Antilisterial effect of citrus essential oils and their performance in edible film formulations

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ABSTRACT

The antimicrobial activity of eight essential oils (EOs) extracted from the fruit peel of *Citrus* genotypes (orange, mandarin and lemon) was evaluated against 76 strains of *Listeria monocytogenes,* previously isolated from different food matrices. EOs showing the most (EO L2 and EO L8) and least (EO O3 and EO M7) effective inhibition activities were chemically characterized by gas chromatography coupled with mass spectrometry (GC/MS) to compare their composition. EO L2 and EO L8 were chosen to determine the MIC and to evaluate the cell viability of the most sensitive strains (*L. monocytogenes* LM35 and LM69) after 1, 2, 4 and 6 hours of exposure. The effectiveness of chitosan (CH) and methylcellulose (MC) edible films, alone and in combination with EO L2 and EO L8, was determined against LM35 and LM69 at 37°C for 0, 8 and 24 h and at 8°C for 0, 1, 3 and 7 days. In addition, the analysis of the microstructure of the films were performed by scanning electron microscope (SEM) to evidence the interactions between the polymers and EOs. Thirty-five and twenty-nine strains were clearly inhibited by EO L2 and EO L8, respectively, while the other *Citrus* EOs showed poor (EO M1, O4, O5, O6) or minimal (EO O3 and M7) antimicrobial activity. A total of 36 chemical volatile substances was identified by GC/MS to detect the compounds that might play an important role in the characterization of the EOs. The chemical characterization points to oxygenated monoterpenes as relevant compounds in inhibiting *Listeria* strains, since they have been detected in lemon EOs in concentrations four/five folds higher than orange EOs. Generally, CH- and MC-based films containing EO L2 and EO L8 showed antilisterial activities, even though, the best performances were observed in case of CH-films at 8°C, with a major reduction up to 3 log (CFU/cm^2) in case of EO L2 incorporation. The microstructures observed by SEM suggested a better incorporation of the EOs in CH matrix, where a higher amount of oil droplets was distinguished. Therefore, lemon EOs incorporated into chitosan films could be an efficient tool to control *Listeria monocytogenes,* especially in refrigerated applied conditions.

Key words: biopreservation, *Citrus*, edible coating, essential oils, GC/MS, *Listeria monocytogenes*

1. Introduction

Listeria monocytogenes is the causative agent of several outbreaks of food-borne listeriosis in America and in Europe (CDC, 2014; ECDC, 2013). This disease primarily affects people with weakened immune systems, such as older adults, pregnant women and newborns. Even though listeriosis is relatively rare and sporadic, it is a disease with high fatality rate (up to 30%) (FAO/WHO, 2004). In the United States, *Listeria* spp. annually induces, on average, 1600 cases of illnesses and 260 deaths, and is the third leading cause of death from food poisoning (Scallan et al., 2011). In 2012, 1642 cases of listeriosis have been reported in Europe with an increasing trend in comparison with previous years (ECDC, 2013). The highest proportions of food samples that exceeded the safety threshold (zero tolerance in all ready-to-eat products) for *L. monocytogenes* in EU, in 2012, were ready-to-eat (RTE) fishery and meat products (ECDC, 2013).

L. monocytogenes is widely present in soil, water, food (McCarthy, 1990; Kaclíková, Kuchta, Kay, & Gray, 2001) and food processing environments (Donnelly, 2001). Its capacity to adhere and colonize inert food contact surfaces such as polypropylenes, rubbers, stainless steel and glass, is well established (Beresford, Andrew, & Shama, 2001; Rieu, Briandet, Habimana, Garmyn, Guzzo, & Piveteau, 2008). Moreover, its ability to grow at a wide range of temperatures (-0.4°C up to 50°C), at a relative low pH (5.0-5.7 at 4°C and 4.3-5.2 at 30°C) and its capacity to form biofilms makes the control of this pathogen very difficult (Luber, Crerar, Dufour, Farber, Datta, & Todd, 2011).

The recent resurgence of listeriosis has prompted the food industry, the public and the government to question the adequacy of the current methods of food safety and preservation. All the recommendations of the Codex Alimentarius to providing guidance on the controls and associated tools that can be adopted by regulators and industry to minimize the likelihood of illnesses arising from the consumption of RTE foods containing *L. monocytogenes* (CAC/GL61, 2007), converge on

71 the reduction of the risk through safe food preparation, consumption and storage practices.
72 Moreover, consumer concern created a demand for more “natural” and “minimally processed” food.
73 As a result, the application of naturally produced antimicrobial compounds, such essential oils
74 (EOs) extracted from plants, has received great attention. EOs are complex mixtures of lipophilic
75 substances which exert different biological properties (Bakkali, Averbeck, Averbeck, & Idaomar,
76 2008) enjoying a “generally recognized as safe” (GRAS) status by the Foods and Drugs
77 Administration (FDA).
78 The antimicrobial properties of EOs depend on their chemical composition (Lanciotti, Gianotti,
79 Patrignani, Belletti, Guerzoni, & Gardini, 2004; Moreira, Ponce, Del Valle, & Roura, 2005; Espina,
80 Somolinos, Lorán, Conchello, García, & Pagán, 2011) which is influenced by raw plant material
81 (genotype and, part of the plant), harvest time, geographical and ecological conditions (Settanni et
82 al., 2014) and extraction method (Burt, 2004).
83 *Citrus* spp. have been extensively investigated for EOs (Tirado, Stashenko, Combariza, & Martinez,
84 1995; Fisher & Phillips, 2008), but the biological activities of the EOs are still under study. Some
85 authors reported EOs to be highly effective, while other stated that the effects are variable (Burt,
86 2004). Recent reports demonstrated that some EOs extracted from *Citrus* in Sicily (south Italy)
87 showed good potential as antimicrobial compounds effective against food spoilage and/or pathogen
88 microorganisms *in vitro* (Settanni et al., 2012; Settanni et al., 2014).
89 Since the intense aroma, the potential toxicity and the extraction costs limit the direct use of EOs in
90 food preservation, the reduction of the doses to be applied to food matrixes is the clue to be pursued
91 to extensively apply EOs. The use of edible coatings as carriers of antimicrobial compounds could
92 be an alternative tool to contrast food spoilage and/or pathogen agents (Aider, 2010; Burt, 2004;
93 Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Sánchez-González, Vargas, González-Martínez,
94 Chiralt, & Cháfer, 2011) and, at the same time, to reduce the amount of EOs to be applied in the
95 food.

96 In this way, the chemico-physical properties of the polymer constituting the film and acting as a
97 selective barrier to gas transport (Vargas, Pastor, Chiralt, McClements, & González-Martínez,
98 2008), together with the antimicrobial properties of EOs included, can be the goal of an hurdle
99 technology applied to food to extend its commercial shelf-life (Park, 1999; Perdones, Sánchez-
100 González, Chiralt, & Vargas, 2012). To this end, the use of biopolymers, such as chitosan (CH) and
101 methylcellulose (MC), piques the interest of food industries and research groups thanks to their
102 excellent film forming properties, non-toxicity, odorless, tasteless, biodegradability and edibility
103 (Krochta & Mulder-Johnston, 1997; Villalobos, Hernández-Muñoz, & Chiralt, 2006; Vargas,
104 Pastor, Chiralt, McClements, & González-Martínez, 2008). Chitosan is a cationic polysaccharide
105 obtained from chitin by deacetylation in the presence of alkali (Sánchez-González, González-
106 Martínez, Chiralt, & Cháfer, 2010) that shows antimicrobial activity itself (Vargas, & González-
107 Martínez, 2010; Zheng & Zhu, 2003) and can also acts in synergy with EOs.
108 The aim of this work was (i) to evaluate the effect of citrus EOs against several *Listeria*
109 *monocytogenes* strains and (ii) to assess the antimicrobial properties of *Citrus* EOs incorporated into
110 chitosan and methylcellulose coatings.
111
112 **2. Materials and methods**
113 *2.1. Listeria monocytogenes strains*
114 Seventy-six strains of *L. monocytogenes* were used in this study. All strains, belonging to the
115 Department of Biotechnology – Microbiology Area, ETSIAMN (Universitat Politècnica de
116 València, Spain), were previously isolated from food matrices including dairy products, fish, meat
117 and vegetables, following the ISO method 11290-1:1996 (ISO 11290-1:1996). Bacterial strains
118 were stored in cryovials (Microbank^{TM} Prolab Diagnostics, Austin, USA) at -80°C. The strains were
119 reactivated and sub-cultured onto Tryptic Soy Agar (TSA, Merck Millipore, Darmstadt, Germany)
120 incubated overnight at 37°C.
121

122 *2.2. Citrus samples and extraction of EOs*

123 The EOs analyzed in this study were obtained from the peels of eight different citrus fruits
124 cultivated in Sicily (Table 2) and collected during March 2014. Samples EO M1 and EO L2 derived
125 from mature trees cultivated in the collection orchard "Parco d'Orleans" of the Agricultural Faculty
126 of Palermo, while samples EO O3, EO O4, EO O5, EO O6, EO M7 and EO L8 from the "Azienda
127 Sperimentale Palazzelli C.R.A. - Centro di ricerca per l'agrumicoltura e le colture mediterranee
128 Contrada Palazzelli Scordia" (CT, Italy).

129 After peeling, the peels were immediately subjected to hydro-distillation for 3 h using a Clevenger-
130 type apparatus (Comandè, Palermo, Italy) collecting the oil in hexane. EOs were dried over
131 anhydrous sodium sulphate and stored at 4°C in air-tight sealed glass vials covered with aluminum
132 foil.

133

134 *2.3. Chemical characterization*

135 GC/MS analysis of the EOs was performed by gas chromatography couple with mass spectrometry
136 (GC/MS) (EI) on a GCMS-QP2010 (Shimadzu, Milan, Italy). NIST 21,107,147 library was used
137 for data acquisition. The analysis was carried out through a fused silica capillary column SLB-5MS
138 (5% diphenyl:95% methylsiloxane) 30 m x 0.25 i.d. x 0.25 mm film thickness (Supelco, Milan,
139 Italy); helium gas was used as the carrier gas at a constant linear rate 30 cm s^{-1} (30.6 kPa);
140 split/splitless injector port; injector temperature 250 °C; injection mode split (split ratio 100:1). The
141 oven temperature was programmed as follows: 50°C, hold 3 min; 3°C/min to 240°C; 15°C/min to
142 280, hold 1 min. MS scan conditions were: source temperature 200 °C, interface temperature 250
143 °C, EI energy 70 eV; mass scan range 40-400 amu. GC/MS analysis was carried out in duplicate.

144

145 *2.4. Screening of antilisterial activity*

146 The antibacterial activity of the eight EOs against *L. monocytogenes* strains was tested by the paper
147 disc diffusion method applied by Kelmanson, Jager, and Van Staden (2000) and with the

148 modifications of Militello et al. (2011). Bacterial cells were grown at 37°C overnight before tests on
149 tryptone soy broth (TSB). A concentration of about 10^7 CFU/mL of each strain was inoculated into
150 7 mL of TSA soft agar (0.7%, w/v) and poured onto TSA. Sterile filter paper discs (Filter-Lab
151 Anoia, Spain) of 6 mm diameter were placed onto the surface of the double agar layer and soaked
152 with 10 µL of each undiluted EO. Sterile water was used as negative control. Antibacterial activity
153 was positive when a definite halo of inhibition (in cm) was detected around the paper disc. Each test
154 was performed in duplicate and the experiments were repeated twice. Resulting data were subjected
155 to statistical analysis using the ANOVA procedure with Statistica 10 (Statsoft, USA) software.
156 Differences between means were determined by Tukey's multiple-range test.
157
158 *2.5. Determination of the minimum inhibitory concentration (MIC)*
159 The minimum inhibitory concentration (MIC) was used to measure the antibacterial activity, since it
160 represents a common method to express the EO antibacterial performances (Burt, 2004). MIC is
161 defined as the lowest concentration of an active compound inhibiting visible growth of the tested
162 organisms (Karapinar & Aktug, 1987). The strength of the antibacterial activity is determined using
163 dilutions of EO in order to determine the end-point by means of the disc diffusion assay as reported
164 above. Serial dilutions (dilution factor = 2) were obtained with dimethyl sulfoxide (DMSO, Sigma-
165 Aldrich, Milan, Italy). DMSO alone was used as negative control. Each test was performed in
166 duplicate and the experiments were repeated twice.
167
168 *2.6. Viability of L. monocytogenes strains by fluorescence microscopy*
169 The viability of the most sensitive *L. monocytogenes* strains after treatment with EOs was evaluated
170 by Viability Kit LIVE/DEAD® *Bac*Light™ (Molecular Probes Inc. Eugene Oregon) and plate
171 counts onto TSA. The viability test was carried out with the strains inoculated at a final density of
172 10^4 CFU/mL in broth containing 1% (v/v) EO. Cells were counted as follows: 500 µl of each broth
173 collected at 0, 1, 2, 4 and 6 h of treatment with EO was added with 0.8 µl of the fluorochromes mix

174 (1:1 v/v, EO/mix) and incubated in darkness at room temperature for 15 minutes. Five microliters of
175 the resulting mixture were placed onto a poly-L-lisina slide (Poly-Prep® slides, Sigma Diagnostics,
176 U.S.A.). After 10 minutes of incubation at room temperature, the counts were carried out by the
177 epifluorescence microscope Olympus BX 50 (with a mercury bulb of 100W) equipped with a
178 double filter (XF 53, Omega) (Olympus Optial Co., Hamburg, Germany). Digital colored photos
179 were taken with Olympus DP10 digital camera (results not shown).
180
181 *2.7. Antilisterial effect of edible EOs-based films*
182 Chitosan-based (CH) and methylcellulose-based (MC) films were used to perform the antilisterial
183 assay. High molecular weight chitosan (1.2 Pa·s viscosity at 1% w/w in 1% w/w glacial acetic acid,
184 acetylation degree: 4.2%, Sigma-Aldrich, USA) was dispersed at 1% w/w in an aqueous solution of
185 acetic acid (1% v/w) and stirred overnight at room temperature. Methylcellulose (0.3-5.6 Pa·s
186 viscosity at 1% w/w in water solution, VWR BDH ProLabo, Spain) was dispersed in distilled water
187 (1% w/w) and heated up to 80°C to promote solubilization.
188 Once the polymer solutions were obtained, each EO was added at a concentration of 0.5%
189 (polymer: EO ratio 2:1) and stirred for 10 minutes. The mixtures were then sonicated by the Vibra
190 Cell VCX750 sonicator (Sonics & Materials, Inc., USA) at 20 kHz and 40% power for 480 s (1 s on
191 and 1 s off) in order to obtain the film forming dispersions (FFD). FFDs were casted in plates
192 (diameter 53 mm), weighted up to 6.7g, to keep polymer amount constant in dry films (30 g
193 polymer/m^2). The films were dried at room temperature and 60% relative humidity (RH).
194 The surface of TSA plates (10 g) was seeded with 0.35 mL of cell suspensions (10^4 CFU/mL) and
195 covered with CH and MC films. Inoculated coated TSA and inoculated non-coated TSA dishes
196 were used as controls. Plates were then sealed with parafilm to avoid dehydration and incubated at
197 37°C for 0, 8 and 24 h and at 8°C for 0, 1, 3 and 7 d. The agar layer was then aseptically removed
198 from each Petri dish and placed into a sterile stomacher bag with 90 mL of Peptone Water (Merck

199 Millipore, Darmstadt, Germany) and homogenized for 60 sec in the stomacher Bag Mixer 400
200 (Interscience, Saint Nom, France).
201 Serial dilutions were set up with Ringer's solution (Sigma-Aldrich, Milan, Italy) and 0.1 mL of cell
202 suspensions were spread plated onto TSA plates. Colonies were enumerated after 24 h at 37°C. The
203 experiment was carried in duplicate.
204
205 *2.8. Microstructure*
206 Film microstructure was observed by Scanning Electron Microscopy in cross-sectioned
207 cryofractured specimens, using a JEOL JSM-5410 (Tokyo, Japan) electron microscope in order to
208 qualitatively assess the EOs incorporation into the polymeric matrix. The films (3 samples per
209 formulation) were equilibrated in P_2O_5 to eliminate water prior cryofracturing them by immersion
210 in liquid nitrogen. Afterwards, cryo-fractured samples were mounted on copper stubs. After gold
211 coating, the images were captured using an accelerating voltage of 10kV.
212
213 **3. Results and discussion**
214 *3.1. Screening of the antilisterial activity*
215 The results of the disc diffusion assay are shown in Table 1. All EOs resulted statistically different
216 ($P \leq 0.001$) in inhibiting the strains tested, confirming previous statements that the sensitivity to
217 natural antimicrobial compounds is strain-dependent (Settanni et al., 2014). EO L2 and EO L8
218 showed the widest spectra of inhibitory activity. In particular, EO L2 inhibited all tested strains and
219 for thirty-five of them the clear halos were larger than 10 mm. Except *L. monocytogenes* LM68, all
220 other strains were sensitive to EO L8 and the halos were registered at diameters larger than 10 mm
221 for twenty-nine indicator strains.
222 Regarding the inhibition by the other EOs, only *L. monocytogenes* LM10, LM16, LM35 and LM69
223 were particularly sensitive. On the contrary, strains LM09, LM29, LM63, LM66, LM68 were not
224 inhibited by at least three EOs. EOs O3 and M7 did not show interesting antilisterial activities.

MICs were calculated only for the most effective EOs (EO L2 and EO L8) against *L. monocytogenes* LM35 and LM69, which were registered as the most sensitive strains. Both strains were equally inhibited and the values registered were 0.625 for EO L2 and 1.25 μL/mL for EO L8.

3.2. Characterization of EOs by GC/MS

Analysis of volatile compounds was carried out after extraction of EOs. Based on the antilisterial activity, EO L2 and EO L8, as most effective, and EO O3 and EO M7, as less effective oils, were chemically analyzed by GC-MS. The identified volatile compounds and their relative amounts are given in Tables 3. A total of 36 compounds were characterized among the four EOs. The phytochemical groups included monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpene hydrocarbons. Monoterpene hydrocarbons were quantitatively relevant, ranging from 88.35% (EO L2) to 98.07% (EO O3). Limonene accounted for the major proportion by quantity in all samples. The oxygenated monoterpenes of lemon EOs were four/five folds those of EO O3 and EO M7, indicating a direct role in the mechanisms of inhibition. Sesquiterpene hydrocarbons were detected in minimal percentages in lemon EOs, only traces were found in EO M7 while they were absent in EO O3.

Monoterpene hydrocarbons such as α-Thujene, p-Cymene and cis-2,6-Dimethyl-2,6-octadiene were found only in lemon EOs. Among the oxygenated monoterpenes, 1-Octanol, Fenchol, Citronellal, cis-Geraniol, α-Citronellol, β-Citral, cis-p-Mentha-2,8-dien-1-ol, Geranyl acetate and Neryl acetate were identified only in EO L2 and EO L8. On the contrary, β-Terpinol was only found in EO O3 and EO M7. Almost all compounds showed statistical differences in quantitative terms among EOs. The higher presence of oxygenated monoterpenes in volatile composition profile of EO L2 and EO L8 could explain the greater inhibitory activity than the EO O3 and EO M7.

3.3. Viability assay

250 Dead and viable cells were detected and counted using epifluorescence microscopy (Fig. 1). Plate
251 counts of the untreated samples showed an increase of 10^3 CFU/ml for both strains within the six
252 hours of treatment. Divergent results were obtained comparing the counts assessed by
253 epifluorescence microscopy and plate counts.
254 Based on epifluorescence microscopy, viable cells amounted to 10^{3-4} CFU/ml for LM35 and 10^{4-5}
255 CFU/ml for LM69, while dead cells reached up to 3 and 4 log CFU/ml in case of LM35 and LM69,
256 respectively. These results are in contrast with those of direct plate counts, where no cultivable cells
257 were detected after 1 hour (or 2 hours in case of LM35 added with EO L8) of incubation. This could
258 be explained by an active but non-culturable (ABNC) state of cells stressed by EOs (Boulos,
259 Prevost, Barbeau, Coallier, & Desjardins, 1999). This was confirmed by Nexmann, Jacobsen,
260 Rasmussen, and Jakobsen (1997) who registered significantly fewer viable *L. monocytogenes* cells
261 counted by culture-based techniques compared to the active bacteria detected using fluorescent
262 direct counts. Similar results were achieved with lactic acid bacteria (Moreno, Collado, Ferrús,
263 Cobo, Hernández, & Hernández, 2006) using fluorescent flow cytometric measurements (Boulos,
264 Prevost, Barbeau, Coallier, J., & Desjardins et al., 1999). According to Joux & Lebaron (2000),
265 bacterial cells cannot be necessarily considered active if they show intact membranes, but it would
266 seem to be more accurate to assume that membrane-compromised cells are dead (Berney,
267 Weilenmann, & Egli, 2006). The EOs antimicrobial activity is due to their hydrophobic nature
268 affecting the lipid bilayer of microbial cells, as confirmed by the evidences of this assays, since the
269 kit used enables differentiation only between bacteria with intact and damaged cytoplasmic
270 membranes, differentiating between active and dead cells (Sachidanandham, Yew-Hoong Gin, &
271 Laa Poh, 2005).
272
273 3.4. *Antilisterial effect of edible EOs-based films and film microstructure*
274 Antilisterial performances of CH- and MC-based edible films determined on TSA, alone and in
275 combination with EO L2 and EO L8, are shown in Figures 1. The overall effect of CH- and MC-

276 based films, in terms of trend, was similar for both strains tested. The addiction of the EOs into the
277 films enhanced their bactericidal activity. The highest antimicrobial effect was obtained for CH
278 films at 8ºC (Fig. 1E and G). When sample EO L2 was added to the films, a reduction in the range
279 of 2-3 Log CFU/cm^2 was obtained as compared to control plates (Fig. 1A and E). This oil sample
280 determined the lowest listeria counts in both film matrices (CH or MC). In general, the EO L2-
281 based films showed the best inhibition activity compared with the CH or MC control films, and
282 also, compared to EO L8-based films.
283 After a storage period of 24 hours at 37°C and 7 days at 8°C, pure MC films showed no significant
284 effect on the growth of both strains. MC films incorporating EO L2 promoted a slight reduction in
285 Listeria counts at 37°C after 8 hours of incubation (≤1-2 log CFU/cm^2) (Fig 1B).
286 A stronger antilisterial effect was evidenced for the CH-based films, alone and in combination with
287 EOs. Specifically, CH-films were more effective in reducing the microbial growth at 8°C rather
288 than 37°C. In fact, CH- films added with EOs led to a reduction up to 3 and 6 log CFU/cm^2, in the
289 case of LM35 and LM69, respectively, when incubated at 8°C for 7 days (Fig. 1 E and G).
290 The highest significant antibacterial effect evidenced in case of the incubation at 8°C may be related
291 to the influence of the temperature in promoting the permeability of cell membranes and, thus,
292 dissolving more easily EOs in the lipid bilayer when low temperatures occur (Sánchez-González,
293 Vargas, González-Martínez, Chiralt, & Cháfer, 2011).
294 Figure 2 shows the SEM microstructures of the cross-sections of CH and MC films. Pure MC and
295 CH films (Fig. 2A, D) exhibited a homogeneous and continued microstructure in line to that
296 observed in previous studies (Vargas, Albors, Chiralt, & González-Martínez, 2011). The addition of
297 the lemon EOs to the film matrix promoted discontinuities (Fig. 2B, C, E, F), in agreement with the
298 results reported by Perdones, Sánchez-González, Chiralt, & Vargas (2012) in CH-based films
299 containing essential oil. The presence of EO droplets is more noticeable in CH-based films (Fig. 2B,
300 C), and especially in films containing EO L2 (droplets size 1-8µm). The observations pointed to a

301 better incorporation of the EOs in CH matrix, where a higher amount of oil droplets was
302 distinguished.
303 Furthermore, the higher inhibition activity recorded for EO L2 included into CH matrix can be due
304 not only to the better incorporation, but also to the subsequent release of the active compounds. A
305 good incorporation of EO into the films slows down the diffusion rate of the antimicrobial
306 compounds, keeping high concentrations of EOs for extended period of time and reducing the levels
307 of microorganisms on the surface.
308
309 **Conclusion**
310 Citrus EOs shown bioactive properties against *L. monocytogenes*, The antibacterial effect of these
311 EOs was maintained when they were incorporated into biodegradable films based on chitosan or
312 methylcellulose. Chitosan films containing EO L2 were the most effective in reducing *L.*
313 *monocytogenes* counts. Chitosan edible films enriched with lemon oils represent an alternative tool
314 to control surface contaminations of *L. monocytogenes*, especially in refrigerated conditions. The
315 reduction in EO concentration needed for film applications as compared to direct contact
316 treatments, can decrease the possible sensory impact on food. Works are being prepared to refine
317 the technology for the production of EO-based films, to evaluate the suitability of the films tested in
318 this study on food matrices, as well as the impact of the EO released on the sensory quality. Hence,
319 the foreseeable potential practical application of this study is to reduce the presence of L.
320 monocytogenes in foods, but also to valorise citrus fruit peel that basically constitutes a waste
321 of the fruit juice industry in Sicily.
322
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327 some of the fruits used for EOs extractions.

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Table 1. Inhibitory activity[a] of citrus EOs against *Listeria monocytogenes* isolated from food tested by disc diffusion assay.

Strain code	EO M1	EO L2	EO O3	EO O4	EO O5	EO O6	EO M7	EO L8	Statistical significance[b]	Source of isolation[c]
LM01	0.6	0.8	0.6	0.6	0.6	0.6	0.7	1	***	M
LM02	0.7	0.8	0	0.6	1	0.8	0.8	1	***	M
LM03	0.8	0.8	0	0	0.8	1	0.6	0.8	**	M
LM04	1	1	0.6	0.6	0.6	0.6	0.6	0.6	***	D
LM05	0	0.8	0	0.8	0.6	1	0.6	1.4	**	D
LM06	0.7	0.8	0.6	0.6	0.6	0.8	0.7	0.8	***	M
LM07	0.6	0.8	0.7	0.6	0.6	0.6	0.7	0.8	***	D
LM08	0.6	0.6	0.8	0.6	0.7	0.8	0.6	1.1	***	F
LM09	0.6	0.8	0	0.8	0	0.8	0	0.8	*	F
LM10	0.8	0.8	1	0.8	1.4	1	1	1	***	D
LM11	0.6	1	0.6	0	0.8	0.8	0.8	0.8	***	F
LM12	0.8	0.9	0.6	0.6	0.6	0.8	0.6	0.8	***	M
LM13	0.8	1	0.6	0.6	0.6	0.8	0.8	1.2	***	D
LM14	0.6	0.8	0	0.6	0.6	0.8	0.6	0.8	***	F
LM15	0.6	1	0	0.8	0.6	0.8	0	0.8	**	D
LM16	0	1	0	1	0.6	1	0.8	1	**	F
LM17	0.8	1.2	0.6	0.8	0.7	0.7	0.6	0.8	***	D
LM18	0.7	1	0.6	0.8	0.6	0.6	0.8	0.8	***	F
LM19	0.6	0.9	0.6	0.6	0.8	0.8	0.7	0.8	***	F
LM20	0.7	1.1	0.6	0.8	0.6	0.8	0.6	1	***	F
LM21	0.7	0.8	0.7	0.7	1	1	0.6	0.8	***	M
LM22	0.6	0.8	0.6	0.6	0.8	1	0.8	1	***	F
LM23	0.7	1	0.6	0.8	0.6	0.6	0	0.7	***	D
LM24	0.6	0.9	0.8	0.8	0.6	0.6	0.6	0.8	***	F
LM25	0.6	0.8	0.6	1	0.8	0.7	0	1	***	F
LM26	0.7	0.8	0.6	0.7	0.8	0.6	0.7	1	***	M
LM27	0.8	1.3	0.8	0.8	0.6	0.8	0.6	1	***	D
LM28	0.7	0.8	0.6	0.6	0.6	0.7	0.6	0.8	***	M
LM29	0	0.8	0	0.6	0.6	0.8	0	1	*	M
LM30	0.6	1	0.6	0.8	0.6	0.6	0.6	1	***	PF
LM31	0.6	0.8	0.6	0.7	0.6	0.6	0.6	0.8	***	M
LM32	0.6	1	0	0.6	0.6	0.6	0.6	0.8	***	F
LM33	0	1	0.6	0.8	0.6	0.8	0.6	1	***	F
LM34	0.6	0.6	0.6	0.6	0.6	0.8	0.6	0.8	***	F
LM35	1	1	0	0.8	0.8	1.2	1	1.4	***	V
LM36	0.8	1	0.8	0.8	0	0.8	0.8	1	***	F
LM37	0.6	1.2	0.6	0.6	0.6	0.6	0.6	0.8	***	F
LM38	1.2	1	0.7	0.7	0.6	0	0	0.8	**	D
LM39	0.8	0.8	0.7	0.7	0.8	0.8	0.6	1	***	D
LM40	0.6	1	0	0.6	0.6	0.6	0.6	1	***	D
LM41	0.7	1.2	0.8	0.6	0.6	0	0.7	1	***	F
LM42	0	0.6	0.6	0.6	0.6	0.8	0	0.8	**	M
LM43	0.6	1.2	0.6	0.8	0.6	0.6	0	0.8	***	D
LM44	0.6	0.8	0.6	0.6	0.6	0.8	0.7	1	***	M
LM45	0.8	0.6	0.6	0.6	0.6	0.6	0.6	0.8	***	PF
LM46	0.7	1	0.6	0.7	0.6	0.8	0.6	1	***	F
LM47	0.6	1.2	0.6	0.8	0.6	0.8	0.6	1	***	M
LM48	0.6	1.2	0.6	0.6	0.8	0.8	0.8	1	***	D
LM49	0.6	1	0.6	0.6	0.7	0.8	0.6	0.8	***	M
LM50	0.6	0.8	0.6	0.6	0.6	0.6	0.6	0.8	***	F
LM51	1.2	1.3	0.8	0.8	0.6	0.6	0.6	0.8	***	M
LM52	0.6	1	0.6	0.6	0	0.6	0.6	0.7	***	F
LM53	0.6	0.9	0.6	0.6	0.6	0.6	0.6	0.8	***	D
LM54	0.9	1	0.6	0.6	0.6	0.8	0	0.8	***	M
LM55	0.6	0.8	0.7	0.6	0.6	0.6	0.6	0.8	***	M
LM56	0.6	0.8	0.6	0.6	0	0.6	0	0.8	**	D
LM57	1	1	0.7	0.6	0.6	0.8	0	1.1	***	D
LM58	0.6	0.8	0.6	0.7	0.7	0.6	0.6	0.8	***	M
LM59	0.6	0.8	0.6	0.6	0.6	0.6	0.6	0.8	***	D
LM60	0.6	1	0.6	0.6	0.6	0.6	0.6	0.8	***	M
LM61	0.9	1	0.9	0.6	0.6	0.6	0.6	0.9	***	PF
LM62	0.6	0.6	0.8	1	0.6	0.6	0.6	1.2	***	D
LM63	0.6	1	0.6	0.6	0	0	0	1	*	F
LM64	0.6	1	0.8	08	0.8	0	0	0.9	**	F
LM65	0.6	1	0.6	0	0.6	0.6	0	0.8	**	PF
LM66	0.6	0.8	0.6	0.6	0	0	0	0.8	*	F
LM67	0.6	0.6	0.6	0.6	0.6	0.7	0.6	0.9	***	F
LM68	0.6	0.6	0	0.6	0	0	0	0	ns	F
LM69	1	1.2	0.8	1	1	1.1	0.8	1.4	***	D
LM70	0.8	0.6	0.8	0.6	0.6	0.8	0.6	1	***	V
LM71	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.8	***	M
LM72	0.6	0.8	0.6	0.6	0.6	0.6	0.6	0.8	***	M
LM73	0.8	0.8	0.8	0.8	0.6	0.6	0.6	0.8	***	F
LM74	0.9	1.1	0.8	0.8	0.8	0.8	0.6	0.8	***	D
LM75	0.8	0.9	0.7	0.7	0.6	0.6	0.6	0.8	***	M
LM76	0.6	0.8	0.6	0.6	0.6	0.6	0.6	0.8	***	D

[a] Results indicate mean value of four determinations (carried out in duplicate and repeated twice). The values are expressed in cm.

[b]P value: *, P≤0.05; **, P≤0.01; ***, P≤0.001.
[c] M, Meat; D, Dairy; F, Fish; V, Vegetable; PF, Packaged food.

429 **Table 2. Sicilian** EOs used in the antilisterial screening.

EO	Species	Variety	Sperimental Orchard
M1	Mandarin (*Citrus reticulata* Blanco)	Mandarino Tardivo di Ciaculli	Campo dei Tigli (Palermo)
L2	Lemon (*Citrus limon* L. Burm.)	Femminello Santa Teresa	Campo dei Tigli (Palermo)
O3	Sweet Orange (*Citrus sinensis* L. Osbeck)	Moro Nucellare	Campo Palazzelli (Acireale)
O4	Sweet Orange (*Citrus sinensis* L. Osbeck)	Lane Late	Campo Palazzelli (Acireale)
O5	Sweet Orange (*Citrus sinensis* L. Osbeck)	Tarocco Tardivo	Campo Palazzelli (Acireale)
O6	Sweet Orange (*Citrus sinensis* L. Osbeck)	Sanguinello Nucellare	Campo Palazzelli (Acireale)
M7	Hybrid of Horoval clementine x Tarocco orange	Alkantara mandarin ®	Campo Palazzelli (Acireale)
L8	Lemon (*Citrus limon* L. Burm.)	Limone KR (Siracusano)	Campo Palazzelli (Acireale)

430

Table 3. Chemical composition[a] of citrus EOs.

Compound	RT	EO L2		EO O3		EO M7		EO L8		Statistical significance[b]
Monoterpene hydrocarbons		88.35		98.07		97.81		90.93		
α-Thujene	9.801	0.215	B	n.d.	A	n.d.	A	0.305	C	***
α-Pinene	10.129	1.290	B	0.340	A	0.410	A	1.325	B	***
Sabinene	11.900	1.105	B	0.210	A	0.220	A	1.135	B	***
β-Pinene	12.155	9.890	C	0.025	A	0.025	A	9.125	B	***
β-Myrcene	12.666	1.105	A	1.695	C	1.890	D	1.425	B	***
α-Phellandrene	13.467	0.185	C	0.105	B	0.055	A	0.065	A	***
3-Carene	13.560	n.d.	ns	0.090	ns	0.040	ns	n.d.	ns	ns
α-Terpinene	13.944	n.d.	ns	0.040	ns	0.040	ns	0.340	ns	ns
p-Cymene	14.275	11.515	C	n.d.	A	n.d.	A	0.440	B	***
D-Limonene	14.854	62.780	A	95.445	C	94.910	C	64.505	B	***
ϒ-Terpinene	16.080	0.025	A	0.075	A	0.180	B	9.525	C	***
(+)-2-Caren	17.315	n.d.	A	0.045	B	0.035	B	0.510	C	***
cis-2,6-Dimethyl-2,6-octadiene	29.716	0.240	B	n.d.	A	n.d.	A	2.225	C	***
Oxygenated monoterpenes		10.770		1.930		2.175		8.275		
1-Octanol	16.736	0.065	C	n.d.	A	n.d.	A	0.050	B	***
Linalol	18.024	0.425	A	1.005	B	1.555	C	0.410	A	***
Nonanal	18.252	0.190	B	0.040	A	0.020	A	0.135	B	**
Fenchol	18.972	0.030	B	n.d.	A	n.d.	A	0.015	B	**
Limonene epoxide	19.608	0.815	B	n.d.	A	n.d.	A	n.d.	A	***
Limonene oxide, trans	19.820	1.000	ns	n.d.	ns	n.d.	ns	n.d.	ns	ns
β-Terpinol	20.507	n.d.	A	0.035	C	0.020	B	n.d.	A	**
Citronellal	20.556	0.065	B	n.d.	A	n.d.	A	0.095	C	***
4-Terpineol	21.971	0.630	B	0.235	A	0.225	A	1.010	C	***
α-Terpineol	22.705	1.445	D	0.415	B	0.265	A	1.100	C	***
Decanal	23.157	0.085	A	0.200	A.C	0.090	A	0.040	A,B	*
trans-Carveol	23.801	0.180	B	n.d.	A	n.d.	A	n.d.	A	***
cis-Geraniol	24.087	0.175	B	n.d.	A	n.d.	A	1.245	C	***
α-Citronellol	24.200	0.070	A	n.d.	A	n.d.	A	0.325	B	**
β-Citral	24.704	1.550	C	n.d.	A	n.d.	A	1.355	B	***
(-)-Carvone	24.947	0.165	B	n.d.	A	n.d.	A	n.d.	A	***
cis-p-Mentha-2,8-dien-1-ol	26.058	0.220	B	n.d.	A	n.d.	A	1.790	C	***
α-Citral	26.090	1.980	B	n.d.	A	n.d.	A	n.d.	A	***
Geranyl acetate	30.116	0.980	C	n.d.	A	n.d.	A	0.325	B	***
Neryl acetate	30.979	0.700	C	n.d.	A	n.d.	A	0.380	B	***
Sesquiterpene hydrocarbons		0.880		n.d.		0.020		0.800		
α-Bergamotene	33.375	0.315	C	n.d.	A	n.d.	A	0.275	B	***
β-Bisabolene	36.474	0.480	C	n.d.	A	n.d.	A	0.385	B	***
Caryophyllene oxide	39.463	0.085	C	n.d.	A	0.020	B	0.140	D	***

[a] Data are means of two replicates expressed as percent area.

Abbreviations: RT, retention time on SLB-5MS column; ns, not significant; n.d., not detectable.

[b] P value: *, P≤0.05; **, P≤0.01; ***, P≤0.001.

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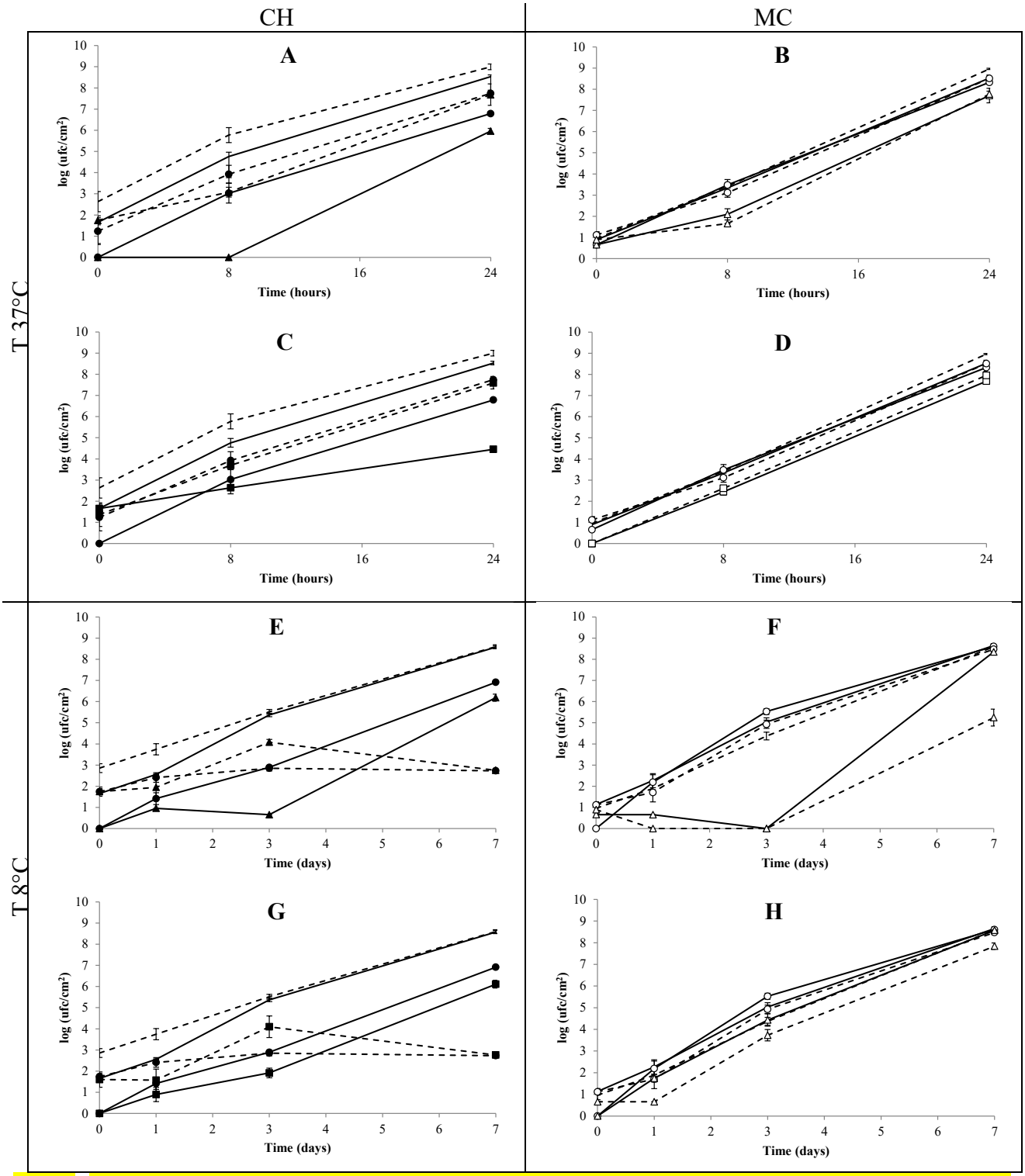

Fig. 1. Effect of incorporation of EOs in chitosan and methylcellulose films on the growth of *L. monocytogenes* at 37°C for 24 h (A, B, C and D) and 8°C for 7 d (E, F, G and H). Symbols:——, strain LM35; – –, strain LM69; black marks indicate chitosan films; empty marks indicate methylcellulose films; unmarked lines indicate control strains; ●,○, indicate control films; ▲, Δ, indicate films with EO L2; ■, □, indicate films with EO L8. A and E, chitosan films with EO L2; B and F, methylcellulose films with EO L2; C and G, chitosan films with EO L8; D and H, methylcellulose films with EO L8.

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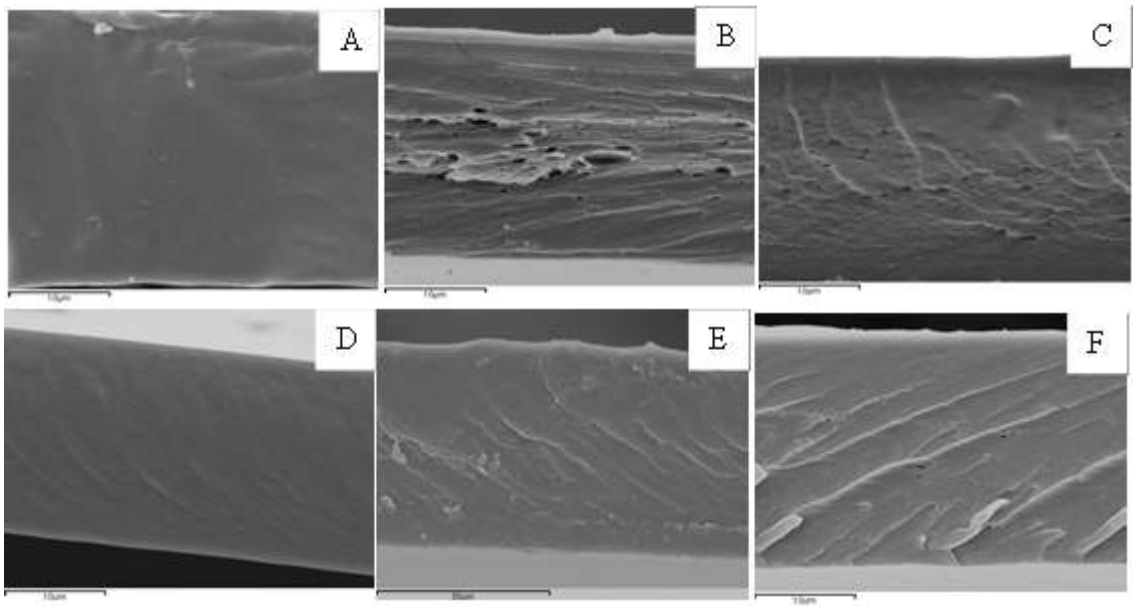

438

439 **Fig. 2.** SEM microstructure of cross sections of chitosan and methylcellulose films with essential oils.
440 Magnification is x3500. A, chitosan films; B, chitosan film with EO L2; C, chitosan film with EO L8; D,
441 methylcellulose film; E, methylcellulose film with EO L2; F, methylcellulose film with EO L8.