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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

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

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

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

1. Introduction.

Microorganisms causing food spoilage are a major concern for the food industry and the extension of shelf-life is an on-going demand for both retailers and consumers. Such extension is mainly achieved by technological improvements and addition of synthetic food preservatives. Natural products in general are an alternative to synthetic preservatives, and among them, essential oils (EOs) are typical antimicrobial agents without harmful residues. Since the 1990s, they have been widely studied for their antimicrobial activity and many EOs (e.g. thyme, oregano, cinnamon, horseradish) and their components have demonstrated antimicrobial efficacy against food-borne pathogenic and spoilage microorganisms (Arsi et al., 2014; Bakkali & Idaomar, 2008; Burt, 2004; Calo, Baker, Park, & Ricke, 2015; Kim & Rhee, 2016; Lang & Buchbauer, 2012; Pinheiro et al., 2015; Tajkarimi, Ibrahim, & Cliver, 2010). Another interesting area of application for EOs is animal nutrition. The prophylactic use of antibiotics in the livestock industry to obtain improvements in growth, feed consumption and decreased mortality caused by bacterial diseases 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 synthetized and fully characterized and their activity against *Clostridium*
72 *perfringens, Salmonella typhimurium, Salmonella enteritidis* and *Escherichia coli* is presented.
73

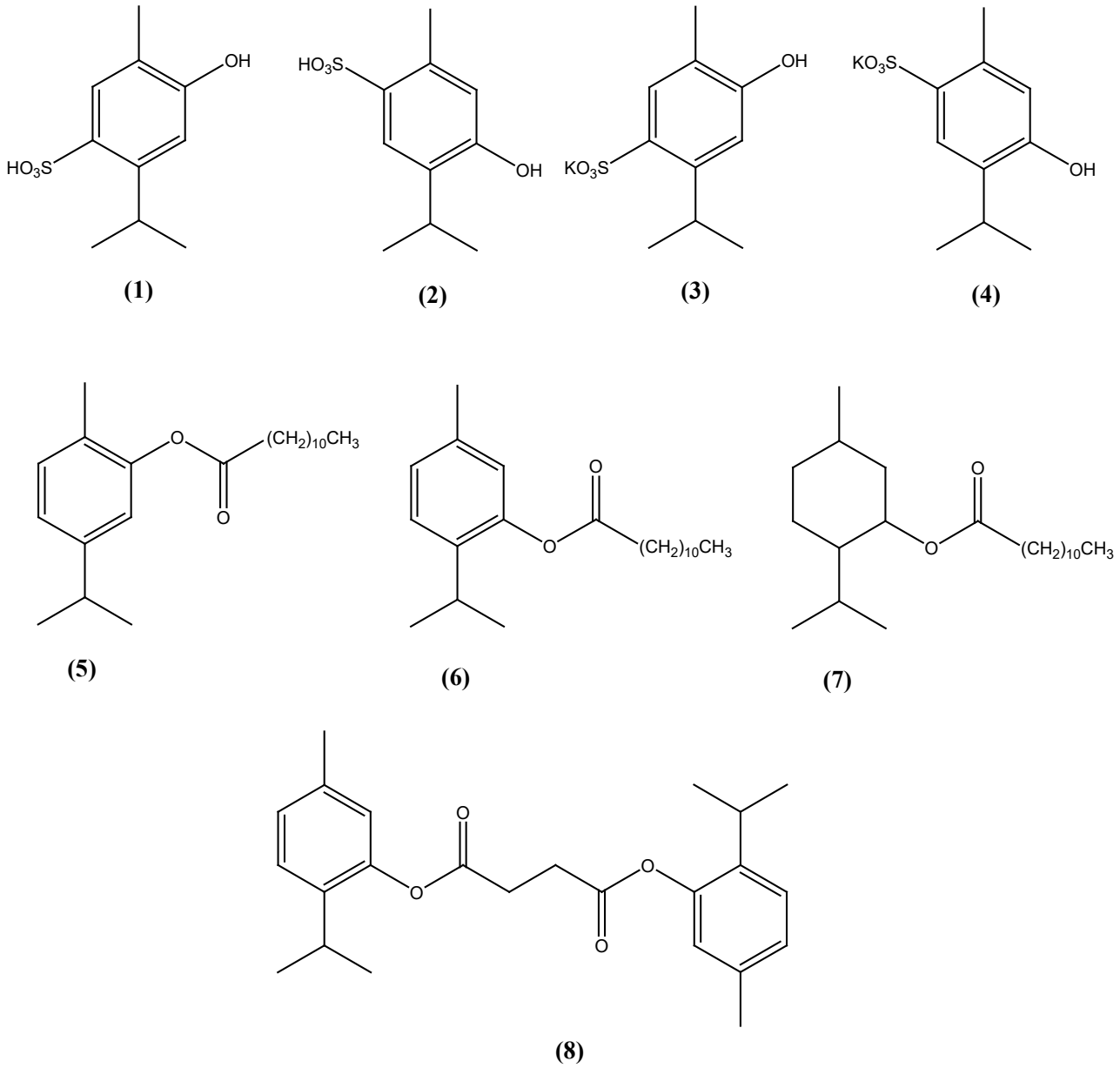

Figure 1. Schematic representation of carvacrol, thymol and menthol derivatives **1-8**.

2. Materials and Methods.

2.1. Chemistry

All reagents and solvents were commercially available. NMR spectra were recorded on Bruker AVANCEIII (FT; 400 MHz, ^{1}H; 75 MHz, $^{13}C\{^{1}H\}$). Chemical shifts (δ) for ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra were referenced using internal solvent resonances and were reported relative to tetramethylsilane (TMS). FTIR spectra (4000−700 cm^{-1}) were recorded on a Nicolet Nexus spectrophotometer equipped with a Smart Orbit HATR accessory (diamond crystal). Melting points (mp) were determined using an Electrothermal melting point or a Köfler apparatus and are uncorrected (see Table 1). For **3-7** mass spectra were acquired in EI mode (positive ions) by mean of a DEP-probe (Direct Exposure Probe) mounting on the tip a Pt-filament with a DSQII Thermo Fisher apparatus equipped with a single quadrupole analyzer. The analyses were conducted in flash mode with an amperage gradient of 100mA/sec up to 1000mA, correspondingly to an estimated temperature of 1000 °C. ESI mass of **8** was registered by using a Waters Acquity Ultraperformance spectrometer equipped with a Single Quadrupole Detector and UPLC Acquity Waters source. Working parameters were set as follows: source temperature 150°C, desolvation temperature 300°C, solvent flow 0.2 mL/min, capillary voltage 3 kV and cone voltage 60V. All mass spectra were recorded in full scan analysis mode in the range 0-1000 m/z. Thermogravimetric analysis (TGA) was performed on a TA Q50 ultramicro balance instrument (ramp rate = 5 °C min^{-1}) and under a N_2 flow rate of 90 mL min^{-1} at atmospheric pressure.

4-hydroxy-2-isopropyl-5-methyl benzene sulfonic acid (1). Carvacrol (2.2 g, 0.013 mol) and sulfuric acid 96%w (2.1 ml, 0.038 mol) were mixed for 2 hours under vacuum at 30°C. The crude solid was recrystallized in water, yielding white crystals. IR (cm^{-1}): 3404br, 3221br, 2966w, 1734br, 1123m, 1164s, 1037s. ^{1}H-NMR (400 MHz, d_6-DMSO, ppm, δ): 1.10 (d, 6H, CH_3(*i*-Pr)); 2.06 (s, 3H, CH_3); 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, $[C_{10}H_{14}O_4S]^+$; 215.0, $[C_9H_{11}O_4S]^+$.

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

recrystallized in water, yielding white crystals. IR (cm^{-1}): 3394br, 3285br, 2964w, 1701br, 1076m, 1011s.^{1}H-NMR (400 MHz, d_6-DMSO, ppm, δ): 1.13 (d, 6H, CH_3(*i*-Pr)); 2.40 (s, 3H, CH_3); 3.14 (m, 1H, CH(*i*-Pr)); 6.56 (s, 1H, CH (Ph)); 7.51 (s, 1H, CH(Ph)). EI-MS (m/z): 230.0, $[C_{10}H_{14}O_4S]^+$; 215.0, $[C_9H_{11}O_4S]^+$.

Potassium 4-hydroxy-2-isopropyl-5-methyl benzene sulfonate (3). **1** (5g) was dissolved in 5 ml of methanol and neutralized with a saturated solution of KOH in water (pH 7). The precipitate was removed by Büchner filtration and the filtrate was vacuum-dried yielding a white solid (3.3g, 95%). IR (cm^{-1}): 3213br, 2966w, 1853w, 1492w, 1410m, 1301w, 1272m, 1164m, 1134m, 1088m, 1042s, 976w, 887w, 717w, 665m, 609m, 580s. ^{1}H-NMR (400 MHz, d_6-DMSO, ppm, δ): 1.09 (d, 6H, CH_3(i-Pr)); 2.04 (s, 3H, CH_3); 4.02 (m, 1H, CH(i-Pr)); 6.72 (s, 1H, CH(Ph)); 7.43 (s, 1H, CH(Ph)); 9.22 (s, 1H, OH). ^{13}C{^{1}H}-NMR (75 MHz, d_6-DMSO, ppm, δ): 15.49 (CH_3), 24.09 (CH_3(i-Pr)), 27.71(CH(i-Pr)), 111.81 (CH(Ph)), 118.98 (CH(Ph)), 129.39 (CH(Ph)), 136.27 (CH(Ph)), 145.16 (CH(Ph)), 155.77 (CH(Ph)). EI-MS (m/z): 230.0, $[C_{10}H_{14}O_4S]^+$; 215.0, $[C_9H_{11}O_4S]^+$.

Potassium 4-hydroxy-5-isopropyl-2-methyl benzene sulfonate (4). **2** (5g) was dissolved in 5 ml of methanol and neutralized (pH 7) with a saturated solution of KOH in water. The precipitate was removed by Büchner filtration and the filtrate was vacuum-dried yielding a white solid (3.2 g, 92%). IR (cm^{-1}): 3415wbr, 2996w, 2871vw, 1611w, 1578w, 1493w, 1459m, 1403w, 1339w, 1259w, 1203m, 1158s, 1130m, 1105w, 1079m, 1038s, 903w, 883w, 867w, 733m, 664s. ^{1}H-NMR (400 MHz, d_6-DMSO, ppm, δ): 1.99 (d, 6H, CH_3(i-Pr)); 2.38 (s, 3H, CH_3); 3.16 (m, 1H, CH(i-Pr)); 6.51 (s, 1H, CH(Ph)); 7.50 (s, 1H, CH(Ph)); 9.19 (s, 1H, OH). ^{13}C{^{1}H}-NMR (75 MHz, d_6-DMSO, ppm, δ): 19.81 (CH_3), 22.63 (CH_3(i-Pr)), 25.91 (CH(i-Pr)), 117.26 (CH(Ph)), 124.71 (CH(Ph)), 129.70 (CH(Ph)), 133.47 (CH(Ph)), 136.02 (CH(Ph)), 155.63 (CH(Ph)). EI-MS (m/z): 230.0, $[C_{10}H_{14}O_4S]^+$; 215.0, $[C_9H_{11}O_4S]^+$.

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

(gradient elution: hexane:dichloromethane 8:2 and then ethyl acetate 100%), yielding a colourless oil (2.2 g, 51%). IR (cm^{-1}): 2954m, 2916vs, 2848s, 1760m, 1701s, 1463m, 1428m, 1411m, 1302m, 1276m, 1247m, 1220m, 1193m, 1168m, 1141m, 1115m, 938m, 720m. ^{1}H-NMR (400 MHz, $CDCl_3$, ppm, δ): 0.92 (t, 3H, CH_3); 1.26 (d, 6H, CH_3(i-Pr)); 1.30-1.47 (m, 14H, CH_2); 1.69 (m, 2H, CH_2); 1.80 (m, 2H, CH_2); 2.15 (s, 3H, CH_3); 2.59 (t, 2H, CH_2); 2.89 (m, 1H, CH(*i*-Pr)); 6.87 (d, 1H,CH (Ph)); 7.03 (dd, 1H, CH(Ph)); 7.16 (dd, 1H, CH(Ph)). EI-MS (m/z): 150, $[C_{10}H_{14}O]^+$; 135, $[C_{10}H_{14}]^+$; 332, $[C_{22}H_{36}O_2]^+$.

2-Isopropyl-5-methyl phenyl dodecanoate (6). Thymol (2 g, 0.013 mol), lauric acid (5.33 g, 0.026 mol) and phosphoric acid 85% (3 drops) are mixed under magnetic stirring at 150°C in vacuum for 12 hours. The crude product is poured in chloroform (4ml) and purified by flash chromatography (silica, n-hexane 100%) yielding a colourless oil (1.2 g, 28%). IR (cm^{-1}): 2957m, 2923s, 2853m, 1709s, 1620w, 1584w, 1505w, 1456m, 1416m, 1378w, 1363w, 1290w, 1226m, 1150s, 1111m, 1087m, 1058w, 946m, 814m, 805m, 721w. ^{1}H-NMR (400 MHz, $CDCl_3$, ppm, δ): 0.91 (t, 3H, CH_3); 1.22 (d, 6H, CH_3(*i*-Pr)); 1.30-1.46 (m, 16H, CH_2); 1.82 (m, 2H, CH_2); 2.34 (s, 3H, CH_3); 2.60 (t, 2H, CH_2); 2.98 (m, 1H, CH(*i*-Pr)); 6.82 (d, 1H, CH(Ph)); 7.04 (dd, 1H, CH(Ph)); 7.21 (dd, 1H, CH(Ph)). EI-MS (m/z): 150, $[C_{10}H_{14}O]^+$; 135, $[C_{10}H_{14}]^+$; 332, $[C_{22}H_{36}O_2]^+$.

2-Isopropyl-5-methyl ciclohexyl dodecanoate (7). Menthol (3 g, 0.019 mol), lauric acid (7 g, 0.35 mol) and phosphoric acid 85% (3 drops) are mixed under magnetic stirring at 100°C under vacuum for 12 hours. The crude was poured in chloroform (4ml) and purified by flash chromatography (silica, n-hexane 100%) yielding a colourless oil (1.6 g, 36%). IR (cm^{-1}): 2956m, 2922s, 2853m, 1731s, 1683m, 1635m, 1558w, 1456m, 1369w, 1248w, 1175m, 1149m, 1107w, 1012m, 983m. ^{1}H-NMR (400 MHz, $CDCl_3$, ppm, δ): 0.78 (d, 6H, CH_3(*i*-Pr)); 0.86-1.09 (m, 11H, CH); 1.28-1.31 (m, 18H, CH_2); 1.51 (m, 1H, CH); 1.61-1.72 (m, 4H, CH), 1.88 (m, 1H, CH(*i*-Pr)); 2.00 (m, 1H, CH(CHO)); 2.29 (t, 2H, CH_2), 4.70 (td, 1H,CHO). ESI-MS (m/z): 700, $[C_{44}H_{84}O_4Na]^+$; 361, $[C_{22}H_{42}O_2Na]^+$.

Bis(2-isopropyl-5-methyl phenyl) succinate (8). Oxalyl chloride (1.7 ml, 0.02 mol) was added under nitrogen to a solution of succinic acid (1g, 0.01 mol) in anhydrous THF (50 ml) in presence of DMF as catalyst (3 drops) and stirred at r.t for 1 hour. Volatiles were removed under vacuum, then dry THF (50 ml) was added and thymol (2.85g, 0.02 mol) was poured in the mixture; the solution was stirred for additional 4 h at r.t.. The volatiles were removed again and ethyl acetate (50ml) was added. The organic phase was washed twice with water (50ml) and with brine. Then it was dried with sodium sulphate, filtered and the filtrate was concentrated, giving rise to a pale yellow oil (1.8 g, 52%). IR (cm^{-1}): 3429br, 2961s, 2921m, 2871w, 1709s, 1619m, 1584m, 1518w, 1458m, 1419s, 1375m, 1336w, 1289s, 1259s, 1227s, 1152s, 1112w, 1087m, 1043m, 1005w, 945m, 855w, 807s, 738m. ^{1}H-NMR (400 MHz, $CDCl_3$, ppm, δ): 1.26 (d, 12H, CH_3(*i*-Pr)); 2.07 (s, 2H, CH_2); 2.30 (s, 6H, CH_3); 3.19 (m, 2H, CH(*i*-Pr)); 6.60 (s, 2H, CH(Ph)); 6.75 (d, 2H, J= 7.6Hz, CH(Ph)); 7.10 (d, 2H, J= 7.6Hz, CH(Ph)). ^{13}C{^{1}H}-NMR (75 MHz, $CDCl_3$, ppm, δ): 20.87 (CH_3); 23.05 (CH_3(i-Pr)); 29.06 (CH_2); 116.03 (CH(Ph)); 121.59 (CH(Ph)); 126.23 (CH(Ph)); 131.48 (CH(Ph)); 136.56 (CH(Ph)); 116.03 (CH(Ph)); 152.62 (CO). ESI-MS (m/z): 405, $[C_{24}H_{30}O_4Na]^+$; 363, $[C_{21}H_{24}O_4Na]^+$; 273, $[C_{14}H_{18}O_4Na]^+$.

2.2. Antimicrobial assays

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

sample of culture of *Clostridium perfringens* was streaked on blood agar base (Oxoid Ltd., Basingstoke, UK) with 5% sheep blood and incubated overnight at 37°C under anaerobic conditions (GENbag anaer, bioMérieux S.A., Marcy l'Etoile, France). For the same reason, samples of cultures of *Escherichia coli*, *Salmonella typhimurium* and *Salmonella enteritidis* were streaked on Hektoen Enteric Agar (Becton Dickinson GmbH, Germany) and incubated overnight at 37°C. Then, one colony of each strain was grown in Brain Heart Infusion (BHI) broth (Becton Dickinson GmbH, Germany) and incubated overnight at 37°C (under anaerobic conditions for *Clostridium perfringens*) and then titrated. For this purpose, serial 10-fold dilutions of each suspension were carried out in Buffered Peptone Water (Oxoid Ltd., Basingstoke, UK); each dilution was streaked on specific media and incubated overnight at 37°C (under anaerobic conditions for *Clostridium perfringens*). Based on the results of the titration each bacterial suspension was diluted in BHI broth to a final concentration of 2X106 cfu/mL. Each bacterial suspension was stored at +4°C until the use as inoculum in the antibacterial test described in the next paragraph.

Minimal Inhibitory Concentration (MIC): MICs were determined using a micro-broth dilution assay. Sterile 96-well microplates U-bottom were used (Cell Star, Greiner Bio-one, Germany) and 100 µl of fresh BHI broth was added to each well of the plate. Tested compounds were dissolved in distilled water and 5% DMSO (v/v, Merck) to obtain a 20% stock solution and 100 µl of this solution were added to each well of the first row. Then 100 µl were removed from the first row and mixed five times with the broth in the corresponding well of the next row. This doubling dilution was performed in column across the plate until the row "H" (100 µl removed from the row "H" were discharged). Then 100 µl of each bacterial suspension were added to each well. This procedure resulted in a final concentration of the bacterial inoculum of 1X106 cfu/mL and in a gradient of two fold dilutions of the tested product ranging from 10% to 0.09% (v/v). All experiments were performed in triplicate. The last three columns of each plate were used as control (growth of the bacterial suspension, absence of bacterial contamination of the BHI broth and of the stock solution of the test product). The microplates were sealed with parafilm and incubated at 37°C

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

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

2.3 Cytotoxicity assay

Human cell line: The HT29 human colorectal carcinoma cell line was obtained from the Northern Ireland Center for Food and Health. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin–streptomycin in a humidified atmosphere at 5% CO_2 and 37 °C. The cultured cells were trypsinized with trypsin/EDTA for a maximum of 5 min and seeded with a subcoltivation ratio of 1:3. Determination of cell numbers and viabilities was performed with the trypan blue exclusion test.

MTS assay: The viability MTS assay (CellTiter96® AQ_{ueous}One Solution Cell Proliferation Assay, Promega Corporation, Madison, WI, USA) was performed to assess the cytotoxicity of the most antimicrobial drugs (carvacrol, thymol and compounds **1**-**4** and **8**) towards HT29 cell line. Tested compounds were dissolved in DMSO to obtain a 10mM solution. 5×10^3 cells/well were seeded in 96-well plates in 100 µL of DMEM medium without phenol red, supplemented with 1% glutamine, 1% penicillin/streptomycin and 5% fetal bovine serum and then incubated at 37 °C in a humidified (95%) CO_2 (5%) incubator. After 24 h, cells were treated, in quadruplicate, with increasing concentrations of the compounds for further 24 h. The cytotoxicity assay was performed by adding 20 µL of the CellTiter96® AQ_{ueous}One Solution Cell Proliferation Assay directly to the culture wells, incubating for 4 h and then recording the absorbance at 485 nm with a 96-well plate reader (SPECTRAFlour, TECAN).

3. Results and Discussion.

3.1. Chemistry

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

In the ^{1}H-NMR spectra of **1-4**, the substitution at the para-position of the phenolic OH can be clearly evinced by the strong modification of the signal pattern in the aromatic region: the two doublets and the singlet of the parent compounds thymol and carvacrol in the range 6.7-7.2 ppm are substituted by two singlets at about 6.7 and 7.4 ppm. Moreover, the signals relative to the methyl and the *i*-propyl substituents are affected in different way by the presence of the sulfonate moiety in **1**, **3** and **2**, **4** respectively. In **1** and the corresponding potassium salt **3**, in fact, the presence of the SO_3 moiety in *orto* to the *i*-propyl implies a shift in the resonances of the CH of about 1 ppm (about 4 ppm vs 3.1 ppm of pure carvacrol), while the methyl signal is little influenced. For **2** and **4**, on the contrary, the signal more influenced by the substitution is the methyl one (about 2.4 ppm vs 2.1 ppm of the parent compound). In the IR of **1-4** it is possible to observe the presence of two strong signals at about 1100 and 1000 cm^{-1}, ascribable to the SO_3 group. Mass data confirmed the proposed stoichiometry. The thermogravimetric analysis on **3** shows a weight loss of 4% at 90°C attributable to a loss of water and a very sharp step at about 270 °C, corresponding to the 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. [a]Lide, 1998
265 [b]Yalkowsky, 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}H-NMR spectra by the resonances in
289 the range 1.0-2.6 ppm, while in the IR spectra the stretching of the C=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

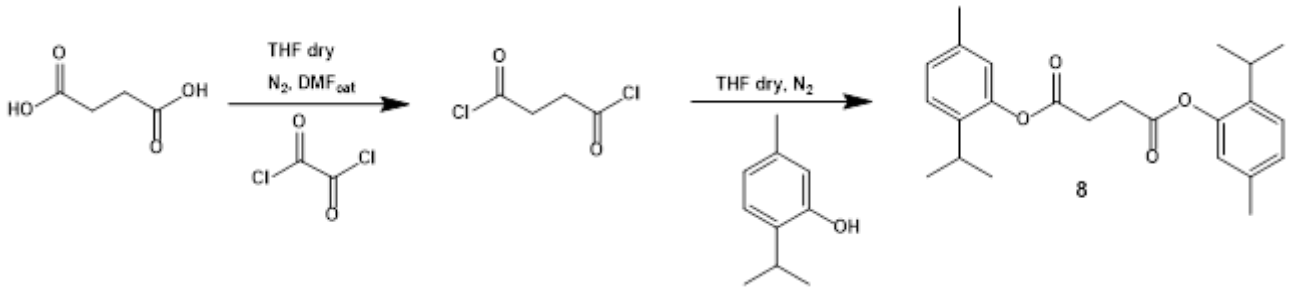

Figure 2. Multistep synthesis of **8**.

3.2. Antimicrobial activity

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

use of EOs and their active principles have demonstrated promising results on animal performance (Zhaikai et al., 2015; Mitsch et al., 2004; Liang et al., 2013), and they represent a very attractive approach, but, again, as feed supplements they present serious limitations: they have stability problems during pelleting processing and their marked odour discourages animal consumption. Compounds **1**-**4**, characterised by low volatility and consequent much less marked odour, can be used instead of thymol and carvacrol as feed supplier and could in principle be better tolerated. Moreover, since they are water soluble, they can be directly used in animal drinking water.

Among all derivatives, the thymol succinate **8** was found to be the more active towards all tested strains, with antibacterial activities analogous to that of the parent compound (MIC and MBC values ≤0.09, **Table 2**). On the contrary, the mono-esters **5**, **6** and **7** did not show significant antimicrobial activity, with MIC≥10%. It is worth noting again that compound **8** has a better organoleptic profile with respect to thymol.

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

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

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

	Inhibitory activity (% v/v) against bacterial strains							
	Escherichia coli		*Salmonella typhimurium*		*Salmonella enteritidis*		*Clostridium perfringens*	
	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

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

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.

Notes

The compounds disclosed in this paper are the subject of the pending International Patent Application N. PCT/IB2015/053591.

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