also prepared without incorporating the essential oils and they were used as control films. The solutions were poured into Teflon circular plates (13.5 cm in diameter) and, after 12 h at room temperature, dried films were peeled off from the casting surface and stored at 4 °C until they were used for the antimicrobial test on cheese slices.

2.6. Antimicrobial assays

2.6.1. Inoculum preparation

Stock cultures of both microorganisms were kept frozen (-25 °C) in Tryptone Soy Broth supplemented with 30% glycerol. Cultures were then regenerated by transferring a loopful of each bacterium into 10 mL of TSB and incubated at 37 °C overnight (ON). Then this culture was diluted to the level of 10⁸ CFU/mL according to 0.5 McFarland method at 600 nm. The culture was centrifuged twice at 4,000×g for 15 min at 4°C. The supernatant was decanted and the pellet was washed using PBS (10mM) followed by vortexing. After the second centrifugation, the

microorganisms were resuspended with a PBS solution and stored at 4°C until use. To verify the number of test bacteria samples in the suspension, dilutions were spread on TSA in triplicate at 37°C for 24 h.

2.6.2. Agar-diffusion disk

The antimicrobial activity was first evaluated by the agar disc diffusion method, according to Clinical and Laboratory Standards Institute (Dannenberg et al., 2017). 100 µL of bacterial cell suspensions (10^8 CFU mL⁻¹) were spread on the surfaces of MHA using sterile cotton swabs. Sterile paper disks (6 mm) were impregnated with 5µl of EOs and were placed on the surface of MHA, and subsequently incubated overnight at 37 °C. After 24 h, the diameter of the resulting inhibition zone was measured and expressed in mm. Experiments were performed in triplicate.

2.6.3. Testing Minimum inhibitory concentration (MIC) of essential oils (EOs) from *Laurus nobilis* and *Rosmarinus officinalis*

MIC is defined as the lowest concentration of samples at which the growth of microorganisms is inhibited. Dilution method was employed to quantitatively determine the minimum inhibitory concentration (MIC) of pure EO and loaded nanofibers, as reported by Wen et al. 2016 with some modifications. Nanofiber samples were first sterilized under UV light for 30 min. Initially, EOs and EO loaded nanofiber samples were dissolved/dispersed in ethanol (50% v/v) at 100 µg/mL and 2500 µg/mL, respectively. 50 µL of the samples were poured into the wells of a 96-well microplate, in which 45 µL of the Mueller Hinton Broth (MHB) solution had already been poured. Then, two-fold serial dilutions of EOs and nanofiber samples were studied being the final diluted concentration 0.2 µg/mL and 0.02 mg/mL, respectively. Then, 5 µL of each solution containing the microorganism was added into the well. Negative controls were performed for EO and nanofiber samples by adding ethanol solution into inoculum and MHB, and positive controls were

carried out just using MHB media. The microplates were incubated at 37° C for 18 hours and turbidity was measured at 600 nm. Three replicates were done for each sample.

2.6.4. Challenge test: antimicrobial test on cheese.

Zein nanofibers (ZNF) and zein films (ZF) obtained by casting were used for direct application onto fresh semi-hard cheese slices. Briefly, 6 g pieces of cheese (40 mm × 40 mm × 3 mm) were sterilized with UV light in a laminar flow safety cabinet for 30 min. Subsequently, they were contaminated with 100 µL of a suspension of the bacterium (1×10^8 CFU mL⁻¹) and spread on their full surfaces with a sterile rod. The inoculated slices were kept 15 min within the laminar flow safety cabinet to let the cheese surface absorb the inoculum and then immediately were coated with sterile zein nanofibers and cast films (4 × 4 cm) and they were put in sterile polyethylene zip bags. Each treatment was performed in triplicate.

Positive controls were uncoated cheese slices and coated cheese with zein nanofibers or cast zein films without EOs in its formulation under the same experimental conditions.

2.7. Statistical analysis

A one-way analysis of variance (ANOVA) followed by Tukey's posthoc test was used to determine significant differences at a significance level of $p < 0.05$, using SPSS software (version 17.0; IBM Corp., Armonk, NY). Results were expressed as mean ± standard deviations (SD). All experiments were performed in triplicate.

3. Results and Discussion

3.1. Characterization of the essential oils (EOs) extracted from *Laurus nobilis* and *Rosmarinus officinalis*

The composition of the essential oils extracted by hydrodistillation from *Laurus nobilis* and *Rosmarinus officinalis* were analysed by GC-MS. As shown in Table 1, almost twenty compounds in LEO and in REO were identified, which represented more than 93% of the total constituents for both EOs. The yields of LEO and REO in the leaves were 2.5 and 1.24%, respectively.

The main compounds detected in REO were 1,8 cineole (55.80%), camphor (7.94%), α -pinene (7.81%), 1,4-cyclohexadiene (3.74%), β -pinene (2.93%), 2-camphanol (2.90%), verbenone (2.82%) and camphene (2.23%), while other compounds were present under 2%. Regarding LEO, the 5 main compounds were 1,8 cineole (69.87%), 1-terpinen-4-ol (3.47%), (+)-limonene (4.77%), β -pinene (2.22%) and methyl eugenol (4.49%) representing 84.82% of the total determined compounds. Both essential oils have highly oxygenated monoterpene and monoterpene hydrocarbon compounds with proved antibacterial and antifungal activity (Okoh et al., 2010).

Our results are in agreement with the findings of previous authors (Dadalioglu & Evrendilek, 2004; Wang, Wu, Zu, & Fu, 2008) who also identified 1,8 cineole as the main component of both essential oils. The qualitative and quantitative differences in the chemical composition of both essential oils if compared with the mentioned research works and with others (Di Leo Lira et al., 2009; Okoh et al., 2010; Vilela et al., 2016) can be related with plant origin, geographical and climate conditions, growing season, harvest time, drying and extraction processes (Dadalioglu & Evrendilek, 2004). Specifically, hydrodistillation has been observed to exert a strong influence on the polyphenol content and important compounds are lost due to solubilisation or degradation processes (Alice et al., 2019).

The antimicrobial activity of the 1,8 cineole-rich EOs against Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*

O157:H7, Salmonella typhimurium, and Salmonella enteritidis) was also evaluated by means of the disk-diffusion method (see Supplementary Material Table S1). The data for the inhibition zones (mm) of various microorganisms indicated that Gram-positive bacteria were more sensitive towards both EOs under study than the Gram-negative ones, being even higher in the case of LEO. For instance, LEO showed inhibition zones of > 17 mm for *Listeria monocytogenes* and *Staphylococcus aureus*, while > 10 - 12 mm inhibition zones for the Gram-negative bacteria. Differences between both 1,8 cineole-rich EOs can be ascribed to the amount of terpenoids and terpenes compounds in the EOs. Terpenoids, which are terpenes that contain additional functional groups like alcohols, esters, carboxylic acid, etc, have been proved to exhibit higher antimicrobial activity than terpenes (Rao, Chen, & McClements, 2019) since properties of terpenoids such as hydrogen binding capacity have been reported to significantly affect their antimicrobial activity. In fact, LEO was found to have a highest amount of terpenoids than REO, especially 1,8-cineole, while REO also contained a higher amount of terpene molecules such as α -pinene, β -pinene, 2-camphanol and verbenone (see Table 1). The antimicrobial mechanism of action of EOs has been widely reported elsewhere (Okoh et al., 2010; Rao et al., 2019). Briefly, EOs compounds are capable of damaging and permeating the lipid structure of the bacterial cell wall and then, the cell death is caused by denaturation of proteins and cell membrane destruction, cytoplasmic leakage and cell lysis. However, it is important to highlight that the 1,8-cineol has been reported to increase the loss of cytoplasmic metabolites and nucleic acids along the damaged cell membrane in microorganism (da Silveira et al., 2014), which could explain the highest antibacterial efficiency of LEO.

3.2 Development and characterization of active fibers containing 1,8 cineole-rich EOs

The apparent viscosity and electrical conductivity of the zein solutions with different EOs concentrations are shown in Table 2. It is well known that solution parameters such as solvent

type, viscosity and electrical conductivity directly affect the morphology of electrospun nanofibers. From Table 2 it can be observed that the average diameter of the fibers decreased with increasing EO content, which is likely related to the decrease in the viscosity of the electrospinning solutions upon addition of EO.

Increasing both EOs content from 1 to 10%, led to a significant ($p < 0.05$) decrease in both the viscosity and conductivity of the solutions. Specially the lower viscosity, favours the formation of thinner fibers during electrospinning as it facilitates jet stretching (Altan, Aytac, & Uyar, 2018; Pérez-Masiá, Lagaron, & López-Rubio, 2014).

As observed in Figure 1 for the LEO-loaded structures as an example, the various zein solutions tested gave rise to smooth and homogeneous bead-free ultrathin fibers, indicating that the incorporation of the EOs in the solutions did not affect fiber formation. The average diameter of pure zein fibers was 201.8 ± 52.2 nm, while incorporation of 1, 5 and 10% LEO led to a decrease in fiber diameter to 178.1 ± 39.8 , 132.3 ± 41.8 and 118.6 ± 43.6 nm, respectively, as commented on above. The decrease in fiber diameter was less pronounced in the case of fibers containing REO, ranging the sizes between $186,30 \pm 52.61$ nm for the structures loaded with 1% REO, to 127.72 ± 44.31 nm for the ones containing 10% REO. These results show that, as previously mentioned, increasing the amount of EOs (from 1 to 10%) incorporated within the zein solutions led to a significant ($p < 0.05$) decrease in the average fiber diameters (cf. Table 1).

Figure 1. Scanning Electron Microscopy (SEM) images of neat zein nanofibers and zein nanofibers loaded with different concentrations of EOs.

From the histograms (cf. Figure 1 and Figure S1 in the Supplementary Material), the loaded fibers had thinner fiber diameters with homogeneous size distributions and the use of an acidic solvent to prepare the starting solutions facilitated the formation of beadless morphologies with smooth surfaces. It is worth mentioning that most of the previous works dealing with zein electrospinning were carried out using ethanolic aqueous solutions that give raise to thicker beaded or flat fibers (Antunes et al., 2017; Silva et al., 2018), while the combination of ethanol and acetic acid used in this work has demonstrated to provide improved fiber morphology, considerably reducing fiber diameter.

As observed from Table 2, there were significant differences between the encapsulation efficiency (EE) values of nanofibers loaded with different EOs concentrations ($p < 0.05$). Zein has demonstrated to be an appropriate matrix for EO encapsulation due to its amphiphilic nature, as

the presence of apolar aminoacids enables hydrophobic interactions with oil constituents, thus exhibiting high encapsulation efficiencies (Tran et al., 2019).

The slightly lower EE value of REO could be associated with its higher volatility (Vishwakarma et al., 2016), although no significant differences between the EE of both essential oils were observed, again highlighting the suitability of electrospinning for encapsulation of volatile substances, as no temperature is applied during the drying of the structures. Similar EE values were reported for electrospun fibers containing flaxseed oil, showing that as concentration increased, EE values decreased, although in the mentioned study, no significant differences were observed (Hadad & Goli, 2019). The decrease in EE upon increasing bioactive concentration is something quite common and it can occur either due to less efficient coating of the bioactive as the ratio matrix:bioactive decreases or due to limitations in the loading capacity of the developed structures (Gomez-Mascaraque, Casagrande Sipoli, de La Torre, & Lopez-Rubio, 2017). In our case and, in contrast with the limited loading capacity of nanoliposomes (Gómez-Mascaraque, Casagrande Sipoli, de La Torre, & López-Rubio, 2017), the loading capacity of the fibers was not reached and an important increase in loading capacity was seen upon increasing bioactive content (see Table 2). Previous studies have shown that it is possible to develop electrospun fibers incorporating up to 30% oil, although quantities above the 10% used in this work resulted in more defective fiber morphologies (Hadad & Goli, 2019). Previous studies dealing with EO encapsulation in electrospun fibers have shown that encapsulation efficiency depends on a number of factors, like electrospinning configuration (uniaxial vs. coaxial), coating material concentration, size distribution of the fibers, EO volatility, and interaction between core and coating materials, amongst others (Ghorani & Tucker, 2015). However, it appears that the chemical nature of the coating material is of outmost importance as great EE differences have been observed depending on the coating material used (Gómez-Mascaraque & López-Rubio, 2016; Tampau, González-Martínez, & Chiralt, 2018; H. Wang et al., 2017; Yao et al., 2017)

368 Infrared spectroscopy was used to investigate potential interactions between EOs and zein matrix.
369 ATR-FTIR spectra of the pure EOs, neat and EO-loaded zein electrospun fibers are gathered in
370 Figure 2. Neat zein fibers, as previously described, exhibited characteristic peaks at 3307 cm^{-1}
371 (hydroxyl and N–H stretching vibrations), 2974 cm^{-1} , 2951 cm^{-1} and 2898 cm^{-1} (C–H stretching
372 vibrations of CH_3 and CH_2 aliphatic groups), the amide I peak (1655 cm^{-1}) mainly ascribed to C=O
373 stretching vibrations, the amide II peak (1544 cm^{-1}), which arises from N–H bending and C–N
374 stretching vibrations and the amide III peak ascribed to C–N stretching band at 1260 cm^{-1}
375 (Alehosseini, Gómez-Mascaraque, Ghorani, & López-Rubio, 2019; Alehosseini, Gómez-
376 Mascaraque, Martínez-Sanz, et al., 2019).

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Figure 2. FTIR spectra of LEO (A) and REO (B), neat zein nanofibers and zein nanofibers loaded with different concentrations of EOs.

In contrast with previous studies, no significant shifts were observed in these spectral bands independently of the final composition of the encapsulation structures and suggesting (from the position of the amide I band) that the conformation of the zein in the fiber structures consisted on α -helices and unfolded protein chains (Aceituno-Medina, Mendoza, Lagaron, & López-Rubio, 2013).

As observed in Figures 2A and 2B, for the LEO and REO spectrum, respectively, showed a broad band centred at $\sim 3465\text{ cm}^{-1}$, associated with hydroxyl groups which may arise from alcohol groups of the chemical compounds present in the essential oils (such as linalool and 1-terpinen-4-ol); the spectral bands at ~ 2965 , 2925 and 2880 cm^{-1} , which are characteristic from alkane asymmetrical and symmetrical C-H stretching vibrations (probably arising from methyl and methylene groups of the EOs); the strong sharp band from the carbonyl (C=O) stretching from terpenoids (centred at $\sim 1744\text{ cm}^{-1}$ and 1731 cm^{-1} for REO and LEO, respectively); and a smaller band around 1680 cm^{-1} related to C=C stretching from terpene compounds (Schulz & Baranska, 2007).

Being 1,8-cineole the most abundant compound in both EOs, it should be expected that the main vibrational bands in the spectra arise from it. In fact, the bands at 1053 and 1079 cm^{-1} can be found in the spectrum of cineole ((Boughendjioua & Boughendjioua, 2017) which correspond to the C-O stretching vibrations of the molecule. Other vibrational bands associated with 1,8-cineole were the symmetrical bending of the methyl CH bonds and -C-O- stretching vibration at 1375 cm^{-1} (Michelina, Naviglio, Gallo, & Severina, 2019), the C-O-C asymmetric and symmetric stretching at 1217 and 1080 cm^{-1} , respectively, and the CH_2 wagging at 988 cm^{-1} corresponding to ring deformation vibrations (González-Rivera et al., 2016).

While the contribution of both EOs was relatively small in the spectra from the loaded zein fibers, some spectral changes could be seen upon addition of EOs at 10% concentration. For instance, the bands at 1746 and 1689 cm^{-1} in the pure REO spectrum were shifted to 1678 cm^{-1} in the spectrum from the hybrid REO-ZNF fibers, probably pointing out interactions between the aromatic rings

of REO and some functional groups from the zein protein. Similar changes were observed in the LEO-loaded fibers. Another obvious change in the spectra was the increase in intensity of the characteristic bands from the essential oils in the range from 1334 to 843 cm^{-1} upon increasing EO concentration.

The thermogravimetric analysis was used to evaluate the thermal stability of the different samples, of outmost importance for practical applications. Figures 3 and 4 show that LEO and REO, respectively had only one degradation stage corresponding to evaporation of volatile compounds. In contrast, the EOs-loaded ZNFs displayed two weight loss stages related to degradation of EOs and protein, respectively.

Figure 3. (A) TGA and (B) DTG thermograms of LEO, neat zein nanofibers and zein nanofibers loaded with different concentrations of LEO.

LEO and REO loaded ZNF showed a similar behaviour. Maximum degradation temperature for both EOs occurred at $\sim 88^{\circ}\text{C}$, thus confirming the similar composition of both LEO and REO, while this maximum shifted to higher temperatures when the EOs were included within the fibers. This confirms the ability of the zein matrix to increase the temperature stability of the volatile compounds, thus broadening the temperature range in which they can be processed (da Rosa et al., 2015). Similar results were found in thymol and carvacrol loaded zein nanoparticles (da Rosa et al., 2015). An enhanced thermal stability of EOs loaded ZNF was attributed to the interaction between zein and essential oils, thus leading to a higher heat resistance of the resulting nanofibers, compared with the unprotected EOs.

Figure 4. (A) TGA and (B) DTG thermograms REO, neat zein nanofibers and zein nanofibers loaded with different concentrations of REO.

It worth mentioning that as EO concentration increased in ZNF samples, the degradation temperatures slightly decreased, fact which is probably related to the greater protection ability as

a function of increasing the ratio encapsulation matrix: active compound and also to the fact that increasing the amount of EO, increases the probability of having more of the oils closer to the surface of the encapsulation structures and, thus, more exposed to the high temperatures. Generally it has been considered that the greater thermal stability of the polymeric/biopolymeric encapsulating matrices play an important role in increasing the thermal stability of essential oils (Silva et al., 2018).

Given that the 1,8 cineole-rich EOs were found to be more active against Gram-positive bacteria, the antimicrobial activity of the loaded fibers was performed against two of the most common pathogenic Gram-positive bacteria involved in foodborne outbreaks, *L. monocytogenes* and *S. aureus*. Table 3 gathers the Minimal Inhibitory Concentration (MIC) of the EOs and loaded-zein fibres. The first clear observation is that the MIC values of the loaded zein nanofibers were significantly higher than those obtained for pure EOs. Taking into account the amount of EOs in the loaded samples, the MIC values were around 20 µg LEO/mL and 32 µg REO/mL in the loaded ZNF, which were higher than the amount required for the neat EOs. This means that the EOs in the loaded fibers had lower antibacterial activity, probably due to both the existing interactions between the EOs and the zein matrix, as observed by FTIR, and the controlled release of the active compounds from the electrospun fibers (Abdollahi, Rezaei, & Farzi, 2012; Alboofetileh, Rezaei, Hosseini, & Abdollahi, 2014). From this table and not surprisingly, it can be observed that inhibition increased with increasing EO concentration in the zein nanofibers, being slightly higher for *S. aureus* than for *L. monocytogenes*. The most effective essential oil was the LEO which indicates that the higher antimicrobial activity was mainly explained by the higher amount of the 1,8-cineole in the LEO (~ 70%).

3.3. Challenge test

Challenge tests on cheese slices at refrigerated temperatures were carried out under conditions of *in vivo* storage; mimicking realistic scenarios of commercially available cheese slices packed and stored under refrigerated conditions (see Supplementary Material Figure S2). For comparative purposes, active films were prepared not only by means of the electrospinning process (zein nanofibers-ZNF-) but also by casting where EOs were directly mixed with the zein matrix without being encapsulated (ZF). The antimicrobial effect against *L. monocytogenes*, *S. aureus* and total mesophilic bacteria are shown in Figures 5, 6 and 7 in which the growth curves of both microorganisms on cheese coated with EOs-loaded zein nanofibers (ZNF), cast films (ZF) and positive control (control treatment of bacteria inoculum in cheese) were compared.

Figure 5. Effect of different loaded-ZNF and ZF films on the growth and survival of *L. monocytogenes* on cheese samples stored at 4 °C.

475 The *L. monocytogenes* counts in the control cheese samples substantially increased from the initial
476 inoculation level by the end of storage time. Indeed, pure ZF and ZNF films (without EO) were
477 not effective at reducing *L. monocytogenes* growth, since no significant differences were observed
478 in microbial growth with respect to the uncovered samples. Antunes et al. (2017) also reported the
479 absence of antibacterial activity of zein films against *L. monocytogenes* strain.

480 After three days of experiments, *L. monocytogenes* counts in the cheese samples covered with the
481 active films were significantly lower than in the uncovered cheese samples and those covered with
482 the neat zein film (ZF) or zein electrospun fibers (ZNF), evidencing the antimicrobial effect of the
483 structures containing the 1,8 cineole-rich EOs. As concern to the type of 1,8 cineole-rich EOs,
484 LEO incorporated into the zein matrix was slightly more effective than REO at the same
485 concentration.

486 As expected, the antimicrobial effect was less marked with 5% EO in the form of cast films where
487 a microbial reduction of approximately ~ 1 log, as compared to the control samples, was observed
488 at the end of the storage time. As it can be observed in Figure 5, EOs-loaded ZF led to greater
489 inhibitions of the bacteria on day 7 when compared to the electrospun structures, indicating the
490 greater release, which took place from the films during this period. Similar reduction of bacterial
491 counts was observed by Dannenberg et al. (2017) in cheese slices covered with cellulose acetate
492 casting films containing pink pepper essential oil as an active compound. In contrast, EOs-loaded
493 ZNF showed a more sustained antimicrobial activity, which could be explained by a more
494 sustained release over time. The fast release of EO from the films rapidly reduced bacterial counts,
495 but then the effect was diminished. After 28 days of storage, the ZNF loaded EOs showed
496 significantly lower counts than the ZF loaded EOs, indicating that, although both contain the same
497 amount of EO, the morphology of the structures had a significant effect in terms of release in
498 longer periods. Concretely, at this final point, the antimicrobial activity was equivalent for 10%
499 EOs prepared by casting (ZF) and 5% EOs prepared by electrospinning (ZNF), thus indicating the

effectiveness of the encapsulation process to preserve the active compounds avoiding their volatilization. In fact, the higher surface to volume ratio and porous structures generated through electrospinning, seem to have a positive effect for the long-term application of antimicrobials when compared to the casting method. Furthermore, the more sustained release from the electrospun structures is also evidenced in the growing curves.

Concerning to *S. aureus* growth, neat ZF and ZNF were not effective at reducing *S. aureus* growth, since no significant differences appeared with respect to the microbial growth on control cheese (uncovered samples). Similarly, Vahedikia et al. (2019) proved that zein films had no bactericidal or bacteriostatic effect against *S. aureus*.

Figure 6. Effect of different loaded-ZNF and ZF films on the growth and survival of *S. aureus* on cheese samples stored at 4 °C.

513

514 1,8 cineole-rich EOs incorporation into ZF and ZNF films reduced the pathogen growth, being

515 REO more effective than LEO as it was also observed for *L. monocytogenes* strain. During the

516 first three days, the highest concentration of LEO and REO added in the form of films (ZF), led to

517 a significant microbial reduction of the initial population in the cheese samples, highlighting the

518 fast release of the 1,8 cineole-rich EOs from the zein films prepared by casting. Throughout the

519 whole storage period, the addition of 1,8 cineole-rich EOs induced a microbial reduction of ~ 1.7

520 and 1.6 logs reduction for LEO and REO, respectively, incorporated in the form of nanofibers,

521 which was significantly higher than in their counterparts prepared by casting, highlighting again

522 the suitability and effectiveness of the electrospinning process for a sustained release of the EOs.

523 Therefore, both LEO and REO incorporated in zein matrices were effective at reducing pathogen

524 growth. Nevertheless, the antibacterial activity of the 1,8 cineole-rich EOs varied as a function of

525 the film formation methodology and the nature of the essential oil. Although 1,8 cineole-rich EOs

526 were effective against the two pathogens considered, they were slightly more effective, at the same

527 concentration, against *L. monocytogenes* than *S. aureus* when incorporated into zein films and this

528 was more marked in films prepared by means of the electrospinning process (ZNF). During the

529 first days of the storage period, the concentration of the REO, rather than the methodology used to

530 prepare the films, was the most relevant parameter in the inhibition of microorganisms' growth.

531 Nevertheless, after 7 days, the methodology used played an important role on the antimicrobial

532 efficiency being higher for ZNF and attributed to a sustained release. Regarding LEO, the

533 methodology used to prepare the films was more relevant during the first three days of storage for

534 *S. aureus* growth, increasing the antimicrobial efficacy for cast films due to the fast release of the

535 main components of the EOs, whereas for longer times, both the LEO concentration and the use

536 of zein nanofibers increased the antimicrobial efficacy.

537

Figure 7. Effect of different loaded-ZNF and ZF films on the growth and survival of aerobic mesophilic bacteria on cheese samples stored at 4 °C.

Concerning the aerobic mesophilic bacteria (Figure 7), LEO-loaded zein films were more effective at controlling aerobic mesophilic bacteria than REO-loaded films. In both cases these loaded films were more effective (2-3 logs reduction) against mesophilic bacteria than for *L. monocytogenes* or *S. aureus* strains. Although several research works have reported the antimicrobial potential of EOs for extending the shelf life of different food products (Oussalah et al., 2004, Fernandes et al., 2017, Ahmed, Hiremath, & Jacob., 2016), a greater effect was found for the 10% LEO loaded NF-based coating, in which a bactericidal effect on undesired spoilage microorganisms for cheese was observed at the end of the storage period. A possible explanation for this would be related to a more sustained release of the bioactive compounds when the essential oil was nanoencapsulated

in comparison with incorporating the EO through the casting method, thereby allowing its antimicrobial action to extend for a longer time.

Conclusions

This work has demonstrated that incorporation of EOs in ZNF constitutes a valuable strategy for enhancing their antimicrobial properties in practical food applications. The strong antimicrobial activity of LEO and REO was mainly ascribed to the presence of 1,8 cineole (69.87 and 55.80%, respectively). Both EOs were incorporated into homogeneous and smooth electrospun fibre mats which showed a significant antimicrobial activity against *L. monocytogenes*, *S. aureus* and aerobic mesophilic bacteria when applied as coatings on cheese slices. LEO exhibited the greatest antimicrobial activity and a complete inhibition of microbial growth was observed for LEO-loaded NFs films for aerobic mesophilic bacteria. When compared to cast films (ZF), the fast release of EO from the ZF rapidly reduced bacterial counts at short storage times, but later on the effect was diminished. In contrast, a greater antimicrobial activity at the end of the storage time was observed for ZNFs, which could be explained by a more sustained release over time due to the higher surface to volume ratio and porous structures generated through electrospinning. Therefore, these active electrospun structures seemed to have a positive effect for long-term application of antimicrobials when compared to the traditionally-used casting method.

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782 **Table 1.** Chemical composition of *Laurus nobilis* (LEO) and *Rosmarinus officinalis* (REO)
783 essential oils

<i>Laurus nobilis</i>			<i>Rosmarinus officinalis</i>		
Chemical compound	Retention time (min)	%	Chemical compound	Retention time (min)	%
α -Phellandrene	8.267	0.12	α -Pinene	8.504	7.61
α -Pinene	8.486	2.21	Camphene	9.026	2.23
Camphene	9.026	0.11	Verbenene	9.264	0.17
β -Pinene	10.083	2.22	β -Pinene	10.083	2.93
α -Terpinene	11.620	0.39	3-Carene	11.359	0.32
1,8-Cineole	12.267	69.87	α -Terpinene	11.626	0.37
1,4-Cyclohexadiene	13.151	0.83	1,8-Cineole	12.267	55.80
γ -Terpinene	14.617	0.35	γ -Terpinene	13.163	0.65
B-phellandrene	15.371	0.56	delta-3-Carene	14.683	1.06
Pinocarveol	16.077	1.02	(-)-Camphor	16.143	7.84
Pinocarvone	16.736	0.93	Pseudolimonene	16.926	0.80
1-Terpinen-4-ol	17.247	3.47	Borneol	17.128	1.58
Linalool	19.912	1.12	1,4-Cyclohexadiene	17.241	3.74
Verbenene	21.033	0.12	beta-phellandrene	17.716	5.39
Pseudolimonene	21.526	0.78	2-Camphanol	20.618	2.90
4-terpineol	21.791	0.56	trans-Caryophyllene	24.476	1.99
(+)-Limonene	22.476	4.77	α -Caryophyllene	25.402	0.32
			delta-Cadinene	27.212	0.19
Total		89.99			95.89

785 **Table 2.** Zein solution properties and subsequent encapsulation efficiency, loading capacity and average electrospun fiber diameter obtained for
786 the various EOs contents (LEO: *Laurus nobilis* essential oil; REO: *Rosmarinus officinalis* essential oil). Mean value $\pm$ standard deviation.

	EO content (%)	Apparent Viscosity (mPa s ⁻¹)	Conductivity (μ S/cm)	Average fiber diameter (nm)	Encapsulation efficiency (%)	Loading capacity (%)
	0	392.7 $\pm$ 7.6 ^a	523.2 $\pm$ 0.8 ^a	201.78 $\pm$ 52.24 ^a	-	-
	1	361.8 $\pm$ 3.7 ^b	509.4 $\pm$ 0.6 ^b	178.11 $\pm$ 39.8 ^a	83.06 $\pm$ 1.1 ^a	0.83
LEO	5	307.8 $\pm$ 8.1 ^c	465.7 $\pm$ 0.8 ^c	132.34 $\pm$ 41.8 ^b	78.40 $\pm$ 1.2 ^b	3.92
	10	272.9 $\pm$ 9.6 ^d	417.6 $\pm$ 0.5 ^d	118.63 $\pm$ 43.8 ^c	74.43 $\pm$ 1.0 ^c	7.44
	1	378.6 $\pm$ 5.2 ^a	515.1 $\pm$ 0.6 ^b	186.30 $\pm$ 52.6 ^a	81.92 $\pm$ 1.1 ^a	0.82
REO	5	343.3 $\pm$ 6.4 ^b	499.7 $\pm$ 0.3 ^c	158.47 $\pm$ 51.6 ^b	76.02 $\pm$ 1.1 ^b	3.80
	10	303.1 $\pm$ 3.4 ^c	477.3 $\pm$ 0.4 ^d	127.72 $\pm$ 44.3 ^c	73.31 $\pm$ 0.8 ^b	7.33

787 Mean values $\pm$ standard deviations. Different letters denote significant differences (p<0.05)

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Table 3. Minimal Inhibitory Concentration (MIC) of LEO, REO and loaded zein nanofibers.

Samples	<i>S. aureus</i>	<i>L. monocitogenes</i>
LEO	15.21±0.62 ^a	10.68±0.31 ^a
LEO5-ZNF	0.417±0.03 ^b	0.359±0.01 ^b
LEO10-ZNF	0.211±0.04 ^c	0.162±0.01 ^c
REO	23.92±0.76 ^a	17.87±0.64 ^a
REO5-ZNF	0.640±0.04 ^b	0.501±0.02 ^b
REO10-ZNF	0.332±0.01 ^c	0.273±0.01 ^c

MIC values are given as mg/mL (for nanofiber) and as µg/mL (for EO). Mean value ± standard deviation. Different superscript letters in each column denote significant differences (p<0.05).

Supplementary Material

Figure S1. Size distribution of unloaded and loaded zein nanofibers.

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