Essential oil nanoemulsions as antimicrobial agents in food

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Abstract

The crescent interest in the use of essential oils (EOs) as natural antimicrobials and preservatives in the food industry has been driven in the last years by the growing consumers' demand for natural products with improved microbial safety, and fresh-like organoleptic properties.

Nanoemulsions efficiently contribute to support the use of EOs in foods by increasing their dispersibility in the food areas where microorganisms grow and proliferate, by reducing the impact on the quality attributes of the product, as well as by enhancing their antimicrobial activity.

Understanding how nanoemulsions intervene on the mass transfer of EOs to the cell membrane and on the mechanism of antimicrobial action will support the engineering of more effective delivery systems and foster the application of EOs in real food systems.

This review focuses on the enabling contribution of nanoemulsions to the use of EOs as natural preservative agents in food, (a) specifically addressing the formulation and fabrication of stable EO nanoemulsions, (b) critically analyzing the reported antimicrobial activity data, both *in vitro* and *in product*, to infer the impact of the delivery system on the mechanisms of action of EOs, as well as (c) discussing the regulatory issues associated with their use in food systems.

Keywords: Nanoemulsion; Essential oil; Antimicrobials; Formulation; Fabrication; Mechanisms

1. Introduction

Essential oils (EOs) are naturally-derived aroma compounds, with wide-spectrum biological activities (Asbahani et al., 2015). To date, EOs have been exploited as flavoring additives, as medicines or cosmetics (Dima and Dima, 2015), as insecticidal, antioxidant, anti-inflammatory, anti-allergic, and anticancer agents (Seow et al., 2014). However, many EOs exert strong antibacterial, antiviral, and antifungal activities, stimulating their application also as natural antimicrobials in food and beverage products (Burt, 2004).

In recent years, the food industry has demonstrated a growing demand for natural compounds to develop novel food preservatives against spoilage and pathogenic microorganisms, as well as to sustain innovation in food packaging (Asbahani et al., 2015).

EOs are complex mixtures of non-volatile and volatile, generally lipophilic, scarcely water-soluble compounds, which can be broadly classified in alkaloids, flavonoids, isoflavones, monoterpenes, phenolic acids, carotenoids and aldehydes (Bakkali et al., 2008; Seow et al., 2014). Among the > 3000 types of known EOs, only ~300 are currently of commercial interest (Dima and Dima, 2015).

EOs are synthesized as secondary metabolites in different plant organs, to provide protection from external agents, such as UV light, herbivores, insects and pathogens (Asbahani et al., 2015; Seow et al., 2014), when released by humidity variation, or mechanical action. Owing to their high molecular reactivity, EOs are accumulated and stored in specialized structures located either on the plant surfaces, such as the secretory glandules or in internal cell organs, such as the vacuoles (Dima and Dima, 2015) to offer an ideal protection as well as to prevent interaction with vital parts.

The use of EOs as a mild preservation technique in the food industry has gained considerable attention in recent years, mainly driven by the concern over the negative perception of consumers on chemical preservatives (Seow et al., 2014).

However, the high reactivity and hydrophobicity of EOs represent a formidable challenge to their direct incorporation in food and beverage products.

To retain their biological activity and minimize at the same time the impact on the organoleptic properties of foods where incorporated, EOs need to be encapsulated in delivery systems, which are compatible with food applications (Buranasuksombat et al., 2011). Emulsion-based delivery systems can be formulated with food-grade ingredients, and can be easily dispersed in those areas of the food, where microorganisms grow and proliferate (Donsì et al., 2011). Moreover, nanometric scale emulsions, or nanoemulsions, offer also additional advantages, such as the minimization of the impact on the organoleptic properties of the food products, as well as an increased bioactivity, due to subcellular size and better diffusion (Donsì et al., 2012a, 2011). The wetting ability of surfactants and emulsifier can also contribute to the antimicrobial and anti-biofilm activities of nanoemulsions (Ferreira et al., 2010; Teixeira et al., 2007). Nevertheless, to date, only pioneering research has been carried out to support the use of nanoemulsions of antimicrobial EOs in food products.

Figure 1 compares the number of publications in the last 20 years on essential oils as antimicrobials, on nanoemulsions, and on essential oil nanoemulsions. While both investigations on essential oils as antimicrobials, and on nanoemulsions can be considered mature research fields, which are in the exponential growth phase (> 500 publications per year), the research field on essential oil nanoemulsions is still in its infancy, with more than 20 publications per year in the last 2 years.

This review will address the enabling contribution of nanoemulsions to the use of EOs as natural preservative agents in food, (a) specifically focusing the formulation and fabrication of stable EO nanoemulsions, (b) critically analyzing the reported antimicrobial activity data, both *in vitro* and *in product*, to infer the impact of the delivery system on the mechanisms of action of EOs, as well as (c) discussing the regulatory issues associated with their use in food systems.

2. Formulation and fabrication of essential oil nanoemulsions

2.1. Definition and properties

Oil-in-water (O/W) nanoemulsions consist of oil droplets, with mean droplet size typically ranging from 20 to 200 nm (Sagalowicz and Leser, 2010), dispersed in an aqueous medium and stabilized by an emulsifier layer. Food-grade surfactants (polysorbates, sugar esters, lecithins) or biopolymers (natural gums, vegetable or animal proteins, modified starches) are frequently used in food applications not only as emulsifying agents, but also to impart to the nanoemulsions some desired features, such as specific interfacial behavior (electrostatic forces, steric repulsion, and rheology), loading capability, as well as response to environmental stresses (Chen et al., 2006).

Due to their nanometric size, nanoemulsions have distinctive and unique properties:

1. Differently from emulsions, which are thermodynamically unstable systems, naturally tending to physical separation, in nanoemulsions, the conditions of physical meta-stability are induced by the Brownian motion effects dominating over gravitational forces. In addition, the strength of the net attractive forces acting between droplets usually decreases with decreasing droplet diameters, reducing aggregation phenomena in nanoemulsions (McClements and Rao, 2011).
2. Moreover, when the droplet diameters are much smaller than the wavelength of light, the nanoemulsions do not scatter light strongly and form transparent or only slightly turbid systems, which are suitable for addition to clear beverages, sauces, soups, and syrups (Salvia-Trujillo et al., 2014a). In particular, nanoemulsions exhibit optical transparency for mean droplet sizes < 40 nm independently on the oil fraction, whereas, in the range between 40 and 100 nm nanoemulsions appear hazy, with a marked dependence on the oil content, and for sizes > 100 nm they appear white due to significant multiple scattering (Mason et al., 2006).
3. As droplets size decreases, also the biological activity of the lipophilic compounds encapsulated in nanoemulsions increases, because of the enhanced transport of active

molecules through biological membranes, as well as of the increased surface area/volume ratio, leading to improved reactivity (Salvia-Trujillo et al., 2015a).

Therefore, in many respects, the encapsulation of lipophilic functional components into nanoemulsions offer several advantages. This is particularly true for EOs, where the issues of physicochemical stability, of minimized impact on the products where they are incorporated, as well as of enhanced antimicrobial activity are of considerable interest for industrial applications as natural antimicrobials.

2.2. Fabrication methods

The fabrication methods of nanoemulsions can be classified in top-down approaches, aimed at the disruption of the oil phase into homogeneously sized, fine droplets, or in bottom-up approaches, aimed instead at driving the direct assembling of molecular building blocks into structured systems.

Figure 2 depicts some of the possible routes to the fabrication of essential oil-in-water nanoemulsions, suggesting the approximate correlation between the process energy involved, the required amount of surfactant with respect to the oil phase, and the expected mean droplet size. The qualitative information given in Figure 2 is supported by the details on different fabrication methods and formulations used in the preparation of EO nanoemulsions, given in Table 1.

2.2.1. Top-down fabrication

Top-down methods mainly consist of mechanical size reduction techniques, based on the focused oil droplet stressing in a process fluid, where fluid-mechanical stresses are generated. Within top-down approaches, the emulsification process occurs in two phases: (a) the break-up of coarse droplets into smaller ones, followed by (b) the absorption of the emulsifier onto the newly formed interfaces, to prevent recoalescence phenomena and promote kinetic meta-stability. The concentration and the properties of the emulsifier hence play a very important role in the efficiency of these emulsification

processes (Donsì et al., 2012b), together with the oil:water ratio and the processing conditions (Silva and Cerqueira, 2015).

The most used systems for the fabrication of essential oil nanoemulsions are colloid milling, ultrasonication, and high pressure homogenization, which requires that large amounts of energy are transferred to the process fluid (50-500 MJ/m^3) (Donsì et al., 2013a). However, less energy-intensive top-down approaches are also possible, such as membrane emulsification.

High pressure homogenization (HPH) is a continuous process, where a process fluid is compressed at high pressures (50-400 MPa), and forced through a specifically designed, micrometric homogenization chamber. The intense shear and elongational stresses, turbulence, and cavitation developed in the homogenization chamber generate the high-intensity fluid-mechanical stresses, responsible for the disruption of the oil droplets dispersed in the fluid. The emulsification process is affected by the efficiency, with which the energy dissipated in the homogenization chamber is transmitted to the oil droplets, which directly depends on the chamber geometry. In particular, among the different chambers equipping commercial HPH devices, ranging from a simple orifice plate to colliding jets and radial diffuser assemblies (Donsì et al., 2009), the orifice valve appears to be one of the most efficient for emulsification (Donsì et al., 2012b; Stang et al., 2001). Orifice valves enable not only an efficient droplet disruption, with high elongational stresses coupled with elevated turbulence, but also a homogeneous volume of interaction downstream of the valve, where emulsifier adsorption at the droplet interface occurs uniformly, reducing the polydispersity of the emulsion droplets (Donsì et al., 2012b).

Table 1 reports the examples of different EOs, which have been prepared by HPH treatment, reaching submicrometric mean droplet sizes with surfactant-to-oil (or emulsifier –to-oil) ratios (SOR) often ≤ 1. It must be remarked that to achieve a uniform, narrow size distribution, often several HPH passes, or number of processing cycles, are recommended, to compensate the uneven distribution of fluid-mechanical stresses in the homogenization chamber (Donsì et al., 2012b). Typical operating pressures

141 for EO nanoemulsions are in the range of 200-300 MPa. However, for high SOR values (≥ 1),
142 significantly smaller pressures can be used (Zahi et al., 2014): for example, no additional droplet size
143 reduction was observed when increasing the pressure from 100 to 150 MPa (Liang et al., 2012).

144 Microfluidization, frequently used at lab-scale for the production of uniformly sized nanoemulsions,
145 designates a specific HPH device, characterized by a proprietary, microchannel, impinging jets,
146 homogenization chamber. Thanks to the high emulsification efficiency of this chamber, lower
147 pressures than other HPH devices are typically used (100 – 150 MPa) (Salvia-Trujillo et al., 2014b).

148 Ultrasonication (US) is another widely used, lab-scale method for the fabrication of nanoemulsions.
149 It is based on the perturbation of the oil-water interface and consequent formation of fine droplets,
150 induced by cavitation phenomenon. The alternating low-pressure and high-pressure waves generated
151 by ultrasounds at high frequency (>18 kHz) induce in liquids the rapid shifts from conditions of vapor
152 bubble formation to vapor bubble collapse. The imploding bubbles lead to the intense shock waves
153 of cavitation, with local pressures in the surrounding liquid rising as high as 1.35 MPa (Maa and Hsu,
154 1999). When associated to high SOR values, US ensures the rapid production of extremely fine
155 nanoemulsions (Ghosh et al., 2014; Salvia-Trujillo et al., 2014b; Sugumar et al., 2014), also in very
156 small processing batches. However, experimental evidence suggests that the local hot spots induced
157 by US might degrade reactive or thermolabile molecules, such as many components of EOs, with a
158 significant reduction in their biological activity, in comparison to other techniques, such as
159 microfluidization (Salvia-Trujillo et al., 2014b).

160 Colloid milling, also known as high shear homogenization (HSH), includes all those devices equipped
161 with high-speed rotor/stator systems, where the emulsification process is controlled by the intense
162 shear stresses, friction, and high-frequency vibrations developed (Schultz et al., 2004). Despite HSH
163 systems are cost-effective, easily scalable methods of emulsification, the resulting emulsion droplet
164 size is limited by the lower intensity of the fluid-mechanical stresses than other systems. Therefore,
165 HSH is extensively used as a preliminary step to produce the coarse emulsions, subsequently

processed by HPH or US (Donsì et al., 2012a, 2011; Sessa and Donsì, 2015). Even when using HSH devices with high emulsification efficiency, such as the Cyclone I.Q.2 microprocessor homogenizer (Pan et al., 2014; Wu et al., 2014), high SOR values are required, and the efficiency of droplet size reduction is limited.

Membrane emulsification is a low shear, low energy process, especially suitable for producing O/W emulsions with a narrow size distribution range, based on the flow of the dispersion oil phase, or of a primary coarse emulsion, through a micrometric membrane in a continuously flowing aqueous phase, containing a hydrophilic surfactant (Joscelyne and Trägårdh, 2000; Liu et al., 2011). Despite optimization of membrane type and processing conditions has been reported to enable the fabrication of nanometric size emulsions (Liu et al., 2011), no relevant applications have been reported to date in the fabrication of essential oil nanoemulsions.

In the perspective of the industrial application, despite the compact, cost-effective, and ease of use of US devices at lab-scale, the industrial application of ultrasonication is limited by the difficult scale-up of the treatment chamber and the sonicator devices (Sessa and Donsì, 2015). HPH, instead, offers significant advantages in terms of ease of operation, industrial scalability, reproducibility, and high throughput (Donsì et al., 2013b). In particular, despite the lower emulsification efficiency (Donsì et al., 2012b), the use of piston valves is recommended, because of the easier and faster handling of the eventual blockage of the homogenization valve, than for fixed geometry valves, such as orifice plates of microfluidizer chambers.

2.2.2. Bottom-up fabrication

Nanoemulsions can also be fabricated through physicochemical processes, based on the spontaneous association of surfactants around the essential oil molecules, driven by the balance of attractive and repulsive forces tending to thermodynamic equilibrium. The self-assembly of oil droplets, hence, depends strongly on the properties of the molecules present, and in particular, their solubility, molecular geometry and surface activity (McClements and Rao, 2011). Environmental factors, such

as temperature, concentration, pH and ionic strength of the system are exploited to control the entity of the involved forces, whereas mechanical energy is required only to ensure thorough system uniformity through agitation, as recently reviewed (Sessa and Donsì, 2015; Silva et al., 2012).

Low energy, bottom-up approaches are able to efficiently produce very fine droplets, using simple to implement and scale-up, low-cost equipment, and preventing the encapsulated molecules from being degraded during processing. However, strict limitations apply to the types of oils and surfactants that can be used to form stable nanoemulsions, as well as to the required surfactant-to-oil ratios (SOR), which are significantly higher than in top-down methods (Sessa and Donsì, 2015).

Solvent demixing is based on the dissolution of the oil phase in a suitable organic solvent, followed by its separation in nanometric droplets through the addition, as an anti-solvent, of an aqueous phase containing stabilizers, such as surfactants or hydrocolloids, as recently reviewed (Sessa and Donsì, 2015; Silva et al., 2012). The rapid diffusion of the organic solvent in the aqueous phase drives the formation of nanoemulsions in one step, at low-energy input, with a high yield of encapsulation. However, this technique requires (a) water-miscible solvents, and (b) the thorough purification of the nanoemulsions from the traces of organic solvents (Silva et al., 2012). Therefore, alternative, more energy-intensive approaches, have been developed. For example, spray-drying oil-in-water emulsions, containing thymol dissolved in hexane emulsified using conjugates of whey protein isolate and maltodextrin, upon rehydration of the dried capsules resulted in transparent and stable thymol nanoemulsions (Shah et al., 2012b).

Spontaneous emulsification consists in the formation of fine oil droplets by mixing an organic phase containing a predominantly hydrophilic surfactant with an aqueous phase. The transition of surfactant molecules from the organic phase to the water phase leads to interfacial turbulence and to the spontaneous formation of nanometric oil droplets (Chang et al., 2013). This concept has been exploited for orange oil (Chang and McClements, 2014) or carvacrol (Chang et al., 2013), mixed with

medium chain triglycerides (MCT) using Tween 40, 60 or 80 as emulsifiers, or clove bud oil using whey protein concentrate, gum arabic, or lecithin (Luo et al., 2014).

Similarly, phase inversion methods are based on spontaneous emulsification induced by controlling the interfacial behavior of the surfactants at the O/W interface, inducing a shift from predominantly lipophilic to predominantly hydrophilic in response to changes in system compositions or environmental conditions, such as temperature (Rao and McClements, 2010), salt concentration, pH value, as well as surfactant concentration (McClements and Rao, 2011). The formed nanoemulsions are kinetically stable and can be considered as irreversible (Silva et al., 2012), as long as they are kept far from phase inversion conditions (Rao and McClements, 2010). For example, in the case of d-limonene, phase inversion was achieved by the slow addition of water/propylene glycol to a d-limonene/Tween 80, resulting in very fine (16 nm) nanoemulsions droplets (Zhang et al., 2014).

2.3. Formulation

Formulation plays a decisive role not only in the minimum achievable droplet size but also in determining several nanoemulsions properties, related to their surface and biological activity. In addition, selection of ingredients and their concentration of use have to meet the constraints coming from current regulations for food formulations as well as economic viability for the food industry.

2.3.1. Role of formulation on mean droplet size

In principle, the selection of a suitable emulsifier, and of its concentration with respect to the oil phase, should take into account several factors, such as (a) the required surface coverage for stable emulsions, (b) the interfacial tension, (c) the kinetics of adsorption at O/W interfaces, as well as the molecular rearrangement in the case of high-molecular weight emulsifiers, such as proteins and polysaccharides, and (d) the hydrophilic-lipophilic balance (HLB) of the surfactant molecules, or concentration and location of hydrophobic moieties in the case of high-molecular weight emulsifiers (Donsì et al., 2012b).

Often, these factors control the final mean droplet size, more than the efficiency of the emulsification process itself. For example, in Table 1 it is evident that when using small surfactant molecules, such as Tween 80 or soy lecithin, mean droplet sizes < 100 nm can be reached, quite independently on the use of HPH, microfluidization, US, self-emulsification or phase inversion methods (Donsì et al., 2011; Sessa and Donsì, 2015; Terjung et al., 2012).

Surfactants with too high HLB numbers, such as Tween 20 (HLB = 16.7) or too low HLB numbers, such as Tween 85 (HLB = 11) or Span 80 (HLB = 8.6) were reported to be unsuitable for the formation of nanoemulsions (Chang and McClements, 2014). Tween 40 (HLB = 15.6), 60 (HLB = 14.9), and 80 (HLB = 15.0) were instead able to form clear nanoemulsions through spontaneous emulsification (Chang and McClements, 2014; Chang et al., 2013). Surfactants with intermediate HLB numbers are reported to form monolayers with low interfacial tensions, which promote the conditions of interfacial turbulence needed for the spontaneous formation of ultrafine droplets at the O/W boundary. At too low HLB numbers, the surfactant tends to remain in the oil phase, whereas at too high HLB number, the curvature of the surfactant monolayer may be too high to form very fine oil droplets (Chang and McClements, 2014). However, despite its being a useful rule of thumb for the preparation of O/W emulsion that the HLB number of the surfactants ranges between 8 and 18, no clear prediction can be derived for the mean droplet size of the emulsion. For example, the combination of Tween 20 with monoolein (HLB = 3.8) enabled the preparation of stable, nanometric emulsions, despite a resulting HLB number of 10.25 (Donsì et al., 2012a, 2011), suggesting that many other factors contribute to control the achievable emulsion size, such as the kinetics of surfactant adsorption onto the O/W interface, as well as the emulsification conditions (Donsì et al., 2012b).

Similarly, surfactant concentration is also extremely important, being directly related to surface coverage of the droplet, as well as to the O/W interfacial tension. For example, it was observed a decrease in droplet size (from > 5000 to < 25 nm) as the Tween 80 concentration was increased from 5 to 20 wt % (Chang et al., 2013). In general, as the interfacial tension decreases, (a) the droplet

break-up efficiency increases (Walstra, 1993), (b) the rate of droplet coalescence increases (Rao and McClements, 2012), and (c) the absorption rate of the emulsifiers onto the newly formed O/W interfaces decreases (Chanamai et al., 2002).

Moreover, the emulsification process is also affected by the viscosity of the disperse oil phase, with respect to the viscosity of the continuous aqueous phase: the closer to unity is the ratio of dispersed phase to continuous phase viscosity, the more efficient is the mechanical emulsification process (Walstra, 1993).

Therefore, several formulations contemplate the blending of the essential oils with other lipids, such as MCT (Chang and McClements, 2014), sesame oil (Terjung et al., 2012), or stearic acid (Zahi et al., 2014), to enable the production of transparent nanoemulsions.

Finally, the properties of the emulsifier layer, including the steric hindrance and electrostatic repulsion between the droplets, together with the affinity of the EO for the oil phase, control the long-term stability of the nanoemulsions, by reducing the tendency to aggregation, coalescence, as well as other instability mechanisms.

2.3.2. Physicochemical stability – Ostwald ripening

The non-negligible solubility in water of many EOs, such as carvacrol (Donsì et al., 2014, 2012a), triggers a specific coalescence phenomenon, named Ostwald ripening (Suriyarak and Weiss, 2014). Preventing Ostwald ripening is one of the main difficulties associated with the formulation of physically stable EOs nanoemulsions (Chang and McClements, 2014; Ziani et al., 2011).

Ostwald ripening can be described as the growth of larger droplets at the expense of the smaller ones, because of molecular diffusion of oil between droplets through the continuous phase, which is induced by the higher local oil solubility around smaller droplets than larger ones (Wooster et al., 2008).

Oils consisting of long-chain triglycerides, which exhibit a negligible aqueous phase solubility, are less prone to Ostwald ripening than oils containing components with an appreciable water solubility (Rao and McClements, 2012). When the use of EOs prone to Ostwald ripening is unavoidable, the rate of Ostwald ripening can be reduced by changing the partitioning of EOs between the lipid droplets and the aqueous phase, for example by mixing with medium or long chain triglycerides oils (Donsì et al., 2014), generating an entropy of mixing effect that counteracts the imbalance of droplet size effect (Chang et al., 2015).

Examples of these "ripening inhibitors" oils include highly non-polar substances, such as corn oil (Chang et al., 2012; Ziani et al., 2011), sunflower oil (Donsì et al., 2014, 2012a), MCT, sesame oil (Terjung et al., 2012), as well as canola oil (Majeed et al., 2016a).

With different EOs and emulsifiers, the optimal weight ratio between EO and the ripening inhibitor may be different (Donsì et al., 2014): for example, Ostwald ripening was inhibited by mixing in the lipid phase thyme oil with ≥ 60 % corn oil (Chang et al., 2012; Ziani et al., 2011), carvacrol with ≥ 50 % MCT (Chang et al., 2013) or sunflower oil (Donsì et al., 2014), orange oil with ≥ 40 % MCT (Chang and McClements, 2014), or eugenol with ≥ 50 % MCT (Ghosh et al., 2014).

In alternative, the incorporation of a slightly water-soluble EO, such as d-limonene, into an organogel-based nanoemulsion also resulted in an improved physical stability. The organogel structure, obtained by adding an oleogelator agent, such as stearic acid, to the lipid phase, effectively contributed to reducing the polarity of EOs and actively prevented the occurrence of Ostwald ripening (Zahi et al., 2014).

Finally, another route reported to inhibit Ostwald ripening is based on the use of macromolecules, able to bind the EO components, as observed for soybean polysaccharide (Wu et al., 2014), or sodium caseinate and lecithin (Xue and Zhong, 2014) with thyme oil, for sodium caseinate with thymol (Pan et al., 2014), for whey protein-maltodextrin conjugates with eugenol (Shah et al., 2013), and for pectins with mandarin or lemongrass oil (Guerra-Rosas et al., 2016). However, when the interaction

between the EOs and the macromolecule is weak, destabilization mechanisms by Ostwald ripening become significant (Guerra-Rosas et al., 2016).

2.3.3. Other nanoemulsion properties affected by formulation

The formulation has a significant effect on different other properties of EO nanoemulsions, of interest for food applications.

One of them is the zeta potential, or surface charge, of nanoemulsions droplets. The electrical charge on the droplets also improves their stability against aggregation (McClements and Rao, 2011). Moreover, the surfaces of microorganisms are typically negatively charged, and therefore positively charged droplets might be desirable to be electrostatically attracted to their surfaces (Ziani et al., 2011). The electrical charge can be developed by using one or more charged emulsifiers, as well as by adsorbing charged substances onto the droplet surfaces (Ziani et al., 2011). Recently, also the use of surfactants with strong antimicrobial activity, such as lauric arginate (a cationic surfactant) and sodium dodecyl sulfate (an anionic surfactant), has been investigated (Ziani et al., 2011). However, their antimicrobial activity was drastically reduced by the presence of emulsion droplets, where they preferentially adsorbed, instead of acting against the microbial surfaces (Ziani et al., 2011).

Another important property of the nanoemulsions, which is controlled through formulation, is the digestibility, which is of particular interest for food application. Recent studies have also shown that nanoemulsion mean particle size and formulation significantly impact the extent of digestion of fats. In particular, the digestion rate is reported to increase with decreasing the oil droplet diameter, due to the increased specific surface area of interaction with lipase enzyme (Majeed et al., 2016a; Troncoso et al., 2012). In addition, the composition of oil droplets affects also the rate of fat digestion, with MCT-based emulsions exhibiting a faster rate than canola oil-based ones (Majeed et al., 2016a).

The use of essential oils, due to their volatility and pungent taste, necessarily impacts also the organoleptic properties of products that contain them, and this should be taken into account in the final product formulation. However, despite the vast body of available literature on the use of EOs as

natural food preservatives, the sensory impact of their addition to foods has been only marginally investigated. The addition of different EOs to tomato juice, vegetable soup, or poultry burgers highlighted very different tolerance limits, in the range 20 – 200 μL/L, depending on the type of EO and the food matrix (Espina et al., 2014). Despite the consideration that encapsulation in nanoemulsions, which is reported to affect the release profile of EOs (Majeed et al., 2016a), is likely to increase these limits, further studies are needed on the topic.

Finally, the most important property that formulation of EO nanoemulsions affects is their antimicrobial efficacy, which is discussed in details in the following section.

3. Antimicrobial activity of essential oil nanoemulsions

The antimicrobial activity of essential oils has been widely recognized for decades and intensively explored in recent years, driven by the search for naturally-derived alternatives to synthetic food additives (Seow et al., 2014). Despite being consolidated that the antimicrobial actions of EOs involve multiple targets within the cell, rather than relying on one specific mechanism, it is also well established that their action is based on their molecular hydrophobicity. EOs, mainly through their phenolic compounds (Salvia-Trujillo et al., 2015a), strongly interact with the lipids of the cell membrane, increasing the membrane permeability, disturbing the original cell structures, breaking homeostasis, and causing the leakage of ions and cytoplasmic content (Seow et al., 2014).

Encapsulation in nanoemulsions, therefore, while enhancing the dispersibility in food matrices and improving the EO physicochemical stability, necessarily has a significant effect on their interaction with microbial cells, as well as on their biological activity.

3.1. Antimicrobial activity against different microbial species

EO nanoemulsions have been tested *in vitro* against different microorganisms, ranging from bacterial cells to fungi, as shown in Table 2, which summarizes the main investigations reported to date, classified in terms of microorganism species, active components, and formulations used.

The effect of EOs on food spoilage and pathogenic bacteria has been widely investigated in the last decades. Gram-negative (Gram-) organisms are believed to be slightly less sensitive to essential oils than Gram-positive (Gram+) bacteria (Seow et al., 2014), because of the differing structures of their respective cell walls. Approximately 90% - 95% of the cell wall of Gram+ bacteria consists of peptidoglycans, whereas the cell wall of Gram- bacteria is more complex, with a thinner peptidoglycan layer and an outer membrane made of a double layer of phospholipids. Hydrophobic molecules can easily penetrate through the thick peptidoglycan layer of Gram+ bacteria, while the outer membrane of Gram- bacteria is almost impermeable to them, limiting the access to the cell membrane (Nazzaro et al., 2013).

Nanoemulsion droplets, thanks to their size and to the exposition of the hydrophilic groups of the emulsifying molecules, can be efficiently transported through the porin proteins of the outer membrane, enabling an effective delivery of EOs also to the cell membrane of Gram- bacteria. For this reason, the comparison of the antimicrobial activity of specific EO nanoemulsions against Gram+ and Gram- bacteria does not display significant differences. For example, while free thyme oil required shorter incubation times to inactivate *Listeria monocytogenes* (Gram +) than *Salmonella Enteritidis* and *Escherichia coli* (both Gram-), no significant difference was observed when the same EO was encapsulated using a soybean polysaccharide (Wu et al., 2014). Similarly, no significant difference was observed in the kinetics of inactivation of encapsulated eugenol against *L. monocytogenes* and *E. coli* (Shah et al., 2013). Conversely, *Lactobacillus delbrueckii* (Gram+) exhibited a greater resistance than *E. coli* to different encapsulated EOs, such as tea tree oil (Donsì et

384 al., 2011), or carvacrol, cinnamaldehyde and d-limonene (Donsì et al., 2012a), suggesting that the
385 nanoemulsions affect also other routes of bacterial inactivation.

386 In general, the antimicrobial efficacy of EOs nanoemulsions strongly depends on EO components,
387 tested microbial strain and emulsion formulation and size.

388 Table 2 shows that recent studies have targeted *in vitro* different Gram- bacteria, such as *E. coli*,
389 *Salmonella enteritidis*, and *Salmonella typhimurium*, as well as different Gram+ bacteria, such as
390 *Staphylococcus aureus*, *L. delbrueckii*, *L. monocytogenes* and *Listeria innocua*.

391 Several studies have also addressed the effect of EO nanoemulsions on yeast cells, among which
392 *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae* are the most investigated. With respect to
393 bacterial inactivation, yeast cells required longer incubation times when exposed to nanoemulsions
394 carvacrol, cinnamaldehyde, and d-limonene (Donsì et al., 2012a), and exhibited lower minimum
395 inhibitory concentration for encapsulated d-limonene (Zhang et al., 2014).

396 Despite the significant interest in the use of EO for food preservation against spore-forming
397 microorganism, only sparse data are available. For example, eugenol nanoemulsions demonstrated to
398 be effective in the inhibition of *Fusarium oxysporum* (Abd-Elsalam and Khokhlov, 2015).

399

400 **3.2. Mechanisms of antimicrobial action**

401 The antimicrobial activity spectrum observed for many EO nanoemulsions is often broader and
402 stronger than for free EOs. Nanoemulsion formulation, mean droplet size and surface charge, in fact,
403 influence (a) the transport of EOs to the cell membrane, as well as (b) their interaction with the
404 multiple molecular sites at the microbial cell membrane. However, nanoemulsions in some cases are
405 also reported to inhibit the EOs antimicrobial activity.

406 Table 3 gathers the main studies that addressed the effects of encapsulation in nanoemulsion on the
407 antimicrobial activity of EOs, qualitatively indicating if the delivery of the EOs by nanoemulsions
408 had a positive (+, ++) or negative (-, --) impact on the resulting antimicrobial activity.

409 Interestingly, the nanoemulsion formulations, which were reported to cause a measurable reduction
410 in microbial inactivation with respect to free EOs, generally involve the use of macromolecules as
411 emulsifiers, with which the EO preferentially interacts. For example, peppermint oil nanoemulsions
412 stabilized by modified starch (Liang et al., 2012) or eugenol nanodispersed by whey protein-
413 maltodextrin conjugates (Shah et al., 2013) exhibited a lower antimicrobial activity than
414 unencapsulated compounds. However, in other cases, the use of macromolecules contributed to
415 enhancing the antimicrobial activity, as for thyme oil emulsified by sodium caseinate (Xue et al.,
416 2015), or lemongrass oil emulsified by a combination of Tween 80 and sodium alginate (Salvia-
417 Trujillo et al., 2014a, 2014b). The apparent contradiction in the different experimental observations
418 listed in Table 3 can be explained on the basis of the different mechanisms of interactions of EOs
419 with the cell membranes, intervening when nanoemulsions are used as delivery systems.

420 First of all, nanoemulsions enable to disperse the EOs in the aqueous phase at a higher concentration
421 than their water solubility. Therefore, when the minimum inhibition concentration of EO is above its
422 water solubility, the use of nanoemulsions enhances the resulting antimicrobial activity (Donsì et al.,
423 2011; Liang et al., 2012).

424 However, several authors have reported that nanoemulsions might enhance the antimicrobial activity
425 of EOs also when used at concentrations below their water solubilities. The different explanations,
426 proposed for the experimental observations described in Table 3, have been rationalized and
427 schematized in Figure 3.

428 The nanoemulsion-based systems of delivery for EOs are likely to promote their interaction with the
429 microbial cell membranes through four main routes:

1. The increased surface area and passive transport through the outer cell membrane improve the interaction with the cytoplasmic membranes (Donsì et al., 2012a). Small nanoemulsion droplets with hydrophilic surfaces are able to pass through the cell membrane via the abundant porin proteins that serve as hydrophilic transmembrane channels for Gram- bacteria (Nazzaro et al., 2013). In the case of Gram+ bacteria and yeast cells, the nanoemulsion droplets contribute to bringing the EO molecules in contact with their action sites (Majeed et al., 2016b). The small nanoemulsion droplets are able to bring the EOs to the cell membrane surface, improving the accessibility to microbial cells, and enabling the disruption of the cell membrane, possibly by altering the phospholipid bilayer integrity or by interfering with active transport proteins embedded in the phospholipid bilayer (Moghimi et al., 2016).

2. The fusion of the emulsifier droplets with the phospholipid bilayer of the cell membrane likely promotes the targeted release of the EOs at the desired sites. Evidence of this route comes from the observation that the use of different surfactants results in a differentiated antimicrobial activity despite the similar droplet size, such as in the case of eugenol emulsified by Tween 80 or SDS against *E. coli* (Li et al., 2015), or of thyme oil emulsified by modified starch or Tween 80 against *S. aureus*, *E. coli*, and *L. monocytogenes* (Majeed et al., 2016b). In addition, specific interactions between emulsifier and cell membranes have also been reported to increase the EO antimicrobial activity (Salvia-Trujillo et al., 2014a, 2014b).

3. The sustained release over time of the EOs from the nanoemulsion droplets, driven by EO partition between the oil droplets and the aqueous phase, prolongs the activity of EOs. Nanoemulsion droplets act as nanotanks, with EOs molecules in dynamic equilibrium between the disperse oil phase and the aqueous phase (Donsì et al., 2012a). In agreement with this hypothesis, several authors have observed (a) an initial lower inactivation rate of encapsulated EOs than free EOs, whereas over prolonged periods of time, the inactivation levels achieved by nanoemulsions were higher (Majeed et al., 2016b), as well as (b) a

dependence of the antimicrobial activity on the concentration in the oil phase of the ripening inhibitor, which becomes a hydrophobic sink. (Chang et al., 2013; Ziani et al., 2011). Moreover, the emulsifier also plays a significant role in affecting, through micellization mechanisms, the solubility of the EOs (Donsì et al., 2012a).

4. The electrostatic interaction of positively charged nanoemulsions droplets with negatively charged microbial cell walls increases the concentration of EOs at the site of action (Chang et al., 2015). However, this hypothesis is still controversial, as in the cases where an enhanced antimicrobial activity was observed, the cationic surfactant used was also a powerful antimicrobial, such as lauric arginate (Chang et al., 2015; Xue et al., 2015; Ziani et al., 2011). In addition, it was also reported that droplet charge, in the case of anionic emulsifier, did not affect the antimicrobial activity of clove oil (Majeed et al., 2016b), or promoted it, as in the case of SDS for eugenol (Li et al., 2015), because of the complex structure of cell walls, bearing bodies with differentiated local charges.

Likely, the different routes described in Figure 3 all coexist and are difficult to single out. Therefore, further studies are required to improve the understanding of the fundamental mechanisms of action of EO nanoemulsions, to help the formulation as a function of the type of essential oils and of the target microorganisms.

3.3. *In product* application

Application of EO nanoemulsions in food products is a challenging task. The large number of spoilage and pathogenic microorganisms contaminating real foods demands a wide spectrum of activity from the antimicrobial systems. However, as discussed in Sections 3.1 and 3.2, often the developed EO nanoemulsions are able to target a microorganism type more than another. In addition, the highly reactive molecules constituting the EOs can be degraded by the interaction with other food ingredients (protein, lipids, and minerals), or can adsorb onto the multiple interfaces present in real

480 foods, resulting in an uneven distribution, losing antimicrobial efficiency (Gyawali and Ibrahim,
481 2014). Generally, a higher concentration of EOs is required to obtain the same efficacy in foods than
482 in synthetic media (Seow et al., 2014).

483 Additionally, the high volatility, reactivity, odor and flavor of EOs might also cause severe
484 interferences with the product sensory properties, resulting in undesired characteristics (Gyawali and
485 Ibrahim, 2014; Kim et al., 2013). For example, clove and oregano oils were reported to react with
486 iron, forming dark pigmentations, which impair product appearance (Burt, 2004; Seow et al., 2014).

487 However, the use of EOs in the preservation of food products is primarily limited by their strong
488 flavor, with the maximum allowable concentration being set by the sensitivity of the olfactory and
489 taste sensors in the specific product (Dima and Dima, 2015).

490 A previous work showed that the impact of EOs on product sensory properties significantly depends
491 on their food compatibility, and in particular on the matching of the EOs components with the
492 physicochemical characteristics of the food product (Espina et al., 2014). For example, panel tests
493 showed that lemon EO was acceptable at higher concentrations than other EOs (mint, thyme, and
494 rosemary) in tomato juice, vegetable soup, or poultry burgers, because of the higher appreciation of
495 sour, citrus-like flavor (Espina et al., 2014).

496 Therefore, the development of undesirable sensory qualities can be prevented by carefully selecting
497 the EOs according to the specific food type (Seow et al., 2014). For example, EOs could be introduced
498 with minor effects on perception into foods prepared with herbs and spices, such as savory meat, fish,
499 cheese, soups, and sauces (Seow et al., 2014).

500 In addition, the use of suitable delivery systems for EOs can be expected to reduce the required EOs
501 concentration by better dispersing them in the food, to partly mask the taste of EOs, by affecting their
502 partition in the aqueous phase and altering their release rate (Donsì et al., 2012a), to reduce the
503 evaporation rate, as well as to prevent interaction with other food components (Shah et al., 2012a).
504 Moreover, the use of different EOs, which are able to develop synergistic antimicrobial effects at low

concentrations, could also contribute to minimizing the alteration of the sensory characteristics of the food (Gyawali and Ibrahim, 2014).

To date, only a limited number of studies has addressed the application of EO nanoemulsions in real food systems, and an even narrower number has considered the inactivation or inhibition of the natural contaminating flora. Table 4 describes the main applications of food preservation through EO nanoemulsions, which are classified in four macro-areas: (a) direct mixing with a liquid food, (b) washing of the food surface with antimicrobial aqueous dispersions, (c) infusion in porous food matrices, and (d) coating with a biopolymeric layer incorporating the EO nanoemulsions. In particular, both direct mixing and coating resulted in a prolonged antimicrobial action of the EOs nanoemulsions over time, with a significant extension of the shelf life of the product. However, embedding the nanoemulsion in a coating biopolymeric matrix appears to be the most promising approach, because of the immobilization of the EOs at the boundary where microorganisms attack the food product, as well as of the minimized amount of antimicrobial compounds needed, in comparison to direct mixing.

3.3.1. Mixing EO nanoemulsions with liquid products

The direct addition of EO nanoemulsions has been described for milk and for fruit juices (Table 4).

In milk of different fat content, the EO nanoemulsions resulted to be more active against an inoculated microbial load than free EOs, because of their even distribution at concentrations above the solubility limit (Shah et al., 2013; Xue et al., 2015). However, a certain dependence on bacterial strain was observed (Xue et al., 2015). In particular, the use of thymol resulted in a prolonged action against *L. monocytogenes* during a shelf life of 7 days at 32 °C, maintaining the bacterial population below detectability limits for skim milk, and constantly decreasing for milk at higher fat content (Pan et al., 2014).

In fruit juices, both tea tree oil and cinnamaldehyde nanoemulsions exhibited a concentration-dependent inhibition of the inoculated microbial load (Donsì et al., 2011; Jo et al., 2015). In particular,

530 in orange and pear juice, the antimicrobial activity of tea tree oil nanoemulsions against *L. delbrueckii*
531 was observed for 16 days of storage at 32 °C, and, in addition, no measurable color variation was
532 observed over the same storage period (Donsì et al., 2011).

533 In orange juice, eugenol nanoemulsions were successfully used to reduce the heterotrophic bacterial
534 population, showing an enhanced *in product* antibacterial activity over same concentration of sodium
535 benzoate (Ghosh et al., 2014).

536 ***3.3.2. Washing the food surface with EO nanoemulsions***

537 The use of washing solutions containing EOs is not new and has been widely investigated. However,
538 this technique is limited by the low water solubility of most EOs (Gutierrez et al., 2009).
539 Nanoemulsions can be profitably exploited not only to increase the EO concentration in the washing
540 solutions, but also to improve the wettability of vegetable leaves.

541 In particular, the observed enhanced antimicrobial effect of oregano oil nanoemulsions in the
542 decontamination of fresh lettuce leaves has been explained by the effect of the formulation in
543 minimizing the interaction with the organic ingredients in the produce (Bhargava et al., 2015). During
544 3 days of storage at 4 °C, no significant growth of *E. coli*, *S. Typhimurium* and of *S. aureus* was
545 observed on the lettuce leaves (Bhargava et al., 2015). On spinach leaves, a significant inhibition of
546 inoculated *E. coli* and *S. enterica* was achieved by washing with carvacrol on eugenol nanoemulsions,
547 with no difference observed between spray and immersion (Ruengvisesh et al., 2015).

548 Further studies are needed to develop formulations, which are able to selectively target spoilage
549 microorganisms while minimizing interaction with the produce and the impact on the organoleptic
550 properties.

551 ***3.3.3. Infusion of EO nanoemulsions into porous food matrices***

552 Another interesting approach to the use of EO nanoemulsions as natural preservatives in foods is their
553 infusion in porous solid foods.

A recent study showed that EO nanoemulsions not only enabled a slow and sustained release of the antimicrobial compounds but also contributed to its incorporation in complex food systems. In particular, the diffusion rate of carvacrol in vegetable or animal tissues resulted to be significantly enhanced when encapsulated in nanoemulsions with mean droplet size below the characteristic size of membrane pores and intercellular interstices (< 200 nm), while the surface composition and charge of emulsion droplets did not play an important role in controlling the infusion process (Donsì et al., 2014). Moreover, the enhanced mass transfer was well correlated with the observed increase in antimicrobial activity of carvacrol, both against *E. coli* inoculated in zucchini samples, and against the endogenous flora present in cooked sausages (Donsì et al., 2014).

A more systematic investigation of infusion of EO nanoemulsions into complex food systems is needed to further support this application.

3.3.4. Coating with of EO nanoemulsions onto food surfaces

In the last decade, several studies have addressed the use of edible films or coatings containing EOs, directly incorporated in the biopolymeric matrix, to extend the shelf life of different food products. Despite this approach offers the advantage of a simple and versatile fabrication process, it (a) significantly reduces the EOs loading capability in the coating, (b) increases the risk of oiling off of EOs, as well as (c) undermines the mechanical properties of the film or coating (Sanchez-Gonzalez et al., 2011).

In contrast, the incorporation of EO nanoemulsions in the biopolymeric matrix enables a better film homogeneity, the EO loading at concentrations above the solubility in the film forming system, as well as enhanced antimicrobial activity (Donsì et al., 2015; Otoni et al., 2014; Severino et al., 2014a, 2014b). Despite the several advantages associated with the use of EO nanoemulsions, to date, no systematic studies have been addressed to optimizing the films and coatings composition, as well as to assessing the effect of EOs nanoemulsion loading on the film forming properties of the biopolymer.

578 The data reported in Table 4 show that a sodium alginate coating layer, containing a lemongrass oil
579 nanoemulsion, significantly extended the shelf life of apple pieces, under refrigerated conditions. The
580 antimicrobial coating not only contributed to maintain the microbial population (either inoculated
581 microorganisms or endogenous flora) of apple pieces significantly below the levels of control
582 samples, but also caused negligible changes in the appearance and texture of the apples, and
583 contributed to a significant reduction in the respiration rate (Salvia-Trujillo et al., 2015b). A modified
584 chitosan coating layer, containing different citrus oil nanoemulsions, was instead used to extend the
585 shelf life of green beans (Donsì et al., 2015; Severino et al., 2015, 2014a) and broccoli florets
586 (Severino et al., 2014b). In the case of green beans, the antimicrobial coating (alone or in combination
587 with other non-thermal technologies) contributed to inhibit the growth of *L. innocua* (Donsì et al.,
588 2015; Severino et al., 2014a) or of *E. coli* O157:H7 and *S. Typhimurium* (Severino et al., 2015) for
589 14 days under refrigerated conditions, with minimal impact on both product color and texture.

590 Edible coatings based on modified chitosan and containing lemon oil were successfully applied
591 against the endogenous flora present on rucola leaves, whose shelf life was prolonged the shelf life
592 of up to 7 days when compared to the untreated samples (Sessa et al., 2015). Methylcellulose coatings
593 containing clove bud oil or oregano oil were instead tested against spoilage microorganisms of sliced
594 bread (Otoni et al., 2014). The sliced bread shelf life was significantly extended, even in comparison
595 to the commercial antifungal agent currently used in bakeries, with the stabilization of the endogenous
596 microbial flora for 15 days of storage at 25 °C (Otoni et al., 2014).

597 The use of EO nanoemulsions in edible coatings is a particularly promising technology for the
598 preservation of several products, because of the small amounts required, the prolonged persistence of
599 the protection layer, as well as of the possible synergism of EO biological activity with the physical
600 barrier offered by the coating.

601

4. Regulatory issues

Finally, another important aspect that needs to be taken into account concerns the safety issues associated with the consumption of foods containing EOs nanoemulsions.

Natural antimicrobials included in foods are generally regulated as food additives or flavorings, with specific databases of safe compounds, permitted concentrations, and permitted food uses provided by different regional authorities, such as the Food and Drug Administration in U.S.A (www.fda.gov), the European Food Safety Authority in Europe (www.efsa.europa.eu), and the China Food Additives & Ingredients Association in China (www.cfaa.cn) (Malhotra et al., 2015). In particular, a different approach is proposed by FDA and EFSA, with the former laying down a list of essential oils, oleoresins, and natural extractives that are generally recognized as safe for their intended use (Code of Federal Regulations, Title 21, Volume 3, 1998), and the latter defining the individual food components, and the eventual restriction of use in different food categories (Regulation (EC) No 2232/96 and Regulation (EC) No 1334/2008). More specifically, due to the batch-wise compositional variability of plant extracts, which reduces the possibility of their standardization, it is not possible to assign them acceptable daily intake (ADI) or no observed adverse effect level (NOEL) values. Therefore, limitations are given on undesirable substances, which should not be added as such to food, but which, due to their natural occurrence, might be present in flavorings and in certain food ingredients with flavoring properties (annex II of Regulation (EC) No 388/1988).

If a compound has not already been approved, the competent regulatory authorities should be addressed (Tajkarimi et al., 2010), through a strict protocol, where the types of foods in which it can be used, the maximum amount allowed, and its proper identification on food labels are defined, based on amount of substance that would normally be consumed, as well as the short- and long-term health effects and other safety considerations (Malhotra et al., 2015).

In addition, the potential risks associated with the use of highly reactive molecules, such as those constituting the EOs, should be continuously monitored. For example, carvacrol, despite being

already included among registered flavorings and foodstuffs for the Council of Europe, the Food and Drug Administration (FDA) and the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO), has been recently showed, by *in vitro* studies, to have a concentration-dependent toxic effect (LLana-Ruiz-Cabello et al., 2014).

In consideration of the great potential of the use of EOs in food preservation, it is desirable that specific ISO standards are developed to assess the legal aspects to set out the definition, the general rules of use, the requirements for labeling and the maximum levels authorized. (Lucera et al., 2012)

In the case of nanoemulsions, the regulatory issues concern both the ingredients and the use of nanotechnology in their engineering. The ingredients used in the formulation of food-grade nanoemulsions are in general of well consolidated use, and have been listed, together with their safe usage levels, by various organizations, including the World Health Organization (www.who.int), the Food and Drug Administration (www.fda.gov) and the European Food Safety Authority (www.efsa.europa.eu) (McClements and Rao, 2011).

However, the lack of a globally recognized regulatory definition of nanotechnology or of nanoscale or nanoengineered materials might originate uncertainty on the safety of the use of nanoscale preparations of existing food ingredients or currently approved food additives (Martins et al., 2015).

FDA has not established, to date, a regulatory definition, but has developed non-binding guidelines (http://www.fda.gov/regulatoryinformation/guidances/ucm257698.htm), which determine that an FDA-regulated product involves the application of nanotechnology, whether at least one of its dimensions falls in the nanoscale range (1–100 nm) or it exhibits physical or chemical properties or biological effects, because of its dimensions falling in the range from 1 to 1000 nm (Martins et al., 2015).

The existing regulatory framework of the European Union (EU) covers the potential uses of nanotechnologies in the food, either by the principles of the general food law (Regulation (EC) No 178/2002) or by specific approval processes. However, EU also gives a definition (Regulation (EU)

No 1363/2013) of engineered nanomaterials as any intentionally manufactured material, which contains 50 % or more of with one or more external dimensions in the nanoscale range (1–100 nm) (Martins et al., 2015).

5. Conclusions and perspectives

The Conclusions section provides a brief summary of the results and discussion, but it should be more than a summary. After showing how each research question posed in the introduction has been addressed, the implications of the findings should be emphasized, explaining how the work is significant. The goal here is to provide the most general claims that can be supported by the evidence. This section should be reader-focused, avoiding a list of all the things that "I" or "we" have accomplished.

The Conclusions section should allow for opportunistic reading. When writing this section, imagine a reader who reads the introduction, skims through the figures, then jumps to the conclusion. The conclusion should concisely provide the key message(s) the author wishes to convey. It should not repeat the arguments made in the results and discussion, only the final and most general conclusions. While the results and discussion section is often quite long, the conclusion section is generally short.

The second goal of the conclusion is to provide a future perspective on the work. This could be recommendations to the audience or a roadmap for future work. A small amount of speculation can be appropriate here, so long as it is relevant and clearly labeled as speculative.

Some common pitfalls when writing the conclusion are **repeating the abstract**, **repeating background information from the introduction**, **introducing new evidence or new arguments** not found in the results and discussion, **repeating the arguments made** in the results and discussion, or **failing to address all of the research questions set out** in the introduction.

Consumers' demand for safe natural products has driven in the last years the search for mild preservation techniques to improve microbial quality and safety, without causing nutritional and organoleptic losses. In this context, natural antimicrobials, such as essential oils, hold potential for providing quality and safety benefits, with a reduced impact on human health (Lucera et al., 2012).

The encapsulation of EOs in food-grade nanoemulsions offers numerous advantages, in terms of effects on EOs biological activity, physicochemical stabilization and *in product* behavior.

However, several challenges still remain for the full exploitation of EO nanoemulsions within a mild strategy for food preservation:

1. The EOs formulations should be developed in agreement with product properties, to efficiently oppose the native endogenous flora, but also to minimize the impact on the organoleptic properties.
2. Controlled or triggered release properties of EOs from the delivery systems are also desirable for higher responsiveness when needed (for example, if pH changes due to microbial growth), and reduced impact when not.
3. In strict association to the controlled release, the encapsulation system should also be developed in consideration of masking the taste of EO compounds, while preserving their antimicrobial efficacy.
4. The combined use of EOs compounds should be exploited to develop a synergistic action to promote the interaction with multiple molecular sites at the microbial cell membrane (Asbahani et al., 2015; Seow et al., 2014). Moreover, the combined use of some natural EOs might eventually induce antagonistic effects (Lucera et al., 2012).
5. A better understanding of the mechanisms by which encapsulated EOs operate is necessary to provide solid grounds for engineering novel antimicrobial systems and strategies.

6. The combination of application of EO nanoemulsions in conjunction with hurdle technologies, for example with gamma ray irradiation, ozone, UV (Severino et al., 2014a), high pressure or pulsed light (Donsì et al., 2015) or with modified-atmosphere packaging (Severino et al., 2015) is likely to contribute to increase the product shelf life, while preserving its fresh-like attributes (Tajkarimi et al., 2010).
7. Costs should be carefully assessed, as the use of essential oils remains expensive, as well as their encapsulation in nanoemulsions, and their eventual application in combination with non-thermal technologies.

References

Abd-Elsalam, K.A., Khokhlov, A.R., 2015. Eugenol oil nanoemulsion: antifungal activity against Fusarium oxysporum f. sp. vasinfectum and phytotoxicity on cottonseeds. Appl. Nanosci. 5, 255–265. doi:10.1007/s13204-014-0398-y

Asbahani, A. El, Miladi, K., Badri, W., Sala, M., Addi, E.H.A., Casabianca, H., Mousadik, A. El, Hartmann, D., Jilale, A., Renaud, F.N.R., Elaissari, A., 2015. Essential oils: From extraction to encapsulation. Int. J. Pharm. 483, 220–243. doi:10.1016/j.ijpharm.2014.12.069

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils - A review. Food Chem. Toxicol. 46, 446–75. doi:10.1016/j.fct.2007.09.106

Bhargava, K., Conti, D.S., da Rocha, S.R.P., Zhang, Y., 2015. Application of an oregano oil nanoemulsion to the control of foodborne bacteria on fresh lettuce. Food Microbiol. 47, 69–73. doi:10.1016/j.fm.2014.11.007

Bilbao-Sainz, C., Chiou, B. Sen, Du, W.X., Gregorsky, K.S., Orts, W.J., 2013. Influence of disperse phase characteristics on stability, physical and antimicrobial properties of emulsions containing cinnamaldehyde. JAOCS, J. Am. Oil Chem. Soc. 90, 233–241. doi:10.1007/s11746-012-2164-1

Buranasuksombat, U., Kwon, Y.J., Turner, M., Bhandari, B., 2011. Influence of emulsion droplet size on antimicrobial properties. Food Sci. Biotechnol. 20, 793–800. doi:10.1007/s10068-011-0110-x

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods--a review. Int. J. Food Microbiol. 94, 223–53. doi:10.1016/j.ijfoodmicro.2004.03.022

Chanamai, R., Horn, G., McClements, D.J., 2002. Influence of oil polarity on droplet growth in oil-in-water emulsions stabilized by a weakly adsorbing biopolymer or a nonionic surfactant. J. Colloid Interface Sci. 247, 167–176. doi:10.1006/jcis.2001.8110

732 Chang, Y., McClements, D.J., 2014. Optimization of orange oil nanoemulsion formation by
733 isothermal low-energy methods: Influence of the oil phase, surfactant, and temperature. J. Agric.
734 Food Chem. 62, 2306–2312. doi:10.1021/jf500160y

735 Chang, Y., McLandsborough, L., McClements, D.J., 2015. Fabrication, stability and efficacy of dual-
736 component antimicrobial nanoemulsions: Essential oil (thyme oil) and cationic surfactant (lauric
737 arginate). Food Chem. 172, 298–304. doi:10.1016/j.foodchem.2014.09.081

738 Chang, Y., McLandsborough, L., McClements, D.J., 2013. Physicochemical properties and
739 antimicrobial efficacy of carvacrol nanoemulsions formed by spontaneous emulsification. J.
740 Agric. Food Chem. 61, 8906–8913. doi:10.1021/jf402147p

741 Chang, Y., McLandsborough, L., McClements, D.J., 2012. Physical properties and antimicrobial
742 efficacy of thyme oil nanoemulsions: Influence of ripening inhibitors. J. Agric. Food Chem. 60,
743 12056–12063. doi:10.1021/jf304045a

744 Chen, L., Remondetto, G.E., Subirade, M., 2006. Food protein-based materials as nutraceutical
745 delivery systems. Trends Food Sci. Technol. 17, 272–283. doi:10.1016/j.tifs.2005.12.011

746 Dima, C., Dima, S., 2015. Essential oils in foods: extraction, stabilization, and toxicity. Curr. Opin.
747 Food Sci. 5, 29–35. doi:http://dx.doi.org/10.1016/j.cofs.2015.07.003

748 Donsì, F., Annunziata, M., Ferrari, G., 2013a. Microbial inactivation by high pressure
749 homogenization: Effect of the disruption valve geometry. J. Food Eng. 115, 362–370.
750 doi:10.1016/j.jfoodeng.2012.10.046

751 Donsì, F., Annunziata, M., Sessa, M., Ferrari, G., 2011. Nanoencapsulation of essential oils to
752 enhance their antimicrobial activity in foods. LWT - Food Sci. Technol. 44, 1908–1914.
753 doi:10.1016/j.lwt.2011.03.003

754 Donsì, F., Annunziata, M., Vincensi, M., Ferrari, G., 2012a. Design of nanoemulsion-based delivery
755 systems of natural antimicrobials: effect of the emulsifier. J. Biotechnol. 159, 342–50.

doi:10.1016/j.jbiotec.2011.07.001

Donsì, F., Cuomo, A., Marchese, E., Ferrari, G., 2014. Infusion of essential oils for food stabilization: Unraveling the role of nanoemulsion-based delivery systems on mass transfer and antimicrobial activity. Innov. Food Sci. Emerg. Technol. 22, 212–220. doi:10.1016/j.ifset.2014.01.008

Donsì, F., Ferrari, G., Maresca, P., 2009. High-Pressure Homogenization for Food Sanitization, Global Issues in Food Science and Technology. doi:10.1016/B978-0-12-374124-0.00019-3

Donsì, F., Marchese, E., Maresca, P., Pataro, G., Vu, K.D., Salmieri, S., Lacroix, M., Ferrari, G., 2015. Green beans preservation by combination of a modified chitosan based-coating containing nanoemulsion of mandarin essential oil with high pressure or pulsed light processing. Postharvest Biol. Technol. 106, 21–32. doi:10.1016/j.postharvbio.2015.02.006

Donsì, F., Sessa, M., Ferrari, G., 2013b. Nanometric-Size Delivery Systems for Bioactive Compounds for the Nutraceutical and Food Industries, in: Bagchi, D., Bagchi, M., Moriyama, H., Shahidi, F. (Eds.), Bio-Nanotechnology: A Revolution in Food, Biomedical and Health Sciences. Blackwell Publishing Ltd., Oxford, UK, pp. 619–666. doi:10.1002/9781118451915.ch37

Donsì, F., Sessa, M., Ferrari, G., 2012b. Effect of emulsifier type and disruption chamber geometry on the fabrication of food nanoemulsions by high pressure homogenization. Ind. Eng. Chem. Res. 51, 7606–7618. doi:10.1021/ie2017898

Espina, L., Garcia-Gonzalo, D., Pagan, R., 2014. Impact of essential oils on the taste acceptance of tomato juice, vegetable soup, or poultry burgers. J. Food Sci. 79. doi:10.1111/1750-3841.12529

Ferreira, J.P., Alves, D., Neves, O., Silva, J., Gibbs, P.A., Teixeira, P.C., 2010. Effects of the components of two antimicrobial emulsions on food-borne pathogens 21, 227–230. doi:10.1016/j.foodcont.2009.05.018

Ghosh, V., Mukherjee, A., Chandrasekaran, N., 2014. Eugenol-loaded antimicrobial nanoemulsion

780 preserves fruit juice against, microbial spoilage. Colloids Surfaces B Biointerfaces 114, 392–
781 397. doi:10.1016/j.colsurfb.2013.10.034

782 Guerra-Rosas, M.I., Morales-Castro, J., Ochoa-Martinez, L.A., Salvia-Trujillo, L., Martin-Belloso,
783 O., 2016. Long-term stability of food-grade nanoemulsions from high methoxyl pectin
784 containing essential oils. Food Hydrocoll. 52, 438–446. doi:10.1016/j.foodhyd.2015.07.017

785 Gutierrez, J., Bourke, P., Lonchamp, J., Barry-Ryan, C., 2009. Impact of plant essential oils on
786 microbiological, organoleptic and quality markers of minimally processed vegetables. Innov.
787 Food Sci. Emerg. Technol. 10, 195–202. doi:10.1016/j.ifset.2008.10.005

788 Gyawali, R., Ibrahim, S.A., 2014. Natural products as antimicrobial agents. Food Control.
789 doi:10.1016/j.foodcont.2014.05.047

790 Jo, Y.J., Chun, J.Y., Kwon, Y.J., Min, S.G., Hong, G.P., Choi, M.J., 2015. Physical and antimicrobial
791 properties of trans-cinnamaldehyde nanoemulsions in water melon juice. LWT - Food Sci.
792 Technol. 60, 444–451. doi:10.1016/j.lwt.2014.09.041

793 Joscelyne, S.M., Trägårdh, G., 2000. Membrane emulsification - A literature review. J. Memb. Sci.
794 doi:10.1016/S0376-7388(99)00334-8

795 Kim, I.H., Lee, H., Kim, J.E., Song, K. Bin, Lee, Y.S., Chung, D.S., Min, S.C., 2013. Plum coatings
796 of lemongrass oil-incorporating carnauba wax-based nanoemulsion. J. Food Sci. 78.
797 doi:10.1111/1750-3841.12244

798 Li, W., Chen, H., He, Z., Han, C., Liu, S., Li, Y., 2015. Influence of surfactant and oil composition
799 on the stability and antibacterial activity of eugenol nanoemulsions. LWT - Food Sci. Technol.
800 62, 39–47. doi:10.1016/j.lwt.2015.01.012

801 Liang, R., Xu, S., Shoemaker, C.F., Li, Y., Zhong, F., Huang, Q., 2012. Physical and antimicrobial
802 properties of peppermint oil nanoemulsions. J. Agric. Food Chem. 60, 7548–7555.
803 doi:10.1021/jf301129k

804 Liu, W., Yang, X.L., Winston Ho, W.S., 2011. Preparation of uniform-sized multiple emulsions and
805 micro/nano particulates for drug delivery by membrane emulsification. J. Pharm. Sci.
806 doi:10.1002/jps.22272

807 LLana-Ruiz-Cabello, M., Maisanaba, S., Puerto, M., Prieto, A.I., Pichardo, S., Jos, Á., Cameán,
808 A.M., 2014. Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol
809 using the Ames Salmonella test and alkaline, Endo III- and FPG-modified comet assays with the
810 human cell line Caco-2. Food Chem. Toxicol. 72, 122–128. doi:10.1016/j.fct.2014.07.013

811 Lucera, A., Costa, C., Conte, A., Del Nobile, M.A., 2012. Food applications of natural antimicrobial
812 compounds. Front. Microbiol. doi:10.3389/fmicb.2012.00287

813 Luo, Y., Zhang, Y., Pan, K., Critzer, F., Davidson, P.M., Zhong, Q., 2014. Self-emulsification of
814 alkaline-dissolved clove bud oil by whey protein, gum arabic, lecithin, and their combinations.
815 J. Agric. Food Chem. 62, 4417–4424. doi:10.1021/jf500698k

816 Maa, Y.F., Hsu, C.C., 1999. Performance of sonication and microfluidization for liquid-liquid
817 emulsification. Pharm. Dev. Technol. 4, 233–240. doi:10.1081/PDT-100101357

818 Majeed, H., Antoniou, J., Hategekimana, J., Sharif, H.R., Haider, J., Liu, F., Ali, B., Rong, L., Ma,
819 J., Zhong, F., 2016a. Influence of carrier oil type, particle size on invitro lipid digestion and
820 eugenol release in emulsion and nanoemulsions. Food Hydrocoll. 52, 415–422.
821 doi:10.1016/j.foodhyd.2015.07.009

822 Majeed, H., Liu, F., Hategekimana, J., Sharif, H.R., Qi, J., Ali, B., Bian, Y.Y., Ma, J., Yokoyama,
823 W., Zhong, F., 2016b. Bactericidal action mechanism of negatively charged food grade clove
824 oil nanoemulsions. Food Chem. 197, 75–83. doi:10.1016/j.foodchem.2015.10.015

825 Malhotra, B., Keshwani, A., Kharkwal, H., 2015. Antimicrobial food packaging: potential and
826 pitfalls. Front. Microbiol. 6, 611. doi:10.3389/fmicb.2015.00611

827 Martins, J.T., Ramos, O.L., Pinheiro, A.C., Bourbon, A.I., Silva, H.D., Rivera, M.C., Cerqueira,

828 M.A., Pastrana, L., Malcata, F.X., Gonzalez-Fernandez, A., Vicente, A.A., 2015. Edible Bio-
829 Based Nanostructures: Delivery, Absorption and Potential Toxicity. Food Eng. Rev. 7, 491–513.
830 doi:10.1007/s12393-015-9116-0

831 Mason, T.G., Wilking, J.N., Meleson, K., Chang, C.B., Graves, S.M., 2006. Nanoemulsions:
832 formation, structure, and physical properties. J. Phys. Condens. Matter 18, R635–R666.
833 doi:10.1088/0953-8984/18/41/R01

834 McClements, D.J., Rao, J., 2011. Food-grade nanoemulsions: formulation, fabrication, properties,
835 performance, biological fate, and potential toxicity. Crit. Rev. Food Sci. Nutr. 51, 285–330.
836 doi:10.1080/10408398.2011.559558

837 Moghimi, R., Ghaderi, L., Rafati, H., Aliahmadi, A., Mcclements, D.J., 2016. Superior antibacterial
838 activity of nanoemulsion of Thymus daenensis essential oil against E. coli. Food Chem. 194,
839 410–415. doi:10.1016/j.foodchem.2015.07.139

840 Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R., De Feo, V., 2013. Effect of essential oils on
841 pathogenic bacteria. Pharmaceuticals (Basel). 6, 1451–74. doi:10.3390/ph6121451

842 Otoni, C.G., Pontes, S.F.O., Medeiros, E.A.A., Soares, N.D.F.F., 2014. Edible films from
843 methylcellulose and nanoemulsions of clove bud (Syzygium aromaticum) and oregano
844 (Origanum vulgare) essential oils as shelf life extenders for sliced bread. J. Agric. Food Chem.
845 62, 5214–5219. doi:10.1021/jf501055f

846 Pan, K., Chen, H., Davidson, P.M., Zhong, Q., 2014. Thymol nanoencapsulated by sodium caseinate:
847 Physical and antilisterial properties. J. Agric. Food Chem. 62, 1649–1657.
848 doi:10.1021/jf4055402

849 Rao, J., McClements, D.J., 2012. Impact of lemon oil composition on formation and stability of model
850 food and beverage emulsions. Food Chem. 134, 749–757. doi:10.1016/j.foodchem.2012.02.174

851 Rao, J., McClements, D.J., 2010. Stabilization of phase inversion temperature nanoemulsions by

surfactant displacement. J. Agric. Food Chem. 58, 7059–7066. doi:10.1021/jf100990r

Ruengvisesh, S., Loquercio, A., Castell-Perez, E., Taylor, T.M., 2015. Inhibition of Bacterial Pathogens in Medium and on Spinach Leaf Surfaces using Plant-Derived Antimicrobials Loaded in Surfactant Micelles. J. Food Sci. 80, M2522–M2529. doi:10.1111/1750-3841.13085

Sagalowicz, L., Leser, M.E., 2010. Delivery systems for liquid food products. Curr. Opin. Colloid Interface Sci. 15, 61–72. doi:10.1016/j.cocis.2009.12.003

Salvia-Trujillo, L., Rojas-Graü, A., Soliva-Fortuny, R., Martín-Belloso, O., 2015a. Physicochemical characterization and antimicrobial activity of food-grade emulsions and nanoemulsions incorporating essential oils. Food Hydrocoll. 43, 547–556. doi:10.1016/j.foodhyd.2014.07.012

Salvia-Trujillo, L., Rojas-Grau, M.A., Soliva-Fortuny, R., Martin-Belloso, O., 2015b. Use of antimicrobial nanoemulsions as edible coatings: Impact on safety and quality attributes of fresh-cut fuji apples. Postharvest Biol. Technol. 105, 8–16. doi:10.1016/j.postharvbio.2015.03.009

Salvia-Trujillo, L., Rojas-Grau, M.A., Soliva-Fortuny, R., Martin-Belloso, O., 2014a. Formulation of Antimicrobial Edible Nanoemulsions with Pseudo-Ternary Phase Experimental Design. Food Bioprocess Technol. 7, 3022–3032. doi:10.1007/s11947-014-1314-x

Salvia-Trujillo, L., Rojas-Graü, M.A., Soliva-Fortuny, R., Martín-Belloso, O., 2014b. Impact of microfluidization or ultrasound processing on the antimicrobial activity against Escherichia coli of lemongrass oil-loaded nanoemulsions. Food Control 37, 292–297. doi:10.1016/j.foodcont.2013.09.015

Sanchez-Gonzalez, L., Chiralt, A., Gonzalez-Martinez, C., Chafer, M., 2011. Effect of essential oils on properties of film forming emulsions and films based on hydroxypropylmethylcellulose and chitosan. J. Food Eng. 105, 246–253. doi:10.1016/j.jfoodeng.2011.02.028

Schultz, S., Wagner, G., Urban, K., Ulrich, J., 2004. High-pressure homogenization as a process for emulsion formation. Chem. Eng. Technol. 27, 361–368. doi:10.1002/ceat.200406111

876 Seow, Y.X., Yeo, C.R., Chung, H.L., Yuk, H.-G., 2014. Plant essential oils as active antimicrobial
877 agents. Crit. Rev. Food Sci. Nutr. 54, 625–44. doi:10.1080/10408398.2011.599504

878 Sessa, M., Donsì, F., 2015. Nanoemulsion-Based Delivery Systems, in: Sagis, L.M.C. (Ed.),
879 Microencapsulation and Microspheres for Food Applications. Elsevier, London, pp. 79–94.
880 doi:10.1016/B978-0-12-800350-3.00007-8

881 Sessa, M., Ferrari, G., Donsì, F., 2015. Novel Edible Coating Containing Essential Oil
882 Nanoemulsions to Prolong the Shelf Life of Vegetable Products. Chem. Eng. Trans. 43, 55–60.
883 doi:10.3303/CET1543010

884 Severino, R., Ferrari, G., Vu, K.D., Donsì, F., Salmieri, S., Lacroix, M., 2015. Antimicrobial effects
885 of modified chitosan based coating containing nanoemulsion of essential oils, modified
886 atmosphere packaging and gamma irradiation against Escherichia coli O157:H7 and Salmonella
887 Typhimurium on green beans. Food Control 50, 215–222. doi:10.1016/j.foodcont.2014.08.029

888 Severino, R., Vu, K.D., Donsì, F., Salmieri, S., Ferrari, G., Lacroix, M., 2014a. Antibacterial and
889 physical effects of modified chitosan based-coating containing nanoemulsion of mandarin
890 essential oil and three non-thermal treatments against Listeria innocua in green beans. Int. J.
891 Food Microbiol. 191, 82–88. doi:10.1016/j.ijfoodmicro.2014.09.007

892 Severino, R., Vu, K.D., Donsì, F., Salmieri, S., Ferrari, G., Lacroix, M., 2014b. Antimicrobial effects
893 of different combined non-thermal treatments against Listeria monocytogenes in broccoli
894 florets. J. Food Eng. 124, 1–10. doi:10.1016/j.jfoodeng.2013.09.026

895 Shah, B., Davidson, P.M., Zhong, Q., 2013. Nanodispersed eugenol has improved antimicrobial
896 activity against Escherichia coli O157:H7 and Listeria monocytogenes in bovine milk. Int. J.
897 Food Microbiol. 161, 53–9. doi:10.1016/j.ijfoodmicro.2012.11.020

898 Shah, B., Davidson, P.M., Zhong, Q., 2012a. Encapsulation of eugenol using Maillard-type
899 conjugates to form transparent and heat stable nanoscale dispersions. LWT - Food Sci. Technol.

900 49, 139–148. doi:10.1016/j.lwt.2012.04.029

901 Shah, B., Ikeda, S., Michael Davidson, P., Zhong, Q., 2012b. Nanodispersing thymol in whey protein
902 isolate-maltodextrin conjugate capsules produced using the emulsion-evaporation technique. J.
903 Food Eng. 113, 79–86. doi:10.1016/j.jfoodeng.2012.05.019

904 Silva, H.D., Cerqueira, M.A., 2015. Influence of surfactant and processing conditions in the stability
905 of oil-in-water nanoemulsions. J. Food Eng. 167, 89–98. doi:10.1016/j.jfoodeng.2015.07.037

906 Silva, H.D., Cerqueira, M.A., Vicente, A.A., 2012. Nanoemulsions for Food Applications:
907 Development and Characterization. Food Bioprocess Technol. doi:10.1007/s11947-011-0683-7

908 Stang, M., Schuchmann, H., Schubert, H., 2001. Emulsification in High-Pressure Homogenizers.
909 Eng. Life Sci. 1, 151. doi:10.1002/1618-2863(200110)1:4<151::AID-ELSC151>3.0.CO;2-D

910 Sugumar, S., Ghosh, V., Nirmala, M.J., Mukherjee, A., Chandrasekaran, N., 2014. Ultrasonic
911 emulsification of eucalyptus oil nanoemulsion: Antibacterial activity against Staphylococcus
912 aureus and wound healing activity in Wistar rats. Ultrason. Sonochem. 21, 1044–1049.
913 doi:10.1016/j.ultsonch.2013.10.021

914 Sugumar, S., Nirmala, J., Ghosh, V., Anjali, H., Mukherjee, A., Chandrasekaran, N., 2013. Bio-based
915 nanoemulsion formulation, characterization and antibacterial activity against food-borne
916 pathogens. J. Basic Microbiol. 53, 677–685. doi:10.1002/jobm.201200060

917 Suriyarak, S., Weiss, J., 2014. Cutoff Ostwald ripening stability of alkane-in-water emulsion loaded
918 with eugenol. Colloids Surfaces A Physicochem. Eng. Asp. 446, 71–79.
919 doi:10.1016/j.colsurfa.2014.01.009

920 Tajkarimi, M.M., Ibrahim, S.A., Cliver, D.O., 2010. Antimicrobial herb and spice compounds in food.
921 Food Control. doi:10.1016/j.foodcont.2010.02.003

922 Teixeira, P.C., Leite, G.M., Domingues, R.J., Silva, J., Gibbs, P.A., Ferreira, J.P., 2007. Antimicrobial
923 effects of a microemulsion and a nanoemulsion on enteric and other pathogens and biofilms. Int.

924 J. Food Microbiol. 118, 15–19. doi:10.1016/j.ijfoodmicro.2007.05.008

925 Terjung, N., Löffler, M., Gibis, M., Hinrichs, J., Weiss, J., 2012. Influence of droplet size on the
926 efficacy of oil-in-water emulsions loaded with phenolic antimicrobials. Food Funct. 3, 290–301.
927 doi:10.1039/c2fo10198j

928 Troncoso, E., Aguilera, J.M., McClements, D.J., 2012. Fabrication, characterization and lipase
929 digestibility of food-grade nanoemulsions. Food Hydrocoll. 27, 355–363.
930 doi:10.1016/j.foodhyd.2011.10.014

931 Walstra, P., 1993. Principles of emulsion formation. Chem. Eng. Sci. 48, 333–349. doi:10.1016/0009-
932 2509(93)80021-H

933 Wooster, T.J., Golding, M., Sanguansri, P., 2008. Impact of oil type on nanoemulsion formation and
934 ostwald ripening stability. Langmuir 24, 12758–12765. doi:10.1021/la801685v

935 Wu, J.E., Lin, J., Zhong, Q., 2014. Physical and antimicrobial characteristics of thyme oil emulsified
936 with soluble soybean polysaccharide. Food Hydrocoll. 39, 144–150.
937 doi:10.1016/j.foodhyd.2013.12.029

938 Xue, J., Michael Davidson, P., Zhong, Q., 2015. Antimicrobial activity of thyme oil co-
939 nanoemulsified with sodium caseinate and lecithin. Int. J. Food Microbiol. 210, 1–8.
940 doi:10.1016/j.ijfoodmicro.2015.06.003

941 Xue, J., Zhong, Q., 2014. Thyme oil nanoemulsions coemulsified by sodium caseinate and lecithin.
942 J. Agric. Food Chem. 62, 9900–9907. doi:10.1021/jf5034366

943 Zahi, M.R., Wan, P., Liang, H., Yuan, Q., 2014. Formation and stability of d -limonene organogel-
944 based nanoemulsion prepared by a high-pressure homogenizer. J. Agric. Food Chem. 62, 12563–
945 12569. doi:10.1021/jf5032108

946 Zhang, Z., Vriesekoop, F., Yuan, Q., Liang, H., 2014. Effects of nisin on the antimicrobial activity of
947 d-limonene and its nanoemulsion. Food Chem. 150, 307–312.

948 doi:10.1016/j.foodchem.2013.10.160

949 Ziani, K., Chang, Y., McLandsborough, L., McClements, D.J., 2011. Influence of surfactant charge
950 on antimicrobial efficacy of surfactant-stabilized thyme oil nanoemulsions. J. Agric. Food
951 Chem. 59, 6247–6255. doi:10.1021/jf200450m

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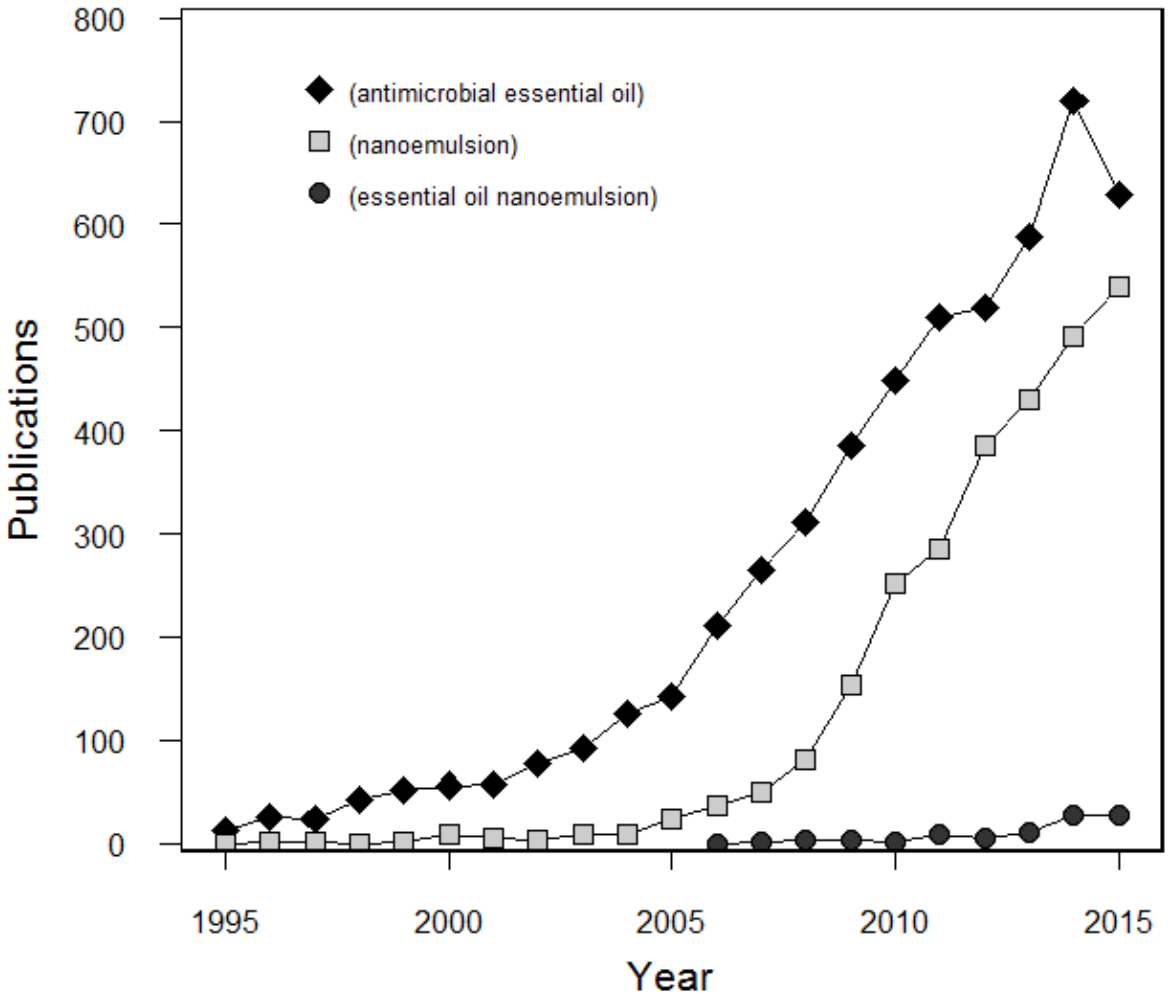
Figure captions

Figure 1. *Number of publications indexed by Scopus (www.scopus.com) based on search strings related to the use of "essential oils as antimicrobials", "nanoemulsions", and "essential oil nanoemulsions" in the title, keywords, and abstract of the publication.*

Figure 2. *Schematics of the different fabrication methods of O/W nanoemulsions, correlating their requirements in terms of energy and surfactant to oil ratio with the expected mean droplet size.*

Figure 3. *Schematics of the different routes promoted by the nanoemulsions for the interaction of EO with the microbial cell membranes.*



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963 Figure 1

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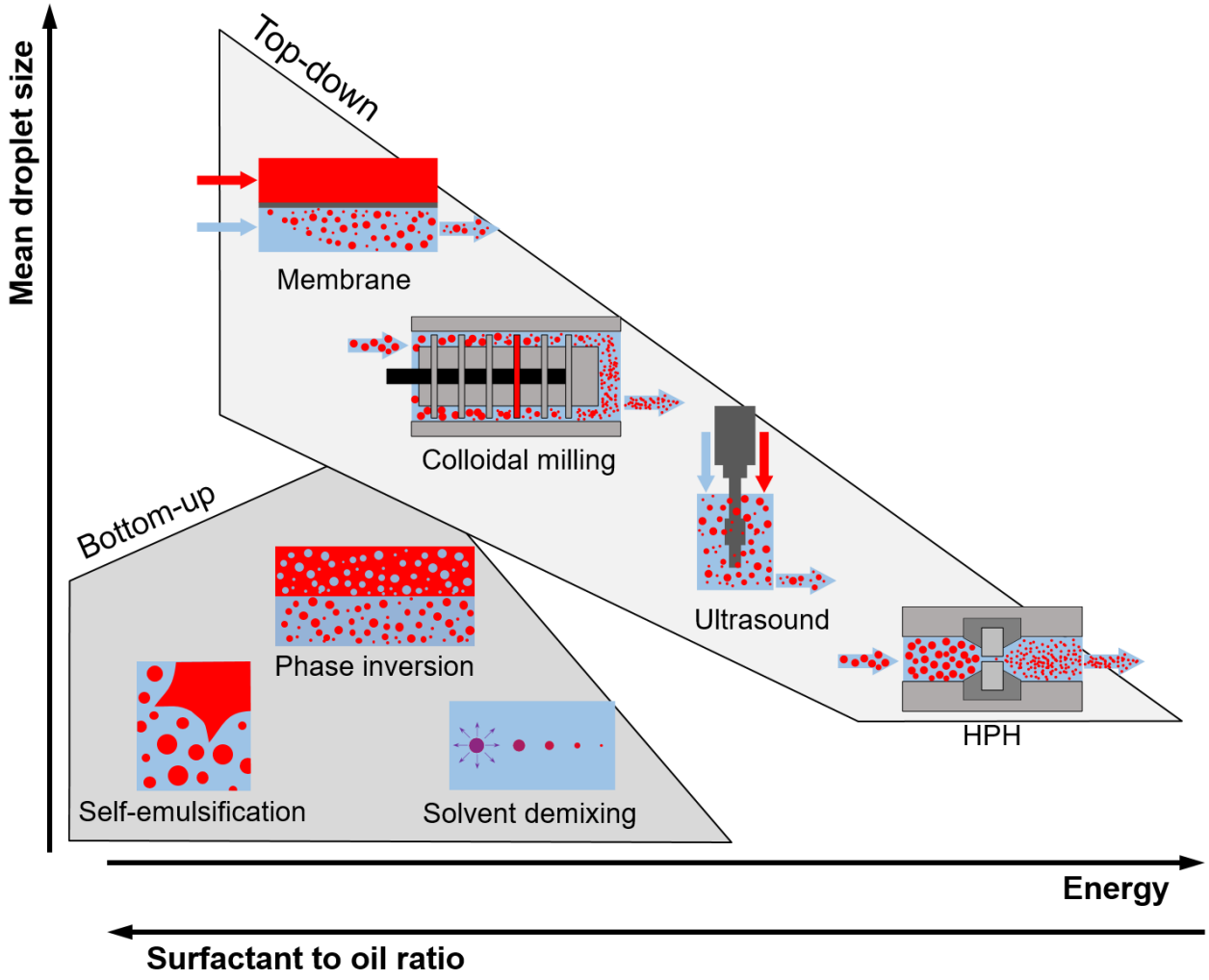

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966 Figure 2

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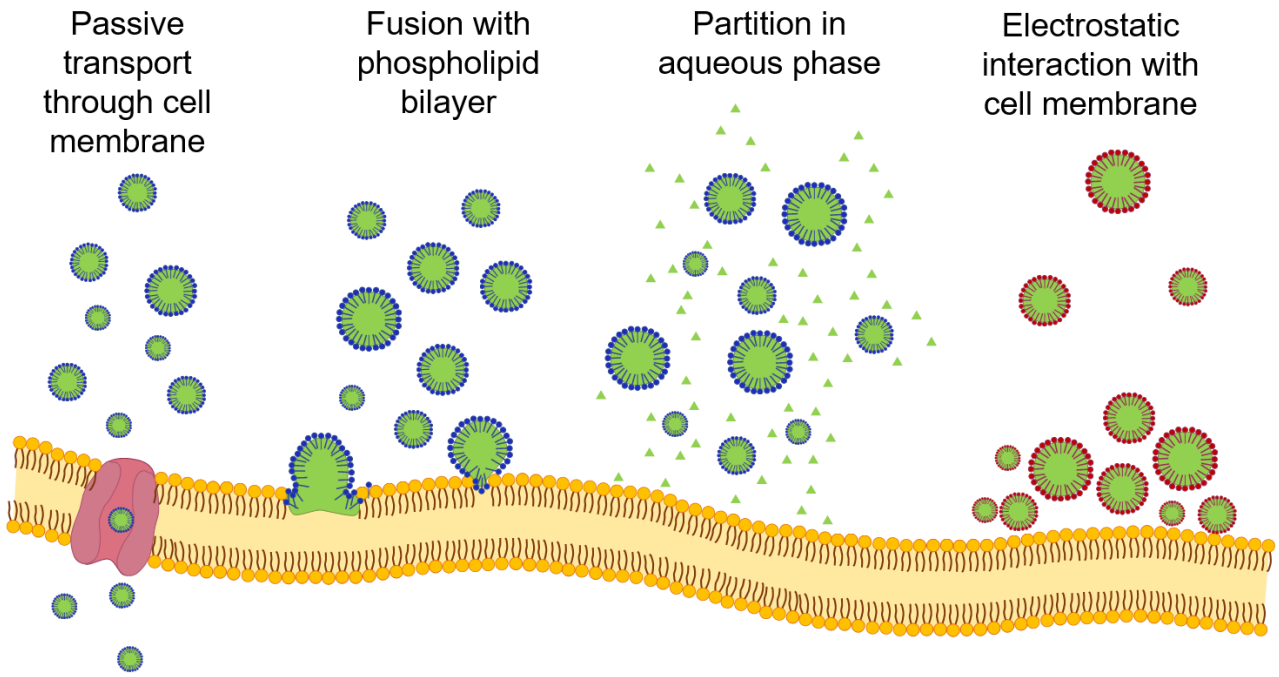

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969 Figure 3

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Table captions

Table 1. *Formulation and fabrication process of EO nanoemulsions, as a function of oil phase composition and fraction (Φ_{Oil}), emulsifier and emulsifier-to-oil or surfactant-to-oil (SOR) ratio, method of emulsification, its operating conditions and the corresponding nanoemulsion mean droplet size.*

Table 2. *Formulation of EO nanoemulsions tested against different microbial species and strains.*

Table 3. *Main hypothesized mechanisms of antimicrobial action of different EO nanoemulsions, correlated with their formulation, mean droplet size and microbial strain, against which have been tested. The impact of the nanoemulsion is classified as slightly positive (+), or positive (++) when the encapsulation of EOs in nanometric size emulsions improves the antimicrobial activity with respect to free EOs or EOs encapsulated in micrometric size emulsions; it is classified as slightly negative (-) or negative (--) when it decreases the antimicrobial activity, and in neutral (+/-) when no significant change is observed.*

Table 4. *Different techniques of application of EO nanoemulsions in real food products, describing EO nanoemulsion formulation, microbial strain contaminating the products, and the conditions of in product antimicrobial tests.*

Table 1.

Oil phase	Φ_{Oil} (%)	Emulsifiers	SOR	Method	Conditions	Mean droplet size (nm)	Reference
Carvacrol and peanut oil	4	Tween 20 + monoolein (1:1)	0.75	HPH	200 MPa, 5 passes	120	(Donsì et al., 2014)
d-limonene	5	Modified starch	2	HPH	300 MPa, 10 passes	367	(Donsì et al., 2011)
d-limonene and sunflower oil	5	Tween 20 + monoolein	0.3	HPH	300 MPa, 10 passes	131	(Donsì et al., 2011)
d-limonene, MCT and stearic acid	10	Tween 80	1	HPH	30 MPa, 10 passes	110	(Zahi et al., 2014)
Peppermint oil and MCT	12	Modified starch	1	HPH	100 MPa, 10 passes	200	(Liang et al., 2012)
Tea tree oil	5	Soy lecithin	0.2	HPH	300 MPa, 10 passes	74	(Donsì et al., 2011)
Cinnamaldehyde	0.2	Tween 80	3	Microfluidization	140 MPa, 10 passes	127	(Jo et al., 2015)
Eugenol and canola oil	10	Modified starch	0.2	Microfluidization	100 MPa (passes: N.A.)	153	(Majeed et al., 2016a)
Lemongrass oil	1	Tween 80 + sodium alginate (1:1)	2	Microfluidization	100 MPa, 10 passes	6	(Salvia-Trujillo et al., 2014b)
Eucalyptus oil	16.7	Tween 80	1	US	20 kHz, 750 W, 30 min	5	(Sugumar et al., 2014)
Eugenol and Sesame oil	6	Tween 80	3	US	20 kHz, 750 W, 30 min	13	(Ghosh et al., 2014)
Lemongrass oil	1	Tween 80 + sodium alginate (1:1)	2	US	24 kHz, 400 W, 3 min	4	(Salvia-Trujillo et al., 2014b)
Tea tree oil	5	Soy lecithin	0.2	HSH	24000 rpm, 5 min	175	(Donsì et al., 2011)
Thyme oil	1	Soluble soybean polysaccharide	5	HSH	15000 rpm, 3 min	300	(Wu et al., 2014)
Thymol	1	Sodium caseinate	5	HSH	15000 rpm, 3 min	110	(Pan et al., 2014)
Thymol (in hexane)	10	Whey proteins-maltodextrin conjugate	1.1	Solvent demixing	15000 rpm, 3 min, spray drying, rehydration	70	(Shah et al., 2012b)
Carvacrol and MCT	10	Tween 40, 60 or 80	1	Self-emulsification	Titration under continuous stirring of the oil and surfactant into an aqueous solution	55	(Chang et al., 2013)

Clove bud oil	1	Whey protein concentrates + gum arabic + lecithin.	1	Self-emulsification	Oil dissolution in hot alkaline solutions, addition into neutral solutions of emulsifier, adjusted to pH 7.0.	240	(Luo et al., 2014)
Orange oil and MCT	10	Tween 40, 60 or 80	2	Self-emulsification	Titration under continuous stirring of the oil and surfactant into an aqueous solution	25	(Chang and McClements, 2014)
d-limonene	4	Tween 80	1.5	Phase inversion	Slow addition of water/propylene glycol to d-limonene/Tween 80 mixture until phase inversion	16	(Zhang et al., 2014)

Table 2.

Classification	Microorganism	Essential oil	Formulation (Emulsifiers, stabilizers, * indicates eventual additional oil)
Gram-bacteria	*Escherichia coli*	Carvacrol	Sunflower oil* and sugar ester / Tween 20 + monoolein / lecithin / pea proteins (Donsì et al., 2012a)
		Cinnamaldehyde	Tween 20 (Jo et al., 2015); Acetem 90-50K* and Tween 60 (Bilbao-Sainz et al., 2013); Sunflower oil* and sugar ester / Tween 20 + monoolein / lecithin / pea proteins (Donsì et al., 2012a)
		Clove oil	Tween 80 + sodium alginate (Salvia-Trujillo et al., 2015a); Whey proteins + gum arabic + lecithin (Luo et al., 2014)
		d-limonene	Tween 80 (Zhang et al., 2014); Palm oil* and lecithin (Donsì et al., 2011); Sunflower oil* and Tween 20 + monoolein (Donsì et al., 2011); Modified starch (Donsì et al., 2011); Sunflower oil* and sugar ester / Tween 20 + monoolein / lecithin / pea proteins (Donsì et al., 2012a)
		Eugenol	Whey proteins + maltodextrin conjugates (Shah et al., 2013); MCT* and Tween 80 (Terjung et al., 2012)
		Lemongrass	Tween 80 + sodium alginate (Salvia-Trujillo et al., 2015a, 2014a, 2014b)
		Tea tree oil	Lecithin (Donsì et al., 2011); Tween 80 + sodium alginate (Salvia-Trujillo et al., 2015a)
		Thyme oil	Tween 80 + lecithin (Moghimi et al., 2016); Sodium caseinate + lecithin (Xue et al., 2015); Tween 80 + sodium alginate (Salvia-Trujillo et al., 2015a); Soluble soybean polysaccharide (Wu et al., 2014)
	Salmonella enteritidis	Clove oil	Whey proteins + gum Arabic + lecithin (Luo et al., 2014)
		Thyme oil	Sodium caseinate + lecithin (Xue et al., 2015); Soluble soybean polysaccharide (Wu et al., 2014)
	Salmonella typhimurium	Cinnamaldehyde	Tween 20 (Jo et al., 2015)
Gram+ bacteria	*Staphylococcus aureus*	d-limonene	Tween 80 (Zhang et al., 2014)
		Eucalyptus oil	Tween 80 (Sugumar et al., 2014, 2013)
		Eugenol	Sesame oil* and Tween 20/80 (Ghosh et al., 2014)
		Peppermint oil	MCT* and modified starch (Liang et al., 2012)
	Lactobacillus delbrueckii	Carvacrol	Sunflower oil* and sugar ester / Tween 20 + monoolein / lecithin / pea proteins (Donsì et al., 2012a)
		Cinnamaldehyde	Sunflower oil* and sugar ester / Tween 20 + monoolein / lecithin / pea proteins (Donsì et al., 2012a)
		d-limonene	Palm oil* and lecithin (Donsì et al., 2011); Sunflower oil* and Tween 20 + monoolein (Donsì et al., 2011); Modified starch (Donsì et al., 2011); Sunflower oil* and sugar ester / Tween 20 + monoolein / lecithin / pea proteins (Donsì et al., 2012a)
		Tea tree oil	Lecithin (Donsì et al., 2011)
	Listeria monocytogenes	Cinnamaldehyde	Acetem 90-50K* and Tween 60 (Bilbao-Sainz et al., 2013)
		Clove oil	Whey proteins + gum Arabic + lecithin (Luo et al., 2014)
		Eugenol	Whey proteins + maltodextrin conjugates (Shah et al., 2013); in MCT + Tween 80 (Terjung et al., 2012)
		Peppermint oil	MCT* and modified starch (Liang et al., 2012)
		Thyme oil	Sodium caseinate and lecithin (Xue et al., 2015); Soluble soybean polysaccharide (Wu et al., 2014)
	Listeria innocua	Carvacrol	MCT* and Tween 80 (Terjung et al., 2012)
		Eugenol	MCT* and Tween 80 (Terjung et al., 2012)
Fungi	*Zygosaccharomyces bailii*	Carvacrol	MCT* and Tween 20 (Chang et al., 2013)
		Thyme oil	Corn oil* and Tween 80 + lauric arginate (Chang et al., 2015, 2012)
	Saccharomyces cerevisiae	Carvacrol	MCT* and Tween 20 (Chang et al., 2013); in sunflower oil and sugar ester / Tween 20 + monoolein / lecithin / pea proteins (Donsì et al., 2012a)

	Cinnamaldehyde	Sunflower oil* and sugar ester / Tween 20 + monoolein / lecithin / pea proteins (Donsì et al., 2012a)
	d-limonene	Tween 80 (Zhang et al., 2014); Palm oil* and lecithin (Donsì et al., 2011); Sunflower oil* and Tween 20 + monoolein (Donsì et al., 2011); Modified starch (Donsì et al., 2011); Sunflower oil* and sugar ester / Tween 20 + monoolein / lecithin / pea proteins (Donsì et al., 2012a)
	Tea tree oil	Lecithin (Donsì et al., 2011)
Fusarium oxysporum	Eugenol	Tween 20 (Abd-Elsalam and Khokhlov, 2015)

Table 3.

Oil phase	Emulsifier	Mean droplet size (nm)	Microbial strain	Impact of nano-emulsion	Mechanism	Reference
Carvacrol / Cinnamaldehyde / d-limonene	Tween 20 – monoolein / Sugar esters / Lecithin / Pea proteins	170 - 240	*S. cerevisiae, E. coli, L. delbrueckii*	+	Effect of formulation on the solubilization of EO, improving their interaction with the cell membrane. Nanoemulsion droplets behave as nanotanks for sustained release of EOs.	(Donsì et al., 2012a)
Clove oil and canola oil	Modified starch	150	*S. aureus, E. coli, L. monocytogenes*	+/-	Higher activity of EO nanoemulsion than free EO only against Gram+ due to the interaction of modified starch with their cell wall.	(Majeed et al., 2016b)
Lemongrass oil	Tween 80 and sodium alginate	5	*E. coli*	++	Association of the emulsifier with some constituents of the biological membrane promotes the antimicrobial activity of EO nanoemulsions.	(Salvia-Trujillo et al., 2014a, 2014b)
d-limonene	Tween 80	16	*S. cerevisiae S. aureus, B. subtilis, E. coli*	++	Higher antimicrobial activity of EO nanoemulsion than free EO	(Zhang et al., 2014)
Peppermint oil and MCT	Modified starch	146	*L. monocytogenes, S. aureus*	-	Nanoemulsions (a) prolong antibacterial activities through sustained release, (b) limit the contact of EO with the membrane of bacteria, decreasing their concentration in aqueous phase	(Liang et al., 2012)
Eugenol	Whey protein and maltodextrin conjugates	127	*E. coli, L. monocytogenes*	--	Binding between emulsifier and EO reduces the resulting antimicrobial activity	(Shah et al., 2013)
Eugenol and bean oil	Tween 80 / SDS	140	*E. coli*	++	Nanoemulsion enhanced antibacterial activity of EO. Anionic surfactant (SDS) was more active than nonionic (Tween 80)	(Li et al., 2015)
Eugenol and MCT	Tween 80	80-3000	*E. coli*	--	Smaller emulsion droplets reduce the EO antimicrobial activity, due to EO accumulation at droplet interfaces	(Terjung et al., 2012)
Tea tree oil	Lecithin	175-74	*S. cerevisiae, E. coli, L. delbrueckii*	++	Nanoemulsions improve the dispersibility of poorly soluble EOs.	(Donsì et al., 2011)
Thyme oil	Tween 80 + lecithin	143	*E. coli*	++	Nanoemulsions improve (a) the access of EO to bacterial cells, (b) their ability to disrupt cell membrane	(Moghimi et al., 2016)
Thyme oil	Sodium caseinate and soy lecithin	82	*E. coli, S. Enteritidis, L. monocytogenes*	+	(a) Binding between emulsifier and EO promotes antimicrobial activity. (b) Nanoemulsions improve the dispersibility of poorly soluble EOs.	(Xue et al., 2015)
d-limonene	Tween 80 + monoolein / modified starch	155-366	*S. cerevisiae, E. coli, L. delbrueckii*	+	Increased solubility of EOs in aqueous phase	(Donsì et al., 2011)

Table 4.

Technique	Product	Contamination	Conditions	Essential oil	Formulation (Emulsifiers, stabilizers, * indicates an additional oil, ** indicates the coating material)
Mixing	Milk	E. coli, L. monocytogenes	48 h at 32/35 °C	Eugenol	Whey proteins and maltodextrins conjugate (Shah et al., 2013)
		E. coli, L. monocytogenes, S. enteritidis,	72 h at 32/35 °C	Thyme oil	Sodium caseinate + lecithin (Xue et al., 2015)
		L. monocytogenes	7 d at 21 °C	Thymol	Sodium caseinate (Pan et al., 2014)
	Orange juice	Native cultivable bacteria	72 h at 4 °C, 24 h at 25 °C	Eugenol	Sesame oil* and Tween 20/80 (Ghosh et al., 2014)
		L. delbrueckii	16 d at 32 °C	Tea tree oil	Lecithin (Donsì et al., 2011)
	Pear juice	L. delbrueckii	16 d at 32 °C	Tea tree oil	Lecithin (Donsì et al., 2011)
	Watermelon juice	E. coli, S. Typhimurium, S. aureus	48 h at 37 °C	Cinnamaldehyde	Tween 20 (Jo et al., 2015)
Washing	Fresh lettuce	E. coli, L. monocytogenes, S. Typhimurium	72 h at 4 °C	Oregano oil	Tween 80 (Bhargava et al., 2015)
	Spinach leaves	E. coli, S. enterica	Immediately	Carvacrol / Eugenol	Tween 20 / Surfynol 485W / SDS / CytoGuardR LA 20 (Ruengvisesh et al., 2015)
Infusion	Pork sausages	Endogenous flora	24 h at 4 °C	Carvacrol	Peanut oil* and lecithin / Tween 20 + monoolein / Sugar ester (Donsì et al., 2014)
	Zucchini	E. coli	24 h at 4 °C	Carvacrol	Peanut oil* and lecithin / Tween 20 + monoolein / Sugar ester (Donsì et al., 2014)
Coating	Apple pieces	E. coli, Endogenous flora	14 d at 4 °C	Lemongrass	Tween 80, sodium alginate ** (Salvia-Trujillo et al., 2015b)
	Broccoli florets	L. monocytogenes	6 d at 4 °C	Citrus oils	Tween 20 + monoolein, native or modified chitosan** (Severino et al., 2014b)
	Green beans	E. coli, S. Typhimurium	13 d at 4 °C	Citrus oils	Tween 20 + monoolein, modified chitosan** (Severino et al., 2015)
	Green beans	L. innocua	14 d at 4 °C	Mandarin oil	Tween 20 + monoolein, modified chitosan** (Donsì et al., 2015; Severino et al., 2014a)
	Plums	E. coli, S. Typhimurium	28 d at 4 °C, 15 d at 25 °C	Lemongrass oil	Tween 80, carnauba wax** (Kim et al., 2013)
	Rucola leaves	Endogenous flora	21 d at 4 °C	Lemon oil	Tween 20 + monoolein, modified chitosan** (Sessa et al., 2015)
	Sliced bread	Endogenous flora	15 d at 25 °C	Clove bud oil / Oregano oil	Tween 80, methylcellulose** (Otoni et al., 2014)