TITLE:

Antimicrobial efficacy of *Thymbra capitata* (L.) Cav. essential oil loaded in self-assembled zein nanoparticles in combination with heat

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HIGHLIGHTS

Carvacrol was identified as the major compound of *Thymbra capitata* oil (TCEO)

Zein, a plant protein isolated from maize, is proposed as biocompatible carrier

Novel self-assembled zein nanocapsules loaded with TCEO (zn-TCEO) are described

Valuable synergistic effects were obtained when combining mild heat and zn-TCEO

The use of zn-TCEO might improve product higienization or surface disinfection
Abstract

This study reports on the chemical composition of *Thymbra capitata* essential oil (TCEO) and its antimicrobial activity when applied with heat either as a suspension (s-TCEO) or loaded in self-assembled zein nanoparticles (zn-TCEO). Zein, a plant protein isolated from corn and maize, is proposed as natural, and biocompatible carrier. TCEO composition was analysed by GC-MS, and 35 components were identified. Carvacrol, a monoterpenoid, was the major constituent (73.8%). zn-TCEO were prepared under low shear conditions and characterized according to droplet size (<180 nm) and encapsulation efficiency (77.8%). The two TCEO formulations (s-TCEO and zn-TCEO) were compared in terms of antibacterial activity against *Escherichia coli* O157:H7 Sakai and *Listeria monocytogenes* EGD-e. The zn-TCEO displayed a greater bacteriostatic activity than s-TCEO, probably due to their improved dispersion in the growth media. However, zn-TCEO exerted a lower bactericidal activity than s-TCEO, probably due to the EO progressive release. The combination of TCEO and heat (53°C) exerted valuable synergistic lethal effects, causing the death of up to 5 log_{10} cycles of both microorganisms. The effectiveness of zn-TCEO was especially improved at pH 4.0. Therefore, the application of this new delivery system, designed to encapsulate and protect EOs, and ensure their controlled release, might represent an advantageous alternative for food, cosmetic or pharmaceutical industries to improve the efficacy of higienization processes or surface cleaning and disinfection procedures when combined with mild heat.

**Keywords:** *Thymbra capitata* essential oil; chemical composition, nanocapsules; zeins; heat; synergism.
Plant essential oils (EOs) are natural compounds extracted mainly from aromatic and medicinal plants. Due to their antimicrobial properties, they have been frequently recommended as biocides, preservatives or for cleaning and disinfection procedures (Burt, 2004; Souza et al., 2016). However, their chemical instability due to oxidation, high reactivity, and hydrophobicity thwarts any attempt to incorporate them directly into cleaning solutions, cosmetic products, or food beverages (Donsì and Ferrari, 2016). New delivery systems have therefore been designed in order to protect their chemical properties and ensure their controlled release (Prakash et al., 2018).

Active encapsulation has mainly been used to protect bioactive compounds from adverse environmental factors, to enhance solubility of poorly soluble actives and to grant them certain specific properties, such as sustained or controlled release. Unfortunately, most of these promising nanoencapsulation methods require expensive equipment and present difficulties in the scale-up phase (spray drying, freeze-drying, etc.). Recent developments in the preparation of nanoparticles have been marked by a series of emerging issues: the requirement for less toxic reagents, a simplification of the procedure with the purpose of allowing economic scale-up, and optimization to improve yield and entrapment efficacy (Reis et al., 2006).

*Thymbra capitata* (L.) Cav. (syn. *Thymus capitatus* (L.) Cav., Lamiaceae) is an aromatic herb that grows in the Mediterranean area and produces an essential oil (TCEO). Thanks to its recognized antimicrobial and antioxidant properties, it can be used as a preservative for foodstuffs and formulations (Delgado-Adámez et al., 2017; Falcó et al., 2018; Neves et al., 2017).
Several natural polymers such as zein (Weissmueller et al., 2016), casein (Peñalva et al., 2018), or chitosan (Yuan et al., 2016) have been proposed as natural, and biocompatible carriers for different actives. Zein is a plant protein isolated from corn or maize belonging to a family of prolamines which are composed of hydrophobic aminoacids (Salman et al., 2013). In this context, the use of zein in conjunction with a controlled self-assembly method for encapsulation of TCEO would allow us to develop a carrier for this natural, low-cost, biocompatible and GRAS (generally recognized as safe) EO (Patel and Velikov, 2014).

Most of the studies that propose the encapsulation of EOs describe how their progressive release from nanocapsules can help inhibit microbial growth by developing a prolonged bacteriostatic activity over time (Chouhan et al., 2017). However, when a bactericidal effect is required in a short treatment, as tends to occur during higienization or surface cleaning and disinfection, the control of the liberation of EOs might entail a limitation, since the doses required to exert a bactericidal effect are not easily achieved.

In this regard, remarkable synergistic lethal effects have been described when EO suspensions (Espina et al., 2010; Guevara et al., 2015; Raybaudi-Massilia et al., 2009) or nanoemulsions (de Carvalho et al., 2018; Mate et al., 2016; Pagán et al., 2018) are applied in combination with physical processes, for instance mild heat treatments (50-60ºC). Such combined treatments have been proposed not only as alternatives to traditional fruit juice preservation methods, but also as a good means of eradicating biofilms of *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus* from plastic surfaces (Espina et al., 2017). The described synergism has enabled the reduction of treatment temperatures and/or antimicrobial doses: in view of the strong flavor of most EOs, this can represent an enormous advantage.
As far as we can ascertain, the behavior of zein nanocapsules under heat treatments or of the antimicrobial properties of encapsulated EOs with zeins in combination with heat against pathogenic microorganisms has not been previously studied.

This research was therefore carried out in order to (i) evaluate the chemical composition of TCEO and its effectiveness *in vitro* on growth and survival of two pathogenic bacteria: *E. coli* O157:H7 Sakai and *L. monocytogenes* EGD-e; ii) obtain and characterize novel self-assembled zein nanocapsules loaded with TCEO (zn-TCEO) for TCEO protection and progressive release, and iii) to assess the antimicrobial efficacy of zn-TCEO as a single hurdle or in combination with heat as a function of treatment medium pH.

2. Material and Methods

2.1. *Thymbra capitata* essential oil (TCEO)

One-hundred-percent pure and natural TCEO was obtained by hydrodistillation of the flower heads of *Thymbra capitata*. TCEO was kindly provided by the TELIC Group (Barcelona, Spain). Once received, the EO was kept at room temperature, in the dark, in sealed glass vials until used.

Following the method described by Friedman et al. (2002), we applied a vigorous shaking procedure to prepare TCEO suspensions (s-TCEO) in McIlvaine buffer at pH 7.0 and at pH 4.0.

2.2. Chemical analysis of *Thymbra capitata* essential oil
α-Pinene, camphene, β-pinene, 1-octen-3-ol, myrcene, α-phellandrene, δ-3-carene, p-cymene, limonene, 1,8-cineole, (E)-β-ocimene, γ-terpinene, terpinolene, linalool, borneol, terpinen-4-ol, α-terpineol, thymol, carvacrol, (E)-caryophyllene, α-humulene, and caryophyllene oxide were purchased from Sigma-Aldrich (Milan, Italy) and used for peak assignment. A mix of \( n \)-alkanes ranging from octane (C\(_8\)) to triacontane (C\(_{30}\)) (Supelco, Bellefonte, CA, USA) was used to calculate the temperature-programmed retention index (RI).

An Agilent 6890N gas chromatograph coupled to a single quadrupole 5973N mass spectrometer (Agilent, CA, USA) was used for the analysis of TCEO. Components were separated on apolar HP-5 MS (30 m x 0.25 mm i.d., 0.1 μm f.t.; Agilent) made up of 5% phenylmethylpolysiloxane. Oven was programmed as follows: 5 min at 60°C raised at 4°C/min up to 220°C, then 11°C/min up to 280°C, held for 15 min. The temperature of injector and detector (single quadrupole) was set to 280°C. Helium (99.99%) was the carrier gas with a flow of 1 mL/min. TCEO was diluted in \( n \)-hexane (6 μL in 594 μL of solvent) and injected (2 μL) in split mode (1:50 ratio) into the GC-MS system. The spectra were acquired in full scan (29–400 \( m/z \)) using the electron-impact (EI, 70 eV) mode.

The software applications used for peak assignment as well as the adopted identification criteria were the same as those reported by Benelli et al. (2018). Semi-quantification of EO components was obtained by peak area normalisation, taking the same response factor for all volatile components into account.

2.3. Encapsulation of TCEO with zeins (zn-TCEO)

The TCEO-loaded zein nanocapsules were prepared according to self-assembly methods, avoiding the use of volatile organic solvent (Salman et al., 2013). We obtained
the oil phase by mixing TCEO, surfactants (Tween 20; Panreac, Madrid, Spain),
cosurfactants (propanediol, Dupont Tate & Lyle Bioproducts, TN, USA; propylene glycol
and denatured alcohol, Guinama, Valencia, Spain), zein plasticizers (Oleic acid, Panreac,
Madrid, Spain), and zein (Flo Chemical Corporation, MA, USA) under magnetic stirring.
The surfactant-oil mixture was subsequently added into water under continuous agitation
to form the nanocapsules.

2.4. Characterization, stability and encapsulation efficiency of zn-TCEO

We measured droplet size distribution and the polydispersity index of the zn-TCEO with dynamic light scattering (DLS) analysis in a particle size analyzer (90S Particle Size Analyzer, Brookhaven Instruments, NY, USA).

Encapsulation efficiency, which corresponds to the percentage of encapsulated TCEO, was determined by carvacrol quantification via an analytical HPLC method on an Agilent 1260 infinity system equipped with a quaternary pump, an auto-sampler with high sensitivity cell, a thermostatized column compartment, and a diode array detector. UV spectra were collected at 220 and 278 nm. Instrument control, data collection, and data processing were carried out with Agilent OpenLab CDS software. The column was a Zorbax SB-C18 (250 x 4.6 mm, 5 µm; Agilent). The mobile phase was an isocratic combination of acetonitrile ACN:H₂O (Merck, Darmstadt, Germany) (50:50) with a flow rate of 2 mL/min. Injection volume for standard solutions and all samples was 10 µL.

In order to separate free from encapsulated TCEO, a 100 kDa Amicon was used (Merck Millipore, Darmstadt, Germany). After centrifugation, the filtrate was collected and quantified. Encapsulation efficiency (EE) was calculated as follows:
To ensure the stability of the zn-TCEO, they were subjected to 3 heating-cooling cycles (1 h at 70ºC and 1 h at 4ºC) and characterized according to their size and encapsulation efficiency as describe above.

2.5. Micro-organisms and growth conditions

*E. coli* O157:H7 Sakai *stx1A/stx2A* was kindly provided by The National Primate Research Center, KRIIBB, Ochang, South Korea (Prof. Kyu-Tae Chang), a strain isolated from an outbreak associated with white radish sprout (Michino et al., 1999), and genetically modified thereafter in order to remove Shiga toxin genes. *L. monocytogenes* EGD-e was kindly provided by the Institute for Medical Microbiology in Giessen, Germany (Prof. Chakraborty). Culture preparation and growth conditions were the same as those reported by Luis-Villaroya et al. (2015).

2.6. Evaluation of the antimicrobial properties of s-TCEO and zn-TCEO

A modified filter paper disc agar diffusion technique (Meena and Sethi, 1994) was applied in order to screen antimicrobial activity of s-TCEO and of zn-TCEO against *E. coli* O157:H7 Sakai and against *L. monocytogenes* EGD-e. We applied filter paper disks (Whatman No. 1; diameter: 6 mm) containing 20 µL of s-TCEO or of zn-TCEO diluted in sterile tryptone soya broth (Oxoid, Hampshire, England) with 0.6% yeast extract added (Oxoid) (TCSYE) (TCEO final concentration: 0.5%) to the surface of agar plates of tryptone soya agar (Oxoid) supplemented with 0.6% yeast extract (TSEYE) that had been previously seeded by spreading one sterile hyssop impregnated with a stationary phase.
culture. For a period of 24 h, the plates were incubated at the appropriate temperature (37°C). The diameter of the resulting zone of partial inhibition was measured in mm.

Moreover, we evaluated the antimicrobial properties of s-TCEO and of zn-TCEO by ascertaining the degree of bacterial inactivation as a function of treatment medium pH. For this purpose we added cells from stationary-phase cultures at final concentrations of 3 x 10^7 CFU/mL to buffers (pH 7.0 and 4.0), both with and without s-TCEO and zn-TCEO (TCEO final concentration: 0.2 µL/mL). Buffer pH was not altered by the addition of antimicrobial compounds. We applied antimicrobial compound treatments for 20 min at 20°C. Samples were taken after intervals of 5, 10, 15, and 20 min, and survivors were counted as described below.

2.7. Measurement of cell inactivation by heat treatment alone, and by heat treatments combined with s-TCEO or zn-TCEO

Heat treatments and combined treatments were carried out at 53°C in an incubator (FX Incubator, mod. ZE/FX, Zeulab, Zaragoza, Spain). To monitor heating temperature, a thermocouple was used (Ahlborn, mod. Almemo 2450, Holzkirchen, Germany). Treatment temperatures were chosen on the basis of preliminary results (data not shown). In order to match previously published data (Espina et al., 2010; Luis-Villaroya et al., 2015; Pagán et al., 2018), the chosen initial bacterial concentration was approximately 3 x 10^7 CFU/mL. As treatment media, we used a sterile McIlvaine buffer of pH 7.0 and 4.0, as well as the same media with s-TCEO or zn-TCEO added (TCEO final concentration: 0.1 and 0.2 µL/mL). Samples were taken and survivors were counted.

2.8. Counts of viable cells
Samples were diluted after treatment in 0.1% w/v peptone water (Oxoid). Subsequently, 0.1-mL samples were pour-plated onto a recovery medium (TSAYE). Plates were incubated at 37°C for 24 h (*E. coli* O157:H7), or for 48 h (*L. monocytogenes* EGD-e). An image analyzer automatic counter (Protos; Analytical Measuring Systems, Cambridge, United Kingdom) was used in order to count the CFUs. Inactivation was expressed in terms of the extent of reduction in log$_{10}$ counts (CFU) after each type of treatment.

2.9. Statistical analysis

Results were obtained from at least three independent experiments carried out on separate working days with different microbial cultures in order to evaluate the disk diffusion assay and the efficacy of lethal treatments. Results were represented as the mean ± standard deviation using the PRISM® program (GraphPad Software, Inc., San Diego, USA). Data were analyzed and submitted to comparison of averages via ANOVA followed by a *post-hoc* Tukey test and *t*-tests with GraphPad PRISM®. Differences were considered significant if $P<$0.05.

3. Results and discussion

3.1. Chemical composition of *Thymbra capitata* essential oil (TCEO)

Qualitative and quantitative analysis of the TCEO is summarized in Table 1. Thirty-five volatile components were identified, representing 98.6% of all detected constituents. The components were grouped into main four classes: monoterpenic hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes.
As shown in Table 1, TCEO contained mostly oxygenated monoterpenes, which accounted for 77.5% of its composition. Carvacrol was the component present in the greatest amount (73.8%). Apart from carvacrol, 4 constituents were present in concentrations greater than 1%: \( p \)-cymene (9.2%), \( \gamma \)-terpinene (5.2%), \((E)\)-caryophyllene (2%) and thymol (1.7%), commonly present in most of species of Thymus genus (Fumiere et al., 2017; Trindade et al., 2018). The total sum of these components represented 93% of the TCEO composition.

These results are similar to those described for the EO of T. capitatus (a synonym of T. capitata) obtained from three different locations in Tunisia (Jendouba, Haouaria and Ain Tounine) (Bounatirou et al., 2007), from Badajoz (Spain) (Delgado-Adámez et al., 2017) and from Sicily (Italy) (Ramos et al., 2017), in which carvacrol was the predominant component, followed by \( p \)-cymene, \( \gamma \)-terpinene and \( \beta \)-caryophyllene.

3.2. Characterization of self-assembled zein nanoparticles loaded with TCEO

The design of the oil-loaded nanocapsules consisted in modifying the concentrations and the type of excipients. For this purpose, different surfactants and co-surfactants were added to TCEO and were mixed in presence of different concentrations of zein. Several formulations were pre-selected (those with an encapsulation efficiency [EE] greater than 70%) and subjected to three heating-cooling cycles (1 h at 70ºC and 1 h at 4 ºC). The final formulation (namely zn-TCEO) was selected for its stability even after the stress conditions. DLS spectra showed low polydispersed particles, with a particle size of 180 nm (Fig.1). As summarized in table 2, it contained 0.5% of TCEO, and its particle size and its EE remained stable (less than 6% variation).

A similarly high EE using zein as vehicle (close to 80%) has been previously described (Bilenler et al., 2015; Wu et al., 2012), reaching values between 60-90%.
However, those studies involved solvent evaporation steps that impede scale-up and industrialization of the nanoparticles. The nanoparticles described in the present study present the advantages of conventional systems, while avoiding the drawbacks of industrial manufacturing. Moreover, they are based on the use of a clear, odorless, non-toxic, biodegradable and water-insoluble vegetable protein, isolated from corn or maize, and compatible with food, cosmetic and pharmaceutical products (Salman et al., 2013).

3.3. Evaluation of the antimicrobial properties of s-TCEO and zn-TCEO

After having prepared and characterized the self-assembled zein nanoparticles loaded with TCEO, the present study’s goal was to evaluate and compare the bacteriostatic and bactericidal effectiveness of s-TCEO and zn-TCEO.

Preliminary screening of the *in vitro* bacteriostatic activity of s-TCEO and zn-TCEO was carried out against one Gram-negative and one Gram-positive pathogenic bacterium, respectively, namely *E. coli* O157:H7 Sakai and *L. monocytogenes* EGD-e, applying a modified version of filter paper disc agar diffusion technique. In this regard, it should be noted that the TCEO concentration in both preparations was much lower (0.5%) than that usually employed when this technique is carried out with pure EOs; thus, the obtained results are not directly comparable with previous works. Table 3 summarizes the antibacterial activity of the two TCEO preparations under study (s-TCEO and zn-TCEO). Despite their low concentration, both EO preparations were partially active against growth of both microorganisms. Inhibitory halos were clearly defined, but isolated colonies grew randomly inside them; therefore, the inhibitory halos were only partial. As a result, the use of TCEO in the form of loaded nanoparticles of zeins displayed a greater diffusion through the growth media and an increased partial inhibition halo from 12.1 (s-TCEO) to 41.7 (zn-TCEO) mm (*P*<0.05) against *E. coli* O157:H7 Sakai and from
10.1 (s-TCEO) to 72.3 (zn-TCEO) mm (P<0.05) against *L. monocytogenes* EGD-e (Table 3). These results indicate that zn-TCEO can inhibit microbial growth more effectively than s-TCEO. The improved bacteriostatic activity of encapsulated TCEO have already been seen in other studies against *E. coli* and moreover against some Gram-positive bacteria (Ben Jemaa et al., 2018; Benjemaa et al., 2018). These results might be explained due to its higher stability and improved dispersion throughout the hydrophilic growth media. The comparison of these results with those obtained by Ait-Ouazzou et al. (2011), where a carvacrol inhibition halo against *E. coli* O157:H7 and *L. monocytogenes* EGD-e was ascertained, allows us to conclude that the greater proportion of the antimicrobial activity of TCEO is due to its high carvacrol content (73.8%).

The encapsulation of EOs has proven to be a good practice when one is seeking to maintain or even enhance their bacteriostatic activity (Donsì and Ferrari, 2016). However, this practice has the drawback that EO is only gradually released (Hu et al., 2018). Thus, when a rapid bioavailability of EOs is required, as is the case in the course of classical inactivating procedures (higienization, surface disinfection, cleaning protocols, etc.), encapsulation and prolonged release of EOs may limit their antimicrobial activity.

As shown in Fig. 2, the measurement of cell inactivation after exposure to 0.2 μL/mL of s-TCEO and zn-TCEO in buffer of pH 7.0 and 4.0 at room temperature for a short treatment time (up to 20 min) indicated that zn-TCEO displayed a lower antimicrobial activity compared with s-TCEO. Whereas the inactivation of 5 log₁₀ cycles of *L. monocytogenes* EGD-e in buffer of pH 4.0 using s-TCEO required 10 min, no significant inactivation (P<0.05) was observed in the presence of zn-TCEO after 20 min (Fig. 2B). In fact, under the treatment conditions investigated, the encapsulated EO did not exert any significant degree of inactivation against *E. coli* O157:H7 Sakai or *L.
monocytogenes EGD-e. This might be associated with the sustained release of TCEO from zein nanocapsules. In fact, our research group has determined that the release of TCEO from the nanocapsules at pH 7.0 starts at 4 h (data not shown).

This EO concentration (0.2 µL/mL) was established for comparative purposes based on previous results (Ait-Ouazzou et al., 2011; Ait-Ouazzou et al., 2013). Thus, E. coli O157:H7 Sakai displayed a greater sensitivity to s-TCEO when the treatment was carried out in buffer at pH 7.0 than at pH 4.0 (Fig. 2A). Whereas the 20-min. treatment at pH 7.0 achieved 2.3 log_{10} cycles of reduction of the cell population, at pH 4.0 only 1.7 log_{10} cycles were inactivated. This effect of pH is not common, although it has already been observed in treatments with citral against E. coli BJ4 (Somolinos et al., 2010). On the contrary, in two studies that evaluated the antimicrobial activity of carvacrol against E. coli O157: H7 (phage type 34) (Ait-Ouazzou et al., 2011) and E. coli BJ4 (Ait-Ouazzou et al., 2013), a greater sensitivity to the compound was observed at pH 4.0, which indicates that microbial resistance to carvacrol under different treatment medium pH is dependent on the strain that is being studied.

Regarding results on L. monocytogenes EGD-e, the latter microorganism was more sensitive at pH 4.0 than at pH 7.0 (Fig. 2B). Whereas at pH 4.0 the treatment caused the inactivation of 4.7 log_{10} cell cycles after 7 min of treatment, at pH 7.0 the degree of inactivation was less than 1 log_{10} cycle after a 20 min-treatment. These results are in agreement with several studies conducted with carvacrol against L. monocytogenes EGD-e (Ait-Ouazzou et al., 2011; Ait-Ouazzou et al., 2013). This may be due to the fact that at acidic pH, the EOs and their individual constituents display a higher hydrophobicity and, consequently, interact better with cell envelopes (Burt, 2004).

3.4. Synergistic effect of heat and s-TCEO and zn-TCEO
On the basis of the hurdle theory introduced by Leistner and Gorris (1995), many researchers have observed synergistic lethal effects when heat is applied in combination with novel chemical preservatives, such as EOs, and have recommended their use for the improvement of food preservation, product higienization or in order to enhance the effectiveness of cleaning and disinfectant methods (Espina et al., 2017; Guevara et al., 2015; Mate et al., 2016; Pagán et al., 2018). These treatments tend to be very brief (sec to min). It is unknown whether the use of zein nanocapsules would hamper the synergism between heat and EOs described above.

Fig. 3 shows the survival curves of *E. coli* O157:H7 Sakai after a combined treatment at 53°C for 12 min in the presence of s-TCEO or zn-TCEO (0.1 μL/mL of TCEO) in buffers of pH 7.0 and 4.0. As can be observed in the figure, the inactivation of *E. coli* O157:H7 Sakai via the combined treatment was more rapid than the inactivation achieved through heat treatment. An outstanding synergism was observed between heat and either s-TCEO or zn-TCEO: while heat or TCEO acting separately scarcely caused the inactivation of <0.5 log10 cycles of *E. coli* O157:H7 Sakai at pH 7.0 or 4.0 after 12- and 10 min-treatments, respectively, their simultaneous use caused the inactivation of up to 5 log10 cycles as a function of EO preparation and treatment medium pH. Whereas no significant differences (*P* >0.05) in lethality were observed when combining heat and s-TCEO or zn-TCEO at pH 7.0 (Fig. 3A), the combination of heat and zn-TCEO was more effective at pH 4.0 (Fig. 3B). The unexpected results obtained at pH 4.0 pointed toward encapsulation with zeins as the most efficient way to enhance synergism between heat and TCEO. These results may be related to cell sensitization caused by zeins at acidic pH. In this regard, since the isoelectric point of zein is 6.8, under low pH conditions (i.e. pH 4.0) the surface of the zein nanoparticles is positively charged. This phenomenon would enhance the interaction of TCEO-loaded zein nanocapsules with the negatively charged
outer membrane of *E. coli*, thereby enhancing the antimicrobial activity of TCEO, and especially of its main constituent, carvacrol.

On the other hand, at pH 7.0, a further experiment was performed to determine the concentration of TCEO loaded in nanocapules of zeins which is required to reach the degree of lethality observed when combining heat and s-TCEO: no significant differences ($P>0.05$) were observed when using 0.1 $\mu$L/mL of s-TCEO and 0.2 $\mu$L/mL of zn-TCEO in combination with mild heat (data not shown). Thus, considering that the use of the nanocapsules is a preferable choice in view of the previously described advantages regarding protection and improved distribution of EOs, the implementation of nanocapsules would require a double concentration of the selected EO.

Regarding the *L. monocytogenes* EGD-e results, Fig. 4 shows the survival curves at 53ºC in the presence of s-TCEO or zn-TCEO (0.1 $\mu$L/mL of TCEO) in buffers of pH 7.0 and 4.0. As one can observe in the figure, the extent of inactivation achieved thanks to combined treatment always exceeded the amount of inactivation obtained by heat treatment at either pH 7.0 or 4.0, which indicates a synergism between both hurdles against *L. monocytogenes* EGD-e. Moreover, unlike the results obtained with *E. coli* O157:H7 Sakai, the synergism achieved against *L. monocytogenes* EGD-e was the same using TCEO in the form of a suspension or loaded in nanocapules of zeins, either at pH 7.0 (Fig. 4A) or 4.0 (Fig. 4B). According to these results, no interference of treatment medium pH or of TCEO preparation was observed when the combined treatment was applied against *L. monocytogenes* EGD-e, probably due to this bacterium’s lack of outer membrane. Thus, the use of mild temperatures might contribute to accelerate the release of TCEO, increasing its bioavailability to interact against the bacterial population.

4. Conclusions
The use of self-assembled zein nanoparticles to encapsulate EOs might represent an alternative preferable to the use of EOs in suspension, not only in order to overcome their high volatility and to improve EO dispersion in hydrophilic solutions, but also in order to enhance their antimicrobial activity. Moreover, the use of a non-toxic, biocompatible, natural polymer isolated from corn or maize (zein) for EO nanoencapsulation, combined with a method that is simple and easy to scale up, permits it to be used in many different applications of interest for food, cosmetic or pharmaceutical industries.

The bacteriostatic activity of TCEO-loaded zein nanoparticles was greater than that of unprotected EO solutions. In terms of bactericidal activity, the combination of mild heat with TCEO nanoparticles displayed a great synergism, especially against *E. coli* O157:H7 Sakai at low pH. These synergistic effects hold promise for the improvement of alternative higienization processes, as well as for cleaning and disinfection procedures.

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**Figure legends**

**Fig. 1.** DLS graph of *Thymbra capitata* essential oil-loaded zein nanocapsules (zn-TCEO).
Fig 2. Survival curves of *Escherichia coli* O157:H7 Sakai (A) and *Listeria monocytogenes* EGDe (B) (initial concentration: $3 \times 10^7$ CFU/mL) after exposure to s-TCEO (●, ●) or zn-TCEO (●, ●) (0.2 μL/mL of TCEO) in buffer of pH 7.0 (●, ●) and 4.0 (●, ●) at room temperature. Data represent the mean ± standard deviation (error bars) of at least three independent experiments. The dotted line represents the detection limit.

Fig. 3. Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration: $3 \times 10^7$ CFU/mL) after a heat treatment at 53ºC for 15 min in buffers of pH 7.0 (A) and 4.0 (B) (●), or after combined treatment with heat and s-TCEO (●, ●) or zn-TCEO (●, ●) (0.1 μL/mL of TCEO). Data represent the mean ± standard deviation (error bars) of at least three independent experiments. The dotted line represents the detection limit.

Fig. 4. Survival curves of *L. monocytogenes* EGD-e (initial concentration: $3 \times 10^7$ CFU/mL) after a heat treatment at 53 ºC for 15 min in buffers of pH 7.0 (A) and 4.0 (B) (●), or after combined treatment with heat and s-TCEO (●, ●) or zn-TCEO (●, ●) (0.1 μL/mL of TCEO). Data represent the mean ± standard deviation (error bars) of at least three independent experiments. The dotted line represents the detection limit.
<table>
<thead>
<tr>
<th>Characteristics before stress condition</th>
<th>Size (nm)</th>
<th>PDI&lt;sup&gt;1&lt;/sup&gt;</th>
<th>EE&lt;sup&gt;2&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active concentration (%)</td>
<td></td>
<td>180</td>
<td>0.25</td>
</tr>
<tr>
<td>Zein concentration (%)</td>
<td></td>
<td>180</td>
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<table>
<thead>
<tr>
<th>Characteristics after stress condition</th>
<th>Size (nm)</th>
<th>PDI&lt;sup&gt;1&lt;/sup&gt;</th>
<th>EE&lt;sup&gt;2&lt;/sup&gt; (%)</th>
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<td></td>
<td></td>
<td>190</td>
<td>0.250</td>
</tr>
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<td>77.8</td>
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</tbody>
</table>

<sup>1</sup>Polydispersity index; <sup>2</sup> Encapsulation efficiency;

**Table 2.** Relevant characteristics of *Thymbra capitata* essential oil (s-TCEO) and TCEO encapsulated with zeins (z-TCEO-z)
Table 1. Chemical composition of the essential oil of *Thymus capitatus*.

<table>
<thead>
<tr>
<th>No</th>
<th>Component*</th>
<th>RI</th>
<th>RI Lit.</th>
<th>%d</th>
<th>ID*</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>alpha-thujene</td>
<td>921</td>
<td>924</td>
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<tr>
<td>2</td>
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<td>932</td>
<td>0.9±0.2</td>
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<tr>
<td>3</td>
<td>camphene</td>
<td>940</td>
<td>946</td>
<td>0.1±0.0</td>
<td>Std,RI,MS</td>
</tr>
<tr>
<td>4</td>
<td>beta-pinene</td>
<td>969</td>
<td>974</td>
<td>0.1±0.0</td>
<td>Std,RI,MS</td>
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<tr>
<td>5</td>
<td>1-octen-3-ol</td>
<td>977</td>
<td>974</td>
<td>0.1±0.0</td>
<td>Std,RI,MS</td>
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<tr>
<td>6</td>
<td>3-octanone</td>
<td>987</td>
<td>979</td>
<td>Trf</td>
<td>RI,MS</td>
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<tr>
<td>7</td>
<td>myrcene</td>
<td>990</td>
<td>988</td>
<td>0.9±0.1</td>
<td>Std,RI,MS</td>
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<tr>
<td>8</td>
<td>3-octanol</td>
<td>999</td>
<td>988</td>
<td>Tr</td>
<td>RI,MS</td>
</tr>
<tr>
<td>9</td>
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<td>Std,RI,MS</td>
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<td>linalool</td>
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<td>borneol</td>
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<td>27</td>
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<td>Tr</td>
<td>RI,MS</td>
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<td>35</td>
<td>caryophyllene oxide</td>
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<td>1583</td>
<td>0.1±0.0</td>
<td>Std,RI,MS</td>
</tr>
</tbody>
</table>

Total identified (%) 98.6±0.4
Grouped compounds (%)

- Monoterpene hydrocarbons 18.6±0.9
- Oxygenated monoterpenes 77.5±2.8
- Sesquiterpene hydrocarbons 2.2±0.5
- Oxygenated sesquiterpenes 0.1±0.0
- Others 0.2±0.0
Compounds are listed in order of their elution from a HP-5MS column. Linear retention index on HP-5MS column, experimentally determined using homologous series of C₆-C₃₀ alkanes. Linear retention index taken from Adams (2007) or NIST 17 (2017). Relative percentage values are means of two determinations ± SD. Identification methods: std, based on comparison with authentic compounds; MS, based on comparison with WILEY, ADAMS, FFNSC2 and NIST 17 MS databases; RI, based on comparison of calculated RI with those reported in ADAMS, FFNSC2 and NIST 08. Tr, % below 0.1%
Figure 3.
Figure 2

A) Log$_{10}$ N/N$_0$

B) Log$_{10}$ N$_0$/N$_0$

Time (min)
Figure 4.

A)

B)