Essential Oils Encapsulated in Polymer-based Nanocapsules as Potential Candidates for Application in Food Preservation

Giuseppe Granata,[a] Stefano Stracquadanio,[b] Marco Leonardi,[a] Edoardo Napoli,[a] Grazia Maria Letizia Consoli,[a] Viviana Cafiso,[b] Stefania Stefani,[b] Corrada Geraci[a]*

[a]Istituto Chimica Biomolecolare - C.N.R., Via Paolo Gaifami 18, 95126, Catania, Italy

[b]Department of Biomedical and Biotechnological Sciences, University of Catania, Via S. Sofia 97, 95123 Catania, Italy

Abstract

The aim of this work is the encapsulation of essential oils (EOs) in polymeric nanocapsules (NCs) in order to enhance their antimicrobial activity against food-borne pathogens. *Thymus capitatus* and *Origanum vulgare* EOs, were selected for their different chemical composition being carvacrol (73%) and thymol (44%) the mayor constituent, respectively. Polymeric poly(ε-caprolactone) (PCL) nanocapsules loaded with EOs were prepared by nanoprecipitation method. The EO-NCs showed monomodal distribution with diameter size 171 and 175 nm, high efficiency of encapsulation and stability with high retention of EOs at both 4 °C and 40 °C, for a period of at least 30 days. The antimicrobial activity of EO-NCs against food-borne pathogens was higher than that of the corresponding pure essential oils and the NCs loaded with *Thymus capitatus* EO were the most active. Interestingly EO-NCs showed a bactericidal activity already at the minimum inhibitory concentrations (MICs). It makes them appealing as natural food preservatives.

Keywords, polycaprolactone nanocapsule; essential oils; antimicrobial activity; food-borne pathogens; natural food preservative.

*Corresponding author: Tel: +39 095 7338318; fax: +39 095 7338310
e-mail address: corrada.geraci@icb.cnr.it

1. Introduction

People today are increasingly oriented towards the consumption of food commodities with characteristics of naturalness and minimal process (Román, Sánchez-Siles, & Siegrist, 2017). These features are perceived by the consumers as synonymous with health and are determining factors for the food acceptance. Nevertheless, the new lifestyle in industrialized countries strongly addresses consumers' attitudes towards the consumption of processed foods (ready-to-eat foods). For this reason, the control of all those reactions (physical, chemical, enzymatic, and microbiological) responsible of food quality deterioration is the principal target for the food preservation. Microbiological contamination is one of the major causes of food deterioration with consequent alteration of the nutritional, healthiness and sensory qualities. Recently, it was observed an increment of foodborne diseases related to microbial contamination. World Health Organization (WHO) in a report published in 2015, estimated that in each year occur globally about 600 million cases (almost 1 in 10 people in the world) of food-borne illnesses and 420,000 associated deaths (WHO, 2005). The control of bacterial contamination in fresh or processed foods is a topic of great interest that needs effective responses. Sanitation by chemical products such as chlorine derivatives, iodophors, peroxyacetic acid and quaternary ammonium compounds, is not always enough. Even though they are used to produce microbial mortality, the possibility of promoting bacterial resistance subsists. In addition, consumers prefer food free from synthetic additives and preservatives and consider the term “natural” as a healthy index. In this context plant essential oils (EOs), generally recognized as safe (GRAS) compounds (FAO/WHO, 2008) and often endowed with antimicrobial activity, could represent a natural potential alternative to the use of chemical preservatives in food (Mith, 2014). Due to the multicomponent nature, the EOs antimicrobial mechanism is multitarget and there is no evidence of the occurrence of essential oils resistance. Additionally, EOs could be used to fight multi-drug resistant of pathogenic microorganisms (Chávez-González, Rodrıguez-Herrera, & Aguilar, 2016).

Among the EOs, *Origanum vulgare* (Or) and *Thymus capitatus* (Th), pervasive aromatic plants of Lamiaceae family, are of great interest. In fact, they are successfully used in medicine (Mendes, 2011), food (Sacchetti et al., 2005), agricultural (Lazar-Baker, Hetherington, Ku, & Newman, (2010), veterinary, and pest control (Jeong, Lim, Kim, & Lee, 2008; La Pergola et al., 2017).

The antimicrobial and the modulating antibiotic activity of these EOs (Schillaci et al., 2013) is due to the synergism of some major components, as phenolic compounds thymol and carvacrol, and other minor components, including monoterpene hydrocarbons as *p*-cymene and γ-terpinene.

In general, EOs possess high volatility, low solubility in aqueous phase, flavor, and sensitivity to oxygen, light, heat (Reineccius, 2016). In addition, their interaction with food matrix, where fats, proteins, and starch are present, makes difficult the use of pure EO in processed food. Nanoencapsulation of EOs represents a valid and efficient strategy to overcome these obstacles. In fact, this technique could mask flavor, decrease volatility, increase solubility and physical stability, and reduce the interaction with the food ingredients, improving bioaccessibility and bioavailability of EOs (Weiss, Gaysinsky, Davidson, & McClements, 2009). Compared to large capsules, the nanocapsules offer the advantages of a subcellular size, a larger surface area per unit volume, and potential enhancement of EOs concentration in food area where the microorganisms are preferentially located, such as water-rich phases or liquid-solid interfaces (Weiss et al., 2009). The nanocapsules can act as reservoir and controlled release systems of the bioactives and favor their delivery to bacterial cell.

In recent years, there has been a great interest in the development of new biodegradable and biocompatible nanoparticles able to encapsulate lipophilic functional food ingredients. Among them the nanoparticles based on the aliphatic polyester poly(ε-caprolactone) (PCL) find interesting applications in the food and medical fields for reason of cost efficiency and favorable

physicochemical properties (Woodruff, & Hutmacher, 2010). PCL is insoluble in water, resistant to oil and solvents, innocuous to the environment, and slow to degrade in aqueous medium. In comparison to hydrophilic polymer, PCL is able to control the release of active ingredient for a longer time (up to several weeks) (Ahlin Grabnar, & Kristl, 2011). Nanocapsules prepared by interfacial deposition of PCL as preformed polymer (nanoprecipitation technique) have a lipid core where the bioactive is confined, surrounded by polymeric wall of PCL in turn covered by a surfactant polysorbate 80 (Venturini et al., 2011).

Numerous examples of natural polymeric nanocapsules containing EOs are known (Vishwakarma, Gautam, Babu, Mittal, & Jaitak, 2016), but only a few examples of nanocapsules based on PCL synthetic polymers loaded with EOs have been reported. In particular, tea tree essential oil (TTO) which has important pharmacological properties was loaded into nanocapsules of PCL prepared by nanoprecipitation (Flores et al., 2011). The nanocapsules (diameter around 200 nm) are homogeneous, display stability during the storage time (two months), ability to protect TTO from evaporation (Flores et al., 2011), and antifungal activity (Flores at al., 2013). Khoobdel et al. (2017) reported stable nanocapsules (average size around 145 nm) obtained by the same method and loaded with *Rosmarinus officinalis* essential oil. This nanosystem showed insecticidal activity in *Tribolium castaneum* (Khoobdel et al., 2017). Rubenick et al. (2017) reported that rosemary essential oil was used as oil core of PCL nanoparticle (mean size around 233 nm) to encapsulate the antibiotic mupirocin in attempt to increase the antibiotic activity. Ephrem et al. (2014) demonstrated that the nanoprecipitation method is also useful to prepare at large scale nanocapsules of PCL loaded with rosemary essential oil. In this case, the nanocapsules (at small and large scale) showed spherical morphology, mean size around 230 nm, stability over time and high encapsulation efficiency.

The objective of our work is the encapsulation with high efficiency and stability of essential oil from *Thymus capitatus* and *Origanum vulgare* in a nanocarrier based on PCL and the evaluation

104 of antimicrobial activity of these nanosystems against food-borne pathogens and food spoilage
105 bacteria respect to pure essential oils, in order to obtain potential candidates for application in
106 food preservation.

107 The EO loaded nanoparticles (EO-NC) here reported are characterized for particle size,
108 encapsulation efficiency, stability over time, and antimicrobial activity.

109

110 **2. Material and Methods**

111 *2.1. Materials*

112 Poly(ε-caprolactone) (PCL) (M_w ~ 65,000) were obtained from Ega-chemie (Steinheim,
113 Germany); sorbitan monostearate (SM) from Farmalabor (Bari, Italy); polysorbate 80 (Tween
114 80) from Merck (Milan, Italy). All chemicals and solvents were of analytic or pharmaceutical
115 grade. EO-NC suspensions were prepared using LC-MS Grade water (LiChrosolv, Merck).

116

117 *2.2 Essential oil preparation and characterization*

118 *2.2.1. Plant material*

119 The starting plant materials were two samples of cultivated plants classified as *Origanum*
120 *vulgare* ssp. *hirtum* and *Thymus capitatus* coming from Aragona (Sicily). The two samples
121 were harvested in the period comprised between the beginning of May and the end of June, and
122 naturally dried in the shadow at the collection point at a temperature between 25 and 30 °C, for
123 about 10-15 days.

124 *2.2.2. Isolation of the Essential Oil*

125 Air dried aerial part of each sample (100 g) was subjected to hydrodistillation in a Clevenger-
126 type apparatus until there was no significant increase in the volume of oil collected (3 h). Each
127 oil was dried over anhydrous sodium sulfate, and stored under N_2 in a sealed vial until required.

128 *2.2.3. GC- and GC-MS Analyses of Essential Oils*

129 Gas chromatographic (GC) analyses were run on a Shimadzu gas chromatograph, Model 17-A
130 equipped with a flame ionization detector (FID), and with an operating software Class VP
131 Chromatography Date System version 4.3 (Shimadzu). Analytical conditions: SPB-5 capillary
132 column (15 m x 0.10 mm x 0.15 μm); helium as carrier gas (1 mL/min); injection in split mode
133 (1:200); injected volume 1 μL (4% essential oil/CH_2Cl_2 v/v); injector and detector temperature
134 250 and 280 °C, respectively; linear velocity in column 19 cm/sec. The oven temperature was
135 held at 60 °C for 1 min, then programmed from 60 to 280 °C at 10 °C/min, then 280 °C for 1
136 min. Percentages of compounds were determined from their peak areas in the GC-FID profiles.

137 Gas-chromatography-mass spectrometry (GC-MS) was carried out in the fast mode on a
138 Shimadzu GC-MS mod. GCMS-QP5050A, with the same column and the same operative
139 conditions used for analytical GC-FID, operating software GCMS solution version 1.02
140 (Shimadzu). Ionization voltage 70 eV, electron multiplier 900 V, ion source temperature 180
141 °C. Mass spectra data were acquired in the scan mode in m/z range 40-400. The same oil
142 solutions (1 μL) were injected with the split mode (1:96).

143 *2.2.4. Identification of Components of Essential Oils*

144 The identity of components was based on their GC retention index (relative to C_9-C_{20} *n*-alkanes
145 on the SPB-5 column), computer matching of spectral MS data with those from NIST MS
146 libraries, the comparison of the fragmentation patterns with those reported in literature (Adams,
147 2012) and, whenever possible, co-injections with authentic samples.

148

149 *2.3. Preparation of essential oil-loaded nanocapsules (EO-NCs)*

150 Th-NC, Or-NC were prepared by adapting the reported method from Venturini et al. (2011).
151 The mixture of sorbitan monostearate (35 mg), PCL (90 mg), and EO (310 mg) in acetone (25
152 mL) was stirred magnetically at 30 °C until complete dissolution of all components. Then, the
153 obtained organic phase was injected into a water solution (50 mL) of polysorbate 80 (75 mg),
154 and the resulting mixture was kept for 10 min at 25 °C. The organic solvent was removed under
155 vacuum carefully, setting the bath at 30 °C, reducing the pressure gradually from 500 to 200
156 mbar in 30 min, subsequently from 200 to 90 mBar in 30 min, and finally keeping that pressure
157 constant for 10 min. At the end, the mixture was kept under a gentle N_2 flow for 30 min at
158 atmospheric pressure to promote complete evaporation of the organic solvent. The EO-NC
159 milky suspension (50 mL) was obtained. In the same condition, but in absence of essential oil,
160 the empty NC suspension was prepared.

161

162 *2.4. Characterization of EO-NCs*

163 *2.4.1. Particle size and polydispersity measurements*

164 The mean diameter (*z*-average) of nanocapsules and polydispersity index (PDI) were
165 determined at 25 °C by dynamic light scattering (DLS) experiments performed on a Zetasizer
166 Nano ZS-90, Malvern Instruments, UK. The EO-NC suspensions were diluted (1:100, v/v) with
167 pure water. Data analysis was performed using Zetasizer Version 7.02 software.

168 *2.4.2. Zeta potential*

169 The zeta potential (ζ) values were determined by electrophoretic mobility using a Zetasizer
170 Nano ZS-90, Malvern Instruments, UK. The EO-NC suspensions were diluted (1:1000, v/v)
171 with pre-filtered (0.45 μm) 10 mM NaCl aqueous solution. Data were analysed using Zetasizer
172 Version 7.02 software.

173 *2.4.3. pH measurements*

174 pH measurements of the EO-NC suspensions were performed on a SevenCompact pH Meter
175 (Mettler Toledo, Milan, Italy) at 25 °C, without previous dilution.

176 *2.4.4. Encapsulation efficiency (EE) of EO-NCs*

177 The total content of essential oil in EO-NC suspensions was determined by UV−vis
178 spectroscopy (8453 UV-Visible Spectrophotometer, Agilent Technologies). In brief, 40 µL of
179 EO-NC suspension were diluted with 2 mL of acetonitrile and the amount of EO was derived
180 by the absorbance at 274 nm, corrected from the small absorbance due the other components
181 and compared to calibration curve of respective EO ($R^2 = 0.9999$). The absorbance of other
182 components of nanocapsule was estimated treating in the same condition the empty NC
183 suspension. The plots were constructed by plotting the absorbance at 274 nm of eleven
184 solutions containing different concentrations of each EO (from 10 to 110 µg/mL). The total
185 content of EO in NC suspensions were 5.9 mg/mL for Th and 5.4 mg/mL for Or.

186 The free essential oil was determined by the ultrafiltration/centrifugation technique (Nanosep
187 30K Omega, Pall Life Science, Milan, Italy; 90 min at 3500 × *g*). Suspension (500 µL) of EO-
188 NC was ultrafiltrated, and then an aliquot (80 µL) of the filtrate was diluted with 2 mL of
189 acetonitrile. The absorbance at 274 nm of resulting solution was registered by Uv-Vis
190 spectrophotometer.

191 The encapsulation efficiency was calculated using the following Eq.:

$$EE\ (\%) = \frac{[EO\ loaded]}{[EO]_{tot}} \times 100$$

192

where the $[EO\ loaded] = [EO]_{tot} - [EO]_{free}$ represents the content of essential oil loaded in EO-NC suspensions; the $[EO]_{tot}$ and the $[EO]_{free}$ are the total and free content of essential oil in EO-NC suspensions, respectively.

2.4.6 Stability over time of essential oil-loaded nanocapsules (EO-NCs)

The EO-NC suspensions were stored at 4 °C and a 40 °C, in dark conditions and in closed vessels. Particle size distribution of EO-NCs and loaded amount retention (%) of EOs in NCs were estimated over a period of 30 days. In particular, EO retention (%) was calculated according to the following Eq.:

$$EO\ retention\ (\%) = \frac{[EO\ loaded]_t}{[EO\ loaded]_0} \times 100$$

where the numerator is the EO loaded concentration at the time *t* and the denominator is EO loaded concentration of freshly prepared suspensions.

2.4.7 Statistical analysis

Each experiment was replicated at least twice and measurements performed at least three times. All data are expressed as mean ± standard deviation (SD). The effect of the storage time (0, 7, 15, 21, and 30 days) at 4 °C and in stressed condition (40 °C) was evaluated, the corresponding results were analyzed by variance analysis (ANOVA) and the mean values were compared by Tukey's test at a significance level of 0.05.

2.5 Microbiological studies

2.5.1.Microorganisms and growth conditions

Staphylococcus aureus ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Lysteria monocytogenes* ATCC 19118 were obtained from the American

type Culture Collection (Manassas, VA, USA). *S. aureus* was grown on MSA (Oxoid, Basingstoke, UK), *E. coli* and *P. aeruginosa* on MacConkey agar (Oxoid, Basingstoke, UK) and *L. monocytogenes* on BHI agar (Oxoid, Basingstoke, UK) at 37 °C for 24 h.

2.5.2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Microtiter plate assays were performed to determine the minimum inhibitory concentration of the Th/Or essential oils and EO-NCs according to the standard method (Wayne, PA: CLSI; 2013), with some modifications. EOs were emulsified with 5% DMSO in a 1:1 ratio and diluted in Mueller Hinton broth (MHB) (Oxoid, Basingstoke, UK) to 32 mg/mL final concentration. Th-NC (5.9 mg/mL of essential oil) and Or-NC (5.4 mg/mL of essential oil) suspensions were diluted in MHB to a 4 mg/mL final concentration. 100 µL of each EO or EO-NCs was inoculated in 96-well microplates containing sterile MHB and the serial two-fold dilutions were performed. The EO concentration range was 16 to 0.015 mg/mL for pure EOs and 2 to 0.002 mg/mL for EO-NCs. Subsequently, 10^5 CFU/mL of the bacteria was inoculated in each well and 100 µL, diluted appropriately, was spotted on MH or BHI plates for the MBC determination. Finally, the microplates were incubated for 18 h at 37 °C. Unloaded-NCs were used as NC antimicrobial activity controls and wells with sterile MHB or BHI as sterility controls. MICs value was determined at the lowest concentrations of the antimicrobial agent inhibiting bacterial growth, MBCs as the lowest concentration of each antimicrobial agent resulting in microbial death as defined by the inability to re-culture bacteria. The MIC and MBC values were expressed in mg/mL. All assays were carried out in triplicate with independently grown cultures. The results are means of three different experiments.

3. Results and discussion

3.1. Volatile composition of Origanum vulgare ssp. hirtum and Thymus capitatus oils

240 The complete analysis and chromatograms of the two tested samples are reported in Table 1,
241 Table S1, Figures S1, and S2 (see Supplementary material). *Origanum vulgare* sample presents
242 a typical Sicilian oregano profile (Tuttolomondo et al., 2015; Napoli & Ruberto, 2012) with
243 high amount of thymol (more than 43%, thymol chemotype) followed by γ-terpinene (15%)
244 and *p*-cymene (14%). All other compounds are below 4%. In terms of chemical families,
245 oxygenated monoterpenes are the main class (52%) followed by monoterpene hydrocarbons
246 (40%) and sesquiterpenes (7%).

247 *Thymus capitatus* sample is characterized by a high content of carvacrol (73%, carvacrol
248 chemotype) and a very low amount of thymol (0.3%) as already reported as typical for wild
249 Sicilian thyme population (Napoli, Curcuruto, & Ruberto, 2010). *p*-cymene (6%) β-
250 caryophyllene (5%) and γ-terpinene (2%) are the other main compounds. Also for thyme, the
251 oxygenated monoterpenes (76%) are the highly represented class of compounds, followed by
252 monoterpene hydrocarbons (14%) and sesquiterpenes (7%).

253 *3.2. Preparation and physochemical characteterization of EO-NCs*

254 *3.2.1 Preparation of EO-NCs*

255 EOs were encapsulated in poly(ε-caprolactone)-based nanoparticles obtained by a modification
256 of the interfacial deposition of the preformed polymer method reported by Venturini et al.,
257 2011. This method led to formation of exclusively of lipid-core nanocapsules by the diffusion
258 of the organic solvent into the aqueous phase, followed by the solvent evaporation on the
259 water/air interface. The inner core of nanocapsule (NC) is formed by the sorbitan monostearate
260 (SM) and, differently from Venturini method, by the EO which replaces the triglycerides. The
261 lipid core is surrounded by PCL, which is in turn covered by polysorbate 80, a food-grade non-
262 ionic surfactant. This latter ensures an adequate hydrophilicity of the system, and prevents the
263 NCs coalescence by a steric stabilization effect (Jäger et al. 2009, Venturini et al., 2011). In the
264 EO-NC preparation process an acceptable loss of EOs equal to 5% for Th-NCs and 13% for Or-

NCs was observed. The use of controlled vacuum and temperature during the organic solvent removal and of a gentle N_2 flow at the end, were crucial to avoid the unwanted loss of encapsulated EOs. The different percentages of loss in EOs could be ascribable to their different composition in volatile components.

3.2.2 Physicochemical characterization of EO-NCs

Size distribution by intensity of Th-NCs and Or-NCs, measured by DLS was indicative of a monomodal distribution in particle size for both the nanosystems (Figure 1).

The *z*-average diameter of nanoparticles (Table 2) is 175 ± 1 nm and 171 ± 2 nm for Th-NCs and Or-NCs, respectively. These values were in agreement with those expected by using the nanocapsule preparation technique reported by Venturini et al. 2011.

Furthermore, the very low PDI values of 0.09 ± 0.03 and 0.10 ± 0.01, for Th-NCs and Or-NCs respectively, agree with an extremely narrow size distribution of nanoparticles. As reported by Patravale, Date, & Kulkarni (2004), PDI values smaller than 0.2 are considered ideal, while PDI values higher than 0.5 are indicative of a very broad size distribution.

Zeta potential is very important parameter that provides useful information about the stability of aqueous nano-suspensions against unpleasant phenomena such as precipitation or flocculation. Both Th-NC and Or-NC suspensions show negative zeta potential (-4.3 ± 0.5 mV for Th-NCs and -3.2 ± 0.4 mV for Or-NCs) due to the free negative charge of carboxylic groups present at the polymer extremities. The low values measured are ascribable to polysorbate 80, covering the nonoparticle wall. This non-ionic surfactant stabilizes the nanocapsules by a steric mechanism (Mora-Huertas, Fessi, & Elaissari, 2010), preventing regrettable physical processes. Moreover, the zeta potential values found for EO-NCs are in agreement with those obtained for other PCL nanoparticles (Granata, Consoli, Lo Nigro, Geraci, 2018; Jäger et al., 2009).

289 The two EOs do not significantly affect the pH values of the resulting EO-NC suspensions,
290 with value of 4.4 ± 0.1 and 4.6 ± 0.1 for Th-NCs and Or-NCs, respectively.

291 *3.2.1 Encapsulation efficiency percentage (EE %)*

292 The *EE* % of different NCs loaded with Th and Or, obtained considering the ratio of quantities
293 between the encapsulated EO and the total EO measured in the suspension, are very high with
294 values of 96 ± 4 and 91 ± 1, respectively (Table 2). These date, indicative of an excellent
295 encapsulation efficiency for both the EOs in poly(ε-caprolactone)-based nanocapsules, are
296 extremely higher than those observed for chitosan-based nanosystems, widely diffused in food
297 industry. For example, the *EE* % (by Uv-vis analysis) of oregano essential oil encapsulated in
298 chitosan nanoparticle ranged from 5.4% to 24.72% (Hosseini, Zandi, Rezaei, &
299 Farahmandghavi, 2013) and thyme essential oil loaded chitosan nanoparticles showed *EE* %
300 value of about 30.67% (Ghaderi-Ghahfarokhi, Barzegar, Sahari, & Azizi, 2016).

301 *3.2.3 Stability assay*

302 The stability of the EO-NC suspensions was evaluated by monitoring parameters as size,
303 polydispersity and zeta potential over time. In particular, the samples were analyzed at 7, 15,
304 21, 30 days of storage (4 °C and 40 °C). As showed in Table 3, no significant changes in
305 particle size, polydispersity index, and zeta potential were observed and the formulations
306 remained essentially stable when the time and temperature of storage were varied. In particular
307 the particle size distribution curves (Figure 1) of the freshly prepared samples and those stored
308 at 4 °C and 40 °C for 30 days, were quite superimposable indicating that the temperature and
309 the time did not affect the particle size. In all the performed experiments, PDI values were
310 much lower than 0.2, confirming an excellent homogeneity of the nanoformulations (Grillo,
311 dos Santos, Maruyama, Rosa, de Lima, & Fraceto, 2012). During the storage time, it was
312 possible to observe an increase of negative zeta potential values. It was attributable to small

enhancement of the negative charges, originating from the PCL, which are able to stabilize the nanoparticles by electrostatic repulsion. The presence of polysorbate 80, covering PCL polymer, promotes the nanoparticles stability by steric effect (Wu, Zhang, & Watanabe, 2011). Therefore, these nanosystems showed no tendency to aggregation when stored at 4 °C or 40 °C for a period of 30 days.

The ability of the nanocapsules to retain inside them the essential oils, varying the time and conditions of storage, is an index of stability. The results, showed in Figure 2, revealed that the NCs kept at 4 °C or 40 °C have a high EO retention capacity, indicating the stability and robustness of the carrier system.

In particular, for Th-NCs kept at 4 °C a decrease in EO retention (%) is not observed up to 7 days, while for Or-NCs this period is protracted to 15 days. After that, the retention in the following 7 days drops to 88% for Th-NCs (15 days) and to 92% for Or-NCs (21 days). These values remain almost unchanged up to 30 days (86% for Th-NCs and 92% for Or-NCs).

For both the formulations kept at 40 °C, it was observed a little decrease of EO retention in the first 15 days with values of 88 and 92% for Th-NCs and Or-NCs, respectively. In the following days, a slower controlled release of EOs takes place and the final retention (30 days) is 84% for Th-NCs and 90% for Or-NCs.

The initial higher rate of EOs release (burst effect) may be generally ascribed to the desorption of the EO from the surface and/or to polymer relaxation mechanism (swelling/shrinking) generated by diffusion and dispersion of water into PCL wall. The following lag phase, with very slow release rate of OE is related to the diffusion process from the NC reservoir core to the aqueous external medium (Mora-Hertas et al., 2010).

3.4. Antimicrobial activity of Th-NC and Or-NC

336 The antimicrobial activity of pure EOs of *Thymus capitatus* and *Origanum vulgare* along with
337 Th-NCs, Or-NCs, and empty NCs were evaluated against a panel of four selected Gram-
338 positive and Gram-negative food poisoning pathogens.
339 MIC and MBC values of EO-NCs against *S. aureus*, *E. coli*, and *L. monocytogenes* were two-
340 or three-fold lower than values of pure EOs, whereas those of Or-NCs against *L.*
341 *monocytogenes* were only one-fold lower. The encapsulated EOs possess higher inhibitory and
342 bactericidal activity than pure EOs (Table 4). In particular, the MIC of Or pure oil against *S.*
343 *aureus* and *E. coli* was reduced from 4 mg/mL to 0.5 mg/mL by Or-NCs, while the MIC of Th
344 pure oil was reduced from 2 mg/mL to 0.25 mg/mL by Th-NCs. This enhanced antimicrobial
345 activity of EOs is attributable to their encapsulation in nanometric systems that can increase the
346 transport mechanisms and diffusion of EO across the bacterial cell membrane (Bilia, Guccione,
347 Isacchi, Righeschi, Firenzuoli, & Bergonzi, 2014). Unlike other bacteria *P. aeruginosa* did not
348 show significant susceptibility to pure or encapsulated oils (data not shown). Moreover, no
349 antimicrobial effects were shown by empty NCs against all microorganisms.

350 With regard to *S. aureus*, *E. coli* and *L. monocytogenes*, EO-NCs displayed MBCs equal to
351 MICs, with the exception of Th-NCs against *L. monocytogenes* where MBC is one-fold higher
352 than MIC. These results indicate that EO-NCs have a bactericidal activity already at minimum
353 inhibitory concentrations or at least at one-fold higher than MIC on tested microorganisms,
354 making their use more interesting as natural food preservatives.

355 Gram-positive bacteria are more susceptible than Gram-negative bacteria to essential oil
356 treatment. The Gram-negative bacteria have an outer membrane containing hydrophylic
357 lipopolysaccharides (LPS) that protect the cell from macromolecules and hydrophobic
358 compounds such as those contained in essential oils (Hyldgaard, Mygind, & Meyer 2012).

359 Carvacrol and thymol are major volatile compounds present in our samples of *Thymus*
360 *capitatus* and *Origanum vulgare* EOs with values of 73% and 44%, respectively. These

compounds are known for antibacterial activity and their mode of action has been extensively studied. They are able to interact with outer cell membrane, increasing fluidity and permeability, and to cause structural and functional damages to cytoplasmic membrane (Hyldgaard, 2012). In particular, for Gram-negative bacteria, the disintegration of the outer membrane with release of LPS was demonstrated (Helander et al., 1998). The EO-NCs antibacterial activity against Gram-positive is similar to that against Gram-negative bacteria. It could be ascribed to the action mode of major components, carvacrol and thymol, present in EO samples.

Moreover, the antimicrobial activity of Th-EO and Th-NCs was higher than that of Or-EO and Or-NCs, respectively. That may be due to the higher amount of bioactive compound in Th-EO.

4. Conclusion

In this study, we have realized stable nanosystems based on poly(ε)caprolactone to encapsulate EOs from *Thymus capitatus* and *Origanum vulgare*, widely used as flavoring agents in foods. The nanoparticles, prepared by nanoprecipitation method by using a biodegradable and biocompatible polymer, show diameter of 171 ± 2 nm (Or-NCs) and 175 ± 1 nm (Th-NCs), monomodal size distribution with a very low PDI (≤0.10) and high encapsulation efficiency (*EE%* 96 ± 4 for Th-NCs and 91 ± 1 for Or-NCs). Dimensional stability of nanoparticles does not change over time (30 days) when the suspensions are kept at temperature of 4 °C and 40 °C to simulate a normal and accelerate storage process. Moreover, the high values of EOs retention (%) for EO-NCs at 30 days of storage are indicative of nanosystems able to provide a sustained and slow release of the EOs. The antimicrobial activity of EO-NCs against food-borne pathogens was higher than that of pure essential oils, pointing out the process effectiveness of their encapsulation in a nanometric structure. Among the EO-NCs those containing Th-EO are the most active probably due to the different chemical composition of bioactives.

385 Our findings suggest these nanosystems as appealing candidates for use in food sector. They
386 could represent a natural alternative to chemical preservatives in preventing the spread of food-
387 borne diseases and ensuring greater safety in processed foods using nanotechnology.

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Figure Captions

Fig. 1. Intensity weighted distribution of the hydrodynamic particle diameter D_H of (a) Th-NC, (b) Or-NC. The solid line is relative to the freshly prepared sample; the dashed and dotted lines are relative to the sample stored for 30 days at 4 °C and at 40 °C, respectively.

Fig. 2. Essential oil retention over time at 4 °C (light colours) and 40 °C (dark colours) of (a) Th-NC; (b) Or-NC.

Table 1

Table 1. List and percentages of identified compounds of *Origanum vulgare* and *Thymus capitatus* essential oils.

#[a]	Class/Compound[c]	Oregano %	Thyme %
	Monoterpene hydrocarbons	**40.31 (±0.095)**	**14.68 (±0.032)**
2	α-thujene	1.26 (±0.005)	0.67 (±0.003)
3	α-pinene[b]	0.76 (±0.003)	0.58 (±0.002)
4	camphene[b]	0.09 (±0.001)	0.17 (±0.001)
6	sabinene[b]	0.16 (±0.001)	0.10 (±0.002)
7	myrcene[b]	2.28 (±0.008)	1.71 (±0.005)
9	α-phellandrene	0.25 (±0.001)	0.16 (±0.001)
11	α-terpinene[b]	2.48 (±0.008)	1.18 (±0.003)
12	*p*-cymene[b]	14.49 (± 0.038)	6.88 (±0.014)
13	limonene[b]	0.55 (±0.002)	0.47 (±0.001)
15	*cis*-ocimene	1.75 (±0.004)	-
16	*trans*-ocimene	0.58 (±0.004)	0.03 (±0.000)
17	γ-terpinene[b]	15.35 (±0.031)	2.50 (±0.006)
19	terpinolene[b]	0.14 (±0.002)	0.15 (±0.000)
	Oxygenated monoterpenes	**52.12 (±0.111)**	**76.61 (±0.052)**
18	*cis*-sabinene hydrate[b]	0.07 (±0.000)	0.33 (±0.003)
20	Linalool[b]	0.63 (±0.001)	1.17 (±0.006)
25	borneol[b]	0.12 (±0.001)	0.55 (±0.005)
26	terpinen-4-ol[b]	0.89 (±0.002)	0.71 (±0.004)
30	thymol methyl ether[b]	1.99 (±0.002)	-
32	carvacrol methyl ether[b]	3.64 (±0.003)	-
35	*p*-cymen-7-ol	0.07 (±0.001)	0.17 (±0.008)
36	thymol[b]	**43.93 (±0.106)**	0.31 (±0.025)
37	carvacrol[b]	0.51 (±0.002)	**73.03 (±0.055)**
	Sesquiterpenes	**7.36 (±0.188)**	**7.15 (±0.013)**
43	α-copaene[b]	0.12 (±0.001)	-
46	β-caryophyllene[b]	1.40 (±0.001)	5.45 (±0.005)
47	β-copaene	0.12 (±0.001)	0.02 (±0.005)
48	α-*trans*-bergamotene	0.04 (±0.000)	0.20 (±0.150)
49	α-humulene[b]	0.17 (±0.011)	0.03 (±0.001)
50	aromadendrene	0.04 (±0.002)	0.27 (±0.025)
51	*allo*-aromadendrene	0.11 (±0.001)	-
53	γ-muurolene	0.43 (±0.005)	0.03 (±0.001)
56	β-selinene	0.15 (±0.045)	-
57	γ-amorphene	0.04 (±0.014)	0.06 (±0.001)
58	α-muurolene	0.14 (±0.016)	-
59	β-bisabolene	1.69 (±0.003)	0.49 (±0.001)
60	γ-cadinene	0.52 (±0.002)	0.02 (±0.000)
61	δ-cadinene	1.04 (±0.003)	0.07 (±0.001)
67	spathulenol	0.11 (±0.001)	0.02 (±0.001)
68	caryophyllene oxide	0.42 (±0.001)	0.29 (±0.001)
	Others	**0.02 (±0.001)**	**0.95 (±0.001)**
5	octen-3-ol	-	0.39 (±0.001)
38	methyl decanoate	-	0.12 (±0.000)
64	geranyl butanoate	-	0.36 (±0.001)

[a] The numbering refers to elution order and values (relative peak area percent) represent averages of 3 determinations. [b]Co-elution with authentic sample. [c]Compounds with a % less than 0.1 were not reported. A complete list of identified compounds is available on Table 1S (see Supplementary material).

Table 2

Table 2. Stability over time of essential oil-loaded nanocapsules (EO-NCs) at 4°C.

EO-NC	Storage time (days)				
	0	7	15	21	30
Th-NC					
Z-average (nm)	175 ± 1[a]	163 ± 1[b]	165 ± 2[b]	163 ± 2[b]	162 ± 1[b]
PDI	0.09 ± 0.03[a]	0.07 ± 0.03[a]	0.08 ± 0.01[a]	0.08 ± 0.02[a]	0.06 ± 0.02[a]
ζ (mV)	-4.3 ± 0.5[a]	-4.5 ± 0.8[a]	-4.1 ± 0.6[a]	-6.5 ± 0.4[b]	-8.4 ± 0.4[c]
Th loaded amount (mg/mL)	5.6 ± 0.2[a]	5.6 ± 0.2[a]	4.9 ± 0.2[b]	4.9 ± 0.2[b]	4.8 ± 0.1[b]
Or-NC					
Z-average (nm)	171 ± 2[a]	178 ± 3[b]	184 ± 2[c]	185 ± 1[c]	184 ± 1[c]
PDI	0.10 ± 0.01[a]	0.09 ± 0.01[a]	0.09 ± 0.02[a]	0.06 ± 0.02[a]	0.09 ± 0.02[a]
ζ (mV)	-3.2 ± 0.4[a]	-7.7 ± 0.5[b]	-3.1 ± 0.3[a]	-6.7 ± 0.9[b]	-9.4 ± 0.7[c]
Or loaded amount (mg/mL)	4.9 ± 0.1[a]	4.9 ± 0.3[a]	4.9 ± 0.2[a]	4.5 ± 0.2[b]	4.5 ± 0.1[b]

Values in the same line with the same superscripts are not significantly different ($p > 0.05$).

Table 3

Table 3. Stability over time of essential oil-loaded nanocapsules (EO-NCs) at 40°C.

EO-NC	Storage time (days)				
	0	7	15	21	30
Th-NC					
Z-average (nm)	175 ± 1[a]	175 ± 1[a]	178 ± 3[a]	176 ± 1[a]	177 ± 1[a]
PDI	0.09 ± 0.03[a]	0.07 ± 0.02[a]	0.08 ± 0.03[a]	0.07 ± 0.02[a]	0.10 ± 0.01[a]
ζ (mV)	-4.3 ± 0.5[a]	-4.1 ± 0.9[a]	-3.5 ± 0.5[a]	-3.9 ± 0.2[a]	-8.8 ± 0.6[b]
Th loaded amount (mg/mL)	5.6 ± 0.2[a]	5.3 ± 0.2[a,b]	4.9 ± 0.2[b,c]	4.8 ± 0.1[c]	4.7 ± 0.2[c]
Or-NC					
Z-average (nm)	171 ± 2[a]	177 ± 1[b]	178 ± 2[b]	177 ± 2[b]	180 ± 2[b]
PDI	0.10 ± 0.01[a]	0.06 ± 0.01[a]	0.10 ± 0.02[a]	0.09 ± 0.03[a]	0.06 ± 0.02[a]
ζ (mV)	-3.2 ± 0.4[a,b]	-4.4 ± 0.9[a]	-2.8 ± 0.5[b]	-5.9 ± 0.2[c]	-8.2 ± 0.3[d]
Or loaded amount (mg/mL)	4.9 ± 0.1[a]	4.7 ± 0.1[a,b]	4.5 ± 0.2[b,c]	4.5 ± 0.1[b,c]	4.4 ± 0.2[c]

Values in the same line with the same superscripts are not significantly different ($p > 0.05$).

Table 4

Table 4. MIC[a] and MBC[a] measurements of pure and encapsulated essential oils on different microbial strains.

	S. aureus ATCC 29213		*E. coli* ATCC 25922		*L. monocytogenes* ATCC 19118	
	MIC	MBC	MIC	MBC	MIC	MBC
Th-EO	2	2	2	2	1	2
Th-NC	0.5	0.5	0.25	0.25	0.25	0.5
Or-EO	4	4	4	4	2	4
Or-NC	0.5	0.5	0.5	0.5	1	1

[a]MIC and MBC were expressed in mg/ml.

Figure 1
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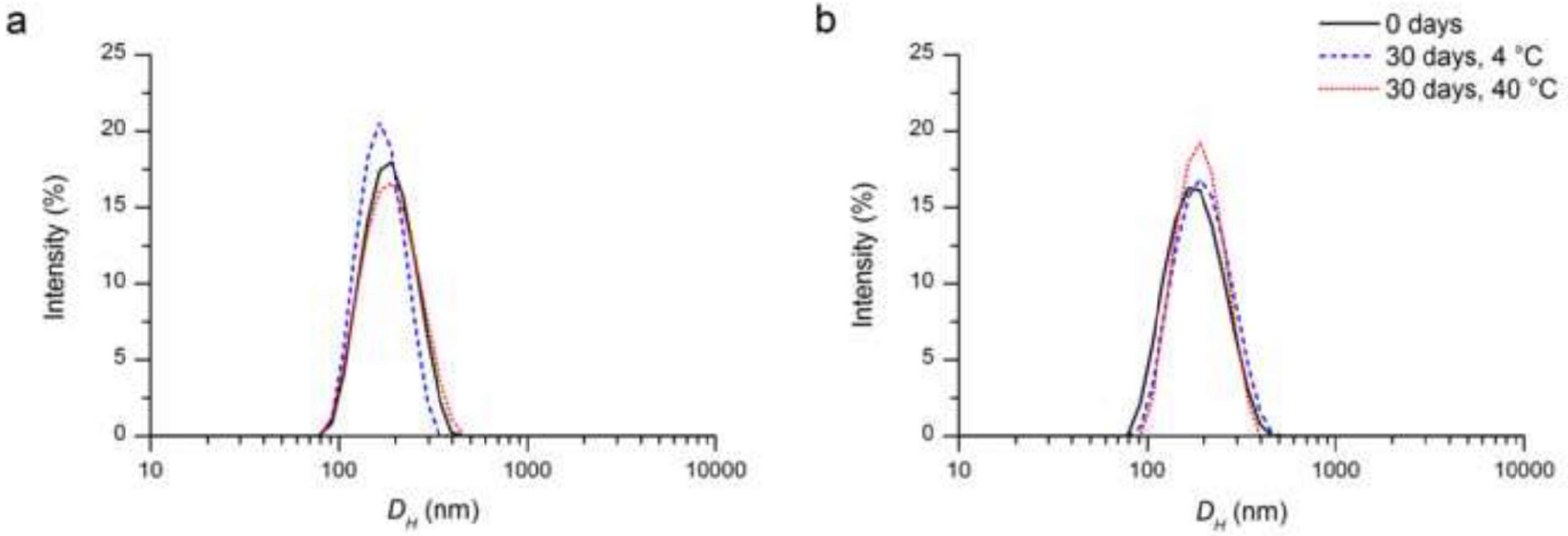

Figure 2
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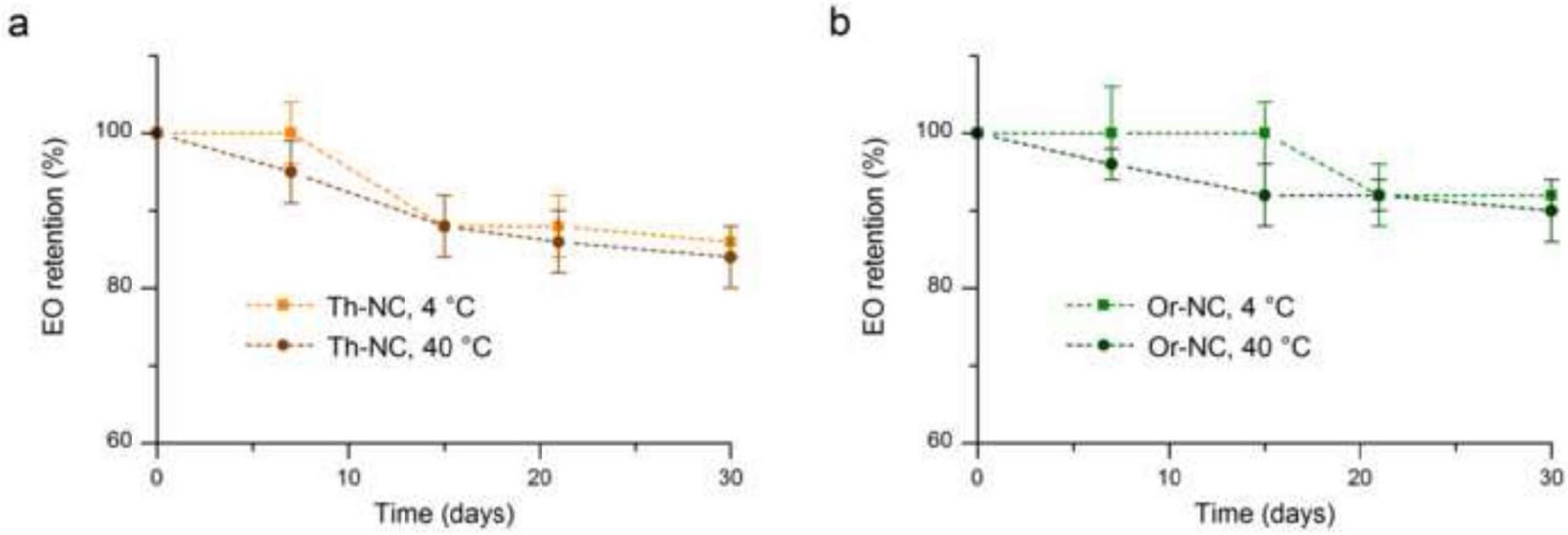

Supplementary Material

Click here to download Supplementary Material: Fig S1-Fig S2-Table S1.docx