1 **Full title: Antibacterial and antifungal activity of essential oils against some**
2 **pathogenic bacteria and yeasts shed from poultry**
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4 **Short title: Antimicrobial activity of essential oils against pathogens of poultry**
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8 Valentina Virginia Ebani [a,c], Simona Nardoni [a,c], Fabrizio Bertelloni [a], Silvia Giovanelli [b],
9 Guido Rocchigiani [a], Luisa Pistelli [b,c], Francesca Mancianti [a,c]
10
11
12
13 [a] Department of Veterinary Science, University of Pisa, viale delle Piagge 2, 56124 Pisa,
14 Italy
15 [b] Department of Pharmacy, University of Pisa, via Bonanno 6, 56126 Pisa, Italy
16 [c] Centro Interdipartimentale di Ricerca "Nutraceutica e Alimentazione per la Salute",
17 University of Pisa, via del Borghetto 80, 56124 Pisa, Italy
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27 Corresponding author:
28 Valentina V. Ebani, DVM, PhD
29 Department of Veterinary Science, University of Pisa
30 Viale delle Piagge, 2
31 56124 Pisa, Italy
32 Tel: +39 0502216968
33 Email: valentina.virginia.ebani@unipi.it
34
35

36 **Abstract**

37 Antibiotics have been used for decades in poultry diets to increase performance and
38 decrease morbidity and mortality. The growing concern over the spreading of antibiotic-
39 resistant bacteria among animals and humans has resulted in the ban of the feed use of
40 antibiotic growth promoters in livestock and in some cases additives derived from plants
41 are used as alternative.

42 Four commercial essential oils, from litsea (*Litsea cubeba* L.), oregano (*Origanum*
43 *vulgare* L. subsp. *hirtum*), marjoram (*Origanum majorana* L.), thymus (*Thymus vulgaris*
44 L.) and their mixtures, were tested against pathogenic bacteria and yeasts that may be shed
45 in feces by poultry. In particular, the analysis were carried out against reference and wild
46 bacterial strains of *Salmonella enterica* serovar Typhimurium, *Yersinia enterocolitica*,
47 *Listeria monocytogenes*, *Enterococcus durans*, *E. faecalis*, and *E. faecium*, and wild
48 isolates of *Candida albicans, C. tropicalis, C. guilliermondii, C. krusei, C. parapsilosis*
49 and *Saccharomyces cerevisiae.*

50 Essential oils had varying degrees of growth inhibition in relationship to the tested
51 bacterial and yeast strains; however the best results were achieved by *O. vulgare* and *T.*
52 *vulgaris*.

53 All mixtures gave good results with reference and field bacterial strains, with MIC values
54 ranging from 1.130 to 0.138 gr/ml. The mixture composed by *O. vulgare, T. serpyllum*
55 and *O. majorana* appeared the most effective against the tested yeast isolates, with MIC
56 1.849 mg/ml

57 *O. vulgare* and *T. vulgaris* showed good antimicrobial activities, thus they seem useful not
58 only to promote poultry growth, but also to control fastidious microorganisms commonly
59 occurring in digestive tract of these animals.

60

61 **Key words:** Poultry; Essential oils; Antibacterial activity; Antifungal activity; Enteric
62 Pathogens

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64

65 **Introduction**

66 Essential oils (EOs) are more or less volatile substances with more or less odorous impact,
67 produced either by steam distillation or dry distillation or by means of a mechanical
68 treatment from one single species. [1]

69 EOs and other plant extracts possess antibacterial, antifungal and antiviral properties and
70 have been screened worldwide as potential sources of novel antimicrobial compounds,
71 alternatives to treat infectious diseases and to promote growth and nutrient utilization. [2, 3,
72 4, 5, 6, 7]

73 Poultry often shed with feces pathogen bacteria and yeasts that can pollute the
74 environment and infect other animals and humans.

75 Salmonellae are Gram-negative zoonotic pathogens of the *Enterobacteriaceae* family.
76 Important diseases of poultry are caused by members of *Salmonella* genus: the host-
77 adapted serovars Pullorum and Gallinarum are the agent of Pullorum disease and Fowl
78 typhoid, respectively, whereas other serotypes, particularly Typhimurium and Enteritidis,
79 are cause of infections in birds and mammals, including humans. [8]

80 *Yersinia enterocolitica*, member of the *Enterobacteriaceae* family, is a zoonotic enteric
81 pathogen usually transmitted by swine, but avian species, in particular poultry, may act as
82 amplifier hosts. [9]

83 *Listeria monocytogenes* is a Gram-positive cocco-bacillary bacterium implicated in
84 diseases of many domestic and wild animal species and humans. *L. monocytogenes* causes
85 septicaemia in poultry and other birds, but avian hosts may harbor and excrete with feces
86 this pathogen without developing diseases.

87 The Gram-positive *Enterococcus* species are enteric streptococci, which are found in the
88 intestinal tract of birds and mammals. They are opportunistic pathogens and may cause
89 both septicaemic and localized infections in chickens, turkeys, ducks, pigeons and other
90 birds. [10]

91 Bacterial resistance to multiple antibiotics is a serious health problem. In fact, pathogenic
92 bacteria often are resistant to one or more antibiotics, representing a severe threat for the
93 successful treatment of animal and human infections. Moreover, multi-drug resistant
94 bacteria act as efficient donors of resistance genes.

95 Yeasts are part of normal microflora and invasive infections arise when barrier leakage or
96 impaired immune function occurs. So both environmental yeasts such as *Candida* non-
97 *albicans* species and endosaprophytes such as *C. albicans* can act as opportunist
98 pathogens leading to mucosal and invasive diseases, in animals and both in
99 immunocompetent and in immunocompromised patients [11] showing different patterns of
100 antimycotic sensitivity.

101 The aim of the present study was to evaluate the *in vitro* antibacterial and antifungal
102 activities of four essential oils, alone and in mixture, against the most frequent pathogens

103 excreted in poultry feces. The choice of the EOs was done on the basis of their
104 antimicrobial activity reported in literature and on their availability on the market.

105 **Experimental**

106 ***Essential oils***

107 The study was carried out using four EOs: litsea (*Litsea cubeba* (Lour.) Pers.), oregano
108 (*Origanum vulgare* L. subsp. *hirtum*), marjoram (*Origanum majorana* L.) and thymus
109 (*Thymus vulgaris* L.). All EOs were purchased directly from the market (FLORA®, Pisa,
110 Italy). They were stored at 4°C in dark glass bottles and were subjected to microbial
111 analysis for quality control before their employment in the tests. Dilutions of each oil
112 carried out in peptone water were spread onto agar plate count (APC) and these were
113 enumerated after incubation at 30 °C for 72 h.

114 On the basis of the effectiveness of these oils, three mixtures were set up and assayed
115 against all selected agents: OT (*O.vulgare* and *T. vulgaris)*, OTM (*O. vulgare, T. vulgaris*
116 and *O. majorana)*, and OTL (*O. vulgare, T. vulgaris* and *L. cubeba*).

117

118 ***Gas Chromatography – Mass Spectrometry Analysis***

119 The GC analysis were accomplished with an HP-5890 Series II instrument equipped with
120 a HP-Wax and HP-5 capillary columns (both 30 m x 0.25 mm, 0.25 μm film thickness),
121 working with the following temperature program: 60°C for 10 min, rising at 5°C/min to
122 220°C. The injector and detector temperatures were maintained at 250°C; carrier gas,
123 nitrogen (2 mL/min); detector, dual FID; split ratio 1:30. The volume injected was 0.5 μL.
124 The relative proportions of the oil constituents were percentages obtained by FID peak-
125 area normalization without the use of a response factor. GC-MS analyses were performed
126 with a Varian CP-3800 gas chromatograph equipped with a DB-5 capillary column (30 m
127 x 0.25; coating thickness, 0.25 μm) and a Varian Saturn 2000 ion trap mass detector.
128 Analytical conditions were as follows: injector and transfer line temperatures, 220 and
129 240°C at 3°C/min, respectively; oven temperature, programmed from 60 to 240°C at
130 3°C/min; carrier gas, helium at 1 mL/min; injection, 0.2 μL (10% hexane solution); split
131 ratio, 1:30. Identification of the constituents was based on comparison of the retention
132 times with those of authentic samples, comparing their linear retention indices relative to
133 the series of n-hydrocarbons, and on computer matching against commercial and home-
134 made library mass spectra built up from pure substances and components of known oils
135 and MS literature data. [12, 13]

136

137

138 ***Antibacterial activity***

139 *Bacterial strains*

140 EOs were individually tested against 6 wild bacterial strains and 6 ATCC (American Type
141 of Culture Collection) strains, belonging to the species *Salmonella enterica* serovar
142 Typhimurium, *Yersinia enterocolitica*, *Listeria monocytogenes*, *Enterococcus durans*, *E.*
143 *faecalis*, and *E. faecium*.

144 The field strains have been previously isolated from poultry fecal samples, typed and
145 stored at -80°C in glycerol broth.

146

147 *Agar disc diffusion method*

148 Kirby-Bauer agar disc diffusion method was used to determine the antibacterial activity of
149 the EOs, following the procedures described by Clinical and Laboratory Standards
150 Institute[14] with some modifications. Briefly, active cultures were prepared by transferring
151 a loopful of bacterial cells from the stock cultures to tubes containing brain hearth infusion
152 broth (BHIB, Oxoid LTD Basingstoke, Hampshire, England) that were incubated for 24 h
153 at 37ºC. The cultures were suspended in sterile saline solution to obtain a turbidity
154 equivalent to a 0.5 McFarland standard, approximately 1 to 2 × 10^7 CFU/ml. The
155 microbial suspension was streaked over the surface of Mueller Hinton agar (MHA, Oxoid)
156 plates using a sterile cotton swab in order to get a uniform microbial growth on test plates.
157 Under aseptic conditions, absorbent paper discs (diameter 6 mm, Whatman paper No.1,
158 Oxoid) were placed on the agar plates and 10 µl from a 1:10 dilution in dimethyl sulfoxide
159 (DMSO, Oxoid) for each oil and each mixture was put on the discs. Negative controls
160 were prepared using a filter paper disc impregnated only with 10 µl of DMSO.

161 The plates were incubated at 37ºC for 24 h, followed by the measurement of the diameter
162 of the growth inhibition zone expressed in millimetres (mm). All tests were performed in
163 triplicate.

164 Bacterial strains were tested by Kirby-Bauer method to evaluate their *in vitro* sensitivity to
165 5 Antibiotics (Oxoid): tetracycline (30 µg), ceftazidime (30µg), rifampicin (30 µg),
166 cephalexin (30 µg), and cefotaxime (30 µg). The results were interpreted on the basis of
167 the indications suggested by the National Committee for Clinical Laboratory Standards
168 (NCCLS). [15]

169

170
171
172 *Minimum inhibitory concentration*
173 The minimum inhibitory concentration (MIC) values were determined for each bacterial
174 strain, which was sensitive to the EOs in Kirby-Bauer assay.
175 MIC was tested with the broth microdilution method on the basis of the guidelines of
176 NCCLS (1990) and the protocol reported by Lević et al. [16] with some modifications. The
177 bacterial inoculates were prepared using overnight cultures and suspensions were adjusted
178 to 0.5 McFarland standard turbidity.
179 The assays were carried out in BHIB. The aliquots of 20 µl of each oil and mixture were
180 added into each well of a 96-well microtitre plate, in dilutions ranging from 1000 to 8
181 µl/ml. Then 160 µl of BHIB were added and 20 µl of each bacterial suspension were
182 inoculated into each well. The test was performed in a total volume of 200 µl with final
183 EOs concentrations of 100 to 0.8 µl/ml.
184 Plates were incubated at 37°C for 24 hours. The same assay was performed
185 simultaneously for bacterial growth control (tested bacteria and BHIB) and sterility
186 control (tested oil or mixture and BHIB). All tests were performed in triplicate.
187 The MIC value was defined as the lowest concentration of EO/mixture at which
188 microorganisms show no visible growth.
189
190 ***Antimycotic activity***
191 *Yeasts species*
192 The efficacy of the selected EOs was tested against 5 *Candida* isolates (*C. albicans, C.*
193 *tropicalis, C. guilliermondii, C. krusei* and *C. parapsilosis*). *Saccharomyces cerevisiae* can
194 be administered to broilers as probiotic for its activity on performance and immune
195 modulatory functions, [17] for this reason N. 1 isolate of this fungal species was tested to
196 evaluate a possible inhibitory activity of tested EOs. All yeasts had been isolated from
197 poultry droppings and identified by their morphological and physiological features.
198 Definitive identification was achieved by ID32C galleries (BioMerieux, Marcy l'Etoile,
199 France).
200
201 *Microdilution test*
202 Antimycotic activity of selected EOs was assessed by broth microdilution method in malt
203 extract broth following the guidelines of EUCAST modified by Budzynska et al., [18] using

204 sweet almond fatty oil (*Prunus dulcis* Mill. D.A. Webb.) instead of Tween 20 for
205 preparing yeast suspension. Dilutions (v/v%) of 10%, 7.5%, 5%, 1%, 0.75%, 0.5% and
206 0.25% of EO solution were employed. All tests were carried out in triplicate. Mixtures in
207 almond oil were dissolved into the medium and assayed at 1%, 0.75%, 0.5% and 0.25%
208 dilutions. Control cultures tested versus sweet almond EO were achieved. Results were
209 expressed as mg/ml.
210
211
212 *Etest*
213 Etest (BioMerieux, Marcy l'Etoile, France) was performed as recommended by the
214 manufacturer. Strips containing anidulafungin, amphotericine B, caspofungine,
215 fluconazole, micafungine, posaconazole and voriconazole were used.
216
217 **Results**
218 ***EOs composition***
219 The chemical composition of the tested EOs is reported in Table 1. All the EOs showed a
220 common aspect: the large predominance of monoterpenes, that constitute nearly the total
221 amount of constituents in the EOs. In fact, they were rich in oxygenated monoterpenes
222 (ranging from 64.2% to 76.8%) followed by monoterpene hydrocarbons (from 15.2% to
223 27.7%). However the main compounds in each EO are quite different, except for *O.*
224 *vulgare* and *O. majorana*, where carvacrol stand out from the others. *O. vulgare* EO was
225 characterized by carvacrol as main compound (65.9%), while thymol was the principal
226 constituent present with high percentage in *T. vulgaris* (52.6%). *O. majorana* showed
227 carvacrol as major compound (20.8%) even though in about half amount if compared with
228 *O. vulgare*, followed by other two oxygenated monoterpenes as Terpinen-4-ol (17.6%)
229 and *trans*-sabinene hydrate (12.8%). The EO of *L. cubeba* evidenced good amount of
230 geranial and neral (36.9% and 32.0% respectively) together with limonene (a monoterpene
231 hydrocarbon, 10.8%). All the analysed EOs presented a chemical composition in
232 agreement with the literature data for the EOs obtained from the same plant material. [19,
233 20, 21]
234
235 ***Antibacterial activity***
236 The results of the present study showed that the selected EOs had varying degrees of
237 growth inhibition against the tested bacterial strains. The diameters of inhibition zone and

238 the MIC values testing each bacterial strain with the different EOs and mixtures are
239 reported in Tables 2 and 3. No inhibition zone was observed when DMSO was tested as
240 negative control.
241 The results of the disc diffusion method revealed that EO from *O. majorana* has a very
242 low or no activity against the tested ATCC bacteria.
243 The most relevant results were obtained with *O. vulgare* against the same strains. MIC
244 values of *O. vulgare* were 2.367 gr/ml with *E. durans*, *E. faecalis, E. faecium* and *S.* ser.
245 Typhimurium, 1.183 gr/ml with *L. monocytogenes,* 0.587 gr/ml with *Y. enterocolitica*.
246 Testing field bacterial isolates, *O. vulgare* and *T. vulgaris* resulted the most active:
247 oregano had a 1.183 gr/ml MIC with *L. monocytogenes* and 0.587 gr/ml with *Y.*
248 *enterocolitica*, while 2.367 gr/ml was the value for the remaining isolates; thymus resulted
249 no active against *E. durans*, but showed a 2.342 gr/ml MIC with the other bacteria.
250 All the prepared mixtures gave good results with reference and field strains, with MIC
251 values ranging from 1.130 gr/ml to 0.138 gr/ml.
252 Table 4 shows the results obtained by Kirby-Bauer test with five antibiotics against all the
253 bacterial strains.
254
255 ***Antimycotic activity***
256 The selected EOs, showed a variable degree of antimycotic activity at tested dilutions,
257 with MICs ranging from 0.937 to 14.055 mg/ml. Sweet almond EO did not inhibit yeasts'
258 growth. MIC values varied among the different fungal species tested. In general terms
259 most effective EOs was *O. vulgare* active against all tested yeasts with a MIC range from
260 0.947 to 4.735. Among all tested fungal species *C. krusei* had the lowest MIC values while
261 *C. tropicalis* appeared to be less sensitive. In general MIC values of different mixtures
262 were lower with respect to the single EO, ranging from 1.844 to 3.768 mg/ml, except for
263 *C. guilliermondii* which showed low MICs for *O. vulgare* (0.947 mg/ml) and *T vulgaris*
264 (0.947 mg/ml). The mixture composed by *O. vulgare, O. majorana* and *.T vulgaris*
265 appeared to be the most effective. *C. krusei* and *C. albicans* had the lowest MIC values
266 when tested versus mixtures.,
267 The anti-yeast activity of conventional antimycotic drugs tested was consistent with data
268 available from literature (Pfaller et al., 2006; Pfaller et al., 2015)
269 [1] More detailed data are reported in Tables 5 and 6.
270
271 **Discussion**

272 The results obtained in the present study show that the examined EOs have different
273 degrees of efficacy in relation to the selected microorganisms. The variations in the EO
274 content and aromatic profile are reflected in the different activities.
275 To the best of our knowledge, this is the first paper comprehending a wide number of both
276 bacteria and yeasts obtained by digestive tract of poultry, acting as true or opportunistic
277 pathogens, tested versus four chemically defined EOs.
278 The antibacterial activities of EOs from different plants have been demonstrated in several
279 *in vitro* studies. [24, 25, 26, 27] However, data obtained were very heterogeneous being related
280 to different plants, climate conditions, cultivation methods or harvesting areas and
281 bacterial strains. Moreover, the results are strongly related to EOs content, aromatic
282 profiles and the used method.
283 In the present study, bacterial isolates obtained from poultry fecal samples together with
284 their corresponding ATCC species, have been tested. Results are largely varying on the
285 basis of bacterial strain and EO tested.
286 Gram-negative bacteria resulted generally more sensitive to EOs when compared to Gram-
287 positive, according to some authors. [28, 29] The difference in sensitivity to EOs could be
288 related to the cell wall structure. In fact, Gram-negative bacteria have a thin peptidoglycan
289 layer and an outer membrane containing lipopolysaccharide (LPS) and phospholipids. On
290 the other hand Gram-positive microorganisms have a thicker peptidoglycan layer.
291 Some authors affirm that carvacrol and thymol are able to disintegrate the outer membrane
292 of Gram-negative bacteria, releasing LPS and increasing the permeability of the
293 cytoplasmic membrane to ATP and depolarize the same membrane. [30, 31]
294 Other authors found EOs more active against Gram-positive bacteria. The structure of the
295 Gram-positive bacteria cell wall allows hydrophobic molecules to easily penetrate the
296 cells and act on both the cell wall and within the cytoplasm. Phenolic compounds, which
297 are also present in the EOs, generally show antimicrobial activity against Gram-positive
298 bacteria. Their effect depends on the amount of the compound present; at low
299 concentrations, they can interfere with enzymes involved in the production of energy, and
300 at higher concentrations, they can denature proteins.
301 However, the degree of susceptibility of Gram-positive and Gram-negative bacteria is
302 strictly related to the bacterial species and the chemical composition of EOs. [32]
303 Gram-negative species tested in the present investigation were *S.* serov. Typhimurium and
304 *Y. enterocolitica* that are frequently excreted in feces by broilers and laying hens. ATCC
305 *Salmonella* resulted quite susceptible to *O. vulgare* (2.367 gr/ml) and *T. vulgaris* (1.171

306 gr/ml), whereas the field *Salmonella* strain showed moderate sensitivity to *O. vulgare*
307 (2.367 gr/ml), *O. majorana* (4.470 gr/ml) and *T. vulgaris* (2.342 gr/ml). Both reference
308 and field *Salmonella* strains resulted sensible to all the mixtures assayed (0.565 - 0.138
309 gr/ml).
310 High antimicrobial activity was observed with *O. vulgare* against *Y. enterocolitica* (0.587
311 gr/ml) in both ATCC and field strains, probably to the high amount of carvacrol, that it is
312 demonstrated as one of the most antibacterial EO components.
313 All the mixtures of the selected EOs showed similar activity against reference and field *Y.*
314 *enterocolitica* strains, with the minimum value of MIC (0.138 gr/ml).
315 Gram-positive bacteria included in the present study are *L. monocytogenes* and three
316 *Enterococcus* species, that are the most frequently found enterococci in poultry.
317 *O. vulgare* showed a good activity against *L. monocytogenes* (1.183 gr/ml) in both ATCC
318 and field isolates.
319 *L. cubeba* resulted active against the field strain of this pathogen (1.107 gr/ml), whereas
320 no activity was observed against the ATCC strain. These results could indicate that the
321 sensitivity of listeriae are strongly related to the strain. There are very few data about the
322 antimicrobial activity of this plant, even though some authors consider its EO with marked
323 antimicrobial activity against *L. monocytogenes.* [33]
324 Enterococci, both reference and field strains, resulted more resistant than the other
325 bacterial species to the tested EOs. Enterococci are often resistant to more antibiotics, as
326 also demonstrated in Table 4; in fact they have intrinsic resistance to many antimicrobial
327 agents and are able to acquire antibiotic-resistance determinants. For these reasons they
328 represent a severe threat for the therapy of animal and human infections. [34] Among the
329 tested enterococcal strains, *E. durans* appeared the most resistant; in fact thymus EO had
330 no activity against this species which is probably intrinsically resistant to the chemical
331 components of *T. vulgaris*.
332 The three mixtures assayed gave good results against both listeria and enterococci, with
333 MIC values ranging from 0.282 to1.130 gr/ml.
334 Data dealing with *in vitro* sensitivity available from the literature cannot be easily
335 compared with results obtained in the present study, due to the different methodologies
336 used, to different origin of tested yeast isolates and to the lack of data about susceptibility
337 of some fungal species to some examined EOs. Our data about *O. vulgare* agree with Cleff
338 et al., [35] who documented an *in vitro* efficacy of this EO versus *C. krusei, C. albicans* and
339 *C. parapsilosis.* Furthermore *O. vulgare* was the sole EO active against *C. albicans.*

340 *O. majorana* EO failed to inhibit growth of all fungal species. The ineffectiveness against
341 *C. albicans* was reported by Leeja and Thoppil [36] and by Kozlowska et al. [37] These
342 authors also reported the inefficacy against *C.parapsilosis*, conversely the same EO
343 appeared to be effective against *C. tropicalis* and partially effective versus *C. krusei* and
344 *C. guilliermondii.*
345 *T. vulgaris* EO has a well-known antimicrobial and antimycotic effect, [38] but in the
346 present study was active against *C. krusei* and *C.guilliermondii*, in disagreement with
347 Farrukh et al. [39]
348 *L. cubeba* was effective against *C. krusei* and *C.guilliermondii*, but did not act against *C.*
349 *albicans, C. tropicalis* and *C.parapsilosis*. The only data available from literature [40]
350 dealed with the effectiveness of this EO against *C. albicans* and referred a good
351 antimycotic activity, not confirmed by our results.
352 From a general point of view the MICs showed by mixtures were lower than the
353 respective values of single EOs.
354 A number of published results concerning the activity of essential oils containing
355 carvacrol and/or thymol against poultry pathogens are present in literature, however our
356 study comprehends also oils with a different composition (i.e. *L. cubeba*) both alone and
357 in mixture. Furthermore several both fungal and bacterial agents have been simultaneously
358 tested.
359
360 In conclusion, *O. vulgare* and *T. vulgaris* showed the highest antimicrobial activity against
361 the Gram positive and Gram negative tested pathogens, according to some previous
362 studies. [41, 42] These EOs, in particular *O. vulgare* seemed to be active against most of the
363 yeast isolates too.
364 The three tested mixtures showed relevant activities against all the selected bacterial
365 strains and even though their effectiveness against yeasts appeared more variable, their
366 MICs versus *C. albicans, C.parapsilosis* and *C. tropicalis* appeared to be strongly lower.
367 The further goals would be to set up EOs mixture for *in vivo* administration, both to
368 promote growth and to control fastidious microorganisms commonly occurring in
369 digestive tract of poultry. [2]
370 Considered that *S. cerevisiae* had a moderate sensitivity against *O. vulgare* EO, in respect
371 to all the examined *Candida* spp., and together with *C. parapsilosis* showed the highest
372 MIC versus *O. vulgare* and *T. vulgaris* in mixture, the use of such EOs in mixture would
373 not interfere with its growth along with its probiotic action.

374
375
376
377 **Conflict of interest**

378 The Authors declare no conflict of interest statement.

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Table 1 – Relative percentage of the main constituents of essential oils detected by GC-MS analysis.

Compounds	LRI§	*Litzea cubeba*	*Origanum majorana*	*Origanum vulgare*	*Thymus vulgaris*
(E)-2-Hexenal	873				0.1
α-Thujene	932		0.8	0.8	0.1
α-Pinene	940	1.1	0.7	1.0	0.8
α-Fenchene	951			0.1	
Camphene	955	0.3			0.3
Sabinene	978	0.9	3.2		
β-Pinene	981	0.9	0.7	0.4	
1-Octen-3-ol	982				0.5
6-Methyl-5-hepten-2-one	990	1.4			
Myrcene	993	1.1	1.5	2.2	0.7
3-Octanol	998		0.1	0.1	0.1
α-Phellandrene	1006		0.2	0.3	
α-Terpinene	1019		4.7	2.1	0.8
p-Cymene	1028		4.2	**9.3**	**15.3**
Limonene	1032	**10.8**	2.1	0.7	0.4
1,8-Cineole	1036	0.6	0.1	0.8	0.7
(E)-*β*-Ocimene	1053		0.1	0.1	
γ-Terpinene	1062		7.9	5.2	2.9
cis-Sabinene hydrate	1072		3.2	0.3	0.1
Terpinolene	1090	0.1	1.5	0.3	0.2
trans-Sabinene hydrate	1098		**12.8**	1.8	3.8
Linalool	1102	1.7			
cis-para-Mentha-2-en-1-ol	1121		0.9		
cis-Pinene hydrate	1144		0.6		
Camphor	1148		0.2	0.1	0.5
Citronellal	1155	1.7			
Borneol	1169		0.2	0.3	1.6
4-Terpineol	1180	0.3	**17.6**	0.9	2.4
iso-Verbanol	1180		0.2		
α-Terpineol	1192	0.8	2.7	0.2	0.4
unknown					1.7
neoiso-Verbanol	1190		0.2		
Verbenone	1205		0.3		
Nerol	1228	0.8			
Thymol methyl ether	1232				1.7
Neral	1242	**32.0**			
Carvone	1248		0.6		
Geraniol	1259	1.5	2.7		
Linalool acetate	1260		3.2		
Geranial	1276	**36.9**			
Citronellyl formate	1280	0.5			

Isobornyl acetate	1285		0.2	0.1	
Thymol	1290		0.2	0.9	**52.6**
Carvacrol	1299		**20.8**	**65.9**	0.2
α-Cubebene	1351	0.3			
Eugenol	1361			0.1	
Carvacrol acetate	1371				0.2
α-Copaene	1376		0.1		
β-Bourbonene	1383				0.1
β-Elemene	1392	0.2			
β-Caryophyllene	1418	2.2	1.7	3.7	6.8
β-Gurjunene	1432				0.4
α-Humulene	1456	0.2		0.1	0.2
Bicyclogermacrene	1495	0.1			
α-Muurolene	1499		1.4		
β-Bisabolene	1509		0.1	0.4	
trans-γ-Cadinene	1513				0.7
δ-Cadinene	1523				1.0
Spathulenol	1577		0.2		
Caryophyllene oxide	1582	0.3	0.2	0.4	
Monoterpene hydrocarbons		15.2	27.7	22.6	21.5
Oxygenated monoterpenes		**76.8**	**66.7**	**71.3**	**64.2**
Sesquiterpenes hydrocarbons		3.0	3.3	4.2	9.2
Oxygenated sesquiterpenes		0.3	0.4	0.4	-
Others		1.4	0.1	0.2	2.4
Total		**96.7**	**98.2**	**98.7**	**97.3**

Table 2- Antibacterial activity: zone of inhibition of the selected EOS and mixtures according to the Kirby-Bauer method against the selected ATCC and field bacterial strains.

Essential oils	STRAINS *Salmonella* Typhimurium ATCC 14028	*Yersinia enterocolitica* ATCC 55075	*Listeria monocytogenes* ATCC 7644	*Enterococcus durans* ATCC 19432	*Enterococcus faecium* ATCC 19434	*Enterococcus faecalis* ATCC 19433
	M SD	M SD	M SD	M SD	M SD	M SD
L. cubeba	7.0 ± 0.0	15.7 ± 0.6	0.0 ± 0.0	7.0 ± 0.0	7.0 ± 0.0	7.0 ± 0.0
T. vulgaris	9.0 ± 0.0	14.7 ± 1.5	10.3 ± 0.6	0.0 ± 0.0	10.3 ± 1.5	8.7 ± 0.6
O. majorana	8.3 ± 0.6	7.0 ± 0.0	7.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
O. vulgare	15.3 ± 0.6	22.7 ± 1.5	11.0 ± 0.0	14.3 ± 0.6	13.7 ± 1.2	11.7 ± 1.5
Mixtures						
OT	13.3 ± 0.6	21.0 ± 3.6	12.7 ± 0.6	22.7 ± 0.6	19.7 ± 0.6	16.3 ± 0.6
OTM	12.3 ± 0.6	18.7 ± 1.2	12.7 ± 0.6	18.3 ± 1.2	15.7 ± 0.6	15.0 ± 1.0
OTL	9.3 ± 1.5	21.7 ± 1.5	15.3 ± 1.2	14.3 ± 0.6	16.7 ± 0.6	12.3 ± 1.2

Essential oils	*Salmonella* Typhimurium	*Yersinia enterocolitica*	*Listeria monocytogenes*	*Enterococcus durans*	*Enterococcus faecium*	*Enterococcus faecalis*
	M SD	M SD	M SD	M SD	M SD	M SD
L. cubeba	8.0 ± 0.0	17.3 ± 0.6	11.7 ± 0.6	7.0 ± 0.0	7.7 ± 0.6	8.3 ± 0.6
T. vulgaris	11.3 ± 0.6	24.7 ± 1.5	10.7 ± 0.6	0.0 ± 0.0	16.0 ± 1.0	14.3 ± 0.6
O. majorana	10.7 ± 0.6	10.3 ± 0.6	8.0 ± 1.0	7.7 ± 0.6	9.0 ± 0.0	0.0 ± 0.0

O. vulgare	11.3 ± 0.6	31.7 ± 1.5	18.0 ± 2.0	12.7 ± 0.6	13.3 ± 0.6	12.0 ± 1.0
Mixtures						
OT	14.7 ± 0.6	30.3 ± 0.6	29.0 ± 1.0	23.7 ± 0.6	16.0 ± 0.0	14.7 ± 0.6
OTM	13.0 ± 0.0	22.3 ± 1.5	21.7 ± 1.5	15.3 ± 0.6	17.7 ± 0.6	13.7 ± 0.6
OTL	10.7 ± 0.6	19.3 ± 0.6	20.0 ± 1.7	15.7 ± 0.6	12.0 ± 0.0	11.7 ± 0.6

Legenda – M: mean expressed in mm; SD: standard deviation; OT: mixture *O.vulgare* and *T. vulgaris*; OTM: mixture *O. vulgare*, *T. vulgaris* and *O. majorana*; OTL: mixture *O. vulgare*, *T. vulgaris* and *L. cubeba*

Table 3 - Antimicrobial activity expressed as the minimum inhibitory concentration (gr/ml) of EOs and mixtures against selected ATCC and field bacterial strains.

	STRAINS					
	Salmonella Typhimurium ATCC 14028	*Yersinia enterocolitica* ATCC 55075	*Listeria monocytogenes* ATCC 7644	*Enterococcus durans* ATCC 19432	*Enterococcus faecium* ATCC 19434	*Enterococcus faecalis* ATCC 19433
Essential oils						
L. cubeba	17.720	1.107	-	17.720	17.720	17.720
T. vulgaris	1.171	1.171	1.171	-	2.342	4.685
O. majorana	17.880	17.880	17.880	-	-	-
O. vulgare	2.367	0.587	1.183	2.367	2.367	2.367
Mixtures						
OT	0.565	0.141	0.282	0.565	1.130	0.565
OTM	0.277	0.138	0.554	0.554	1.108	0.554
OTL	0.276	0.138	0.553	0.553	1.106	0.553
	Salmonella Typhimurium	*Yersinia enterocolitica*	*Listeria monocytogenes*	*Enterococcus durans*	*Enterococcus faecium*	*Enterococcus faecalis*
Essential oils						
L. cubeba	17.720	8.860	1.107	17.720	17.720	17.720
T. vulgaris	2.342	2.342	2.342	-	2.342	2.342
O. majorana	4.470	4.470	17.880	17.880	17.880	-
O. vulgare	2.367	0.587	1.183	2.367	2.367	2.367
Mixtures						
OT	0.565	0.141	0.565	0.565	0.565	0.565
OTM	0.138	0.138	0.554	0.554	0.554	0.554

OTL	0.138	0.138	0.553	0.553	0.553	0.553

Legenda – OT: mixture *O.vulgare* and *T. vulgaris*; OTM: mixture *O. vulgare*, *T. vulgaris* and *O. majorana*; OTL: mixture *O. vulgare*, *T. vulgaris* and *L. cubeba*

Table 4 – The inhibition zones resulted from the application of different antibiotics against selected bacterial strains.

	ANTIBIOTICS				
STRAINS	**Tetracycline (30 µg/disc)**	**Ceftazidime (30 µg/disc)**	**Rifampicin (30 µg/disc)**	**Cephalexin (30 µg/disc)**	**Cefotaxime (30 µg/disc)**
S. ser. Typhimurium ATCC14028	18 (S)	22 (S)	15 (R)	20 (S)	26 (S)
Y. enterocolitica ATCC 55075	26 (S)	23 (S)	17 (I)	0 (R)	24 (S)
L. monocytogenes ATCC7644	26 (S)	0 (R)	32 (S)	24 (S)	20 (I)
E. durans ATCC19432	24 (S)	0 (R)	18 (I)	13 (R)	0 (R)
E. faecium ATCC19434	24 (S)	0 (R)	17 (I)	10 (R)	0 (R)
E. faecalis ATCC19433	10 (R)	0 (R)	20 (S)	14 (R)	17 (I)
S. ser. Typhimurium	18 (S)	19 (S)	15 (R)	21 (S)	25 (S)
Y. enterocolitica	22 (S)	27 (S)	17 (I)	0 (R)	32 (S)
L. monocytogenes	26 (S)	0 (R)	28 (S)	21 (S)	10 (R)
E. durans	24 (S)	0 (R)	33 (S)	14 (R)	0 (R)
E. faecium	7 (R)	0 (R)	30 (S)	0 (R)	0 (R)
E. faecalis	10 (R)	0 (R)	15 (R)	13 (R)	18 (I)

Legenda – S: susceptible; R: resistant; I: intermediate

Table 5 - Minimum inhibitory concentration (% v/v) of essential oils and their mixtures against selected yeasts.

	STRAINS					
	Candida albicans	*Candida guilliermondii*	*Candida tropicalis*	*Candida parapsilosis*	*Candida krusei*	*Saccharomyces cerevisiae*
Essential oils						
L. cubeba	7.5	0.75	7.5	7.5	1	1
T. vulgaris	7.5	0.5	7.5	7.5	1	1
O. majorana	7.5	7.5	7.5	7.5	7.5	1
O. vulgare	1	0.5	2	1	1	1
Mixtures						
OT	1	1	1	1	1	1
OTM	0.5	1	1	1	0.5	0.75
OTL	0.5	0.5	0.5	1	0.5	1

Legenda – OT: mixture *O.vulgare* and *T. vulgaris*; OTM: mixture *O. vulgare*, *T. vulgaris* and *O. majorana*; OTL: mixture *O. vulgare*, *T. vulgaris* and *L. cubeba*

Table 6 - *In vitro* sensitivity of selected yeasts against conventional antimycotic drugs.

Yeast	**AND**	**AB**	**CS**	**FL**	**MYC**	**PO**	**VO**
Candida albicans	S	S	S	R	S	S	S
Candida guilliermondii	S	S	S	S	S	S	S
Candida krusei	S	S	R	R	S	S	S
Candida parapsilosis	R	S	R	R	S	S	S
Candida tropicalis	R	S	S	S	S	S	S
Saccharomyces cerevisiae	S	S	S	S	S	S	S

Legenda: AND- anidulafungin; AB-amphotericin b; CS-caspofungin; FL-fluconazole; MYC- micafungin; PO-posaconazole; VO-voriconazole; S-sensitive; R-resistant