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Investigation of antibacterial activity of new classes of essential oils derivatives

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Abstract.

Essential oils (EOs) have deserved much attention in the past decades for their antimicrobial activity, since many of them have demonstrated efficacy against food-borne pathogenic and spoilage microorganisms. Moreover, they have potential application in animal nutrition as multifunctional feed supplements, avoiding or diminishing the use of antibiotics in livestock. However, low solubility and bioavailability as well as volatility and marked aromatic note are important limitations in food and feed applications. In this study we present the synthesis, characterization and evaluation of the antibacterial activity of new thymol, carvacrol and menthol derivatives. The new compounds have been designed to overcome the limitations of the precursors, such as poor water solubility and volatility, still maintaining a good antimicrobial profile. We

24 evaluated the activity of the synthesized compounds against pathogens causing important foodborne
25 diseases, *i.e.* *Clostridium perfringens*, *Salmonella typhimurium*, *Salmonella enteritidis* and
26 *Escherichia coli*. The low MICs and MBCs values for some of the studied compounds, combined
27 with water solubility and negligible cytotoxicity towards HT-29 human cells, confirmed the
28 potential use for EOs derivatives in the food industry.

29 **Abbreviations:** EOs, essential oils; TGA, thermogravimetric analysis; HIA, heart infusion agar;
30 BHI, brain heart infusion; MIC, minimal inhibitory concentration; MBC, minimal bactericidal
31 concentration; GI%, growth inhibition percent.

32 **Keywords.** essential oils derivatives; antibacterial activity; bioavailability; food-borne pathogens

33

34 1. Introduction.

35 Microorganisms causing food spoilage are a major concern for the food industry and the extension
36 of shelf-life is an on-going demand for both retailers and consumers. Such extension is mainly
37 achieved by technological improvements and addition of synthetic food preservatives. Natural
38 products in general are an alternative to synthetic preservatives, and among them, essential oils
39 (EOs) are typical antimicrobial agents without harmful residues. Since the 1990s, they have been
40 widely studied for their antimicrobial activity and many EOs (e.g. thyme, oregano, cinnamon,
41 horseradish) and their components have demonstrated antimicrobial efficacy against food-borne
42 pathogenic and spoilage microorganisms (Arsi et al., 2014; Bakkali & Idaomar, 2008; Burt, 2004;
43 Calo, Baker, Park, & Ricke, 2015; Kim & Rhee, 2016; Lang & Buchbauer, 2012; Pinheiro et al.,
44 2015; Tajkarimi, Ibrahim, & Cliver, 2010). Another interesting area of application for EOs is
45 animal nutrition. The prophylactic use of antibiotics in the livestock industry to obtain
46 improvements in growth, feed consumption and decreased mortality caused by bacterial diseases
47 has been a common practice for decades, especially for swine and poultry. However, the concern

48 over the transmission and the proliferation of resistant bacteria via the food chain has led to the ban
49 of the feed use of antibiotic growth promoters in livestock within the European Union since 2006. A
50 wide range of EOs have the potential to act as multifunctional feed supplements for animals. Some
51 EOs, in fact, are reported to have multiple actions in monogastric animals, including effects on
52 performance, digestive systems, lipid metabolism, prevention of tissue oxidation and modulation of
53 microbial populations (Zhaikai et al., 2015; Mitsch et al., 2004; Liang et al., 2013).

54 EOs antimicrobial activity has been attributed mainly to phenolic compounds (Pesavento et al.,
55 2015), such as carvacrol and thymol (Cherallier, 1996; Lambert, Skandamis, Coote, & Nychas,
56 2001; Valero, 2006; Xu, Zhou, Ji, Pei, & Xu, 2008). They are additives generally recognized as safe
57 and they are widely used as food preservatives (Goñi et al., 2009; Lv, Liang, Yuan, & Li, 2011).
58 They can be directly incorporated into or coated onto packaging films, in order to enhance shelf-life
59 (Calo et al., 2015). However, their low solubility and bioavailability limit the cytotoxic potential on
60 bacteria, virus, fungi and parasites and their delivery is still a challenge (Kaur, Darokar, & Ahmad,
61 2014; Suntres, Coccimiglio, & Alipour, 2015). Additionally, the volatility and marked aromatic
62 note of a lot of EOs, which are appreciable features in applications such as aromatherapy or
63 perfume production, are conversely major limitations in food and feed applications. In fact, high
64 concentrations are needed to ensure food safety, but effective concentrations usually result in
65 negative flavour and in sensory changes, which discourages the consumption. The purpose of this
66 study is the synthesis, characterization and evaluation of the antibacterial activity of new carvacrol,
67 thymol and menthol derivatives (**Figure 1**). The compounds have been designed with the aim of
68 overcoming limitations, such as poor water solubility and volatility, still maintaining a good activity
69 against pathogens. In this way, it would be possible to exploit the antibacterial properties of EOs
70 active principles (i.e., menthol, carvacrol, thymol), but with more manageable compounds.
71 Compounds **1-8** were synthesized and fully characterized and their activity against *Clostridium*
72 *perfringens*, *Salmonella typhimurium*, *Salmonella enteritidis* and *Escherichia coli* is presented.

73

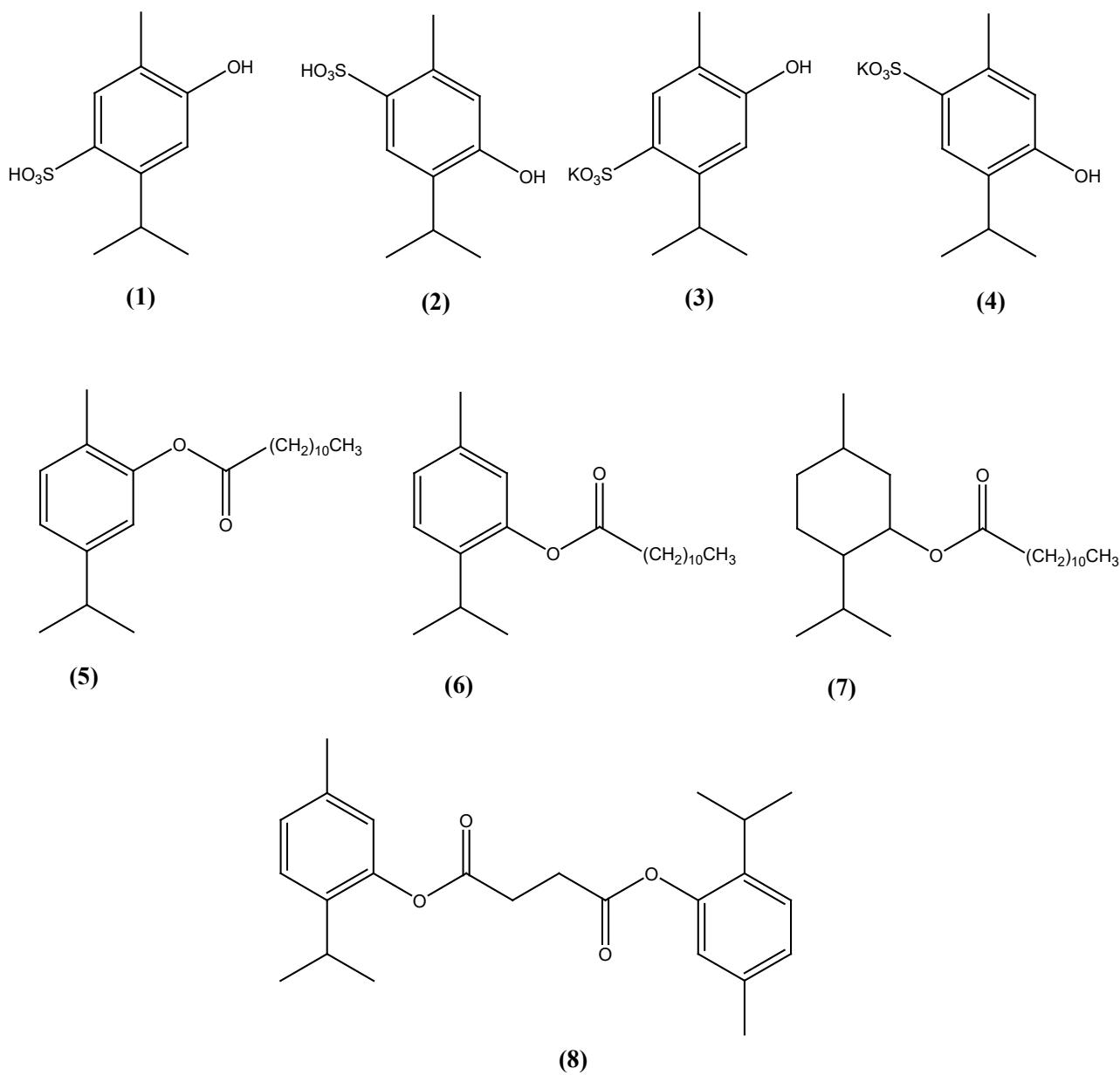


Figure 1. Schematic representation of carvacrol, thymol and menthol derivatives **1-8**.

79 **2. Materials and Methods.**

80 *2.1. Chemistry*

81 All reagents and solvents were commercially available. NMR spectra were recorded on Bruker
82 AVANCEIII (FT; 400 MHz, ^1H ; 75 MHz, $^{13}\text{C}\{^1\text{H}\}$). Chemical shifts (δ) for ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR
83 spectra were referenced using internal solvent resonances and were reported relative to
84 tetramethylsilane (TMS). FTIR spectra ($4000\text{--}700\text{ cm}^{-1}$) were recorded on a Nicolet Nexus
85 spectrophotometer equipped with a Smart Orbit HATR accessory (diamond crystal). Melting points
86 (mp) were determined using an Electrothermal melting point or a K fller apparatus and are
87 uncorrected (see Table 1). For **3-7** mass spectra were acquired in EI mode (positive ions) by mean
88 of a DEP-probe (Direct Exposure Probe) mounting on the tip a Pt-filament with a DSQII Thermo
89 Fisher apparatus equipped with a single quadrupole analyzer. The analyses were conducted in flash
90 mode with an amperage gradient of 100mA/sec up to 1000mA, correspondingly to an estimated
91 temperature of 1000  C. ESI mass of **8** was registered by using a Waters Acquity Ultrapformance
92 spectrometer equipped with a Single Quadrupole Detector and UPLC Acquity Waters source.
93 Working parameters were set as follows: source temperature 150 C, desolvation temperature
94 300 C, solvent flow 0.2 mL/min, capillary voltage 3 kV and cone voltage 60V. All mass spectra
95 were recorded in full scan analysis mode in the range 0-1000 m/z. Thermogravimetric analysis
96 (TGA) was performed on a TA Q50 ultramicro balance instrument (ramp rate = 5  C min⁻¹) and
97 under a N₂ flow rate of 90 mL min⁻¹ at atmospheric pressure.

98
99 ***4-hydroxy-2-isopropyl-5-methyl benzene sulfonic acid (1)***. Carvacrol (2.2 g, 0.013 mol) and
100 sulfuric acid 96%w (2.1 ml, 0.038 mol) were mixed for 2 hours under vacuum at 30 C. The crude
101 solid was recrystallized in water, yielding white crystals. IR (cm⁻¹): 3404br, 3221br, 2966w, 1734br,
102 1123m, 1164s, 1037s. $^1\text{H-NMR}$ (400 MHz, d₆-DMSO, ppm, δ): 1.10 (d, 6H, CH₃(*i*-Pr)); 2.06 (s,
103 3H, CH₃); 4.01 (m, 1H, CH(*i*-Pr)); 6.67 (s, 1H, CH(Ph)); 7.43 (s, 1H, CH(Ph)). EI-MS (m/z): 230.0,
104 [C₁₀H₁₄O₄S]⁺; 215.0, [C₉H₁₁O₄S]⁺.

105 ***4-hydroxy-5-isopropyl-2-methyl benzene sulfonic acid (2)***. Thymol (2.2 g, 0.013 mol) and sulfuric
106 acid 96%w (2.1 ml, 0.038 mol) were mixed for 2 hours under vacuum at 40 C. The crude solid was

107 recrystallized in water, yielding white crystals. IR (cm⁻¹): 3394br, 3285br, 2964w, 1701br, 1076m,
108 1011s. ¹H-NMR (400 MHz, d₆-DMSO, ppm, δ): 1.13 (d, 6H, CH₃(i-Pr)); 2.40 (s, 3H, CH₃); 3.14 (m,
109 1H, CH(i-Pr)); 6.56 (s, 1H, CH (Ph)); 7.51 (s, 1H, CH(Ph)). EI-MS (m/z): 230.0, [C₁₀H₁₄O₄S]⁺;
110 215.0, [C₉H₁₁O₄S]⁺.

111 **Potassium 4-hydroxy-2-isopropyl-5-methyl benzene sulfonate (3).** **1** (5g) was dissolved in 5 ml of
112 methanol and neutralized with a saturated solution of KOH in water (pH 7). The precipitate was
113 removed by Büchner filtration and the filtrate was vacuum-dried yielding a white solid (3.3g, 95%).
114 IR (cm⁻¹): 3213br, 2966w, 1853w, 1492w, 1410m, 1301w, 1272m, 1164m, 1134m, 1088m, 1042s,
115 976w, 887w, 717w, 665m, 609m, 580s. ¹H-NMR (400 MHz, d₆-DMSO, ppm, δ): 1.09 (d, 6H,
116 CH₃(i-Pr)); 2.04 (s, 3H, CH₃); 4.02 (m, 1H, CH(i-Pr)); 6.72 (s, 1H, CH(Ph)); 7.43 (s, 1H, CH(Ph));
117 9.22 (s, 1H, OH). ¹³C{¹H}-NMR (75 MHz, d₆-DMSO, ppm, δ): 15.49 (CH₃), 24.09 (CH₃(i-Pr)),
118 27.71(CH(i-Pr)), 111.81 (CH(Ph)), 118.98 (CH(Ph)), 129.39 (CH(Ph)), 136.27 (CH(Ph)), 145.16
119 (CH(Ph)), 155.77 (CH(Ph)). EI-MS (m/z): 230.0, [C₁₀H₁₄O₄S]⁺; 215.0, [C₉H₁₁O₄S]⁺.

120 **Potassium 4-hydroxy-5-isopropyl-2-methyl benzene sulfonate (4).** **2** (5g) was dissolved in 5 ml of
121 methanol and neutralized (pH 7) with a saturated solution of KOH in water. The precipitate was
122 removed by Büchner filtration and the filtrate was vacuum-dried yielding a white solid (3.2 g,
123 92%). IR (cm⁻¹): 3415wbr, 2996w, 2871vw, 1611w, 1578w, 1493w, 1459m, 1403w, 1339w,
124 1259w, 1203m, 1158s, 1130m, 1105w, 1079m, 1038s, 903w, 883w, 867w, 733m, 664s. ¹H-NMR
125 (400 MHz, d₆-DMSO, ppm, δ): 1.99 (d, 6H, CH₃(i-Pr)); 2.38 (s, 3H, CH₃); 3.16 (m, 1H, CH(i-Pr));
126 6.51 (s, 1H, CH(Ph)); 7.50 (s, 1H, CH(Ph)); 9.19 (s, 1H, OH). ¹³C{¹H}-NMR (75 MHz, d₆-DMSO,
127 ppm, δ): 19.81 (CH₃), 22.63 (CH₃(i-Pr)), 25.91 (CH(i-Pr)), 117.26 (CH(Ph)), 124.71 (CH(Ph)),
128 129.70 (CH(Ph)), 133.47 (CH(Ph)), 136.02 (CH(Ph)), 155.63 (CH(Ph)). EI-MS (m/z): 230.0,
129 [C₁₀H₁₄O₄S]⁺; 215.0, [C₉H₁₁O₄S]⁺.

130 **5-isopropyl-2-methyl phenyl dodecanoate (5).** Carvacrol (2 g, 0.013 mol), lauric acid (2.6 g, 0.013
131 mol) and phosphoric acid (3 drops) were mixed under magnetic stirring at 150°C under vacuum for
132 12 hours. The crude product was poured in chloroform (4ml) and purified by flash chromatography

133 (gradient elution: hexane:dichloromethane 8:2 and then ethyl acetate 100%), yielding a colourless
134 oil (2.2 g, 51%). IR (cm⁻¹): 2954m, 2916vs, 2848s, 1760m, 1701s, 1463m, 1428m, 1411m, 1302m,
135 1276m, 1247m, 1220m, 1193m, 1168m, 1141m, 1115m, 938m, 720m. ¹H-NMR (400 MHz, CDCl₃,
136 ppm, δ): 0.92 (t, 3H, CH₃); 1.26 (d, 6H, CH₃(i-Pr)); 1.30-1.47 (m, 14H, CH₂); 1.69 (m, 2H, CH₂);
137 1.80 (m, 2H, CH₂); 2.15 (s, 3H, CH₃); 2.59 (t, 2H, CH₂); 2.89 (m, 1H, CH(i-Pr)); 6.87 (d, 1H, CH
138 (Ph)); 7.03 (dd, 1H, CH(Ph)); 7.16 (dd, 1H, CH(Ph)). EI-MS (m/z): 150, [C₁₀H₁₄O]⁺; 135,
139 [C₁₀H₁₄]⁺; 332, [C₂₂H₃₆O₂]⁺.

140 **2-Isopropyl-5-methyl phenyl dodecanoate (6)**. Thymol (2 g, 0.013 mol), lauric acid (5.33 g, 0.026
141 mol) and phosphoric acid 85% (3 drops) are mixed under magnetic stirring at 150°C in vacuum for
142 12 hours. The crude product is poured in chloroform (4ml) and purified by flash chromatography
143 (silica, n-hexane 100%) yielding a colourless oil (1.2 g, 28%). IR (cm⁻¹): 2957m, 2923s, 2853m,
144 1709s, 1620w, 1584w, 1505w, 1456m, 1416m, 1378w, 1363w, 1290w, 1226m, 1150s, 1111m,
145 1087m, 1058w, 946m, 814m, 805m, 721w. ¹H-NMR (400 MHz, CDCl₃, ppm, δ): 0.91 (t, 3H, CH₃);
146 1.22 (d, 6H, CH₃(i-Pr)); 1.30-1.46 (m, 16H, CH₂); 1.82 (m, 2H, CH₂); 2.34 (s, 3H, CH₃); 2.60 (t,
147 2H, CH₂); 2.98 (m, 1H, CH(i-Pr)); 6.82 (d, 1H, CH(Ph)); 7.04 (dd, 1H, CH(Ph)); 7.21 (dd, 1H,
148 CH(Ph)). EI-MS (m/z): 150, [C₁₀H₁₄O]⁺; 135, [C₁₀H₁₄]⁺; 332, [C₂₂H₃₆O₂]⁺.

149 **2-Isopropyl-5-methyl cyclohexyl dodecanoate (7)**. Menthol (3 g, 0.019 mol), lauric acid (7 g, 0.35
150 mol) and phosphoric acid 85% (3 drops) are mixed under magnetic stirring at 100°C under vacuum
151 for 12 hours. The crude was poured in chloroform (4ml) and purified by flash chromatography
152 (silica, n-hexane 100%) yielding a colourless oil (1.6 g, 36%). IR (cm⁻¹): 2956m, 2922s, 2853m,
153 1731s, 1683m, 1635m, 1558w, 1456m, 1369w, 1248w, 1175m, 1149m, 1107w, 1012m, 983m. ¹H-
154 NMR (400 MHz, CDCl₃, ppm, δ): 0.78 (d, 6H, CH₃(i-Pr)); 0.86-1.09 (m, 11H, CH); 1.28-1.31 (m,
155 18H, CH₂); 1.51 (m, 1H, CH); 1.61-1.72 (m, 4H, CH), 1.88 (m, 1H, CH(i-Pr)); 2.00 (m, 1H,
156 CH(CHO)); 2.29 (t, 2H, CH₂), 4.70 (td, 1H, CHO). ESI-MS (m/z): 700, [C₄₄H₈₄O₄Na]⁺; 361,
157 [C₂₂H₄₂O₂Na]⁺.

158 ***Bis(2-isopropyl-5-methyl phenyl) succinate (8)***. Oxalyl chloride (1.7 ml, 0.02 mol) was added
159 under nitrogen to a solution of succinic acid (1g, 0.01 mol) in anhydrous THF (50 ml) in presence
160 of DMF as catalyst (3 drops) and stirred at r.t for 1 hour. Volatiles were removed under vacuum,
161 then dry THF (50 ml) was added and thymol (2.85g, 0.02 mol) was poured in the mixture; the
162 solution was stirred for additional 4 h at r.t.. The volatiles were removed again and ethyl acetate
163 (50ml) was added. The organic phase was washed twice with water (50ml) and with brine. Then it
164 was dried with sodium sulphate, filtered and the filtrate was concentrated, giving rise to a pale
165 yellow oil (1.8 g, 52%). IR (cm⁻¹): 3429br, 2961s, 2921m, 2871w, 1709s, 1619m, 1584m, 1518w,
166 1458m, 1419s, 1375m, 1336w, 1289s, 1259s, 1227s, 1152s, 1112w, 1087m, 1043m, 1005w, 945m,
167 855w, 807s, 738m. ¹H-NMR (400 MHz, CDCl₃, ppm, δ): 1.26 (d, 12H, CH₃(*i*-Pr)); 2.07 (s, 2H,
168 CH₂); 2.30 (s, 6H, CH₃); 3.19 (m, 2H, CH(*i*-Pr)); 6.60 (s, 2H, CH(Ph)); 6.75 (d, 2H, J= 7.6Hz,
169 CH(Ph)); 7.10 (d, 2H, J= 7.6Hz, CH(Ph)). ¹³C{¹H}-NMR (75 MHz, CDCl₃, ppm, δ): 20.87 (CH₃);
170 23.05 (CH₃(*i*-Pr)); 29.06 (CH₂); 116.03 (CH(Ph)); 121.59 (CH(Ph)); 126.23 (CH(Ph)); 131.48
171 (CH(Ph)); 136.56 (CH(Ph)); 116.03 (CH(Ph)); 152.62 (CO). ESI-MS (m/z): 405, [C₂₄H₃₀O₄Na]⁺;
172 363, [C₂₁H₂₄O₄Na]⁺; 273, [C₁₄H₁₈O₄Na]⁺.

173

174 2.2. Antimicrobial assays

175 ***Bacterial strains:*** the test microorganisms used in this study were isolated in poultry (at farm level).
176 *Clostridium perfringens* (strain 191999/2014) was isolated from broiler chickens affected by
177 necrotic enteritis. *Salmonella typhimurium* (strain 198306/2014) was isolated from viscera of egg-
178 table layers. *Salmonella enteritidis* (strain 226620/20149) was isolated from faeces taken from an
179 egg-table layer farm. *Escherichia coli* serotype O45 (strain 184049/2014) was isolated from broiler
180 chickens affected by avian colibacillosis. The bacterial strains were isolated and identified using
181 standard procedures adopted by IZSLER Forlì and maintained on slants with heart infusion agar
182 (HIA) (Becton Dickinson GmbH, Germany) at +4°C. To ensure culture purity, before the assays, a

183 sample of culture of *Clostridium perfringens* was streaked on blood agar base (Oxoid Ltd.,
184 Basingstoke, UK) with 5% sheep blood and incubated overnight at 37°C under anaerobic conditions
185 (GENbag anaer, bioMérieux S.A., Marcy l’Etoile, France). For the same reason, samples of cultures
186 of *Escherichia coli*, *Salmonella typhimurium* and *Salmonella enteritidis* were streaked on Hektoen
187 Enteric Agar (Becton Dickinson GmbH, Germany) and incubated overnight at 37°C. Then, one
188 colony of each strain was grown in Brain Heart Infusion (BHI) broth (Becton Dickinson GmbH,
189 Germany) and incubated overnight at 37°C (under anaerobic conditions for *Clostridium*
190 *perfringens*) and then titrated. For this purpose, serial 10-fold dilutions of each suspension were
191 carried out in Buffered Peptone Water (Oxoid Ltd., Basingstoke, UK); each dilution was streaked
192 on specific media and incubated overnight at 37°C (under anaerobic conditions for *Clostridium*
193 *perfringens*). Based on the results of the titration each bacterial suspension was diluted in BHI broth
194 to a final concentration of 2×10^6 cfu/mL. Each bacterial suspension was stored at +4°C until the
195 use as inoculum in the antibacterial test described in the next paragraph.

196 **Minimal Inhibitory Concentration (MIC):** MICs were determined using a micro-broth dilution
197 assay. Sterile 96-well microplates U-bottom were used (Cell Star, Greiner Bio-one, Germany) and
198 100 µl of fresh BHI broth was added to each well of the plate. Tested compounds were dissolved in
199 distilled water and 5% DMSO (v/v, Merck) to obtain a 20% stock solution and 100 µl of this
200 solution were added to each well of the first row. Then 100 µl were removed from the first row and
201 mixed five times with the broth in the corresponding well of the next row. This doubling dilution
202 was performed in column across the plate until the row “H” (100 µl removed from the row “H”
203 were discharged). Then 100 µl of each bacterial suspension were added to each well. This
204 procedure resulted in a final concentration of the bacterial inoculum of 1×10^6 cfu/mL and in a
205 gradient of two fold dilutions of the tested product ranging from 10% to 0.09% (v/v). All
206 experiments were performed in triplicate. The last three columns of each plate were used as control
207 (growth of the bacterial suspension, absence of bacterial contamination of the BHI broth and of the
208 stock solution of the test product). The microplates were sealed with parafilm and incubated at 37°C

209 for 24 hours (under anaerobic conditions for *Clostridium perfringens*). MIC was defined as the
210 lowest concentration of the test product that prevented visible bacterial growth in the triplicate
211 wells. The determinations were repeated three times and results were expressed as average values.

212 **Minimal Bactericidal Concentration (MBC):** MBC was determined inoculating the content of non-
213 growth wells on plates of Brain Heart Infusion Agar (Becton Dickinson GmbH, Germany). All
214 plates were incubated for 24 hours at 37°C (under anaerobic conditions for *Clostridium*
215 *perfringens*). The MBC was recorded as the lowest concentration without bacterial growth.

216 2.3 Cytotoxicity assay

217 **Human cell line:** The HT29 human colorectal carcinoma cell line was obtained from the Northern
218 Ireland Center for Food and Health. Cells were cultured in Dulbecco's Modified Eagle's medium
219 (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin-
220 streptomycin in a humidified atmosphere at 5% CO₂ and 37 °C. The cultured cells were trypsinized
221 with trypsin/EDTA for a maximum of 5 min and seeded with a subcultivation ratio of 1:3.
222 Determination of cell numbers and viabilities was performed with the trypan blue exclusion test.

223 **MTS assay:** The viability MTS assay (CellTiter96® AQueousOne Solution Cell Proliferation Assay,
224 Promega Corporation, Madison, WI, USA) was performed to assess the cytotoxicity of the most
225 antimicrobial drugs (carvacrol, thymol and compounds **1-4** and **8**) towards HT29 cell line. Tested
226 compounds were dissolved in DMSO to obtain a 10mM solution. 5×10^3 cells/well were seeded in
227 96-well plates in 100 µL of DMEM medium without phenol red, supplemented with 1% glutamine,
228 1% penicillin/streptomycin and 5% fetal bovine serum and then incubated at 37 °C in a humidified
229 (95%) CO₂ (5%) incubator. After 24 h, cells were treated, in quadruplicate, with increasing
230 concentrations of the compounds for further 24 h. The cytotoxicity assay was performed by adding
231 20 µL of the CellTiter96® AQueousOne Solution Cell Proliferation Assay directly to the culture
232 wells, incubating for 4 h and then recording the absorbance at 485 nm with a 96-well plate reader
233 (SPECTRAFlour, TECAN).

234

235 **3. Results and Discussion.**

236 *3.1. Chemistry*

237 The strategy used to overcome the problems of both solubility and volatility of thymol, carvacrol
238 and menthol, is to properly functionalize the aromatic ring (compounds **1-4**) or the hydroxyl moiety
239 (compounds **5-8**), developing two different classes of derivatives (**Figure 1**). The sulfonic
240 derivatives **1** and **2** have been obtained in a solvent free, one pot reaction by using an excess of
241 sulfuric acid (96% in water). Both the products are white, hygroscopic solids and are water soluble.
242 The corresponding potassium salts **3** and **4** were obtained through neutralization of a methanolic
243 solution of the sulfonic acid, with an aqueous solution of KOH. It is worth of note that these
244 syntheses, almost quantitative, do not require complex purification steps.

245 In the ¹H-NMR spectra of **1-4**, the substitution at the para-position of the phenolic OH can be
246 clearly evinced by the strong modification of the signal pattern in the aromatic region: the two
247 doublets and the singlet of the parent compounds thymol and carvacrol in the range 6.7-7.2 ppm are
248 substituted by two singlets at about 6.7 and 7.4 ppm. Moreover, the signals relative to the methyl
249 and the *i*-propyl substituents are affected in different way by the presence of the sulfonate moiety in
250 **1, 3** and **2, 4** respectively. In **1** and the corresponding potassium salt **3**, in fact, the presence of the
251 SO₃ moiety in *orto* to the *i*-propyl implies a shift in the resonances of the CH of about 1 ppm (about
252 4 ppm vs 3.1 ppm of pure carvacrol), while the methyl signal is little influenced. For **2** and **4**, on the
253 contrary, the signal more influenced by the substitution is the methyl one (about 2.4 ppm vs 2.1
254 ppm of the parent compound). In the IR of **1-4** it is possible to observe the presence of two strong
255 signals at about 1100 and 1000 cm⁻¹, ascribable to the SO₃ group. Mass data confirmed the
256 proposed stoichiometry. The thermogravimetric analysis on **3** shows a weight loss of 4% at 90°C
257 attributable to a loss of water and a very sharp step at about 270 °C, corresponding to the
258 decomposition of the product. Compounds **3** and **4** are white, crystalline solids stable at room

259 temperature with a high water solubility (**Table 1**) and without the pungent odour typical of their
260 precursor oils. These features are particularly attractive from a practical point of view, for potential
261 applications as food and feed additives.

262

	3	4	Carvacrol ^a	Thymol ^b
Solubility in water, t= 25°C	~250g/l	~190g/l	~0.8g/l	~0.9g/l
Melting point	>270°C	220°C	3-4°C	48-51°C

263

264 **Table 1:** Water solubility and melting point of **3** and **4** and their precursors. ^aLide, 1998

265 ^bYalkowsky, He & Jain, 2010.

266

267 The higher melting points of **3** and **4**, compared with those of the pure EOs, ensure better handling.
268 In fact, the volatility of EOs can involve a loss of the active ingredients during the production
269 processes (dosing, mixing, hot pelleting, transporting and packaging), storage and administration,
270 resulting in final amounts of EOs unavoidably and uncontrollably reduced. Furthermore, higher
271 flash points (lower flammability) result in more safety working conditions.

272 Another convenient strategy to modify the physico-chemical properties of EOs is represented by the
273 esterification of the phenolic moiety. The choice of the proper ester group could in principle allow
274 to couple the biological properties of EOs with the activity of other types of molecules, such as
275 caprylic acid (C8:0), capric acid (C10:0), and lauric acid (C12:0), medium chain fatty acids whose
276 antimicrobial properties have been demonstrated against various pathogens (Arnfinnsson, Steingri,
277 & Bergsson, 2001; Bergsson & Thormar, 2002; Desbois & Smith, 2010; Jang & Rhee, 2009; Nair,
278 Kumar, Jennifer, & Venkitanarayan, 2004; Thormar, Hilmarsson, & Bergsson, 2006; Wang &

279 Johnson, 1992). A synergic effect can be expected when such types of compounds are coupled with
280 EOs, resulting in enhanced bactericidal effects and consequent reduced quantity of antimicrobials
281 needed for food and feed treatment. With this strategy in mind, laurate esters of carvacrol, thymol
282 and menthol were obtained (compounds **5-7**). The synthesis is an esterification between the
283 hydroxyl group of the EO and the carboxyl group of the lauric acid. If in the classic Fisher
284 esterification the alcohol is in excess, conversely in this synthesis the carboxylic acid is in excess
285 over the alcohol. The solvent-free reaction is conducted in presence of an acid catalyst (H_3PO_4)
286 under vacuum, in order to remove water and promote the reaction. For all the substrates the crude
287 products were purified by flash chromatography on silica gel, yielding compounds **5-7** as colourless
288 oils. The presence of the alkyl moiety can be inferred in the $^1\text{H-NMR}$ spectra by the resonances in
289 the range 1.0-2.6 ppm, while in the IR spectra the stretching of the $\text{C}=\text{O}$ is evident at about 1700
290 cm^{-1} . Mass data confirmed the proposed stoichiometries. Contrary to compounds **1-4**, **5-7** are not
291 water soluble, but their lipophilicity can be very useful, for example for encapsulation in solid lipid
292 particles. The bioactive components dispersed throughout a solid lipid matrix can be applied in the
293 delivery of therapeutic agents, include oral, parenteral, and topical drug delivery (Bondi et al., 2007;
294 Liedtke, Wissing, Mu, & Ma, 2000; Mehnert & Mader, 2001; Radtke & Wissing, 2002).

295 The synthesis of the symmetric succinate ester of thymol **8** has been carried out by a multistep
296 procedure (**Figure 2**): first, the synthesis of the acyl chloride of succinic acid in dry THF was
297 performed, and then, after addition of thymol, the pure product **8** was obtained as an odourless
298 yellowish oil soluble in alcoholic solvents but not in water. Interestingly, esters **5-8** qualitatively
299 present an attenuated odour respect to the parent compounds.

300

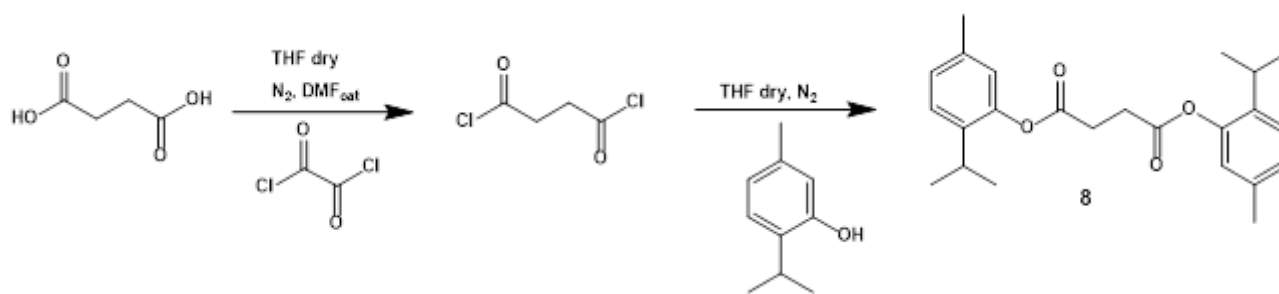


Figure 2. Multistep synthesis of **8**.

301

302

303

304 3.2. Antimicrobial activity

305 The *in vitro* antimicrobial activity of the EOs derivatives **1-8** has been tested and is presented in
 306 **Table 2**. Both the minimum inhibitory concentration (MIC) and minimum bactericidal
 307 concentration (MBC) are shown. Compounds **1-4** revealed an interesting antimicrobial profile. In
 308 particular, the sulfonic acids **1** and **2** are characterized by MIC values in the range 0.37%-0.75%
 309 towards the four tested strains. Their potassium salts (**3** and **4**) show good antibacterial activities as
 310 well, although slightly lower with respect to their precursors **1** and **2**; against *Clostridium*
 311 *perfringens* compound **3** is active at the lowest concentration (0.37%, **Table 2**). Even if the
 312 antibacterial activities of **1-4** are lower to the ones shown by carvacrol and thymol, the remarkable
 313 solubility of these compounds in water, compared to the insolubility of the parent compounds, is a
 314 matter of great interest. It has to be taken into account, in fact, that the use of thymol and carvacrol
 315 as preservatives, directly added to the food or coated in active packaging, is hampered by their both
 316 water insolubility and marked odour, which can alter food taste (Calo et al., 2015). The use of
 317 derivatives like **1-4** could in principle overcome these problems, since they can directly be added to
 318 the food or used in coating packaging with better handling and diminished taste modifications.
 319 Another important point regards animal nutrition. There is a diffused need to find effective
 320 alternatives to the use of antibiotics as growth promoters, since in many countries they have been
 321 restricted or banned because of the emergence of resistance bacterial strains via the food chain. The

322 use of EOs and their active principles have demonstrated promising results on animal performance
323 (Zhaikai et al., 2015; Mitsch et al., 2004; Liang et al., 2013), and they represent a very attractive
324 approach, but, again, as feed supplements they present serious limitations: they have stability
325 problems during pelleting processing and their marked odour discourages animal consumption.
326 Compounds **1-4**, characterised by low volatility and consequent much less marked odour, can be
327 used instead of thymol and carvacrol as feed supplier and could in principle be better tolerated.
328 Moreover, since they are water soluble, they can be directly used in animal drinking water.
329 Among all derivatives, the thymol succinate **8** was found to be the more active towards all tested
330 strains, with antibacterial activities analogous to that of the parent compound (MIC and MBC
331 values ≤ 0.09 , **Table 2**). On the contrary, the mono-esters **5**, **6** and **7** did not show significant
332 antimicrobial activity, with $MIC \geq 10\%$. It is worth noting again that compound **8** has a better
333 organoleptic profile with respect to thymol.

334 Generally, the EOs possessing the strongest antibacterial properties contain a high percentage of
335 phenolic compounds such as carvacrol and thymol. Both substances appear to make the cell
336 membrane permeable (Lambert et al., 2001). The significance of the phenolic ring itself is
337 demonstrated by the lack of activity of menthol compared to carvacrol (Ultee et al., 2002). This was
338 confirmed in our study, since the MICs values of compound **7** were $\geq 10\%$ (v/v). Interestingly, for
339 all tested strains, MICs and MBCs are the same, indicating the bactericidal role of these
340 compounds.

341 Most studies investigating the action of EOs against food spoilage organisms and food borne
342 pathogens agree that, generally, EOs are slightly more active against gram-positive than gram-
343 negative bacteria. In our study, the MICs and MBCs against the gram-positive *Clostridium*
344 *perfringens* were equal to or less than those observed against the tested gram-negative bacteria.
345 However, it has to be considered that previous *in vivo* studies indicated that the effects of the EOs
346 on the proliferation of *Clostridium perfringens* in the intestines of broiler chickens are only partially
347 due to a direct inhibition of the bacteria: digestive enzymes induced by EOs could also increase

348 nutrient digestibility and improve the regulation and stabilization of the gut microbiota (Mitsch, Ko,
 349 Gabler, Losa, & Zimpernik, 2004). Moreover, it is known that the chemical composition of EOs
 350 from a particular plant species can vary according to the geographical origin and harvesting period.
 351 It is therefore possible that variation in composition between batches of EOs is sufficient to cause
 352 variability in the degree of susceptibility of gram-negative and gram-positive bacteria (Dorman,
 353 Deans, Merr, & Myrtaceae, 2000).

354

	Inhibitory activity (% v/v) against bacterial strains							
	<i>Escherichia coli</i>		<i>Salmonella typhimurium</i>		<i>Salmonella enteritidis</i>		<i>Clostridium perfringens</i>	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	0.75	0.75	0.75	0.75	0.37	0.37	0.37	0.37
2	0.75	0.75	0.37	0.37	0.37	0.37	0.37	0.37
3	5	5	1.25	1.25	1.25	1.25	0.37	0.37
4	2.5	2.5	5	5	5	5	1.25	1.25
5	>10	>10	>10	>10	>10	>10	>10	>10
6	>10	>10	>10	>10	>10	>10	>10	>10
7	>10	>10	>10	>10	>10	>10	>10	>10
8	≤0.09	≤0.09	≤0.09	≤0.09	≤0.09	≤0.09	≤0.09	≤0.09
Thymol	≤0.09	≤0.09	≤0.09	≤0.09	≤0.09	≤0.09	≤0.09	≤0.09
Carvacrol	≤0.09	≤0.09	0.37	0.37	≤0.09	≤0.09	≤0.09	≤0.09

355

356 **Table 2.** MIC and MBC (% v/v) of the essential oils derivatives **1-8**.

357

358

359 *3.3 Cytotoxicity*

360 The good antimicrobial profile of compounds **1-4** and **8** allows to envisage the possibility to replace
 361 thymol, carvacrol or menthol, actually used as preservatives and in animal nutrition. In order to
 362 identify a possible risk for human health, we analyzed their cytotoxic profile on HT-29 human cells.
 363 Cytotoxic activity was detected through the MTS assay, a colorimetric method for determining the
 364 number of viable cells in proliferation and results are collected in **Table 3**. In the range of the doses
 365 used (1-100 μM), compounds **1-4** did not show any antiproliferative effects, with a profile even
 366 better of the parent compounds. Carvacrol and thymol, in fact, induced a mild cytotoxicity at the
 367 highest dose tested (100 μM). Compound **8** showed the highest antiproliferative effect on HT-29
 368 human cells, inducing a reduction of viability of approximately 50% at 100 μM . Therefore, the
 369 sulfonic derivatives and their potassium salts (**1-4**) are very promising candidates as novel
 370 antimicrobial compounds to be used as food preservatives as well as feed supplement.

371

Dose (μM)	GI%						
	Carvacrol	Thymol	1	2	3	4	8
0	100	100	100	100	100	100	100
1	92	100	100	100	100	100	100
5	100	100	97	100	100	100	93
10	88	100	100	100	100	100	100
50	92	100	95	100	100	100	83
100	72	79	100	100	100	100	56

372

373 **Table 3.** Antiproliferative effects induced by increasing concentrations of **1-4** and **8** (24h-treatment)
374 on HT-29 human cells, detected by MTS assay. GI% (Growth Inhibition percent). Dose 0: DMSO
375 at the highest concentration tested (1%).

376

377 **4. Conclusions**

378

379 The demand for natural alternatives to synthetic additives is rapidly increasing and the replacement,
380 in foodstuffs, of synthetic antimicrobials by EOs and their active principles is getting considerable
381 attention. Moreover, EOs have the potential to act as multifunctional feed supplements, avoiding or
382 limiting the use of antibiotic growth promoters in livestock, with consequent important applications
383 in the animal nutrition field. In the present work, we presented a series of carvacrol, menthol and
384 thymol derivatives with promising MICs and MBCs values against some pathogens causing
385 important foodborne diseases, *i.e.* *Clostridium perfringens*, *Salmonella typhimurium*, *Salmonella*
386 *enteritidis* and *Escherichia coli*. In order to identify a possible risk for human health, the most
387 active compounds (**1-4** and **8**) were tested for their cytotoxic activity on HT-29 human cells. While
388 **8** showed a not negligible toxicity, **1-4** are not cytotoxic, with a profile even better than the parent
389 compounds thymol and carvacrol.

390 Therefore, the low MICs and MBCs values of compounds **1-4**, combined with their water solubility,
391 absence of toxicity towards human cells and improvement of the organoleptic properties compared
392 to the parent compounds, confirmed the potential use for these derivatives in the food industry for
393 preservation of foodstuffs and increase of shelf life.

394 Further studies involving the incorporation of these compounds into foodstuffs are in progress. In
395 the same way, further studies are in progress to elucidate the *in vivo* effects of the most active
396 thymol and carvacrol derivatives in reducing the colonization of intestinal pathogenic bacteria and
397 in modulating the microbiota of the gastrointestinal tract.

398

399 **Notes**

400 The compounds disclosed in this paper are the subject of the pending International Patent

401 Application N. PCT/IB2015/053591.

402

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407

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