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Effect of drying and interfacial membrane composition on the antimicrobial activity of emulsified citral

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Abstract

Citral-in-water emulsions were prepared with two different essential oil concentrations of 2.5 and 5.0% (w/w), then spray-dried in the presence of the same amount of maltodextrins (20%). The microcapsules were prepared with two different emulsifier compositions: monolayer microcapsules (ML) stabilized by sodium caseinate alone and layer-by-layer microcapsules (LBL) stabilized by sodium caseinate and pectin. The encapsulation efficiency was higher for LBL microcapsules (*e.g.* 99.6 ± 0.4 % for 2.5% citral) than that for ML ones (*e.g.* 78.6 ± 0.6 % for 2.5% citral) which confirm that the additional pectin layer was able to protect citral during the spray-drying process whatever citral concentration. Furthermore, our results showed that the antibacterial activity of the obtained microcapsules significantly depends on both citral concentration and interfacial membrane composition. The presence of two layers surrounding the citral droplets may result in a progressive and controlled release of the encapsulated citral.

Keywords: Citral; Spray-drying; Microencapsulation; Antibacterial activity; Interfaces.

1. Introduction

Essential oils, as secondary metabolites of plants, have many applications in cosmetics, food flavoring and preservation as well as in pharmaceutical industries. To satisfy the growing demand for new and natural antimicrobials for preventing microbial food spoilage and bacterial infections, many research work are carried out on essential oils (Kayode et al., 2018; Khorshidian, Yousefi, Khanniri, & Mortazavian, 2018; Prakash, Baskaran, Paramasivam, & Vadivel, 2018).

Due to the common features of most essential oils as their hydrophobic, volatile and chemically unstable nature, many studies focused on improving their dispersion in aqueous-based product. The challenge is also to maintain their flavor and their potential bioactivity by developing delivery systems containing essential oils (Prakash, Baskaran, Paramasivam, & Vadivel, 2018). The delivery system which encapsulate essential oils can be prepared by emulsification, spray-drying, coaxial electrospray system, freeze-drying, coacervation, *in situ* polymerization, melt-extrusion, supercritical fluid technology, and fluidized-bed-coating (Bakry, Abbas, Ali, Majeed, Abouelwafa, Mousa, et al., 2016).

Citral (3,7-dimethyl-2,6-octadienal) is one of the most important compounds of citrus flavors, and is widely used as flavoring agent in food, beverage and cosmetics industries. As a monoterpene aldehyde, citral consists of two geometrical isomers (geranial and neral). Geranial has a strong lemon odor, and neral's odor is less intense, but sweeter. Besides the appetite stimulating property of citral as flavoring agent, citral has good antifungal and antibacterial activities (Espina, Berdejo, Alfonso, García-Gonzalo, & Pagán, 2017; Li, Wu, Yin, Long, & Li, 2015; Saddiq & Khayyat, 2010), and can be used as insecticide and deodorant (Tak & Isman, 2016). In the pharmaceutical industry, citral is used as antispasmodic, analgesic, anti-inflammatory, antipyretic, diuretic and sedative, due to its spasmolytic, anti-inflammatory, expectorant and weak diuretic effects (Maswal & Dar, 2014; Nishijima, *et al.*, 2014). However, citral is highly vulnerable to acid and oxygen. In fact, acid-catalyzed

67 cyclization and oxidative degradation of citral can reduce the intensity of the fresh
68 lemon flavor and antimicrobial activity that limit the shelf-life of acidic citrus flavored
69 foods, beverages, deodorant and insecticide (Maswal & Dar, 2014). For solving these
70 problems, there are continuous research reports focused on encapsulation of citral in
71 the last ten years. Choi et *al.* (2009) prepared oil-in-water emulsions and encapsulated
72 citral with medium-chain triacylglycerols and triacetin to improve its chemical stability
73 (Choi, Decker, Henson, Popplewell, & McClements, 2009). The authors used cationic,
74 non-ionic and anionic surfactants to prepare emulsions and investigated the influence
75 of droplet charge on the chemical stability of citral in oil-in-water emulsions. They
76 showed that emulsions prepared by using non-ionic or cationic surfactants were more
77 stable than those prepared by using anionic surfactants (Choi, Decker, Henson,
78 Popplewell, & McClements, 2010). Citral retention after spray-drying in sucrose or
79 trehalose matrices was similar for both matrices; however, physical stability of
80 trehalose formulations was better as compared to sucrose (Sosa, Zamora, Chirife, &
81 Schebor, 2011). Therefore, the authors put focus on the emulsions properties and
82 stability before and after spray-drying. The properties of both emulsions and spray-
83 dried powders were similar for the formulations containing sucrose or trehalose which
84 confirmed that trehalose could be used as a replacer for sucrose (Sosa, Schebor, &
85 Pérez, 2014). Yang *et al.* (2015) stabilized citral with a soy protein-polysaccharides
86 Maillard reaction product to enhance the physical stability of oil-in-water emulsions.
87 Lu et al. (2018) encapsulated citral in nanoemulsions and evaluated the antimicrobial
88 activity. Tian, Lu, Li, & Hu (2018) prepared solid lipid nanoparticles loaded with citral
89 by a high-pressure homogenization method. Afzal, Maswal, & Dar (2018) utilized
90 hydrogels prepared by chitosan and alginate to encapsulate citral.

91 In the present study, Layer-by-Layer (LBL) emulsions and Monolayer (ML)
92 emulsions were prepared to encapsulate two different amounts of citral (2.5% and 5%),
93 then the emulsions were spray-dried to obtain citral microcapsules. The antimicrobial
94 activity of citral microcapsules against both Gram-negative and Gram-positive bacteria
95 were evaluated. Sodium caseinate and pectin were chosen as emulsifiers for

96 emulsification and maltodextrins as carrier material for spray-drying because they are
97 natural ingredients already widely used in food industry. In a previous work, the system
98 of sodium caseinate and pectin was already studied for its ability to form composite
99 films (Eghbal *et al.,* 2016). The aim of this work was to investigate the effect of drying
100 and interfacial membrane composition on the antimicrobial activity of emulsified citral.

101 # 2. Materials and methods

102 ## 2.1. Materials: chemicals and bacteria

103 Sodium caseinate was purchased from Fisher Scientific (United Kingdom). Protein
104 content in CAS determined by the Kjeldahl method was 93.20% (nitrogen conversion
105 factor N=6.38). Pectin was from Cargill (Baupte, France). The degree of esterification
106 was from 22% to 28% and degree of acetylation was from 20% to 23%. Maltodextrins
107 DE 19 (dextrose equivalent value of 19) was obtained from Roquette-freres SA,
108 (Lestrem, France). Citral (mixture of *cis* and *trans* isomers, 95% pure) was purchased
109 from Sigma Aldrich Chimie. Analytical grade imidazole ($C_3H_4N_2$), acetic acid, sodium
110 hydroxide (NaOH), hydrochloric acid (HCl), n-hexane (98% of purity), and ethanol
111 were purchased from Sigma-Aldrich Chimie (St Quentin Fallavier, France). Distilled
112 water was used for the preparation of all solutions.

113 The target strains used in this study were: *Listeria innocua* (ATCC 33090), *Kocuria*
114 *rhizophila* (ATCC 9341), *Salmonella enterica* (CIP 8297) and *Staphylococcus aureus*
115 (CIP 4.83). All stains were maintained at −80 °C in Tryptone Soy Broth (TSB, Biokar
116 Diagnistics, France) supplemented with 20% (v/v) glycerol.

117 ## 2.2. Solution preparation

118 Imidazole-acetate buffer solutions (5 mM) were prepared by dispersing weighed
119 amounts of imidazole and acetic acid into distilled water and then adjusting the pH to
120 3.0. The stock solutions were prepared by dispersing sodium caseinate, pectin and
121 maltodextrins powders to unadjusted imidazole-acetate buffer and stirring at room
122 temperature until total hydration, and then the pH was adjusted to 3.0. The pH was
123 adjusted by using HCl (0.1 or 1.0 M) or NaOH (0.1 or 1.0 M). The concentration of
124 each stock solution (w/w) was: sodium caseinate 1.667%, pectin 4%, and maltodextrins
125 50%. The pH of each solution was adjusted again to 3.0.

126 ## 2.3. Emulsion preparation

127 Coarse emulsions were prepared by adding weighted amount of citral to sodium
128 caseinate solution followed by homogenization using an Ultra Turrax PT 4000
129 homogenizer (Polytron, Kinematica, Switzerland) at 20 000 rpm for 5 min. The
130 obtained emulsions (primary emulsions) were further homogenized at 500 bar and five
131 recirculations using a high pressure homogenizer (SPX Brand, APV Model 1000).
132 Monolayer emulsions (ML) were obtained by diluting primary emulsions with pH 3.0
133 buffer. Layer by layer (LBL) emulsions were obtained by slowly adding pectin solution
134 to the primary emulsions and stirring for at least 2 h. All the emulsions were readjusted
135 to pH 3.0 before measurements.

136 **2.4. Spray-drying process**

137 Stock maltodextrins solutions were added to the prepared emulsions, in order to
138 have a final composition (w/w) of 20% maltodextrins DE 19, 0.5% sodium caseinate,
139 0.8% pectin (only for LBL emulsions) and 2.5% or 5.0% citral. The mixtures were
140 stirred for 30 min and then spray-dried using a laboratory scale device equipped with a
141 0.5 nm nozzle atomizer (Mini Spray-Dryer Büchi B-290, Switzerland). The operational
142 conditions of the drying process were: feed flow rate 0.5 L/h, inlet air temperature 180
143 ± 2 °C, outlet air temperature 80 ± 5 °C, and air pressure 3.2 bar. After spray-drying,
144 the powders were collected in sealed containers and stored at 4 °C until analysis.
145 Reconstituted emulsions were prepared by dispersing weighted amounts (the same dry
146 matter as before spray-drying) of spray-dried powders in imidazole-acetate buffer
147 solutions (5 mM, pH 3.0) and stirring for 1 h.

148 **2.5. ζ-potential measurement**

149 The electric charge (ζ-potential) of emulsions were determined using a Zetasizer
150 Nano ZS90 (Malvern Instruments, Malvern, UK). If necessary, the samples were
151 diluted with pH 3.0 imidazole-acetate buffer. Each test was repeated at least three times.
152 The mean ζ-potential (ZP) values (±SD (standard deviation)) were obtained from the
153 instrument.

154 **2.6. Particle size distribution**

155 Particle size distribution of emulsions and spray-dried powders were assessed by
156 a laser diffraction instrument (Mastersizer 3000, Malvern Instruments, Malvern, UK)
157 with a value of 1.5 for relative refractive index (droplet to solvent), 0.1 absorption, and
158 an index of refraction of 1.33 for water phase, 1.36 for ethanol. For measuring the
159 droplet size distribution of emulsions, the emulsions were injected into the
160 measurement chamber containing imidazole-acetate buffer (5 mM; pH 3.0). For
161 measuring the particle size distribution of spray-dried powders, the powder was slowly
162 added into the measurement chamber containing ethanol. The emulsions and spray-
163 dried powders were continuously stirred throughout the measurement to ensure that the
164 samples were homogeneous. The volume mean particle diameter (D_{43}) was calculated
165 by the software from the three injections of three separate samples with five readings
166 per sample.

167 **2.7. Encapsulation Efficiency (EE)**

168 A weighted amount (the same dry matter as in 1 mL of emulsion) of each spray-
169 dried powder was dissolved in 1 mL distilled water in a centrifuge tube and 10 mL
170 hexane were then added. After vortexing the tube for 10 minutes, centrifugation at
171 5 000 g at 4 °C for 30 min was applied to separate the organic phase. After dilution
172 (1:10 or 1:100) of the organic phase in hexane, the amount of citral was quantified by
173 measuring the absorbance by spectrophotometry (Jenway 3705, Villepinte, France) at
174 252 nm using a prepared standard calibration curve of citral in hexane. The
175 encapsulation efficiency was calculated as follows (Lu *et al.*, 2018):

176 $$\text{Encapsulation Efficiency (\%)} = \frac{\text{Amount of encapsulated citral}}{\text{Total amount of added citral}} \times 100$$

177 **2.8. Minimum inhibitory concentration (MIC)**

178 *Listeria innocua* (ATCC 33090), *Kocuria rhizophila* (ATCC 9341), *Salmonella*
179 *enterica* (CIP 8297) and *Staphylococcus aureus* (CIP 4.83) were pre-cultured in TSB
180 at 10% (v/v) for 24 h and then cultured by inoculating 10^4 CFU/L (from the pre-culture)
181 in 50 mL of TSB and incubation for 16 h at 37 °C under shaking (160 rpm). Cells were

182 harvested by centrifugation (5 000 *g* for 5 min at 25 °C) then washed twice with
183 Potassium Phosphate Buffer (PPB; 100 mM, pH 7) before being diluted in Tryptone
184 Salt (TS) broth to 1×10^6 CFU/mL. The minimum inhibitory concentration (MIC) of
185 citral microcapsules was determined by measuring kinetically, the development of
186 turbidity by vertical photometry using a Bioscreen C (Labsystems, Helsinki, Finland).
187 100 µL of bacterial suspension (10^6 CFU/mL) were added to the plate wells containing
188 serial 2-fold dilutions from citral microcapsules stock solution in TSB. Each test plate,
189 included a growth control (bacteria with microcapsule-free TSB), and a sterility control
190 with only microcapsule-free TSB. The plates were incubated at 37 °C under continuous
191 shaking in the Bioscreen C which measured the $OD_{600\ nm}$ every 2 h during 24 h. The
192 MIC was defined as the lowest citral microcapsules concentration preventing the
193 bacterial growth, as measured by optical density. The experiment was repeated three
194 times and mean $OD_{600\ nm}$ values were plotted versus time.

195 **2.9. Growth rate and lag time**

196 The lag time, of each growth curve, was estimated by extrapolating the linear
197 portion of $OD_{600\ nm}$ versus time plot back to the initial $OD_{600\ nm}$. The growth rate (µ)
198 was calculated during the exponential growth according to Hall, Acar, Nandipati, &
199 Barlow (2014).

200 **2.10. Measurement of inhibition zone**

201 *Kocuria rhizophila* (ATCC 9341) was pre-cultured in TSB at 10% (v/v) for 8 h and
202 then pre-cultured in TSB at 10% (v/v) for 16 h at 37 °C. One milliliter of this pre-culture
203 was transferred into 9 mL of TSB and incubated for 5 h at 37 °C. The cells were then
204 diluted in Tryptone Salt (TS) broth to final concentrations of 1×10^6 CFU/mL,
205 incorporated at 5% (v/v) in melted (~50 °C) Tryptone Soy Agar (TSA), and cooled in
206 Petri dishes. A sterilized filter paper with a hole (15 mm in diameter) was placed on the
207 TSA Petri plates inoculated with *K. rhizophila*, and weighted amounts of citral
208 microcapsules were added in the hole. After spreading the powder, the paper was gently
209 removed. The plates were then incubated for 2 h at 4 °C and then incubated at 37 °C.

210 Inhibition zones were measured after 24 h and each experiment was repeated three
211 times.

212 **2.11. Statistical analysis**

213 All experiments were performed using at least three freshly prepared samples. The
214 results presented are the averages and standard deviations that were calculated from
215 these replicate measurements. Statistical differences between samples were calculated
216 using Student's t test for independent samples (Microsoft Excel, Microsoft Corporation,
217 Redmond, WA).

3. Results and discussion

3.1. Physicochemical properties of emulsions

3.1.1. Droplet size distribution of emulsions

The average droplet size of ML and LBL emulsions containing 2.5% or 5.0% citral before and after spray-drying is shown in **Fig. 1A**. Through comparing the droplet size of LBL emulsions with ML emulsions, the effect of interfacial membrane composition was evaluated. Before spray-drying, the mean droplet size of LBL emulsions containing 2.5% or 5.0% citral was around 15 μm, which was significantly ($p < 0.05$) bigger than the mean droplet size of ML emulsions (~ 4 μm).

The effect of spray-drying on the properties of emulsions was evaluated by comparing the droplet size of fresh emulsions before spray-drying and reconstituted emulsions after spray-drying. After spray-drying, the droplet size of ML emulsions increased, whatever the citral concentration. Indeed, ML emulsions containing 2.5% citral increased from 3.5 ± 1.2 μm to 14.5 ± 2.6 μm and for ML emulsions containing 5.0% citral, the droplet size increased from 4.2 ± 0.6 μm to 11.6 ± 1.4 μm. This could be mainly caused by flocculation and/or coalescence. On the other hand, the droplet size of LBL emulsions slightly decreased after spray-drying. For LBL emulsions containing 2.5% citral, the droplet size decreased from 15.3 ± 2.3 μm to 9.7 ± 1.8 μm. For LBL emulsions containing 5.0% citral, the droplet size decreased from 14.6 ± 1.4 μm to 9.1 ± 0.5 μm. The size decrease of LBL emulsions could be due to the dissociation of flocculated droplets during the shearing inside the atomization nozzle, the collapse of surface hairy layers and the shrinkage of overall structures (Liu *et al.,* 2019). The final droplet size of reconstituted LBL emulsions (~ 9 μm) was significantly ($p < 0.05$) smaller than reconstituted ML emulsions (~ 13 μm).

Compared to ML emulsions, the addition of the second layer (pectin) made the droplet size of LBL emulsions increase and improved the stability of LBL emulsions against the shearing and thermal stresses during the spray-drying process. Increasing

245 the amount of citral from 2.5% to 5.0% did not significantly changed the droplet size
246 of each emulsion.

247 *3.1.2. ζ-potential of emulsions*

248 The ζ-potential of ML and LBL emulsions containing 2.5% or 5.0% citral before
249 and after spray-drying was also measured as shown in **Fig. 1B**. For ML emulsions, the
250 ζ-potential was positive (about 25 mV) because of the positively-charged caseinate
251 layer which covered droplets. A decrease of ζ-potential value (from ~25 mV to ~12
252 mV) occurred after spray-drying. In fact, the spray-drying process would cause some
253 structure changes of caseinate on the droplet surface followed by flocculation and/or
254 coalescence. Compared to positively-charged ML emulsions, the ζ-potential value of
255 LBL emulsions was negative (about -10 mV), this transition was due to the addition of
256 negatively-charged pectin which formed the second layer to cover citral droplets
257 (Sejersen *et al.,* 2007). Indeed, the absolute values of zeta potential in LBL systems
258 were lower than in ML systems, which indicated that the electrostatic repulsions in LBL
259 systems were weaker than that in ML systems. However, the stability of emulsions can
260 be attributed to many factors like viscosity, density, size… The change of ζ-potential
261 indicated also that the structure of droplets in the emulsion was changed (Kovacevic *et*
262 *al.,* 2011). No significant change of ζ-potential value was measured before and after
263 spray-drying for LBL emulsions, which confirmed the stability of LBL emulsions. The
264 ζ-potential of each emulsion was also independent of the amount of citral (2.5% or
265 5.0%).

266 **3.2. Properties of citral microcapsules**

267 In this section, the physicochemical properties of spray-dried powder were
268 characterized by measuring particle size and encapsulation efficiency, as shown in **Fig.**
269 **2**. The particle size of citral microcapsules obtained by spray-drying ML emulsions
270 containing 2.5% or 5.0% citral were both around 8.0 μm. However, the powder obtained
271 by spray-drying LBL emulsions had a slight larger size (~12 μm). It is important to note

272 also that the increase of citral amount did not influence the particle size in both ML and
273 LBL cases.

274 Encapsulation efficiency is an important parameter in the evaluation of a
275 microencapsulation technique (Jelvehgari, Valizadeh, Rezapour, & Nokhodchi, 2010).
276 Among the four kinds of citral microcapsules, the spray-dried 2.5% citral LBL
277 emulsion showed the highest encapsulation efficiency (99.6 ± 0.4 %), which was closed
278 to 100%, followed by 5.0% citral LBL (91.2 ± 5.6 %), 2.5% citral ML (78.6 ± 0.6 %)
279 and 5.0% citral ML (62.3 ± 2.4 %). According to these results, with increase of citral
280 amount, the encapsulation efficiency decreased, which is expected because of the limit
281 of loading capacity in microencapsulation system. Compared to the citral
282 microcapsules based on ML emulsions, the spray-dried powder based on LBL
283 emulsions had a higher encapsulation efficiency in both concentrations of citral, which
284 further confirmed the protection of added pectin against the stress sustained during the
285 spray-drying process.

286 **3.3. Antimicrobial activity assessments**

287 *3.3.1. Minimum Inhibitory Concentration (MIC) in liquid media*

288 The minimum inhibitory concentration of the four kinds of citral microcapsules
289 was measured against four target strains as shown in **Table 1**. The results showed that
290 *Salmonella enterica* CIP 8297 (Gram-negative) was more resistant to encapsulated
291 citral, compared to the other 3 Gram-positive bacteria, which could be due to the
292 diffusion restriction of hydrophobic compounds by means of the lipopolysaccharide
293 outer membrane barrier (Belda-Galbis, Pina-Pérez, Leufvén, Martínez, & Rodrigo,
294 2013). This result was consistent with the properties of most essential oils and their
295 components (Tajkarimi, Ibrahim, & Cliver, 2010). In these 4 tested citral microcapsules,
296 5.0 % citral LBL microcapsules showed the lowest MIC (2 mg/mL for *Salmonella*
297 *enterica*, 1 mg/mL for *Staphylococcus aureus*, *Listeria innocua* and *Kocuria*
298 *rhizophila*), which was two times less than the MICs measured for the other 3 citral
299 microcapsules. This could be due to their good encapsulation efficiency combined with

300 the proper concentration of entrapped citral that resulted in controlled release of
301 encapsulated citral.

302 *3.3.2. Growth rate and lag time*

303 For all tested bacteria, the increase of citral capsule concentration reduced the growth
304 rates (**Fig. 3**) while increasing the lag time (**Fig. 4**). This behavior was also described
305 in other studies (Shi *et al.*, 2016; Silva-Angulo *et al.*, 2015). Compared to the other
306 strains, *Salmonella enterica,* was 2-fold more resistant to citral microcapsules and
307 presented higher growth rates at 37 °C whatever the sub-MIC. In fact, it has been
308 reported that fast-growing cells have been recognized to be more sensitive to the
309 stresses than slow-growing cells (Berney, Weilenmann, Ihssen, Bassin, & Egli, 2006).
310 Moreover, when grown under sub-MIC, *Salmonella enterica* showed longer lag times
311 before starting a fast and exponential growth phase (**Fig. 4**). The increase of the lag
312 time is synonym of the time needed by bacteria to adapt to the non-inhibitory doses of
313 citral (Silva-Angulo *et al.*, 2015). Thus, the presence of *Salmonella enterica* in food
314 products represents a real threat for the consumer because of its great proliferation and
315 adaptation ability to unfavorable conditions. Nevertheless, the formulated citral
316 microcapsules seem to be good food preservatives even when used at non-inhibitory
317 doses since they slowdown the bacterial growth. The application of such citral
318 microcapsule-based delivery systems can be combined with cold storage to prolong the
319 shelf life of several food products. The obtained citral microcapsules were able to allow
320 a reduction in the concentration of citral and maximize the effectiveness of a given
321 dose, which could reduce the alteration of the organoleptic properties of foods.
322

323 **3.4. Inhibition zone measurements**

324 Citral microcapsules were added on the surface of a medium containing bacteria.
325 A photograph of inhibition zones of citral microcapsules on medium containing
326 bacteria are shown in **Fig. 5**. After using the same amount of powder (30 mg) for each
327 type of microcapsules inhibition zones with of different sizes were observed: 19.2 ± 0.2
328 mm for 2.5% citral ML microcapsules; 21.8 ± 0.8 mm for 2.5% citral LBL

329 microcapsules; 25.7 ± 1.9 mm for 5.0% citral ML microcapsules; and 32.5 ± 3.1 mm
330 for 5.0% citral LBL microcapsules (these results were significantly different ($p < 0.05$,
331 n = 3)). For the same initial concentration of citral (2.5% or 5.0%), LBL microcapsules
332 had a larger inhibition zone than ML microcapsules. 5% citral microcapsules showed a
333 larger inhibition zone than 2.5% citral microcapsules for both LBL and ML cases. The
334 largest inhibition zone observed for 5.0% citral LBL microcapsules, was due to the high
335 initial citral loaded concentration and high encapsulation efficiency (91.2%). As a
336 result, 5.0% citral LBL microcapsules contained more citral than the other three types
337 of microcapsules.

338 The size of inhibition zone can be affected by several parameters, like the amount
339 of citral microcapsules, the spread area of citral microcapsules, the amount of citral
340 contained in citral microcapsules, the mechanism of citral's release, the number of
341 bacteria in the medium, the time of measuring inhibition zone…. To evaluate the effect
342 of the release rate of encapsulated citral in these four kinds of citral microcapsules, the
343 amount of citral in each citral microcapsule was calculated according to the
344 encapsulation efficiency (Lu *et al.*, 2018). Then, different amount of the four types of
345 microcapsules containing the same quantity of citral were tested. The inhibition zones
346 of the 4 kinds of citral microcapsules were 24.5 ± 0.5^{a} mm for 2.5% citral ML
347 microcapsules; 25.4 ± 0.6^{a} mm for 2.5% citral LBL microcapsules; 30.8 ± 1.0^{b} mm for
348 5.0% citral ML microcapsules; and 34.5 ± 1.5^{c} mm for 5.0% citral LBL microcapsules
349 (different letters followed by the data are significantly different ($p < 0.05$, n = 3)). For
350 this test with the same amount of citral, 5.0% citral LBL microcapsules still had the
351 largest inhibition zone.

352 4. Conclusion

353 Through preparing and spray-drying ML and LBL emulsions containing 2.5% or
354 5.0% citral, we studied the effect of interfacial membrane composition and spray-
355 drying on the properties of emulsions. The results illustrated that the addition of the
356 second layer (pectin) could improve the stability of emulsions against the stress

sustained during the spray-drying process. With increase of the citral amount, the encapsulation efficiency slightly decreased and particle size had no change in both ML and LBL cases. Microbiological tests demonstrated that 5.0% citral LBL microcapsules had the highest antimicrobial activity, which was further confirmed by the application of the dried powders on a medium surface (inhibition zone measurement). Therefore, our formulated microcapsules seem to be a good food preservative even when used at non-inhibitory doses since they slowdown the bacterial growth. The application of such essential oils microcapsule-based delivery systems combined with storage at low temperatures is a promising technique to prolong the shelf life of perishable food products.

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464

465 **Figure captions**

466 **Fig. 1.** Physicochemical properties of emulsions: (**a**) Droplet size distribution of
467 emulsions before and after spray-drying; (**b**) Zeta-potential of emulsions before and
468 after spray-drying.

469 **Fig. 2.** Particle size distribution and encapsulation efficiency of spray-dried powders.

470 **Fig. 3.** Growth rate of 4 tested bacteria (*Salmonella enterica*; *Staphylococcus aureus*;
471 *Listeria innocua*; *Kocuria rhizophila*) treated with 4 kinds of spray-dried powders.

472 **Fig. 4.** Lag time of four tested bacteria (*Salmonella enterica*; *Staphylococcus aureus*;
473 *Listeria innocua*; *Kocuria rhizophila*) treated with 4 kinds of spray-dried powders.

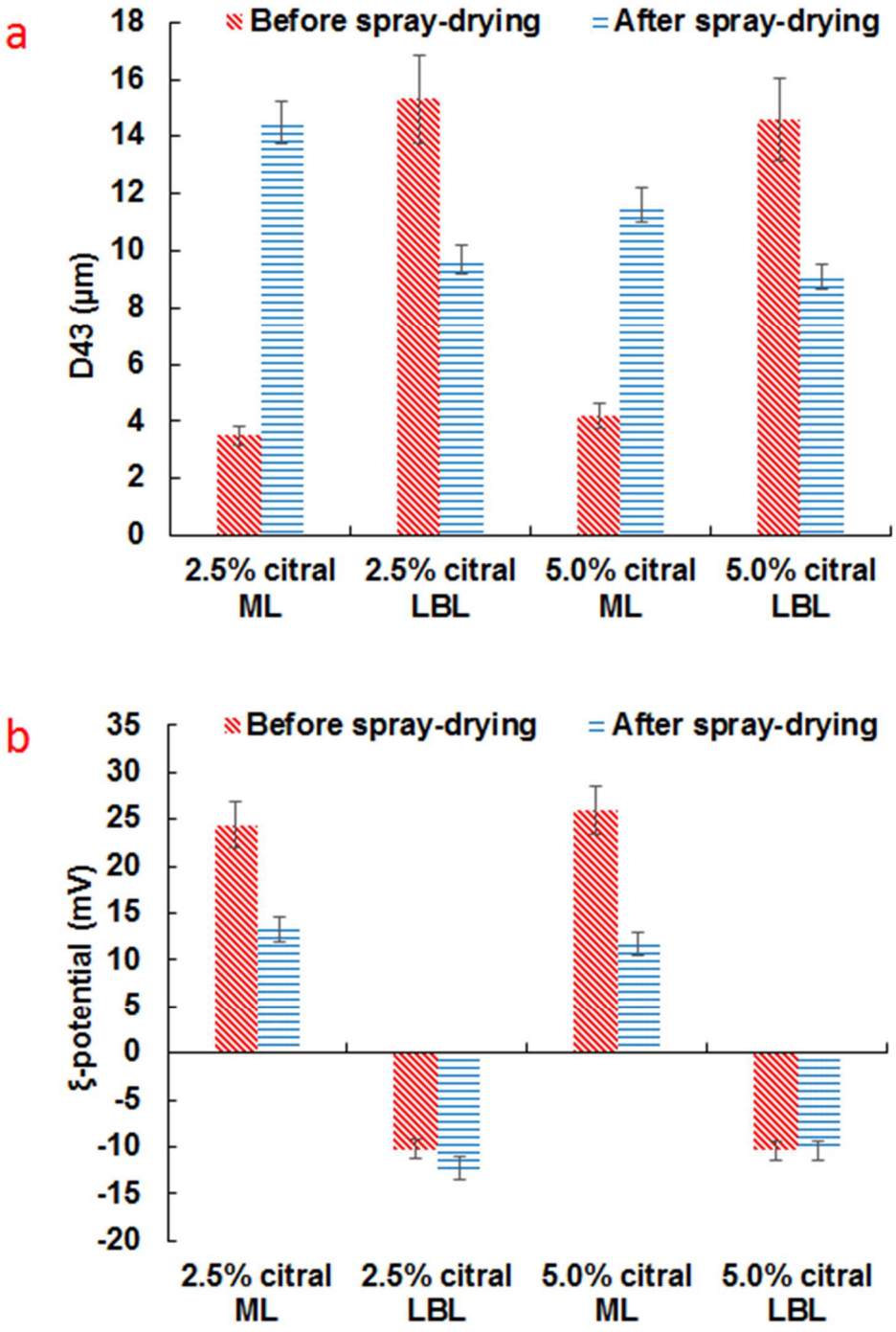
474 **Fig. 5.** Inhibition zones on TSA containing *Kocuria rhizophila* (1×10^6 CFU/mL) with
475 four kinds of spray-dried powders: (**a**) 2.5% citral ML microcapsules; (**b**) 2.5% citral
476 LBL microcapsules; (**c**) 5.0% citral ML microcapsules; (**d**) 5.0% citral LBL
477 microcapsules.

478 **Table 1.** Minimum inhibitory concentration (mg/mL) of encapsulated citral

	2.5% citral ML	**2.5% citral LBL**	**5.0% citral ML**	**5.0% citral LBL**
***Salmonella enterica* CIP 8297**	4	4	4	2
***Staphylococcus aureus* CIP 4.83**	2	2	2	1
***Listeria innocua* ATCC 33090**	2	2	2	1
***Kocuria rhizophila* ATCC 9341**	2	2	2	1

479

480 **Figure 1**



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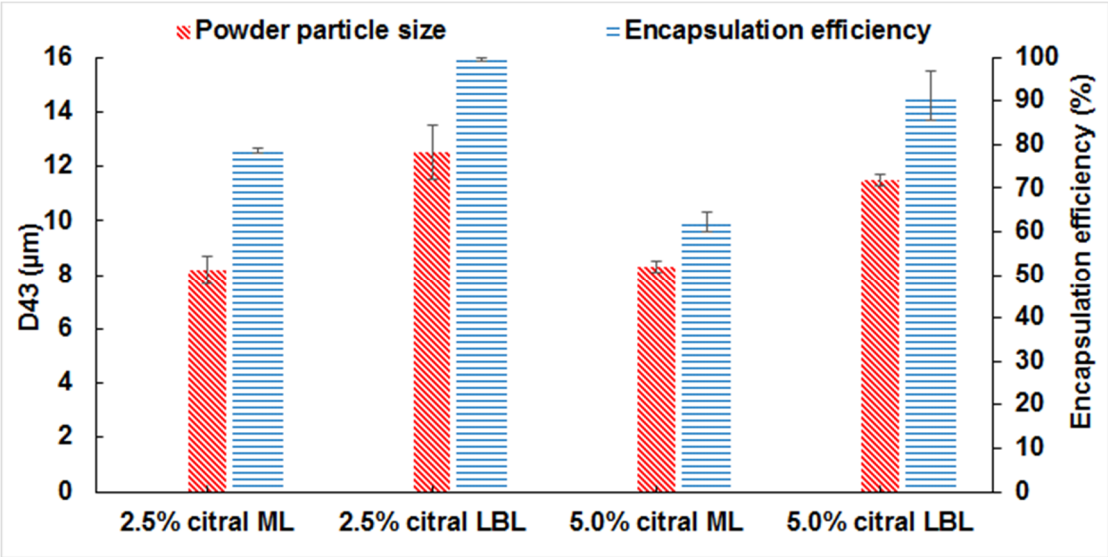
482 **Figure 2**



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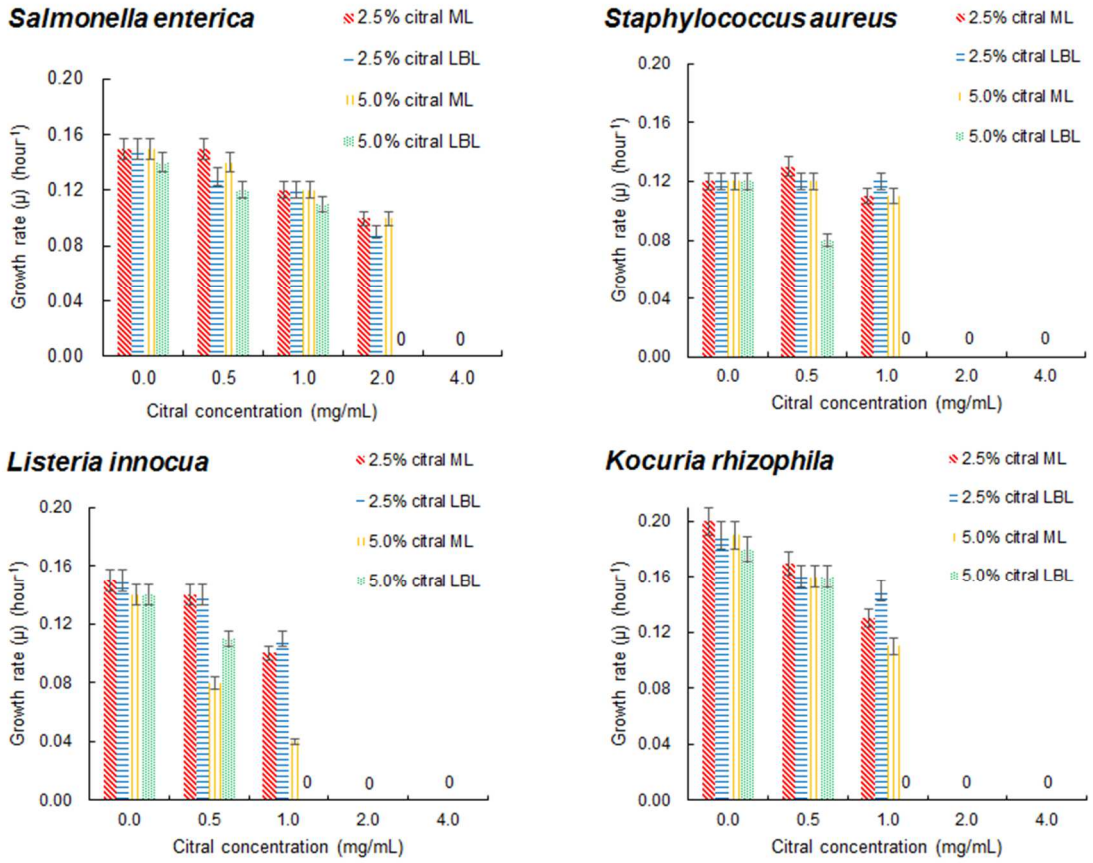
484

485 **Figure 3.**



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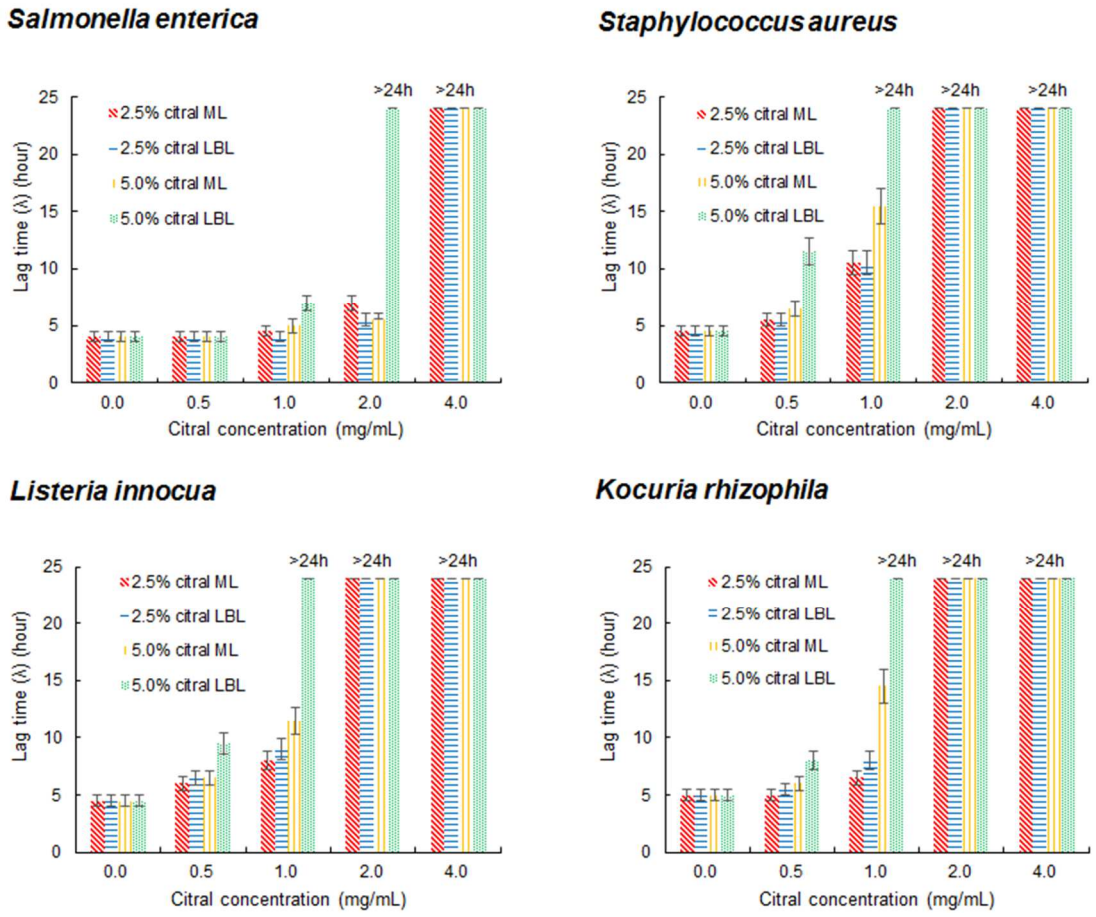
487 **Figure 4.**



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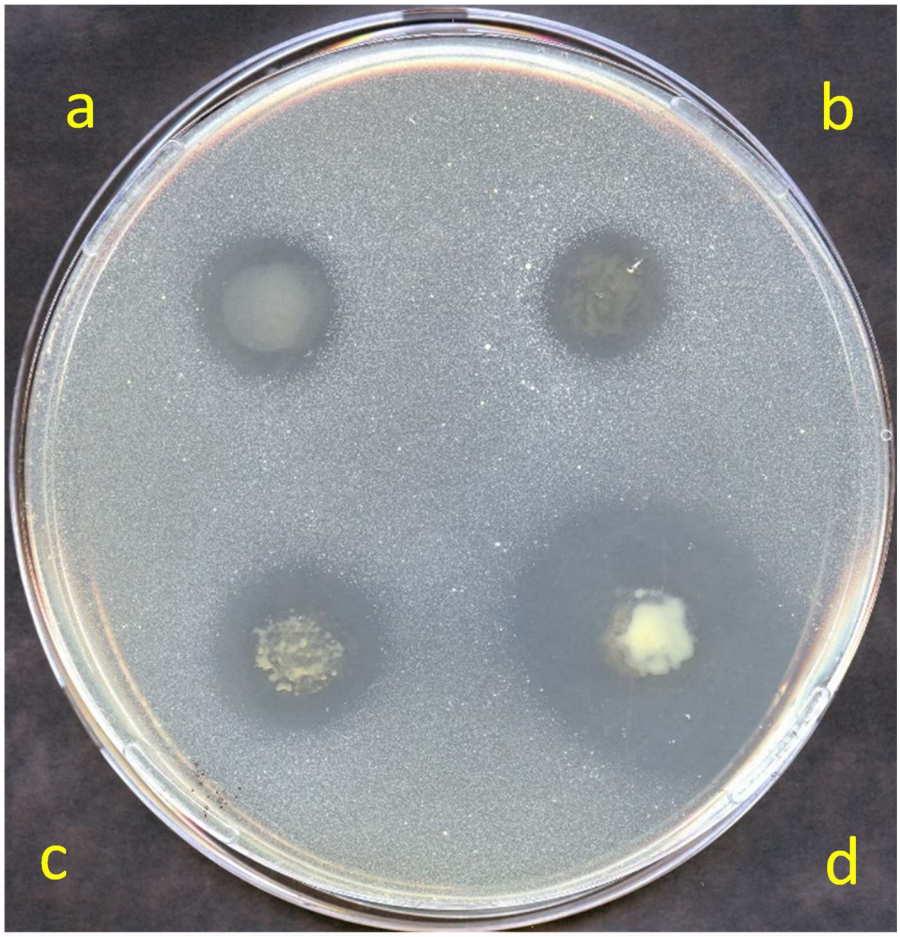
489 **Figure 5.**



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