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Effect of oregano (*Origanum vulgare* L. ssp. *hirtum*) and clove (*Eugenia spp.*) nanoemulsions on *Zygosaccharomyces bailii* survival in salad dressings

Susana Ribes, Ana Fuentes*, Jose Manuel Barat

Departamento Tecnología de Alimentos, Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain

Corresponding author:

Ana Fuentes: Telephone. +34 96 387 90 00; ext. 73664; fax: +34963787369;

Susana RIBES: surillop@gmail.com

Ana FUENTES*: anfuelo@upvnet.upv.es

José Manuel BARAT: jmbarat@tal.upv.es

Abstract

This work aimed to evaluate the *in vitro* effect of encapsulated oregano and clove essential oils on oil-in-water nanoemulsions against *Zygosaccharomyces bailii*. The antifungal efficacy of these nanoemulsions and their sensory acceptance were tested in salad dressings. Both essential oils were effective inhibitors against the target yeast, with minimal inhibitory and fungicidal concentrations of 1.75 mg/mL. In the *in vitro* assay done with the nanoemulsions, no yeast growth was observed for any tested essential oil concentration. In the salad dressings, all the formulations were able to reduce *Z. bailii* growth compared to the control, and only those samples with 1.95 mg/g of essential oil were capable of inhibiting yeast development after 4 inoculation days. The sensory acceptance of the dressing containing the nanoemulsions was similar to the control dressing in appearance, consistency and colour terms. These results evidence the antifungal activity of oregano and clove nanoemulsions against *Z. bailii*.

Keywords: essential oil; oregano; clove; oil-in-water nanoemulsions; sauces

1. Introduction

As healthier eating trends grow, salads, raw veggies and snacks are preferred by consumers, who usually choose dressings and dips to flavour these food products. Commercial salad dressings are based on a mixture of weak organic acids, salts, sugar, chelators, thickening agents, surfactant and preservatives (Castro, Rojas, Campos, & Gerschenson, 2009). Potassium sorbate and sodium benzoate are employed to prevent salad dressing deterioration, which is usually caused by *Lactobacillus, Saccharomyces* and *Zygosaccharomyces.* Of these microorganisms, the most notorious is *Zygosaccharomyces bailii* given its outstanding degree of preservative resistance (Stratford et al., 2013), and its remarkable tolerance to stressful conditions, such as low pH, low water activity (a_w), presence of acetic and lactic acid and fermentable sugars, which are challenges for manufacturers of acidified food and beverages (Erickson & McKenna, 1999). Nevertheless, quantitative data about economic loss caused by this spoilage yeast are hard to achieve, probably due to the food industry's confidential reasons and the limited availability of incidences and economical costs related to spoilage outbreaks (Vermeulen et al., 2008).

Essential oils (EOs) are complex mixtures of often hundreds of individual volatile aroma compounds that are derived from plants with antifungal properties (Monu, Techathuvanan, Wallis, Critzer, & Davidson, 2016). The most widely used EOs as natural food preservatives are cinnamon, clove, lemon grass, oregano, thyme, nutmeg and basil (Ribes, Fuentes, Talens, & Barat, 2017c). Using EOs has several drawbacks, such as their poor solubility in water, and their highly volatility and intensive aroma, which can have unpleasant organoleptic properties after being applied to food commodities. Encapsulation of EOs in delivery systems, such as oil-in-water (O/W) nanoemulsions, overcomes these problems (Merino et al., 2019). Encapsulation improves the antifungal effectiveness of EOs thanks to increased surface areas that come into

contact with microorganisms and diminish their volatility by cushioning unpleasant impacts on organoleptic food properties (Donsì & Ferrari, 2016; Pavela et al., 2019).

For this reason, this research aimed to evaluate the *in vitro* effect of encapsulated oregano and clove EOs on O/W nanoemulsions against *Zygosaccharomyces bailii*. The antifungal efficacy of these nanoemulsions and their sensory acceptance were tested in salad dressings.

2 Materials and Methods

2.1 Strains, media and reagents

Z. bailii was selected as the target yeast because it has been isolated from spoiled salad dressing and can grow in products with a pH of 3.6 and water activity of 0.89 (Monu et al., 2106; Stratford et al., 2013). The *Z. bailii* strain (CECT 12001) was supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain).

Yeast peptone dextrose broth (YPDB), nutrient agar, tryptone phosphate water and acetic acid were supplied by Scharlab (Barcelona, Spain). The oregano EO was obtained from Ernesto Ventós S.A. (Barcelona, Spain). The clove EO and Tween 80 were acquired from Sigma-Aldrich (St. Louis, USA). Xanthan gum (XG, Satiaxane^{TM} CX 911) was purchased from Cargill (Barcelona, Spain). EOs were used as natural antifungal agents and Tween 80 was incorporated as a non-ionic surfactant into nanoemulsions. XG was employed as a stabiliser to extend the stability of nanoemulsions by viscosity modification over time.

2.2 Oregano and clove EOs

2.2.1 In vitro antifungal activity of oregano and clove EOs

86 The *in vitro* antifungal activity of oregano and clove EOs was tested against *Z. bailii* based
87 on the methodology described by Ribes et al. (2017b), with minor changes. Before being used,
88 the target yeast was grown in YPDB at 25ºC for 48 h with continuous orbital agitation at 180
89 rpm. The inoculum was diluted in 10 mL of distilled water containing Tween 80 (0.1% w/v) to
90 a population of 10^7 Colony Forming Units (CFU)/mL. The minimal inhibitory concentration
91 (MIC) values were obtained by a macrodilution test (CLSI, 2007). Erlenmeyer flasks with 15
92 mL of YPDB and 1% (w/v) of Tween 80 (to guarantee that each EO was completely dispersed)
93 were used. The tested concentrations of oregano and clove EOs were 0.25, 0.50, 0.75, 1.00,
94 1.25, 1.50 and 1.75 mg/mL. The control samples (without EOs) were prepared following the
95 same procedure. Each Erlenmeyer flask containing the EO was inoculated with 100 µL of the
96 yeast suspension (10^7 CFU/mL) and incubated at 25 °C for 48 h with orbital agitation at 180
97 rpm (Ribes et al., 2017b). The results were expressed as log CFU/mL.
98 The MIC was established as the smallest amount of oregano and clove EOs that inhibited
99 visible *Z. bailii* growth after 48 h of incubation at 25 °C. The minimal fungicidal concentration
100 (MFC) was determined by spreading 100 µL of the visible non-growth suspension onto Petri
101 plates prepared with 15 g of YPDB with nutritive agar (15 g/L, YPDA). The MFC was denoted
102 as the lowest concentration at which no colonies had grown after 48 h of incubation at 25 °C,
103 based on the procedure described by Ribes et al. (2017b). All the tests were run in triplicate.
104
105 *2.2.2 Gas chromatography-mass spectrometry analysis of oregano and clove EOs*
106 The compositions of oregano and clove EOs were analysed by GC/MS. The analysis was
107 performed in a 6890/5975 inert GC–MS (Agilent Technologies, Santa Clara, CA, USA),
108 equipped with a HP-5 fused silica capillary column (30 m x 0.25 mm x 0.25 µm). The oven
109 temperature was held at 60 ºC for 3 min before being raised to 100 ºC at 10 ºC/min, to 140 ºC

at 5 ºC/min, and finally to 240 ºC at 20 ºC/min. Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. The injector and MS transfer line temperatures were set at 250 ºC and 230 ºC, respectively. The MS analysis parameters were the EI Ion source, electron energy 70 eV, solvent delay of 3 min and m/z 40–550 amu. The EO components were identified according to their retention index and by matching mass spectra with the standard mass spectra from the NIST MS Search 2.0 library. The relative amounts of the individual components of each EO were expressed as percentages of the peak area of total ion chromatograms.

2.3 Oregano and clove nanoemulsions

2.3.1 Preparation

Oregano and clove EOs were encapsulated in O/W nanoemulsions and produced by high-pressure homogenisation (HPH) (Ribes, Fuentes, Talens, & Barat, 2018). The EOs, Tween 80 and XG were mixed for 15 min by magnetic stirring, followed by one single pass at 50 MPa in an HPH system (Panda Plus 2000, Gea Niro Soavi S.p.A., Parma, Italy). The O/W nanoemulsions contained 0, 1.75, 1.85, and 1.95 mg/g of either the oregano or clove EO, 10 mg/g of Tween 80 and 5 mg/g of XG. Both concentrations were defined after considering previous studies (Ribes, Fuentes, Talens, & Barat, 2016; Salvia-Trujillo, Rojas-Graü, Solvia-Fortuny, & Martín-Belloso, 2013). Moreover, the EOs concentrations were established after considering the results obtained from the *in vitro* antifungal activity of EOs.

2.3.2 Physico-chemical characterisation

A Crison Basic 20+ pH meter (Crison S.A. Barcelona, Spain) was used to measure the pH of the oregano and clove nanoemulsions. Particle size analysis was carried out in a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK) according to

Ribes et al. (2016), by using the Mie theory (refractive index of 1.50 and absorption index of 0.01). The ζ-potential was measured, as described by Ribes et al. (2016), by a Zetasizer nano-Z (Malvern Instruments, Worcestershire, UK). The electrophoretic mobility measurements were converted into ζ-potential values by employing the Smoluchowsky mathematical model. Each measurement was taken in triplicate.

2.3.3 Nanoemulsions stability

Five millilitres of the clove and oregano nanoemulsions, which were adjusted to pH 3.3 (by simulating the pH of the salad dressings) and pH 6.5 (which comes closes to the pH value of each nanoemulsion) with acetic acid (10%, v/v), were transferred to a test tube and maintained at 8 ºC or 25 ºC for 0, 1, 4, 7 and 11 days. During the storage period, the creaming index (%) was calculated by employing Equation 1 (Hong, Kim, & Lee, 2018):

$$\text{Creaming index (\%)}: [H_S/H_T] \times 100 \quad (1)$$

where H_S is the height of a serum layer and H_T is the total sample height.

In order to determine the kinetic stability of these samples, the particle size and the ζ-potential analyses were carried out as described in Section 2.3.2. The assays were conducted in triplicate.

2.3.4 In vitro antifungal activity of the oregano and clove nanoemulsions

The antifungal activity of the oregano and clove nanoemulsions against *Z. bailii* was evaluated following the methodology described in Section 2.2, with some modifications. Each nanoemulsion (0.50 g) was added to the media (14.50 mL of YPDB) inoculated with 100 µL of the yeast inoculum (10^7 CFU/mL) and incubated at 25 °C for 48 h with orbital stirring at 180 rpm. The final concentrations of each encapsulated EO in broth were 1.75, 1.85 and 1.95 mg

156 EO/mL. The MIC and MFC were assessed as previously indicated for the antifungal activity of
157 the *in vitro* evaluation of EOs. The results were expressed as log CFU/mL and the test was
158 conducted in triplicate.

159

160 2.4 Salad dressings

161 *2.4.1 Preparation*

162 Salad dressings were prepared by mixing (speed 6, 2 min) in a kitchen robot (Thermomix
163 TM 31, Vorwerk & Co., GmbH, Wuppertal, Germany) the following ingredients: deionised
164 water (50 wt. %), sunflower oil (30 wt. %), acetic acid (10 wt. %), egg yolk (3 wt. %), starch
165 (5 wt. %), sugar (1 wt. %), NaCl (0.50 wt. %) and citric acid (0.50 wt. %). The oregano or clove
166 nanoemulsion (0.50 g) was added to the salad dressing (14.50 g) before being homogenised. As
167 mentioned earlier, the final concentrations of each encapsulated EO in the dressing were 1.75,
168 1.85 and 1.95 mg EO/g. Samples were stored at 8 °C for 1 h before running the analysis.

169

170 *2.4.2 Antimicrobial activity of the oregano and clove nanoemulsions against Z. bailii*

171 Fifteen grams of each salad dressing (control and samples containing the nanoemulsions
172 with 1.75, 1.85 and 1.95 mg EO/g) were inoculated with 100 µL of the yeast inoculum (10^7
173 CFU/mL). Samples were stored at 8 ºC and analysed for 0, 1, 4, 7 and 11 days after their
174 inoculation. Ten grams of samples were placed in sterile plastic bags containing 90 mL of
175 tryptone phosphate water, and were homogenised for 1 min in a Stomacher blender (Masticator
176 IUL, S.A. Instruments, Germany). Serial dilutions were prepared and 0.1 mL of each dilution
177 was spread on the surface of YPDA plates. Finally, they were incubated at 25 °C for 72 h, and
178 yeast cell populations were determined by counting the plates containing between 15 and 150

179 colonies. Counts were expressed as log CFU/g (Pascual & Calderón, 2000). All the assays were
180 run in triplicate.

181

182 *2.4.3 Sensory evaluation*

183 The sensory evaluation of salad dressings was made by a semi-trained panel formed by 30
184 assessors (14 men and 16 women). They were recruited following general guidelines UNE-ISO
185 8586:2012. To introduce the panellists to the sensory analysis, and to identify and score the
186 quality attributes that define each sample, different preparatory sessions were carried out. Tests
187 were run on a structured 9-point hedonic scale (1: very unpleasant and 9: very pleasant) (UNE-
188 ISO 4121:2003) to evaluate the appearance, consistency, colour, flavour, taste, mouth texture
189 and general acceptance attributes. The assessors tested three different samples: i) the control
190 samples (no nanoemulsion included); ii) the samples containing the nanoemulsion prepared
191 with the oregano EO (1.95 mg EO/g); iii) the samples composed of the nanoemulsion prepared
192 with the clove EO (1.95 mg EO/g). The amount of EOs was selected according to the results
193 obtained in the previous test. The sensory analysis was carried out 1 h after preparing the salad
194 dressings. During this time, samples were stored at 8 °C in sealed glass jars. Each sample was
195 presented to the panellists in a transparent plastic glass coded with three arbitrary digits. The
196 panellists were asked to eat an unsalted cracker and drink water between samples to avoid
197 aftertaste (adapted from Ribes et al., 2017a). Sensory evaluations were made by considering
198 the IFST Guidelines for Ethical and Professional Practices for the Sensory Analysis of Foods
199 (Institute of Food Science and Technology, 2015).

200

201 2.5 Statistical analysis

The data obtained in the physico-chemical characterisation, the stability of the O/W nanoemulsions and the *in vitro* antifungal activity of the EOs and O/W nanoemulsions were analysed by a multifactor analysis of variance (multifactor ANOVA) to evaluate the differences among EOs concentrations and between EO types. The results of the impact of the O/W nanoemulsions on the salad dressings' sensory characteristics were studied by a one-way ANOVA. The least significance procedure (LSD) was employed to test for any differences between averages at the 5% level of significance. The results were statistically processed by the Statgraphics Centurion XVI software.

3. Results and Discussion

3.1 *In vitro* antifungal activity and characterisation of the oregano and clove EOs

The antifungal activity of the oregano and clove EOs against *Z. bailii* during storage at 25 °C for 48 h is shown in Figure 1. Yeast counts significantly lowered when increasing concentrations of each EO were used. The treatments with 0.50 mg/mL of the oregano and clove EOs led to an almost 1 log reduction of *Z. bailii* after the 48-hour incubation at 25 °C. Moreover, the *Z. bailii* counts lowered by at least 3 log in those samples with up to 1.00 mg/mL of the oregano and clove EOs. Both EOs exhibited effective inhibition against the target yeast with the MIC and MFC values of 1.75 mg EOs/mL. Monu et al. (2016) studied the efficacy of different EOs and their compounds against several spoilage yeasts with a modified agar dilution assay. For the clove EO, the results obtained by Monu et al. (2016) showed more marked activity against *Z. bailii* (MIC of 200 mg/L) than that found in our study. The difference between the above-cited authors' findings and our own could be attributed to the assay type used to determine the antimicrobial activity of EOs.

It is important to highlight that despite the composition of the clove and oregano EOs being quite different, their antifungal activity was similar. The main oregano EO compound was carvacrol (63.3%) (Table 1, Supplementary Figure S1A), while the clove EO was characterised by a high eugenol concentration (85.5%) (Table 2, Figure S1B). Other oregano EO components included p-cymene (13.0%), γ-terpinene (5.9%) and caryophyllene (5.7%). These data agree with the results obtained in other studies (; Silva, Duarte-Almeida, Pérez, & Franco, 2010; Morshedloo, Salami, Nazeri, Maggi, & Craker, 2018). Morshedloo et al. (2018) observed considerable qualitative and quantitative variability among oregano EOs depending on the harvest year, genetic factors and geographical origin. In line with this, Silva et al. (2010) analysed a commercial oregano EO and found that the carvacrol concentration varied from 61.66% to 93.42%, and from 1.88% to 23.85% for thymol. As indicated by Hernández-González et al. (2017), thymol and carvacrol are responsible for the antimicrobial activity of oregano EO and, hence, variations in EO composition could lead to a different antimicrobial effect. The clove EO contained carvacrol (85.53%), β–caryophyllene (7.4%), eugenol acetate (2.7%) and α-humulene (1.5%). These data coincide with the results reported in other studies, which still demonstrated the variability of natural oil (Chaieb et al., 2007; Prashar & Thaker., 2006). The clove EO, with its main compound eugenol, have been reported as one of the most effective natural antimicrobial agents (Singh, Maurya, de Lampasona & Catalan, et al., 2007; Amiri, Dugas, Pichot, & Bompeix, 2008; Ribes et al., 2016).

The mode of action of the main compounds of the oregano and clove EOs is related to their hydrophobicity. Due to this phenomenon, the carvacrol and eugenol partition in the lipids of the cell membrane modify membrane permeability and lead to the leakage of cell contents when they exert their antibacterial action (Burt, 2004).

249 3.2 Oregano and clove nanoemulsions

250 *3.2.1 Physico-chemical characterisation*

251 The nanoemulsions formulated with the different contents of the oregano and clove EOs
252 were characterised in terms of pH, particle size ($d_{3,2}$ and $d_{4,3}$) and ζ-potential (Table 1).
253 Concentrations were selected according to the data obtained in the *in vitro* antifungal activity
254 assays of EOs (Section 3.1) and after considering that higher EO concentrations are needed to
255 achieve the same effectiveness in both the *in vitro* and *in vivo* tests (Burt, 2004). The pH
256 measurements for the nanoemulsions containing the oregano EO were between 6.78±0.05 and
257 7.05±0.03, while the pH values obtained from the nanoemulsions prepared with the clove EO
258 varied between 6.24±0.06 and 6.98±0.08. Thus the higher the EOs contents in nanoemulsions,
259 the lower the pH value. Sánchez-González, Vargas, Gonzalez-Martínez, Chiralt and Cháfer
260 (2009) also reported that incorporating higher concentrations of EOs significantly lowered the
261 pH of samples owing to the acid nature and dissociation in the aqueous solution of some of their
262 compounds.

263 Regarding particle size, the EO concentrations had a strong impact on $d_{3,2}$ and $d_{4,3}$. As
264 observed, the mean size values ($d_{3,2}$) significantly ($p<0.05$) rose as the amount of EO increased
265 in the nanoemulsion, and this effect was stronger for the oregano EO. The same trend was also
266 noted for the $d_{4,3}$ values. This fact can be explained by an incremented concentration in the
267 disperse phase, which promotes the droplet flocculation ratio and, consequently, lowers the rate
268 in the interfacial stabilising material and the dispersed phase (McClement, 2005).

269 All the ζ-potential values of the nanoemulsions were lower than -30 mV despite the
270 employed concentration of EOs (Table 1). The ζ-potential values of >30 mV or <−30 mV
271 indicated that the repulsive electrostatic forces among droplets likely contributed to prevent
272 their aggregation (Harwansh et al., 2015). It is worth mentioning that the negative charge of the

different nanoemulsions was influenced mainly by XG owing to its anionic nature, regardless of using a non-ionic surfactant like Tween 80 (Salvia-Trujillo et al., 2013).

3.2.2 Nanoemulsions stability

The creaming index exhibits indirect data about the extent of droplet aggregation, coalescence and flocculation in O/W emulsions (Ye & Singh, 2006). Supplementary Figure S2 shows the creaming index results (%) of the oregano and clove nanoemulsions on storage days 7 and 11. The oregano nanoemulsions adjusted to pH 3.3 showed more instability at 8 ºC than at 25 ºC, with a creaming index of 2.0±0.0% when 1.95 mg/g of oregano EO was used. This instability significantly ($p<0.05$) increased over storage time (11 days) to values of 5.0±0.0%, 11.2±4.1% and 15.1±1.1% for the nanoemulsions prepared with 1.75, 1.85 and 1.95 mg/g of the oregano EO and after remaining at 8 ºC (Figure S2A). Similar behaviour was observed for the nanoemulsions with pH values of 6.5. As previously mention, higher concentrations in the disperse phase can explain this phenomenon (McClement, 2005). Lastly, in the clove nanoemulsions at pH 6.5 after 7 storage days at 8 ºC, the migration of droplets was observed; however, at 25 ºC this effect was not observed, indicating that the temperature favoured the instability of these nanoemulsions (Figure S2C). For the clove nanoemulsions, at pH 3.3 the stability of the samples containing 1.95 mg/mL was lower after 7 storage days, which was clearly noted after 11 storage days, regardless of the storage temperature. Furthermore, the samples containing the clove EO, and adjusted to pH 6.5, gave higher creaming index values at 8 ºC than at 25 ºC (Figure S2C-D).

It is worth mentioning that the effect of pH on the creaming index was evidenced only for the clove nanoemulsions. Acid pH values provoke the destabilisation of the samples prepared with 1.95 mg/g of the clove EO after 7 storage days (creaming index: 2.0±0.0%), which was

297 greater after 11 days of storage (the creaming index values of the clove nanoemulsions with
298 1.75, 1.85 and 1.95 mg/g of the EO were 10.0±2.0%, 15.4±1.0% and 20.3±3.2%, respectively).
299 Guerra-Rosas et al., (2016) pointed out that the total rates, degree of creaming and serum
300 separation depend on the overall oil volume fraction of the nanoemulsion, its droplet-size
301 distribution, and the nature of the inter droplet interplays, including any effects of non-absorbed
302 polymers and surfactants. Wang, Feng, Jia, Xu & Zhou (2018) observed that more stable O/W
303 emulsions were formed at higher pH values, which lessened their susceptibility to gravitational
304 separation.
305 Regarding the kinetic stability of the oregano and clove nanoemulsions, Figures S3 and S4
306 (Supplementary Material) provide the mean size values ($d_{3,2}$ and $d_{4,3}$, respectively) of the
307 samples adjusted to pH 3.3 and 6.5, kept at 8 ºC and 25 ºC for 11 days. The oregano
308 nanoemulsions presented higher values of $d_{3,2}$ (nm) than the clove nanoemulsions,
309 irrespectively of pH and temperature. By the end of storage, droplet size significantly up to
310 1338±161 nm, 1654±62 nm and 1698±39 nm when 1.75, 1.85 and 1.95 mg/g of the oregano
311 EO were respectively used. While these samples were kept at 25 ºC for 11 days, their mean
312 droplet size values varied from 1100±23 nm to 1722±177 nm, and the instability effect was
313 enhanced when larger amounts of EO were used. A similar behaviour was observed in the
314 oregano nanoemulsions adjusted to pH 6.5 and kept at 8 ºC or 25 ºC for 11 days. The significant
315 droplet size seen during storage was probably due to the capacity of oil droplets to migrate from
316 smaller to larger droplets in the aqueous phase. Similarly, Guerra-Rosas et al., (2016) noticed
317 that the EOs containing carvacrol were particularly inclined to Ostwald ripening because of its
318 relatively good solubility in water. Conversely, the droplet size of the clove nanoemulsion
319 formulated with the smaller amount of EO remained practically uniform throughout the study,
320 and was considered physically stable, excluding those samples with pH values of 3.3 and kept
321 at 25 ºC for 11 days (see Figure S3B for details). This tendency was generally similar for the

322 $d_{4,3}$ values, and the instability of samples was greater for those nanoemulsions prepared with
323 the oregano EO than with the clove EO, regardless of pH and storage temperature (Figure S4).
324 These results agree with those reported for the creaming index, where the greater stability of
325 clove nanoemulsions rather than oregano nanoemulsions, with pH values of 3.3 and 6.5, and
326 kept at 8 ºC for 11 days, was probably observed due to their small droplet size.
327 In relation to the ζ-potential, in this study the initial interfacial electrical charge data for the
328 oregano nanoemulsions were between -50 mV and -60 mV in all cases, but came close to -30
329 mV for the clove nanoemulsions (Supplementary Figure S5). The ζ-potential of the samples
330 containing the clove EO remained stable during the storage time, and the non-dependence of
331 the pH and storage temperature was noted. However, the interfacial electrical charge of those
332 samples formulated with the oregano EO decreased over time. This effect could be related to
333 the creaming observed in the samples adjusted to pH 6.5 and stored at 8 ºC and 25 ºC for 11
334 days. Despite the constant ζ-potential values of the clove nanoemulsions over time, their
335 instability was observed by the end of the storage period.
336

337 *3.2.3 In vitro antifungal activity of the oregano and clove nanoemulsions*

338 The *in vitro* effectiveness of the antifungal activity of the encapsulated oregano and clove
339 EOs within nanoemulsions in inhibiting *Z. bailii* growth was evaluated (data not shown). No
340 yeast growth was observed for any tested EO concentration, which indicates that the
341 nanoemulsions studied at the lowest oregano and clove concentrations were capable of
342 inhibiting *Z. bailii* growth in culture media. Even though HPH processing led to losses of
343 volatile compounds of around 50% (Ribes, Fuentes, Talens, & Barat, 2017a), the same MIC
344 and MFC values were obtained after evaluating the *in vitro* antifungal activity of both the non-
345 encapsulated and encapsulated EOs in the nanoemulsions against *Z. bailii*. Other authors have

346 evidenced that the encapsulation of EOs enhances their antimicrobial activity (Donsì & Ferrari,
347 2016). Previous works have reported a faster penetration in microbial cells when reducing the
348 droplet size of antifungal agents (formation of nanoemulsions), which would explain the
349 behaviour observed herein (Weiss, Takhistov, & McClements, 2006).

350 3.3 Salad dressings

351 *3.3.1 In situ antifungal activity of the oregano and clove nanoemulsions*

352 Figure 3 shows the *in vivo* growth of *Z. bailii* inoculated in different salad dressings and
353 incubated at 8 ºC for 11 days by simulating refrigerated storage after conventional
354 pasteurisation. The reference samples exhibited constant microbial counts (5 log CFU/g).
355 Incorporation of the oregano and clove EO nanoemulsions into salad dressings lowered the
356 fungal count compared to the reference sample, of 1 and 2 log CFU/g, respectively.

357 In the control dressing, the *Z. bailii* counts remained constant for the first 7 storage days, and
358 increased target yeast growth was noted after 11 inoculation days (6 log CFU/g). This trend can
359 be explained by intrinsic factors and refrigeration temperatures. Karaman, Sagdic and Yilmaz
360 (2016) suggested that *Z. bailii* counts were strongly influenced by storage temperature as these
361 authors found higher yeast counts at increasing temperatures. Other factors related to salad
362 dressing composition, such as water activity and pH, among others, could also affect yeast
363 growth (Monu et al., 2016).

364 The limit of microbial growth used to examine the shelf-life of the salad dressings was a
365 yeast count of 10^2 CFU/g after considering one of the most restrictive levels encountered in
366 food commodities (Pascual & Calderón, 2000). According to this limit, the salad dressings
367 prepared with 1.85 mg/g of the encapsulated clove and oregano EOs were able to maintain the
368 hygienic quality of samples up to 4 and 7 days after their inoculation under the assayed
369 conditions, respectively. The samples formulated with the nanoemulsions containing 1.95 mg/g

of the oregano or clove EO displayed fungicidal activity. In both cases, total growth inhibition took place after 4 days of *Z. bailii* inoculation. The fungicidal effect of EO has been associated with ATPase activity on the cytoplasmic membrane being inhibited by disturbing the transport of nutrients (Cerutti & Alzomora, 1996). In spite of the creaming index observed at the end of the storage period, the fungicidal effect of the O/W nanoemulsions was observed.

Nevertheless, it is worth mentioning that the *in vivo* antifungal activity of the EOs significantly differed from the findings noted in the *in vitro* tests, which was most likely due to the complex environment of foods (Gutierrez, Barry-Ryan & Bourke, 2008; Monu et al., 2016; Omidbeygi, Barzegar, Hamidi, & Naghdibadi, 2007). It has been demonstrated that some food matrix components interfere with antimicrobials by diminishing their activity. Indeed lipids can interact with EOs or solubilise them, which hinders their ability to react with microorganisms and, therefore, decreases their antimicrobial efficacy. The inverse relation between the amount of fat in the food matrix and the antimicrobial of the EOs has been demonstrated in some studies (Gutierrez et al., 2008; Cava, Nowak, Taboada & Marin-Iniesta, 2007).

3.3.2 Sensory analysis

A sensory analysis was performed to assess the acceptance of the salad dressings formulated with the oregano and clove EO nanonoemulsions. Addition of nanoemulsions to dressings did not modify the acceptance of samples in terms of their appearance, consistency and colour attributes, compared to the reference dressing (Figure 3). However, mouth texture was affected when the nanoemulsion was incorporated, regardless of the employed EO. The salad dressing that contained the oregano EO received a similar evaluation score for the taste attribute. However, its general acceptance scored lower than the control sample, probably due to the effect of nanoemulsion on mouth texture, as mentioned above. The samples formulated with

394 the clove EO nanoemulsion received the lowest flavour, taste and mouth texture scores, which
395 affected the final acceptance of these dressings. All these data indicate that the sensory
396 acceptance of incorporating EOs is strongly affected by the type and amount of the EO, and
397 also by the food product. These findings indicate the importance of choosing EO type according
398 to the sensory features that consumers expect.

399 Although the antimicrobial activity of these EOs has been widely studied against different
400 microorganisms, very few studies have evaluated the sensory impact when they are added to
401 food products. According to the available literature, a wide range of tolerance limits can be
402 established depending on the EO type and food matrix (Donsì, & Ferrari, 2016; Espina et al.,
403 2014; Burt, 2004). It is important to point out that the encapsulation of EOs into nanoemulsions
404 modifies the release profile of EOs (Donsì & Ferrari, 2016), which can be employed as a
405 strategy to reduce undesirable modifications in the food product's sensory profile.

406

407 **4. Conclusions**

408 Non-encapsulated oregano and clove EOs displayed good antifungal properties against a
409 well-known food spoilage yeast, *Zygosaccharomyces bailii*. Higher essential oil concentrations
410 led to rising inhibition rates. After encapsulating the EOs in O/W nanoemulsions, the results of
411 the stability assays revealed their creaming over storage.

412 The experimental data of the *in vitro* antifungal assays suggested that the oregano and clove
413 nanoemulsions were able to inhibit yeast development during the storage period, which
414 evidences better antifungal activity compared to the non-encapsulated EOs.

415 The use of these nanoemulsions in salad dressings can control *Zygosaccharomyces bailii*
416 growth. Even the salad dressings containing 1.95 mg/g of each encapsulated EO displayed
417 fungicidal action after 4 days of *Zygosaccharomyces bailii* inoculation.

The use of the oregano EO in food products was better accepted than the clove EO, which indicates that oregano flavour is suitable for formulating salad dressings.

Hence the present work provides the food industry with stable natural systems that ensure the safety of minimally processed foods free of chemical additives. Nevertheless, more detailed studies in other food commodities should be conducted to guarantee the antifungal effect of the O/W nanoemulsions. Special attention should be paid to find appropriate EOs that inhibit the growth of the target microorganism to make it compatible with the food product's sensory profile at the same time.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure captions

548 **Figure 1.** Microbial counts of *Zygosaccharomyces bailii* against the non-encapsulated oregano
549 and clove EOs at 25 °C for 48 h. Mean value (n=3) ± SD. Different lowercase superscripts (a,
550 b, c, d, e, f) indicate significant differences among EO concentrations ($p<0.05$) and different
551 uppercase superscript (A, B) indicate significant differences between EO types ($p<0.05$).

552 **Figure 2.** Effect of incorporating the oregano and clove nanoemulsions into salad dressings on
553 *Zygosaccharomyces bailii* growth, expressed as log CFU/g. Mean value (n=3) ± SD. (NE:
554 nanoemulsion).

555 **Figure 3.** Average score of the attributes tested in the control salad dressing and the salad
556 dressings formulated with the O/W nanoemulsions. 0: very unpleasant and 9: very pleasant.
557 *Indicates significant differences among samples ($p<0.05$) (n=30). (NE: nanoemulsion).

558