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1 **Use of free and encapsulated nerolidol to inhibit the survival of *Lactobacillus fermentum* in**
2 **fresh orange juice**

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11 **Abstract**

12 *Lactobacillus fermentum* is commonly responsible for fruit juice fermentation and spoilage. The
13 aim of this study was to investigate the potential use of nerolidol to control the spoilage of fresh
14 orange juice by *L. fermentum*. Nerolidol was incorporated into hydroxypropyl-  -cyclodextrin
15 inclusion complex, conventional liposome, and drug-in-cyclodextrin-in liposome systems. The
16 systems were lyophilized and characterized with respect to their nerolidol content, size, and
17 morphology. The effects of the acidity and cold storage of orange juice on the survival of *L.*
18 *fermentum* were evaluated. Subsequently, the antibacterial activity of nerolidol in refrigerated
19 orange juice was assessed at pH 3.3. Nerolidol showed a faster antibacterial activity at 4 000   M
20 (5 days) compared to 2 000   M (8 days). Under the same conditions, the inclusion complex
21 completely killed bacteria within 6 days of incubation at 4 000   M, suggesting its potential

22 application in fruit juices. Nerolidol-loaded liposomes did not exhibit an antibacterial activity
23 and altered the appearance of juice.

24 **Keywords:** Cyclodextrin; fruit juice; lactic acid bacteria; liposome; sesquiterpene.

25 **Abbreviations:** CFU: colony forming unit; CL: conventional liposome; DCL drug-in-
26 cyclodextrin-in-liposome; DLS: dynamic light scattering; DMSO: dimethyl sulfoxide; HP- β -CD:
27 hydroxypropyl- β -cyclodextrin; HPLC: high performance liquid chromatography; Ner: nerolidol;
28 MRS: de Man, Rogosa, and Sharpe; PDI: polydispersity index; SEM: scanning electron
29 microscopy; TA: titratable acidity; TEM: transmission electron microscopy.

30 **1 Introduction**

31
32 Orange juice is consumed worldwide and has a number of functional attributes, being rich in
33 bioactive compounds such as polyphenols, carotenoids, and vitamin C. Protecting orange juice
34 from chemical and microbiological spoilage has been a major focus of the juice industry for
35 years. Orange juice is susceptible to spoilage by a wide range of acid-tolerant microorganisms,
36 including fungi, lactic acid bacteria, and spore-forming *Alicyclobacillus* (Ephrem et al., 2018).
37 Different strategies have been adopted to prevent this spoilage, including the use of food
38 additives with or without the use of different pasteurization techniques. Various preservatives are
39 currently added to orange juice, primarily nitrates, sorbates, and benzoates. However, because
40 these preservatives can cause allergic responses and be converted to potential carcinogens
41 (Anand and Sati, 2013), there is currently a need to identify alternative bioactive agents for
42 orange juice preservation.

43 Nerolidol (Ner) is a sesquiterpene alcohol that is present in the essential oils of plants such as
44 lemon grass (*Cymbopogon* spp.), lavender (*Lavandula* spp.), and tea tree (*Melaleuca*

45 *alternifolia*). Ner has been approved for use as a flavouring agent in food products by the United
46 States Food and Drug Administration (Chan et al., 2016) and has been shown to exhibit
47 antimicrobial activity against different microorganisms, including *Streptococcus mutans*,
48 *Staphylococcus aureus*, and *Salmonella enterica* (Chan et al., 2016). However, Ner has a low
49 aqueous solubility and photostability (Azzi et al., 2018; McGinty et al., 2010), requiring its
50 incorporation into appropriate encapsulation systems to promote its use in food preservation.

51 The use of encapsulation systems (e.g., liposomes, nanoemulsions, and cyclodextrin inclusion
52 complexes) to protect natural antimicrobials and enhance their aqueous solubility in food
53 systems has been described in a number of previous studies (Astray et al., 2009; Blanco-Padilla
54 et al., 2014; Weiss et al., 2009). Antimicrobial-loaded encapsulation systems have also been
55 demonstrated to be effective for fruit juice preservation in many studies (Ghosh et al., 2014;
56 Shah et al., 2012; Sugumar et al., 2016; Truong et al., 2010). Azzi et al. (2018) previously
57 prepared hydroxypropyl- β -cyclodextrin (HP- β -CD)/Ner inclusion complex, Ner-loaded
58 conventional liposome (CL), and Ner-in-cyclodextrin-in-liposome (DCL) systems. The prepared
59 aqueous formulations were optimized and characterized in terms of size, encapsulation
60 efficiency, loading rate, release rate, photostability, and storage stability and yielded preparations
61 with high encapsulation efficiencies and loading rates. Moreover, the DCL system exhibited the
62 lowest release rate of Ner, followed by CLs and the inclusion complexes. Significant
63 enhancements of the photostability and storage stability (4 °C) of Ner were also observed.

64 Ner has been previously shown to exhibit outstanding activity against *Lactobacillus fermentum*
65 in de Man, Rogosa, and Sharpe (MRS) broth (pH 6.2, 37 °C) (Ephrem et al., 2019). In this study,
66 *L. fermentum* ATCC 9338, an obligate heterofermentative, gram-positive, anaerobic, thermo-
67 acidophilic lactic acid bacterium linked to fruit juice spoilage was used as a model strain.

68 According to Lawlor et al. (2009), heterofermentative lactic acid bacteria are the major
69 contributors to fruit juice spoilage. These bacteria can produce different products in fruit juices,
70 including lactic acid, acetic acid, ethanol, and carbon dioxide, which are primarily responsible
71 for the alteration of the organoleptic properties of juice (Ashurst, 2016). Aneja et al. (2014)
72 reported 23.3% occurrence of lactic acid bacteria in orange juice. Garcia et al. (2016) identified
73 up to 24 *L. fermentum* strains in different fruit pulp processing byproducts, including pineapple,
74 mango, strawberry, soursop, and Barbados cherry. *L. fermentum* was also isolated from
75 unpasteurized orange juice (Parish and Higgins, 1988) and from pasteurized mixed fruit juice
76 (pineapple, orange, paw-paw, and guava) stored in the presence of sodium benzoate (0.08%)
77 (Uzoukwu et al., 2015).

78 In this study, HP- β -CD/Ner inclusion complexes and Ner-loaded CLs and DCLs were prepared
79 as previously reported by Azzi et al. (2018). The efficacy of free Ner, Ner solubilized in
80 dimethyl sulfoxide (DMSO), HP- β -CD/Ner inclusion complexes, and Ner-loaded CLs and DCLs
81 systems was investigated in orange juice against *L. fermentum*.

82 **2 Materials and methods**

83 **2.1 Chemical and reagents**

84

85 Nerolidol (98%, mixture of *cis* (40%) and *trans* (60%) isomers), thymol (>99%), absolute
86 ethanol, methanol (HPLC-grade), and DMSO were purchased from Sigma-Aldrich (Missouri,
87 United States). HP- β -CD was purchased from Wacker-Chemie (Lyon, France). De
88 Man, Rogosa, and Sharpe (MRS) broth and MRS agar were purchased from Laboratorios Conda
89 (Madrid, Spain). Lipoid E80 (80-85% egg phosphatidylcholine, 7-9.5%
90 phosphatidylethanolamine, 2% water, and 0.2% ethanol) was purchased from Lipoid GmbH

91 (Ludwigshafen, Germany). Citric acid was purchased from Sigma-Aldrich (Steinheim,
92 Germany).

93 **2.2 Bacterial strain and culture conditions**

94
95 *L. fermentum* ATCC 9338 was purchased from the American Type Culture Collection (Virginia,
96 USA). *L. fermentum* cultures were routinely maintained on MRS agar at 4 °C. Before each
97 antimicrobial assay, fresh cultures were prepared in sterile MRS broth and incubated at 37 °C for
98 22 h under anaerobic conditions. A bacterial suspension was prepared by diluting the bacterial
99 culture in MRS broth to a final concentration of 25×10^5 CFU(colony forming unit)/mL.

100 **2.3 Preparation of HP-β-CD/Ner inclusion complexes, conventional liposomes and drug-** 101 **in-cyclodextrin-in-liposomes loaded with Ner**

102 HP-β-CD/Ner inclusion complexes as well as liposomes (CL and DCL systems) loaded with Ner
103 were prepared as previously described by Azzi et al. (2018). Ner inclusion complexes were
104 prepared using the freeze-drying method. First, an aqueous solution (300 mL) containing HP-β-
105 CD (25 mM) and an excess of Ner (667 mg) was magnetically stirred at 300 rpm for 24 h at
106 room temperature. Then, the suspension was filtered (0.45 μm, cellulose acetate membrane) to
107 remove excess Ner, after which it was frozen at -80 °C and lyophilized. Blank HP-β-CD was
108 prepared similarly without Ner. The obtained powders were stored at 4 °C until usage.

109 CLs and DCLs loaded with Ner were prepared using the ethanol injection method as previously
110 described by Azzi et al. (2018). Briefly, to prepare CLs, E80 phospholipids were dissolved in
111 ethanol at 50 mg/mL (0.066 M), after which Ner was added to the organic phase at an E80
112 phospholipid to Ner molar ratio of 100:10. The organic phase (10 mL) was then added to 20 mL
113 of ultrapure water at an injection flow rate of 1 mL/min and under magnetic stirring (400 rpm) at
114 room temperature. The obtained suspension was magnetically stirred for 15 min, after which

115 ethanol and a small volume of water were evaporated under reduced pressure at 25 °C, and a
116 final volume of 17 mL was obtained. Blank CLs were prepared similarly without Ner.
117 Ner-loaded DCLs were prepared following the same method as CL except that the organic phase
118 (10 mL) was added to 20 mL of the HP- β -CD/Ner inclusion complex aqueous solution, which
119 was prepared as described above. The obtained DCL system had an E80:HP- β -CD:Ner molar
120 ratio of 100:75:30. For the preparation of blank DCLs, the organic phase was added to an
121 aqueous solution of HP- β -CD (25 mM).
122 The CL and DCL suspensions were centrifuged at 15 000 rpm for 30 min at 4 °C to eliminate the
123 excess aqueous media. HP- β -CD, used as cryoprotectant, was added to the suspensions at a
124 concentration of 25 mM (Gharib et al., 2017; Sebaaly et al., 2016), after which the samples were
125 frozen at -80 °C and lyophilized. The obtained powders were stored at 4 °C until usage.

126 **2.4 Characterization of delivery systems**

127

128 **2.4.1 Determination of Ner incorporation in the delivery systems by HPLC analysis**

129

130 The total concentration of Ner in the encapsulation systems was determined by HPLC as
131 previously described (Azzi et al., 2018). The HPLC instrument used was a Hitachi LaChrom
132 Elite® HPLC system (L-2130 pump, L-2200 autosampler) coupled to a L-2455 diode array
133 detector. Samples were prepared at a concentration of 1 mg/mL in water. To generate a
134 calibration curve, standard solutions of Ner in methanol were prepared at concentrations ranging
135 from 2.5 to 250 μ g/mL, with thymol used as an internal standard at a concentration of 100 μ g/mL
136 in methanol. For HPLC analyses, 100 μ L of the Ner standard solutions, liposomal suspensions,
137 or inclusion complex solutions were added to 200 μ L of methanol and 100 μ L of thymol solution
138 (100 μ g/mL). The samples were then sonicated for 10 min at 4 °C and centrifuged at 15 000 rpm
139 for 30 min at 4 °C. The supernatant (20 μ L) was then analysed using a reversed-phase C18

140 Agilent column (150 × 4.6 mm, 5 μm), using a mobile phase of methanol/ultrapure water (75/25
141 v/v) at a flow rate of 1 mL/min. The absorbance was measured at 212 nm and used to calculate
142 the mass of Ner per mg of powder.

143 **2.4.2 Size, PDI, and zeta potential of liposome formulations**

144
145 The mean size and polydispersity index (PDI) values of the freeze-dried CLs and DCLs
146 suspended in water were determined by dynamic light scattering (DLS) using a Malvern
147 Zetasizer (3600, Orsay, France). The zeta potential was determined using a Malvern Zetasizer
148 nanoSeries. Each sample was diluted to obtain a count rate between 150 and 300 kilo counts per
149 second. The measurements were repeated in triplicates at 25 °C.

150 **2.4.3 Transmission electron microscopy analysis of liposome formulations**

151
152 The morphologies of the liposomal formulations were determined by transmission electron
153 microscopy (TEM) using a Philips CM120 microscope (Eindhoven, Netherlands) at the “Centre
154 Technologique des Microstructures” (CTμ) at the University of Lyon (Villeurbanne, France).
155 The analyses were performed at an acceleration of 80 kV. One drop of each sample was placed
156 on a copper grid coated with carbon. After 2 min, the excess sample was removed using a filter
157 paper to obtain a thin film on the grid. Negative staining was performed using a 1% sodium
158 silicotungstate solution. After 30 s, the excess sodium silicotungstate solution was removed with
159 filter paper, and the sample was then left to dry at room temperature.

160 **2.4.4 Scanning electron microscopy analysis**

161
162 Scanning electron microscopy (SEM) analyses were performed using an FEI Quanta 250 FEG
163 microscope at the “Centre Technologique des Microstructures” (CTμ) at the University of Lyon
164 (Villeurbanne, France). Freeze-dried samples of HP-β-CD, HP-β-CD/Ner complexes, blank

165 liposomes (CLs or DCLs), or Ner-loaded liposomes (CLs or DCLs) were deposited on a flat steel
166 holder. The samples were then coated under vacuum by cathodic sputtering with copper and
167 observed by SEM under an accelerating voltage of 10 kV.

168 **2.5 Preparation and quality evaluation of orange juice samples**

169
170 Valencia oranges (*Citrus sinensis*) were purchased from a Lebanese local market. Unblemished
171 fruits were selected, washed, and squeezed using a manual hand juicer. Physicochemical
172 parameters (pH, titratable acidity, and Brix degree) were assessed to evaluate the quality of the
173 orange juice during the experimental procedures. The pH of orange juice was determined using a
174 pH meter (Orion Star A111, Thermo Scientific, USA). The pH measures the total concentration
175 of free H⁺ ions in a solution. The titratable acidity (TA) was determined by titration with a
176 sodium hydroxide (0.1 N) solution. The TA represents the total concentration of organic and
177 inorganic acids present in the juice and is expressed as grams of citric acid per litre of orange
178 juice, as citric acid is the major organic acid in orange juice. The total soluble solids (Brix
179 degree) was determined by refractometry using a hand refractometer (713528, Carl Zeiss Jena,
180 Germany). The Brix degree is an indicator of the sugar content in juice, where 1 Brix degree is
181 expressed as 1 g of sucrose in 100 g of juice.

182 **2.6 Bacterial studies**

183

184 **2.6.1 Bacterial survival and proliferation in orange juice**

185

186 The ability of *L. fermentum* to survive or proliferate in orange juice was investigated at two
187 different storage temperatures (4 and 25 °C). In addition, the sensitivity of *L. fermentum* to the
188 pH of orange juice was investigated at pH values between 2.5 and 3.9. When needed, the pH of
189 the orange juice samples was lowered using a citric acid solution (3 M). A 200- μ L volume of a

190 diluted bacterial suspension (25×10^5 CFU/mL) was added to 4.8 mL of orange juice in glass
191 culture tubes (20×100 mm) under sterile conditions to yield an initial bacterial count of 10^5
192 CFU/mL in juice. Control tubes contained orange juice without *L. fermentum*. The bacterial
193 counts were then determined at 0, 24, 48, and 96 h using the plate count method. To this end,
194 samples were serially diluted in peptone water, and 100 μ L of each dilution was spread onto
195 MRS agar. The bacterial counts were then determined after incubating the plates at 37 °C for 22
196 h. Each experiment was performed in triplicate.

197 **2.6.2 Antibacterial activity of Ner solubilized in DMSO in refrigerated orange juice**

198
199 Ner was solubilized in DMSO and tested against *L. fermentum* in orange juice (pH adjusted to
200 3.3) at different concentrations. Twenty five microliters of DMSO-solubilized Ner was added to
201 4.8 mL of orange juice (pH 3.3, TA of approximately 12 g/L) in glass culture tubes (20×100
202 mm) under sterile conditions to yield a range of Ner concentrations (between 50 and 4 000 μ M).
203 Subsequently, 200 μ L of a diluted *L. fermentum* suspension (25×10^5 CFU/mL) was added, and
204 the initial bacterial count in the culture medium was approximately 10^5 CFU/mL. Each test was
205 performed in triplicate under sterile conditions. Orange juice supplemented with DMSO (25 μ L),
206 with or without *L. fermentum*, served as controls for bacterial growth. In addition, control tubes
207 containing orange juice alone were used to exclude the possibility of contamination with lactic
208 acid bacteria. The prepared tubes were then incubated at 4 °C, and changes in the bacterial
209 counts were followed over 10 days of incubation at 4 °C by the plate count method as described
210 above.

211

212

213 **2.6.3 Antibacterial activity of encapsulated Ner in refrigerated orange juice**

214
215 HP- β -CD/Ner complex and Ner-loaded CLs and DCLs were tested against *L. fermentum* in
216 orange juice. Each freeze-dried formulation was added to fresh orange juice (4.8 mL) under
217 sterile conditions to yield the desired concentration of Ner (2 000 or 4 000 μ M). The tubes were
218 then inoculated with *L. fermentum* as previously described and incubated at 4 °C. The bacterial
219 count was assessed over time (20 days) using the plate count method. The experiment was
220 repeated in triplicate.

221 **2.7 Statistical analysis**

222
223 Statistical analysis was performed using Student t-test, and *P* values equal to or less than 0.05
224 were considered to be significant.

225 **3 Results and discussion**

226 **3.1 Characterization of the HP- β -CD/Ner complex, CL, and DCL systems**

227 **3.1.1 Determination of the mass of Ner/mass of freeze-dried encapsulation system**

228 The HPLC analysis results showed that the retained mass of Ner in the HP- β -CD/Ner complexes,
229 CLs, and DCLs was 40, 30, and 15 μ g Ner/mg, respectively.

230 **3.1.2 Size, homogeneity, and morphology of freeze-dried liposomes reconstituted with** 231 **water**

232
233 The DLS analysis of the four liposomal formulations (blank CLs, Ner-loaded CLs, blank DCLs,
234 Ner-loaded DCLs) showed three populations, **in contrast to the two populations obtained by laser**
235 **granulometry analysis of non-freeze dried suspensions** (Azzi et al., 2018). The PdI values of
236 these samples were higher than 0.48 (Table 1), highlighting the low homogeneity of the
237 suspensions. Moreover, the second population represents the major population and had a mean

238 particle size of approximately 1 μm , which is higher than that previously reported by Azzi et al.
 239 (2018). Indeed, the lyophilization process may promote liposome aggregation and fusion. Gharib
 240 et al. (2017) previously reported on the increment of the size and the PDI of the unsaturated
 241 lipoid S100 liposomal suspensions during the freeze drying process, unlike liposomes prepared
 242 with saturated phospholipids (phospholipon 90 H). Similarly, in this study the liposomal
 243 formulations prepared with the unsaturated lipoid E80 demonstrated an increase of the size
 244 following the freeze-drying process.

245 The incorporation of Ner in CL increased the size of particles, similarly to the results reported by
 246 Azzi et al. (2018). In contrast, Ner had no effect on the size of DCL particles. On the other hand,
 247 blank CL and DCL showed a negative zeta potential, which was not greatly affected by the
 248 presence of Ner. Also, the obtained zeta potential values were similar to those previously
 249 reported in literature for lipoid E80 liposomes (Karn et al., 2013).

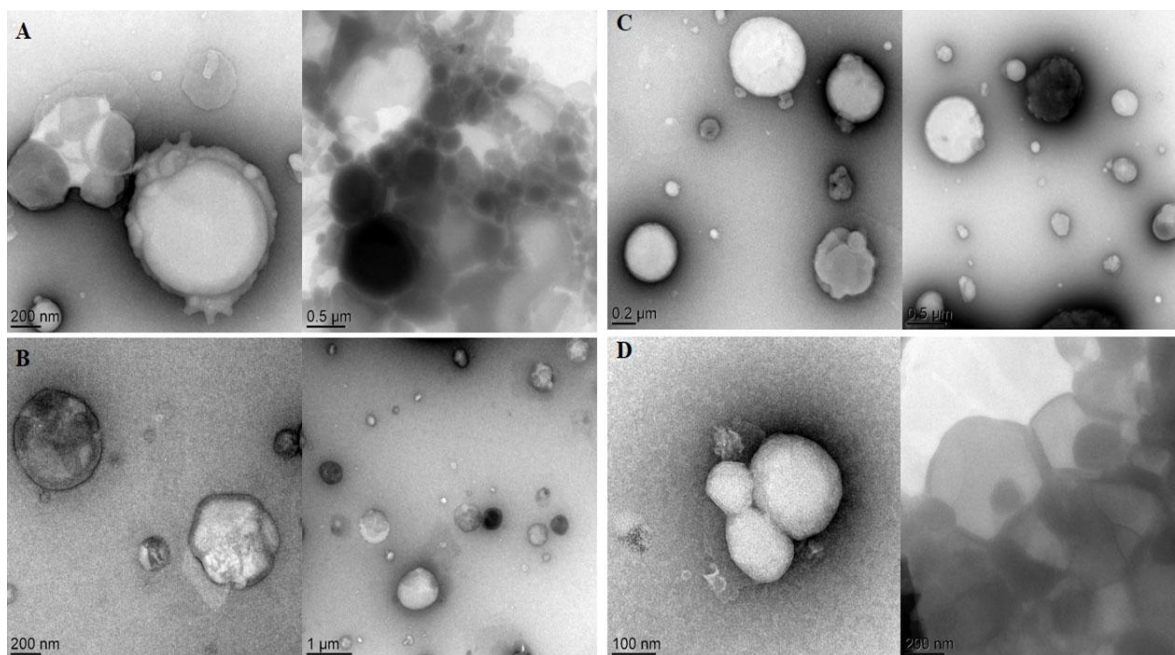
250 **Table 1: Mean size, PDI, and zeta potential values of blank CL and DCL, and Ner-loaded**
 251 **CL and DCL.**

Delivery system	Population 1		Population 2		Population 3		PDI	Zeta potential (mV)
	(%)	Mean size (nm)	(%)	Mean size (nm)	(%)	Mean size (μm)		
Blank CL	24.6	176.3 \pm 55.1	72.9	866.3 \pm 392.4	2.6	5.0 \pm 0.6	0.51	- 32.7 \pm 4.49
Ner-loaded CL	2.3	60.9 \pm 15.2	97.7	1114 \pm 917	-	-	0.52	- 33.1 \pm 3.79
Blank DCL	7.3	130.4 \pm 33.1	76.2	925 \pm 531.5	16.5	4.2 \pm 0.9	0.50	-

								34.1±5.39
Ner- loaded	6.6	119.3±21.6	74.0	925.7±318.4	19.4	4.7±0.7	0.49	-
DCL								40.2±5.42

252

253 TEM analysis supported the results obtained by DLS, as two populations were observed for
 254 blank and Ner-loaded CL and DCL systems (Fig. 1). However, particles with a mean diameter
 255 close to or higher than 2 µm were not observed by TEM for all formulations (Fig. 1).

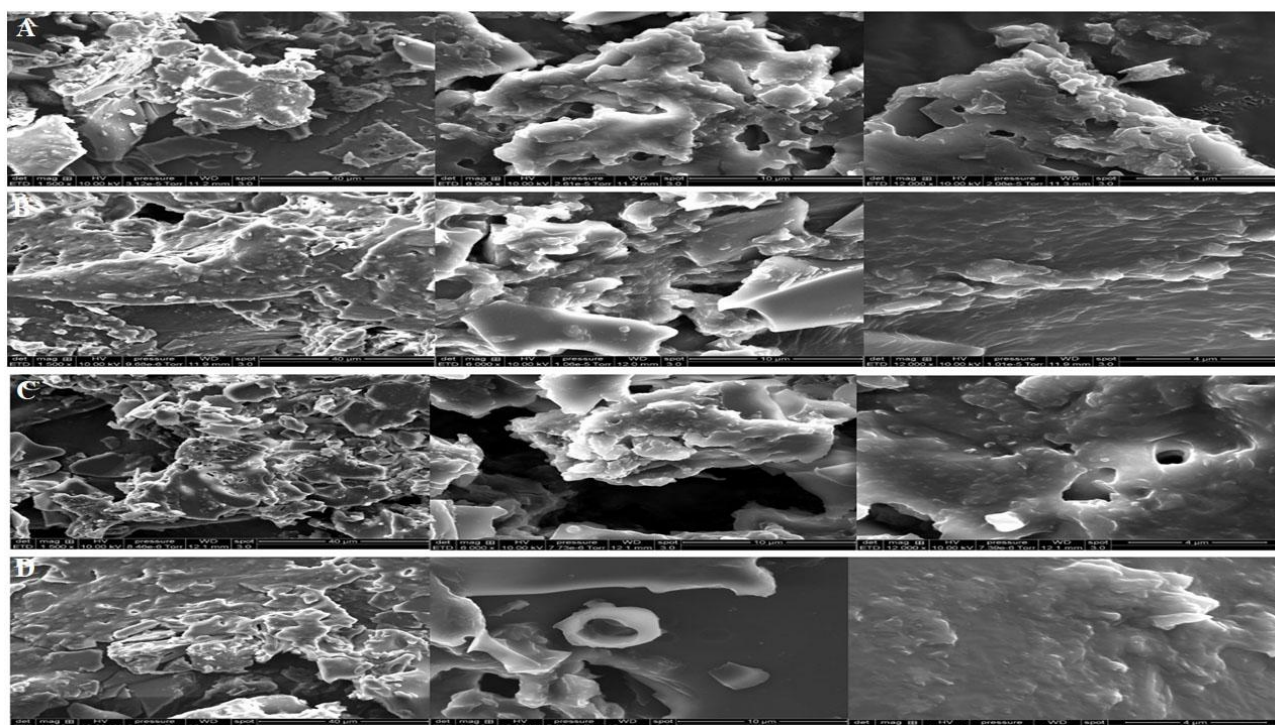


256

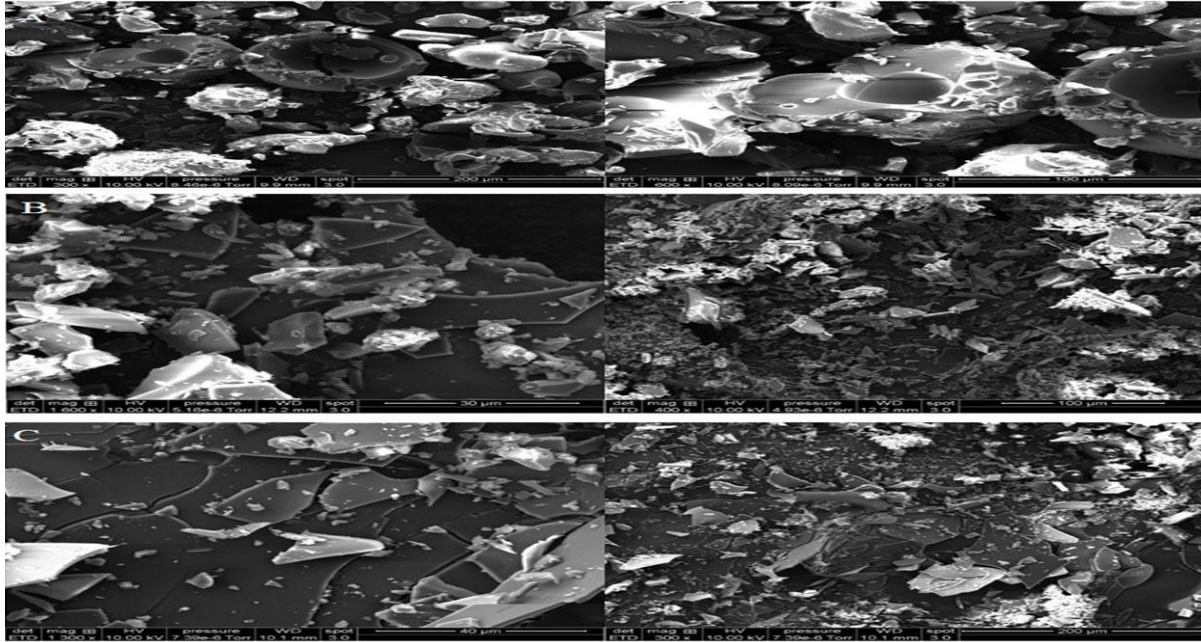
257 **Fig. 1** TEM images of blank CL (A), Ner-loaded CL (B), blank DCL (C), and Ner-loaded DCL
 258 (D).

259 SEM analysis of the freeze-dried CL and DCL showed irregular particles structures (Fig. 2). On
 260 the other hand, untreated HP-β-CD sample appeared as amorphous spherical particles, and the
 261 HP-β-CD/Ner inclusion complexes were observed as irregular particles (Fig. 3). Similar results
 262 were obtained by Figueiras et al. (2007) and Li et al. (2016) for untreated CD powder,

263 CD/omeprazole, and CD/catechin inclusion complexes prepared by freeze-drying. The SEM
264 images of HP- β -CD prepared in the same manner as the HP- β -CD/Ner complexes (dissolution,
265 filtration, and lyophilization) were similar to those of the inclusion complexes. The SEM
266 analysis results could not demonstrate the formation of the inclusion complexes, as the newly
267 observed structures are a direct consequence of the preparation method.



268
269 **Fig. 2** SEM images of blank CL (A), Ner-loaded CL (B), blank DCL (C), and Ner-loaded DCL
270 (D).



271

272 **Fig. 3** SEM images of untreated HP-β-CD (A), HP-β-CD treated as HP-β-CD/Ner complex (B),
 273 and HP-β-CD/Ner inclusion complex (C).

274 **3.2 Bacterial studies**

275

276 **3.2.1 Evaluation of orange juice quality parameters**

277

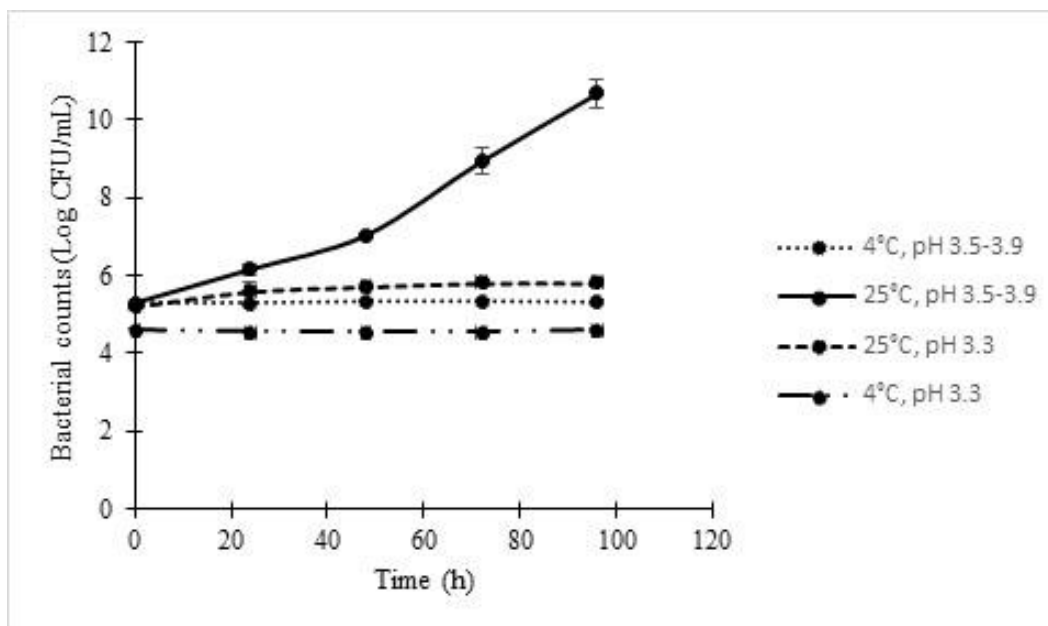
278 Physicochemical quality parameters were assessed for all of the freshly-squeezed orange juice
 279 samples, which exhibited pH values between 3.5 and 3.9, titratable acidity (TA) values between
 280 5 and 6 g/L of citric acid, and Brix degree values of approximately 11-12 °Brix. When orange
 281 juice was stored at 4 °C, the values of these parameters were maintained and were unaffected by
 282 the presence of *L. fermentum* due to the inhibition of bacterial proliferation and metabolic
 283 activity at this temperature. At 25 °C and after 96 h of incubation, the TA increased to 9.4 ± 0.45
 284 g/L of citric acid, equivalent to that of orange juice spiked with *L. fermentum*, and the Brix
 285 degree was decreased by approximately 1 °Brix, whereas no significant changes were noted in
 286 the pH value (data not shown). In addition, similar variations in TA and Brix degree values were

287 observed in the tubes without *L. fermentum* at 96 h due to the proliferation of naturally occurring
288 spoilage bacteria or fungi in orange juice.

289 **3.2.2 *L. fermentum* survival and proliferation in orange juice**

290
291 *L. fermentum* was able to survive in freshly-squeezed orange juice (pH 3.5-3.9; TA 5-6 g/L citric
292 acid; 11-12 °Brix) at 4 °C until the end of the experiment (96 h) (Fig. 4) and exhibited
293 exponential growth at 25 °C, with an approximately 5.5 log increase of the bacterial count after
294 96 h of incubation (Fig. 4). To further investigate the tolerance of *L. fermentum* to acidity in
295 orange juice, the survival and proliferation of *L. fermentum* was tested at 4 and 25 °C in orange
296 juice samples in which the pH was adjusted with citric acid (3 M) to values ranging from 2.5 to
297 3.3. At a pH value of 3.3 (TA of approximately 12 g/L citric acid), the bacteria were able to
298 survive at 4 °C, and only a slight proliferation was observed at 25 °C (Fig. 4). At pH values
299 lower than or equal to 3.0 (TA > 22 g/L citric acid), *L. fermentum* was unable to survive in
300 orange juice at 4 and 25 °C (data not shown). These results indicate that a low pH and/or a high
301 organic acid content in juice may inhibit bacterial growth and even lead to cell death.
302 Furthermore, the effectiveness of organic acids as preservatives is enhanced at lower pH values,
303 as they are active when present in their undissociated form. In the latter state, organic acids can
304 easily penetrate the cytoplasmic membrane of bacteria and alter their internal pH. The pKa
305 values of citric and malic acids, which are the primary organic acids of orange juice, are 3.13 and
306 3.40, respectively (Kim and Rhee, 2013). Thus, at pH values below 3.5, the two organic acids are
307 primarily in their antibacterial, undissociated forms.

308 To avoid possible interference by other microorganisms and to perform the experiments under
309 the storage conditions used for fresh orange juice, the antibacterial activity of Ner was assessed
310 at 4 °C.



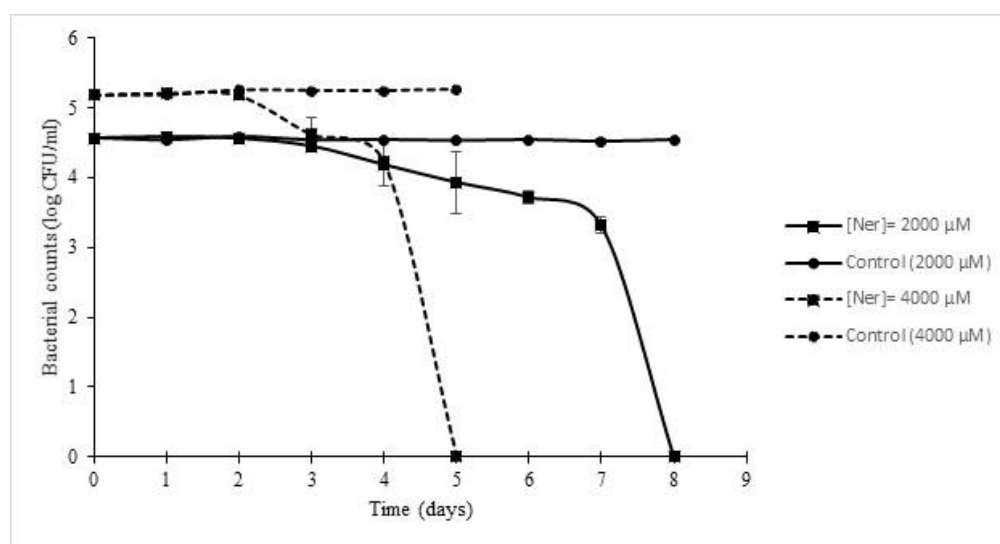
311
 312 **Fig. 4** *L. fermentum* survival and proliferation in orange juice at 4 and 25 °C, at different initial
 313 pH values. TA 5-6 g/L corresponds to a pH of 3.5-3.9; TA of 12 g/L corresponds to pH value of
 314 3.3.

315 3.2.3 Antibacterial activity of Ner in refrigerated orange juice

316
 317 Based on the ability of *L. fermentum* to survive in orange juice at a pH values higher than 3.0, the
 318 antibacterial activity of Ner was assessed in orange juice with a pH value of 3.3 (TA
 319 approximately 12 g/L citric acid) at 4 °C. Ner solubilized in DMSO was tested at concentrations
 320 between 50 and 4 000 μM. At concentrations lower than 2 000 μM (50-1 000 μM), Ner showed
 321 no antibacterial activity for up to 10 days of incubation (data not shown). At 2 000 and 4 000
 322 μM, total bacterial death was obtained within 8 and 5 days of incubation, respectively (Fig. 5).
 323 Surprisingly, the effective concentration of Ner and the time needed to reach total bactericidal
 324 activity in orange juice (pH 3.3, 4 °C) increased by 40- and 12-fold, respectively, compared to
 325 that observed in MRS broth (pH 6.2, 37 °C) (Ephrem et al., 2019). The less potent and delayed
 326 effect of Ner in orange juice may be due to the influence of the incubation temperature and the

327 rich matrix of the juice. Interestingly, Friedman et al. (2004) reported that the antibacterial
328 activity of essential oil components against *Escherichia coli* and *Salmonella enterica* in apple
329 juice was enhanced as the incubation temperature increased from 4 to 37 °C. Some orange juice
330 components may also be major suppressors of Ner activity. For instance, the interaction between
331 essential oils and food components may decrease the bioactivity of essential oil components in
332 food products (Perricone et al., 2015).

333 Effective concentrations (2 000 and 4 000 µM) of Ner were directly added without DMSO to
334 orange juice, and the antibacterial activities of solubilized and unsolubilized Ner in orange juice
335 were compared. The results showed that similar antibacterial activities were obtained.

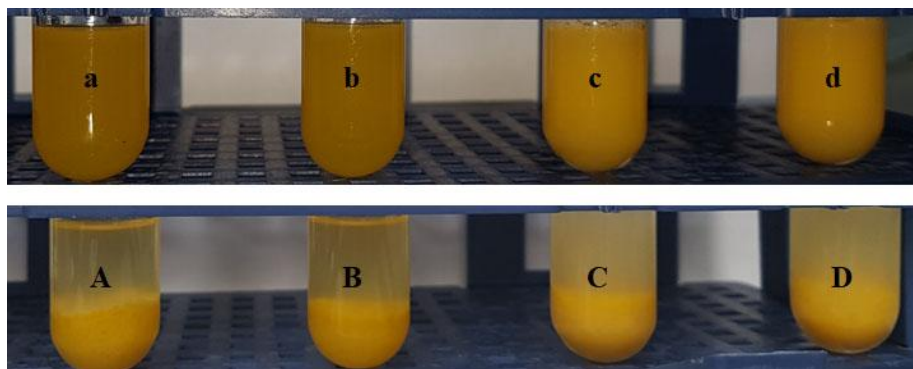


336
337 **Fig. 5** Antibacterial activity of Ner at 2 000 and 4 000 µM in orange juice (pH 3.3; TA:
338 approximately 12 g/L citric acid; 11-12 °Brix) at 4 °C against *L. fermentum*.

339 Nevertheless, at 2 000 and 4 000 µM, undissolved Ner particles were observed in the juice stored
340 at 4 °C, demonstrating the need to solubilize Ner for use in juice. Thus, the antibacterial
341 activities of the HP-β-CD/Ner inclusion complexes, Ner-loaded CL, and Ner-loaded DCL
342 systems were investigated in fresh orange juice stored at 4 °C.

343 3.2.4 Antibacterial activity of encapsulated Ner in refrigerated orange juice

344
345 The HP- β -CD inclusion complex, CL, and DCL systems were tested for their ability to maintain
346 or improve the antibacterial activity of Ner in orange juice (pH 3.3; TA: approximately 12 g/L
347 citric acid; 11-12 °Brix). When Ner-loaded CLs and DCLs were added at a final Ner
348 concentration of 2 000 μ M, the antibacterial activity was considerably delayed, and no
349 antibacterial activity was observed for up to 20 days of storage (data not shown). Compared to
350 the CD inclusion complexes, the CL and DCL systems extended the release of Ner in water at 25
351 °C (Azzi et al., 2018). This result could be due to the strong retention of the loaded agent in
352 liposomes (CLs and DCLs) compared to the inclusion complex system. Furthermore, to be
353 released, Ner needs to overcome the bilayered membrane of CLs and the inclusion complex in
354 addition to the membrane in DCL. Even though this approach increases the photostability of Ner
355 and enhances its stability during storage (Azzi et al., 2018), the delayed release abrogates the
356 antibacterial activity of Ner in orange juice. Thus, the maintained Ner concentration in contact
357 with *L. fermentum* is not effective against the bacterial cells. Moreover, the CL and DCL systems
358 may have specific limitations in fruit juice applications at the tested concentration due to their
359 milky appearance (Fig. 6). However, the HP- β -CD/Ner inclusion complexes did not alter the
360 appearance of the juice (Fig. 6) and showed a total bactericidal activity in orange juice against *L.*
361 *fermentum* within 17 days of incubation at 2 000 μ M and 4 °C (Fig. 7). Therefore, only the
362 inclusion complex was assessed in subsequent investigations.

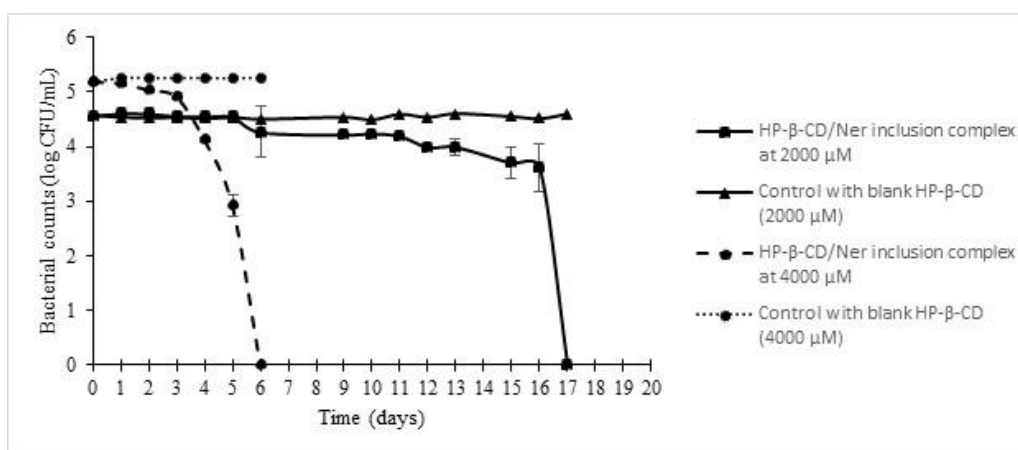


363

364 **Fig. 6** Appearance of orange juice stored at 4 °C in the absence (a and A) and presence of HP-β-
365 CD/Ner complex (b and B), Ner-loaded CL (c and C), and Ner-loaded DCL (d and D), at t= 0
366 and after 24 hours of incubation, respectively. All delivery systems were added to give a final
367 Ner concentration of 2 000 μM.

368 When the HP-β-CD/Ner inclusion complexes were added at a final Ner concentration of 4 000
369 μM, the appearance, pH (3.3), and TA (approximately 12 g/L citric acid) of the juice were not
370 altered. However, a slight change was observed in the Brix degree value, which increased from
371 11-12 to 13 °Brix. Moreover, HP-β-CD/Ner inclusion complex killed all bacteria within 6 days
372 of incubation at 4 °C (Fig. 7). Thus, the inclusion complex showed a bactericidal activity against
373 *L. fermentum* approximately that was 3-fold faster at 4 000 μM than at 2 000 μM (Fig. 7). This
374 result may highlight the higher aqueous solubility of Ner incorporated in inclusion complexes
375 compared to free Ner. Furthermore, the incorporation of Ner into inclusion complexes was
376 observed to increase its aqueous solubility by more than 100-fold at 25 °C (Azzi et al., 2018).
377 However, HP-β-CD/Ner inclusion complexes showed delayed antibacterial activity compared to
378 the free form (8 versus 17 days at 2 000 μM and 5 versus 6 days at 4 000 μM). This delay was
379 even more pronounced at 2 000 μM (nine-days delay) compared to 4 000 μM (one-day delay).
380 This delay may be due to the release kinetics of Ner from the inclusion complexes. Azzi et al.
381 (2018) reported the release of approximately 45% of Ner from inclusion complexes within 8 h,

382 followed by a slow release over 7 days at 25 °C in water. Therefore, at 4 000 µM, the available
 383 concentration of Ner for interacting with *L. fermentum* in juice is approximately 2 000 µM after
 384 8 to 10 h of incubation, with complete bacterial killing observed within 6 days compared to 5
 385 days for free Ner. Shah et al. (2012) also reported a better antibacterial activity of free thymol
 386 compared to thymol nanodispersion. However, different factors limit the application of free
 387 hydrophobic molecules in food rich in water, such as fruit juice, including the appearance of
 388 undissolved particles (Shah et al., 2012) and the low physicochemical stability of the added
 389 agent. In this study, agglomerates of free Ner were observed in orange juice at the assayed
 390 concentrations. On the other hand, Ner has a poor photostability, which was overcome by its
 391 incorporation into HP-β-CD inclusion complexes (Azzi et al., 2018).



392
 393 **Fig. 7** Antibacterial activity of HP-β-CD/Ner complex in orange juice (pH 3.3; TA:
 394 approximately 12 g/L citric acid; 11-12 °Brix) at 4 °C against *L. fermentum*. HP-β-CD/Ner
 395 complex is added to orange juice to give a final concentration of 2 000 µM and 4 000 µM of Ner.

396 4 Conclusions

397
 398 In this study, the use of HP-β-CD/Ner inclusion complexes was observed to be effective against
 399 *L. fermentum* in orange juice (pH 3.3; TA: approximately 12 g/L citric acid; 11-12 °Brix) at 2

400 000 and 4 000 μM , although faster bacterial killing was obtained at 4 000 μM . The addition of
401 the inclusion complexes to the juice at 4 000 μM did not alter the appearance, pH, and TA of
402 juice, and only a slight increase in the Brix degree value was observed. In contrast, the Ner-
403 loaded CL and DCL systems (2 000 μM) strongly altered the physical appearance of the juice
404 and did not exhibit an antibacterial effect within 20 days. According to the results obtained in
405 this study, we can suggest that a combination of cold storage and HP- β -CD/Ner inclusion
406 complexes limits the bacterial spoilage of fresh orange juice. In addition, assessing the use of a
407 combination of the three systems may be interesting, as the inclusion complexes exhibits rapid
408 activity against the bacterium, whereas the liposomal formulations extended the presence of Ner
409 in the juice.

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414 **Conflict of interest**

415 The authors declare that there are no conflicts of interest regarding the publication of this work.

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