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

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22     **Abstract**

23     Citral-in-water emulsions were prepared with two different essential oil  
24     concentrations of 2.5 and 5.0% (w/w), then spray-dried in the presence of the same  
25     amount of maltodextrins (20%). The microcapsules were prepared with two different  
26     emulsifier compositions: monolayer microcapsules (ML) stabilized by sodium  
27     caseinate alone and layer-by-layer microcapsules (LBL) stabilized by sodium  
28     caseinate and pectin. The encapsulation efficiency was higher for LBL  
29     microcapsules (*e.g.* 99.6 ± 0.4 % for 2.5% citral) than that for ML ones (*e.g.* 78.6 ±  
30     0.6 % for 2.5% citral) which confirm that the additional pectin layer was able to  
31     protect citral during the spray-drying process whatever citral concentration.  
32     Furthermore, our results showed that the antibacterial activity of the obtained  
33     microcapsules significantly depends on both citral concentration and interfacial  
34     membrane composition. The presence of two layers surrounding the citral droplets  
35     may result in a progressive and controlled release of the encapsulated citral.

36

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

## 39 **1. Introduction**

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

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

55 Citral (3,7-dimethyl-2,6-octadienal) is one of the most important compounds of  
56 citrus flavors, and is widely used as flavoring agent in food, beverage and cosmetics  
57 industries. As a monoterpene aldehyde, citral consists of two geometrical isomers  
58 (geranial and neral). Geranial has a strong lemon odor, and neral's odor is less intense,  
59 but sweeter. Besides the appetite stimulating property of citral as flavoring agent, citral  
60 has good antifungal and antibacterial activities ([Espina, Berdejo, Alfonso, García-  
61 Gonzalo, & Pagán, 2017](#); [Li, Wu, Yin, Long, & Li, 2015](#); [Saddiq & Khayyat, 2010](#)),  
62 and can be used as insecticide and deodorant ([Tak & Isman, 2016](#)). In the  
63 pharmaceutical industry, citral is used as antispasmodic, analgesic, anti-inflammatory,  
64 antipyretic, diuretic and sedative, due to its spasmolytic, anti-inflammatory,  
65 expectorant and weak diuretic effects ([Maswal & Dar, 2014](#); [Nishijima, et al., 2014](#)).  
66 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 (Baupre, 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<sub>3</sub>H<sub>4</sub>N<sub>2</sub>), 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 Diagnostics, 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 mm 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  $\pm 2$  °C, outlet air temperature  $80 \pm 5$  °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. $\zeta$ -potential measurement**

149 The electric charge ( $\zeta$ -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  $\zeta$ -potential (ZP) values ( $\pm$ 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):

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

## 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  $1 \times 10^6$  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  $\mu$ L 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<sub>600 nm</sub> 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<sub>600 nm</sub> 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<sub>600 nm</sub> versus time plot back to the initial OD<sub>600 nm</sub>. The growth rate ( $\mu$ )  
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  $1 \times 10^6$  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).

## 218 **3. Results and discussion**

### 219 **3.1. Physicochemical properties of emulsions**

#### 220 *3.1.1. Droplet size distribution of emulsions*

221 The average droplet size of ML and LBL emulsions containing 2.5% or 5.0% citral  
222 before and after spray-drying is shown in [Fig. 1A](#). Through comparing the droplet size  
223 of LBL emulsions with ML emulsions, the effect of interfacial membrane composition  
224 was evaluated. Before spray-drying, the mean droplet size of LBL emulsions containing  
225 2.5% or 5.0% citral was around 15  $\mu\text{m}$ , which was significantly ( $p < 0.05$ ) bigger than  
226 the mean droplet size of ML emulsions ( $\sim 4 \mu\text{m}$ ).

227 The effect of spray-drying on the properties of emulsions was evaluated by  
228 comparing the droplet size of fresh emulsions before spray-drying and reconstituted  
229 emulsions after spray-drying. After spray-drying, the droplet size of ML emulsions  
230 increased, whatever the citral concentration. Indeed, ML emulsions containing 2.5%  
231 citral increased from  $3.5 \pm 1.2 \mu\text{m}$  to  $14.5 \pm 2.6 \mu\text{m}$  and for ML emulsions containing  
232 5.0% citral, the droplet size increased from  $4.2 \pm 0.6 \mu\text{m}$  to  $11.6 \pm 1.4 \mu\text{m}$ . This could  
233 be mainly caused by flocculation and/or coalescence. On the other hand, the droplet  
234 size of LBL emulsions slightly decreased after spray-drying. For LBL emulsions  
235 containing 2.5% citral, the droplet size decreased from  $15.3 \pm 2.3 \mu\text{m}$  to  $9.7 \pm 1.8 \mu\text{m}$ .  
236 For LBL emulsions containing 5.0% citral, the droplet size decreased from  $14.6 \pm 1.4$   
237  $\mu\text{m}$  to  $9.1 \pm 0.5 \mu\text{m}$ . The size decrease of LBL emulsions could be due to the dissociation  
238 of flocculated droplets during the shearing inside the atomization nozzle, the collapse  
239 of surface hairy layers and the shrinkage of overall structures ([Liu et al., 2019](#)). The  
240 final droplet size of reconstituted LBL emulsions ( $\sim 9 \mu\text{m}$ ) was significantly ( $p < 0.05$ )  
241 smaller than reconstituted ML emulsions ( $\sim 13 \mu\text{m}$ ).

242 Compared to ML emulsions, the addition of the second layer (pectin) made the  
243 droplet size of LBL emulsions increase and improved the stability of LBL emulsions  
244 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. $\zeta$ -potential of emulsions

248 The  $\zeta$ -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  $\zeta$ -potential was positive (about 25 mV) because of the positively-charged caseinate  
251 layer which covered droplets. A decrease of  $\zeta$ -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  $\zeta$ -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  $\zeta$ -potential  
261 indicated also that the structure of droplets in the emulsion was changed (*Kovacevic et*  
262 *al., 2011*). No significant change of  $\zeta$ -potential value was measured before and after  
263 spray-drying for LBL emulsions, which confirmed the stability of LBL emulsions. The  
264  $\zeta$ -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  $\mu\text{m}$ . However, the powder obtained  
271 by spray-drying LBL emulsions had a slight larger size (~12  $\mu\text{m}$ ). 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 ( $99.6 \pm 0.4 \%$ ), which was closed  
278 to 100%, followed by 5.0% citral LBL ( $91.2 \pm 5.6 \%$ ), 2.5% citral ML ( $78.6 \pm 0.6 \%$ )  
279 and 5.0% citral ML ( $62.3 \pm 2.4 \%$ ). 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:  $19.2 \pm 0.2$   
328 mm for 2.5% citral ML microcapsules;  $21.8 \pm 0.8$  mm for 2.5% citral LBL

329 microcapsules;  $25.7 \pm 1.9$  mm for 5.0% citral ML microcapsules; and  $32.5 \pm 3.1$  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  $24.5 \pm 0.5^a$  mm for 2.5% citral ML  
347 microcapsules;  $25.4 \pm 0.6^a$  mm for 2.5% citral LBL microcapsules;  $30.8 \pm 1.0^b$  mm for  
348 5.0% citral ML microcapsules; and  $34.5 \pm 1.5^c$  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



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

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373 financial supports.

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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* ( $1 \times 10^6$  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

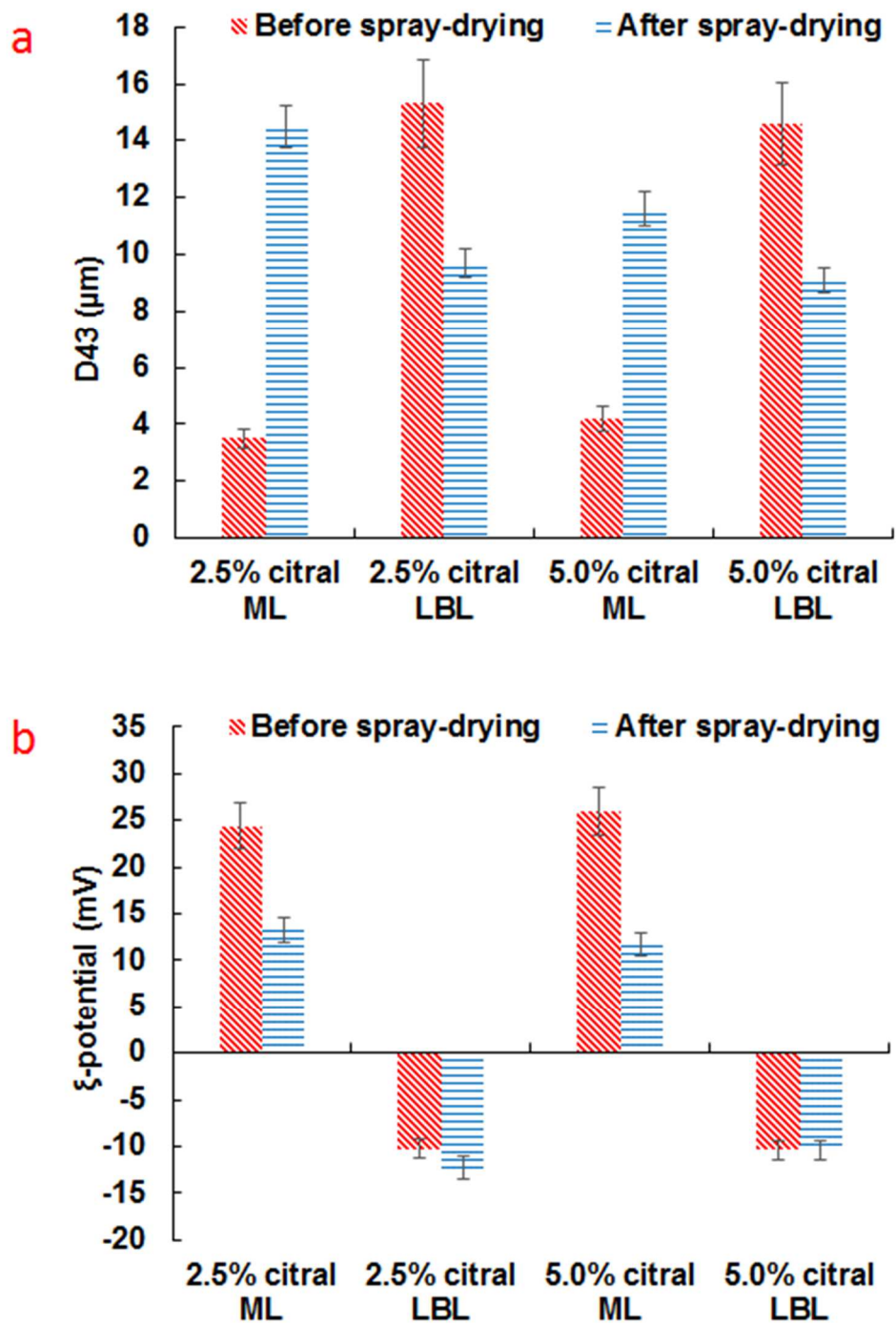
**Table 1.** Minimum inhibitory concentration (mg/mL) of encapsulated citral

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

479

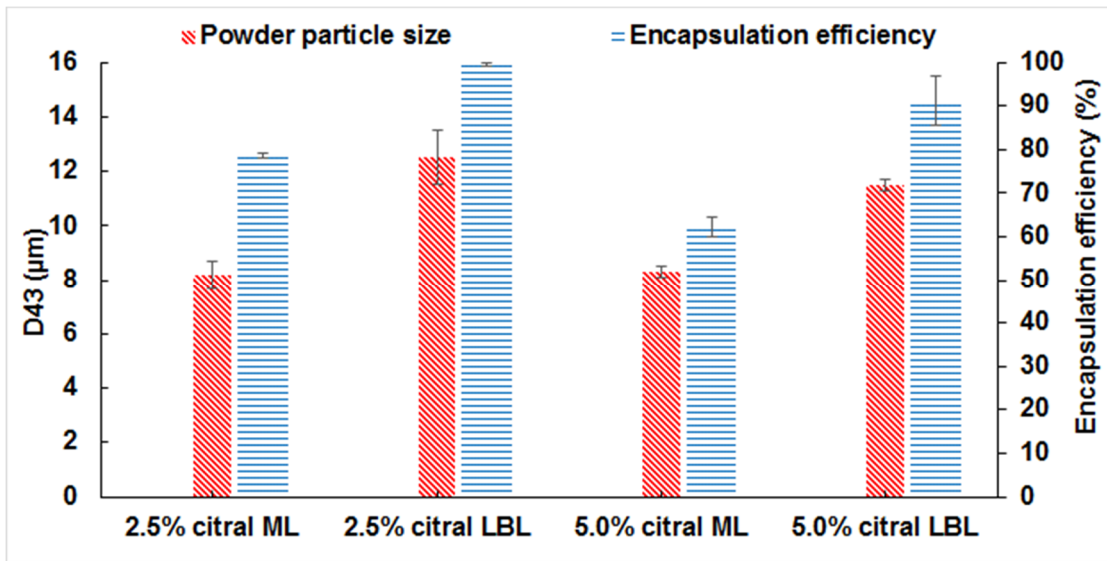


480 **Figure 1**



481

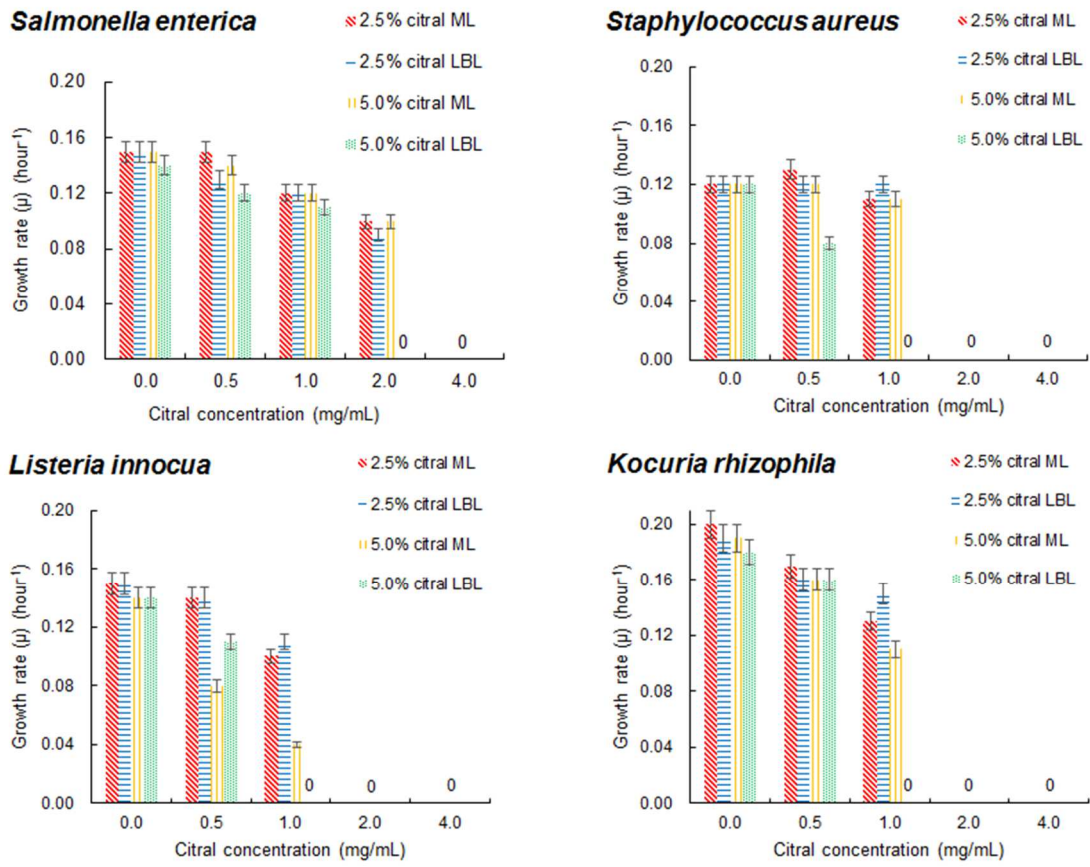
482 **Figure 2**



483

484

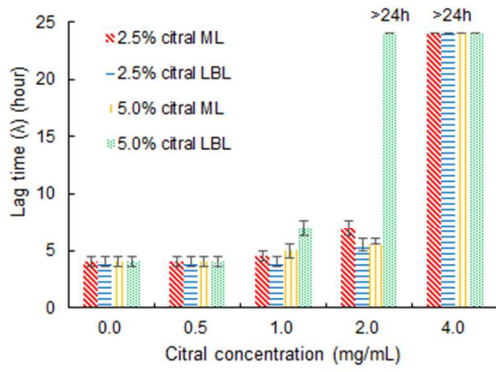
485 **Figure 3.**



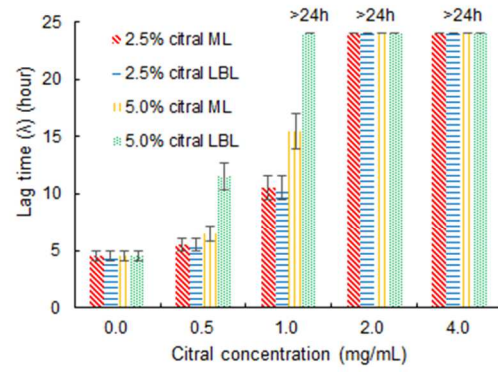
486

487 **Figure 4.**

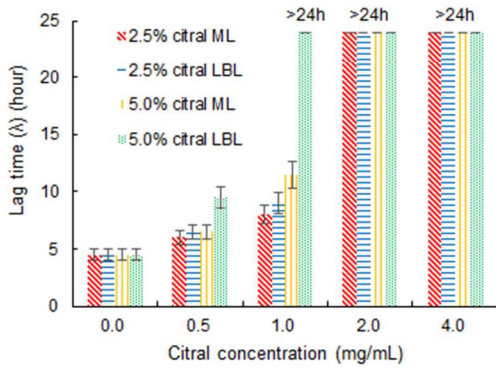
***Salmonella enterica***



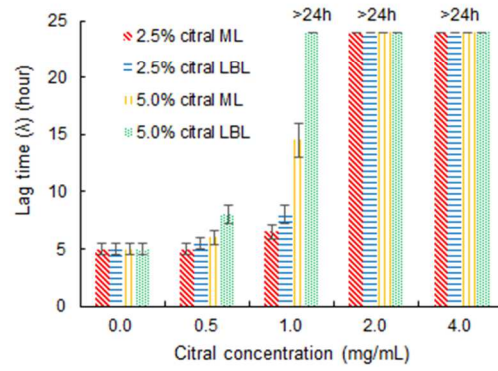
***Staphylococcus aureus***



***Listeria innocua***

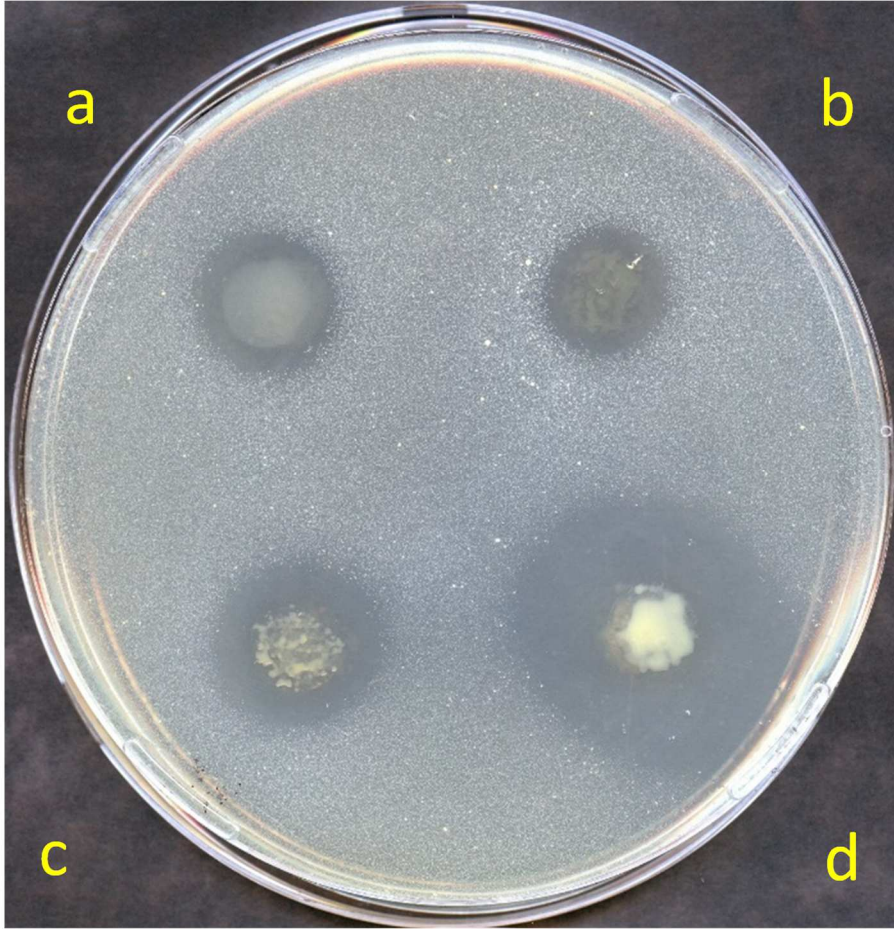


***Kocuria rhizophila***



488

489 **Figure 5.**



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