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Elissa Ephrem, Amal Najjar, Catherine Charcosset, H el ene Greige-Gerges

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1 **Selection of nerolidol among a series of terpenic and phenolic compounds for**  
2 **its potent activity against *Lactobacillus fermentum* ATCC 9338**

3 **Elissa Ephrem<sup>a,b</sup>, Amal Najjar<sup>a</sup>, Catherine Charcosset<sup>b</sup>, H  l  ne Greige-Gerges<sup>a,\*</sup>**

4 <sup>a</sup> Bioactive Molecules Research Laboratory, Faculty of Sciences, Lebanese University, B.P.  
5 90656 Jdaidet El-Matn, Lebanon.

6 <sup>b</sup> Laboratoire d'Automatique et de G  nie des Proc  d  s, Universit   Claude Bernard Lyon I,  
7 France, 43 Boulevard du 11 Novembre 1918, B  timent CPE 69 622, Villeurbanne Cedex,  
8 France.

9 \*Corresponding author: Faculty of Sciences, Bioactive Molecules Research Laboratory,  
10 Lebanese University, B.P. 90656 Jdaidet El-Matn, Lebanon.

11 Tel.: +961 3 341011; Fax: +961 1 689647

12 E-mail addresses: greigegeorges@yahoo.com, hgreige@ul.edu.lb (H  l  ne Greige-Gerges).

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22 **Abstract**

23 Essential oil components are widely used for their antibacterial activity against spoilage  
24 microorganisms in food. *Lactobacillus fermentum* is a Gram-positive lactic acid bacteria  
25 responsible for the deterioration of various food products, including beverages and dairy  
26 products. In this study, 17 terpenic and 11 phenolic compounds were screened against the food  
27 spoilage microorganism *Lactobacillus fermentum* ATCC 9338. The antibacterial activity of the  
28 tested compounds was dependent on hydrophobicity and particular chemical features. Nerolidol  
29 solubilized in dimethylsulfoxide exhibited the highest antibacterial activity and showed low  
30 minimal inhibitory (MIC: 25  $\mu$ M) and minimal bactericidal (MBC: 50  $\mu$ M) concentrations.  
31 Moreover, no viable cells were detected within 16 h of incubation at 50  $\mu$ M. The important  
32 antibacterial activity of nerolidol against *L. fermentum* is probably related to the high  
33 hydrophobicity, the aliphatic chain length, and the presence of the hydroxyl group.  
34 Hydroxypropyl- $\beta$ -cyclodextrin/nerolidol inclusion complex showed MIC and MBC values of  
35 100 and 200  $\mu$ M, respectively. The total bacterial kill was observed after 12 h of incubation. The  
36 results obtained with the inclusion complex are probably due to the time required to allow  
37 nerolidol to be released from the inclusion complex.

38 **Keywords:** Cyclodextrins; *Lactobacillus fermentum*; nerolidol; phenolic compounds; terpenes.

39 **List of abbreviations:** CFU: colony forming unit; DMSO: dimethylsulfoxide; HP- $\beta$ -CD:  
40 hydroxypropyl- $\beta$ -cyclodextrin; HPLC: high performance liquid chromatography; LAB: lactic  
41 acid bacteria; MBC: minimal bactericidal concentration; MIC: minimal inhibitory concentration;  
42 Ner: nerolidol.

## 44 **1. Introduction**

45 *Lactobacillus fermentum* is an ubiquitous Gram-positive, rod-shaped, anaerobic, thermo-  
46 acidophilic lactic acid bacteria (LAB), which can originate from plants, animals, meat, dairies,  
47 fruits, and cereals. *L. fermentum* is an obligate heterofermentative bacteria which ferments  
48 various types of sugars (lactose, fructose, maltose, sucrose) under anaerobic conditions. In  
49 addition to lactic acid, *L. fermentum* produces acetic acid, ethanol, carbon dioxide, and minor by-  
50 products, such as diacetyl, hydrogen peroxide, and different alcohols (e.g. 3-methylbutanol),  
51 esters (e.g. ethyl acetate), and carbonyls (e.g. nonanal) compounds. *L. fermentum* is not harmful  
52 to humans and its presence is necessary for the fermentation of different food products, including  
53 sourdough, cocoa, and certain beverages. Certain *L. fermentum* strains are probiotic agents and  
54 express outstanding health-promoting characteristics when consumed [1]. Nonstarter lactic acid  
55 bacteria, including *L. fermentum*, cause defects in certain food products, such as slits or cracks in  
56 hard cheeses, lack of flavors, or bloated packaging in dairy products [2–4]. *L. fermentum* also  
57 spoils various types of beverages and can grow in fruit juice, leading to the production of  
58 undesirable compounds [5–9]. It is one of the LABs responsible for the desired malolactic  
59 fermentation in wine, resulting in the conversion of malic acid into lactic acid, acetate, succinate,  
60 and carbon dioxide [10]. However, the presence of LABs could lead to wine spoilage as the  
61 control of bacterial growth and malolactic fermentation is difficult to achieve, thus altering the  
62 wine organoleptic properties [10–12]. The growth of heterofermentative LABs (such as *L.*  
63 *fermentum*) in wine causes an increased acidity, cloudiness, and mousy odor [13]. On the other  
64 hand, *L. fermentum* can grow in beer, as it is resistant to hop-compounds [14], leading to beer  
65 spoilage [14,15] and aroma alteration [14].

66 Different strategies have been adopted to overcome the microbial spoilage of food, including the  
67 addition of chemical additives and physical treatments. Physical treatments include various  
68 preservation techniques, such as thermal, ultraviolet light, ultrasound, pulsed electric field, and  
69 high hydrostatic pressure technologies [16]. However, the application of these treatments is  
70 limited due to changes in the organoleptic properties of the food product [17–19] and sometimes  
71 due to high cost [20]. On the other hand, chemical preservatives used against food microbial  
72 spoilage, including benzoates, sorbates, propionates, nitrates, and nitrites, can cause allergic  
73 responses and could be converted to potential carcinogens [21]. The high demand of fresh and  
74 “safe” food, free of synthetic additives and contaminants, have increased the interest of using  
75 natural products for food preservation. Natural antimicrobials may derive from plants, animals,  
76 and microorganisms. Plant essential oils are largely exploited due to their wide spectrum of  
77 antimicrobial activity against spoilage bacteria and food-borne pathogens [22]. Plant  
78 antimicrobials include different chemical classes, among which are saponins, tannins, flavonoids,  
79 terpenes, simple phenols, and phenolic acids [23,24].

80 The majority of natural antimicrobials are hydrophobic and poorly stable, which limit their use in  
81 aqueous media. Solvents, such as dimethylsulfoxide (DMSO) [25], dimethylformamide [26], and  
82 ethanol [27, 28], are used to dissolve hydrophobic compounds in aqueous solutions. However,  
83 the use of organic solvents in food products is not desirable. Various encapsulation systems  
84 (nanoemulsions, liposomes, nanoparticles, solid lipid nanoparticles, cyclodextrins, etc.) were  
85 introduced to the food industry as a novel strategy to overcome the poor water solubility of food  
86 antimicrobials and to enhance their stability in food matrices [29,30]. Cyclodextrins are natural  
87 oligosaccharides widely used in food products for their safety and their ability to deliver  
88 hydrophobic compounds (e.g. antioxidants, antimicrobials, etc.) [31]. Moreover, different

89 derivatives of cyclodextrin have been synthesized to enhance the aqueous solubility of native  
90 cyclodextrins [32].

91 The objective of this study is to select a natural potent antibacterial agent against *L. fermentum*.

92 The selected antibacterial is then complexed with hydroxypropyl- $\beta$ -cyclodextrin (HP- $\beta$ -CD) and  
93 the obtained inclusion complex is tested against the chosen bacterium in culture medium.

94 Therefore, a wide range of terpenic and phenolic phytochemicals was screened against the  
95 bacterium, under its optimal growth conditions. The tested compounds belong to different

96 subclasses and possess variable structures. Seventeen terpenes were tested, among which eight  
97 monoterpene hydrocarbons (camphene, *p*-cymene, limonene,  $\alpha$ -phellandrene,  $\alpha$ -pinene,  $\beta$ -

98 pinene,  $\alpha$ -terpinene,  $\gamma$ -terpinene), a sesquiterpene hydrocarbon ( $\beta$ -caryophyllene), four  
99 monoterpene alcohols (borneol, geraniol, linalool, menthol), a sesquiterpene alcohol (nerolidol),

100 two monoterpene esters (bornyl acetate, linalyl acetate), and a monoterpene ketone (camphor)  
101 (Fig. 1). In addition, eleven phenols were tested, among which four phenylpropenes (*trans*-

102 anethole, eugenol, isoeugenol, estragole), a phenylpropene ester (eugenyl acetate), two  
103 hydroxycinnamic acids (*p*-coumaric acid, *trans*-ferulic acid), a phenol ether (anisole), a

104 methylphenol (*o*-cresol), a flavonoid (quercetin), and a stilbenoid (resveratrol) (Fig. 2).  
105 Nerolidol (Ner) showed the most potent antibacterial activity against *L. fermentum* among the 28

106 tested compounds. The potency of nerolidol solubilized in DMSO and hydroxypropyl- $\beta$ -  
107 cyclodextrin/nerolidol (HP- $\beta$ -CD/Ner) complex was assessed against *L. fermentum* by

108 determining the minimal inhibitory concentration (MIC) and the minimal bactericidal  
109 concentration (MBC) values under the optimal conditions for *L. fermentum* growth. The time

110 required for both forms of nerolidol to cause a total bacterial kill was determined by time-kill  
111 analysis.

## 112 2. Materials and methods

### 113 2.1. Materials

114  $\beta$ -Caryophyllene, geraniol, isoeugenol (98% mixture *cis* and *trans*), nerolidol (98%, mixture of  
115 *cis* (40%) and *trans* (60%) isomers), (-)- $\beta$ -pinene, ethanol, and DMSO were purchased from  
116 Sigma-Aldrich (Missouri, United States). *trans*-Anethole (99%) and linalyl acetate were  
117 purchased from Sigma-Aldrich (Madrid, Spain). Anisole, camphene, *o*-cresol, eugenol, eugenyl  
118 acetate, (-)-menthol, and  $\alpha$ -phellandrene were purchased from Sigma-Aldrich (Schnelldorf,  
119 Germany). (-)-Borneol, (-)-bornyl acetate, *p*-cymene, (R)-(t)-limonene, linalool, (+)- $\alpha$ -pinene,  $\alpha$ -  
120 terpinene, and  $\gamma$ -terpinene were purchased from Sigma-Aldrich (Buchs, Switzerland). ( $\pm$ )-  
121 Camphor and *trans*-ferulic acid were purchased from Sigma-Aldrich (Hong Kong, China).  
122 Quercetin was purchased from Sigma-Aldrich (Haryana, India). *p*-Coumaric acid was purchased  
123 from Sigma-Aldrich (Irvine, United kingdom). Hydroxypropyl- $\beta$ -cyclodextrin was purchased  
124 from Wacker-Chemie (Lyon, France). De Man, Rogosa, and Sharpe (MRS) broth and MRS agar  
125 were purchased from Laboratorios Conda (Madrid, Spain).

### 126 2.2. Bacterial strain and culture

127 *L. fermentum* ATCC 9338 was purchased from American Type Culture Collection (*Manassas*,  
128 Virginia, USA). *L. fermentum* cultures were routinely maintained at 4 °C on MRS agar. Before  
129 each antimicrobial assay, fresh cultures were prepared in sterile MRS broth and incubated at 37  
130 °C for 22 h under anaerobic conditions. A bacterial suspension was prepared by diluting the  
131 bacterial culture in MRS broth to a final concentration of  $25 \times 10^5$  colony forming unit (CFU)/ml.

## 132 2.3. Screening of natural terpenic and phenolic compounds for antibacterial activity

### 133 2.3.1. Screening rounds

134 Twenty-eight natural terpenic and phenolic compounds were screened for their antibacterial  
135 activity against *L. fermentum* at 3500, 500, 250, and 100  $\mu\text{M}$ . The tested molecules were  
136 dissolved in DMSO, except  $\beta$ -caryophyllene and camphene which were dissolved in ethanol, and  
137 the obtained solutions were homogenized by hand agitation prior to each test. Antibacterial agent  
138 solutions (25  $\mu\text{l}$ ) were mixed with MRS broth (4.8 ml) in glass culture tubes (20 x 100 mm). The  
139 tubes were then inoculated with 200  $\mu\text{l}$  of a diluted *L. fermentum* suspension ( $25 \times 10^5$  CFU/ml)  
140 to yield a bacterial concentration of  $10^5$  CFU/ml at baseline. Bacterial cultures (5 ml) exempt of  
141 any agent, or containing 25  $\mu\text{l}$  of DMSO or ethanol, served as controls. All cultures were  
142 incubated at 37 °C for 22 h under anaerobic conditions. Screening rounds were conducted with  
143 bioactive compounds at 3500, 500, and 250  $\mu\text{M}$ , successively. After each screening round,  
144 molecules demonstrating an anti-proliferative activity against *L. fermentum* at a given  
145 concentration were identified. Whereas, compounds demonstrating a bactericidal effect against  
146 *L. fermentum* were selected for another screening round at a lower concentration. The  
147 compounds exhibiting a total bactericidal activity or a total anti-proliferative activity against *L.*  
148 *fermentum* at 250  $\mu\text{M}$  were screened at 100  $\mu\text{M}$ . Each test was performed in triplicate and under  
149 sterile conditions.

### 150 2.3.2. Determination of the anti-proliferative activity of molecules

151 The anti-proliferative activity of the molecules against *L. fermentum* was assessed by UV-visible  
152 spectroscopy at 660 nm using Uviline 9100-9400 spectrophotometer (GmbH, Germany). The  
153 optical density of each tube was measured and compared to the control. The percentage of  
154 bacterial proliferation inhibition was calculated as follows:



155 
$$\text{Bacterial proliferation inhibition (\%)} = 100 \times \left[ 1 - \frac{OD_{660m}}{OD_{660c}} \right],$$

156 where  $OD_{660m}$  and  $OD_{660c}$  are the optical densities of the tubes containing the molecule and the  
157 control tube, respectively. Each test was done in triplicate.

### 158 2.3.3. Determination of the bactericidal activity of the natural molecules

159 Cultures showing a total inhibition of bacterial proliferation were analyzed in duplicate by  
160 spreading an aliquot of 100  $\mu$ l on MRS agar. The bacterial concentration in the control was  
161 determined by enumeration. The bactericidal capacity was then evaluated according to the  
162 decrease in the initial bacterial concentration and calculated as follows:

163 
$$\text{Bacterial kill (\%)} = 100 \times \left[ 1 - \frac{[bac]_m}{[bac]_c} \right],$$

164 where  $[bac]_m$  and  $[bac]_c$  are the bacterial concentrations in the tube containing the molecule and  
165 the control tube, respectively.

### 166 2.4. Preparation of HP- $\beta$ -CD/Ner inclusion complex

167 HP- $\beta$ -CD/Ner inclusion complex was prepared by freeze-drying, as previously described by Azzi  
168 et al. [33]. Briefly, an aqueous solution of HP- $\beta$ -CD (25 mM) containing an excess of nerolidol  
169 was kept under magnetic stirring at 300 rpm for 24 h at room temperature. The suspension was  
170 then filtered (0.45  $\mu$ m, cellulose acetate membrane) to remove the excess of nerolidol. The  
171 filtrate was frozen at -80  $^{\circ}$ C, and lyophilized. HP- $\beta$ -CD/Ner complex in powder form was stored  
172 at 4  $^{\circ}$ C until usage. The amount of nerolidol encapsulated in the cyclodextrin cavity was  
173 determined by HPLC as described previously by Azzi et al. [33], and the result was expressed as  
174 mass of nerolidol ( $\mu$ g) per mg of powder ( $\mu$ g<sub>Ner</sub>/mg<sub>powder</sub>).

175 2.5. Study of the antibacterial activity of nerolidol and HP- $\beta$ -CD/Ner complex

176 2.5.1. Determination of MIC and MBC values

177 Bacterial cultures were prepared as previously described in section 2.3.1, in presence of nerolidol  
178 dissolved in DMSO, and added at a final concentration ranging from 0.1 to 3500  $\mu$ M. The MIC  
179 was determined as the minimal concentration at which no bacterial growth was observed in MRS  
180 broth, whereas the MBC was determined as the minimal concentration at which no bacterial  
181 growth was observed on agar. Each experiment was performed in triplicate and under sterile  
182 conditions. On the other hand, HP- $\beta$ -CD was investigated for its capacity to replace DMSO for  
183 nerolidol solubilization in aqueous solution. The inclusion complex was tested at a final  
184 concentrations of nerolidol ranging between 50 and 4000  $\mu$ M. Bacterial cultures (5 ml) exempt  
185 of any agent, or containing DMSO (25  $\mu$ l) or blank HP- $\beta$ -CD added in similar amounts to that of  
186 the inclusion complex, served as controls.

187 2.5.2. Time-kill analysis

188 Time-kill assay was performed in triplicate on nerolidol and HP- $\beta$ -CD/Ner complex. Cultures  
189 were prepared as described in section 2.3.1. Nerolidol solubilized in DMSO and the complex  
190 were added to cultures at their respective MBC values. Starting from an initial bacterial  
191 concentration of  $10^5$  CFU/ml (5 log CFU/ml), bacterial growth was followed during 22 h in  
192 cultures maintained at 37 °C under anaerobic conditions. The viable plate count was determined  
193 at different time intervals using the spread plate method [34]. Therefore, 100  $\mu$ l of samples with  
194 appropriate dilutions was spread on MRS agar. The plates were incubated under *L. fermentum*  
195 optimal growth conditions for 22 h. The colonies were then counted and the bacterial  
196 concentration was determined.

## 197 2.6. Statistical Analysis

198 Statistical analysis was performed using the Student T test. *P* values equal or less than 0.05 were  
199 considered statistically significant.

## 200 3. Results

### 201 3.1. Antibacterial activity of terpenic and phenolic compounds

202 Terpenic compounds exhibited a significantly higher antibacterial activity against *L. fermentum*  
203 compared to phenolic compounds, with  $\alpha$ -terpinene being the only exception. In fact at 3500 $\mu$ M,  
204 16 among the 17 tested terpenes were bactericidal, whereas  $\alpha$ -terpinene strongly inhibited the  
205 proliferation of *L. fermentum* (89.1%) (Table 1). Eugenol and its ester derivative eugenyl acetate  
206 were the only phenolic compounds exhibiting a strong bactericidal activity against *L. fermentum*  
207 at 3500  $\mu$ M. Indeed, eugenol and eugenyl acetate exhibited a bactericidal activity of 100% and  
208 95%, respectively, within 22 h of incubation at 37 °C under anaerobic conditions (Table 2).  
209 Among the phenolic compounds, *trans*-anethole, estragole, and isoeugenol showed a significant  
210 anti-proliferative activity against *L. fermentum* at the highest concentration (3500  $\mu$ M), where  
211 bacterial growth inhibition was higher than 88% (Table 2). At this concentration, *p*-coumaric  
212 acid, *trans*-ferulic acid, and anisole showed a weak bacterial growth inhibition of 21.9, 17.3, and  
213 16.1%, respectively (Table 2). Whereas, quercetin, *o*-cresol, and resveratrol possessed no  
214 antibacterial activity against *L. fermentum* (Table 2). In fact, quercetin and resveratrol were  
215 insoluble in MRS at this concentration, which was marked by their strong precipitation.

216 The stronger antibacterial property of terpenes compared to phenolic compounds was highlighted  
217 by the strong bactericidal and anti-proliferative effect of different studied terpenes at 500  $\mu$ M.  
218 Indeed, at this concentration, the two phenolic compounds, eugenol and eugenyl acetate, showed  
219 no antibacterial activity against *L. fermentum* (Table 2). On the other hand,  $\beta$ -pinene, bornyl

220 acetate, linalyl acetate, and nerolidol exhibited a total bactericidal activity at 500  $\mu$ M (Table 1).  
221 Camphene, *p*-cymene, limonene,  $\alpha$ -phellandrene,  $\alpha$ -pinene, and  $\gamma$ -tepinene showed a total  
222 inhibition of *L. fermentum* proliferation but were not bactericidal (Table 1). Furthermore,  $\beta$ -  
223 caryophyllene, geraniol, and menthol showed a bacterial inhibition percentage higher than 75%,  
224 compared to control, whereas borneol demonstrated a weak anti-proliferative activity (28.5%)  
225 (Table 1). However, linalool and camphor, which exhibited a bactericidal activity against *L.*  
226 *fermentum* at 3500  $\mu$ M, showed no antibacterial activity at 500  $\mu$ M (Table 1).

227 When  $\beta$ -pinene, bornyl acetate, linalyl acetate, and nerolidol were tested at 250  $\mu$ M, nerolidol  
228 was the only compound exhibiting a bactericidal activity (100%) (Table 1). Whereas,  $\beta$ -pinene,  
229 bornyl acetate, and linalyl acetate showed a strong anti-proliferative activity against *L.*  
230 *fermentum* at 250  $\mu$ M, as no bacterial growth was observed in MRS broth within 22 h of  
231 incubation (Table 1). Nerolidol maintained a strong bactericidal activity (100%) against *L.*  
232 *fermentum* at 100  $\mu$ M, whereas,  $\beta$ -pinene strongly inhibited bacterial proliferation (~91%), and  
233 bornyl acetate and linalyl acetate showed no antibacterial activity (Table 1). Thus, among the 28  
234 tested compounds, nerolidol was retained for further studies as it was the most effective studied  
235 compound against *L. fermentum*.

### 236 3.2. Antibacterial activity of nerolidol

237 Nerolidol was the only molecule that exhibits a bactericidal activity against *L. fermentum* at a  
238 low concentration (100  $\mu$ M) (Tables 1 and 2). Therefore, the study was taken further to  
239 determine the MIC and MBC values of nerolidol. Nerolidol exhibited a strong antibacterial  
240 activity against *L. fermentum* with low MIC (25  $\mu$ M; 5.56 mg/l) and MBC (50  $\mu$ M; 11.12 mg/l)  
241 values. Moreover, *L. fermentum* survival was approximately 1% at a nerolidol concentration of

242 35  $\mu\text{M}$ . However, the adopted MBC was 50  $\mu\text{M}$ , as no bacterial growth was observed at this  
243 concentration.

244 The antibacterial activity profile (bactericidal or bacteriostatic) of nerolidol was evaluated using  
245 the MBC to MIC ratio. The *in vitro* antimicrobial activity of nerolidol can be described as  
246 bactericidal as the MBC to MIC ratio (MBC/MIC= 2) is lower than 4 [35].

### 247 3.3. Antibacterial activity of HP- $\beta$ -CD/Ner complex

248 The amount of nerolidol in the freeze-dried inclusion complex, determined by HPLC, was 40  
249  $\mu\text{g}_{\text{Ner}}/\text{mg}_{\text{powder}}$ . The antibacterial activity of HP- $\beta$ -CD/Ner complex was investigated for the first  
250 time against *L. fermentum* in culture medium under the optimal conditions for bacterial growth.  
251 HP- $\beta$ -CD without nerolidol had no effect on the bacterial growth (data not shown). The MIC and  
252 MBC values of HP- $\beta$ -CD/Ner against *L. fermentum* were 100 and 200  $\mu\text{M}$ , respectively. At 50  
253  $\mu\text{M}$ , a 4 log increment was observed in the bacterial concentration within 22 h of incubation  
254 (data not shown).

### 255 3.4. Nerolidol and HP- $\beta$ -CD/Ner complex time-kill analysis

256 A time-kill analysis was conducted to determine the time required to achieve a total bacterial kill  
257 in presence of nerolidol solubilized in DMSO at 50  $\mu\text{M}$  and HP- $\beta$ -CD/Nero complex at 200  $\mu\text{M}$   
258 (MBC). Free nerolidol exhibited a bactericidal activity against *L. fermentum* within the first few  
259 hours of incubation (Fig. 3). Indeed, a 1.44 log reduction of *L. fermentum* concentration was  
260 observed within 4 h of incubation. The bacterial concentration continued to decrease over time,  
261 as a 2.82 log reduction was obtained after 10 h. At 16 h of incubation, no viable cells were  
262 observed.

263 In presence of HP- $\beta$ -CD/Ner complex (200  $\mu$ M), the bacterial concentration decreased by 1.22  
264 log and 2.44 log after 4 and 10 h, respectively, and a total bacterial death was obtained within 12  
265 h (Fig. 3).

## 266 **4. Discussion**

### 267 4.1. Antibacterial efficiency of nerolidol

268 Plants produce a wide range of antimicrobial agents highly desired by consumers due to their  
269 natural origin. However, many of these antimicrobials are only effective at high concentrations  
270 (for example at millimolar range), thus exhibiting a weak activity compared to common  
271 antibiotics [36]. In this study, nerolidol exhibited the highest antibacterial activity among the 28  
272 tested terpenic and phenolic compounds. The outstanding antibacterial potency of nerolidol  
273 against *L. fermentum* was marked by the low MIC (25  $\mu$ M; 5.56 mg/l) and MBC (50  $\mu$ M; 11.12  
274 mg/l) values, as well as the rapid bactericidal activity (Fig. 3). This compound acts by disrupting  
275 and by damaging the bacterial cell membrane, and by interfering with genes responsible for the  
276 microbe pathogenicity [37]. Besides, Brehm-Stecher and Johnson [27] demonstrated the  
277 permeabilizing effect of nerolidol on *L. fermentum* membrane. The permeabilization of the  
278 bacterial membrane leads to the leakage of the cytoplasmic molecules, thus causing cell lysis  
279 [38]. Also, the disruption of the bacterial membrane would allow the permeation of exogenous  
280 molecules into the bacterial cytoplasm [27]. Moreover, Brehm-Stecher and Johnson [27]  
281 suggested that the permeabilizing activity of nerolidol may be due to its structural resemblance to  
282 the lipids of the bacterial membrane. This was previously highlighted by Cornwell and Barry  
283 [39] which attributed the enhancement of skin penetration by nerolidol to its long hydrocarbon  
284 tail which promotes the interaction of the molecule with the interior of the cell bilayer.

285 Nerolidol is a sesquiterpene alcohol widely used in the food industry as a flavoring agent. It  
286 demonstrated a potent antimicrobial activity against some fungi, in addition to different Gram-  
287 positive and Gram-negative bacterial strains, such as *Staphylococcus aureus* (MIC: 3.9 mg/l; 50  
288 mg/l) [26,40,41], *Streptococcus mutans*, *Propionibacterium acnes* (MIC: 25 mg/l) [26],  
289 *Salmonella enterica* (MIC: 15.6 mg/l) [41], *Trichophyton mentagrophytes* (MIC: 12.5 mg/l) [26],  
290 and *Aspergillus niger* (15.6 mg/l) [41]. On the other hand, nerolidol was able to enhance the  
291 susceptibility of *S. aureus* and *Escherichia coli* to antibiotics, including ciprofloxacin,  
292 erythromycin, gentamicin, vancomycin [42], and amoxicilline/clavulanic acid [43]. In this study,  
293 nerolidol antibacterial potency against *L. fermentum* (MIC: 25  $\mu$ M; 5.56 mg/l) was weaker than  
294 that of gentamicin [44] and chloramphenicol [45], and close to that of novobiocin [46] and N-  
295 alkyl dimethylbenzyl ammonium chloride [47] (Table 3). On the other hand, *L. fermentum* strains  
296 were less sensitive to well-known antibiotics including vancomycin [45,48], teicoplanin [45],  
297 streptomycin [44,45,48], erythromycin, tobramycin, clindamycin, kanamycin, polymixin B  
298 [44,46,48], metronidazole, and nitrofurantoin [49] (Table 3).

#### 299 4.2. Antibacterial assay of HP- $\beta$ -CD/Ner complex

300 Cyclodextrin inclusion complexes have been widely studied for their capacity to enhance the  
301 stability and solubility of antimicrobials [28,50,51].

302 Some studies reported similar antibacterial activities of free and complexed antimicrobials (e.g.  
303 clarithromycin, chlorogenic acid, peptide CM4) [28,50,52], while others proved modifications of  
304 the potency of the antimicrobial agent following complexation with cyclodextrins. Compared to  
305 the free form, the inclusion complex of *Hyptis martiusii* essential oil [53] and coriander essential  
306 oil [51] showed a lower antimicrobial activity, whereas that of carvacrol showed a higher  
307 antimicrobial activity [54].

308 The incorporation of nerolidol into HP- $\beta$ -CD inclusion complex increased the MIC and MBC  
309 values by 4 fold. Azzi et al. [33] studied the release of nerolidol from the inclusion complex in  
310 water and at room temperature by dialysis. Around 45% of nerolidol were released from HP- $\beta$ -  
311 CD/Ner complex within 8 h, followed by a slow release rate over 7 days [33]. In fact, when HP-  
312  $\beta$ -CD/Ner complex is added at 50  $\mu$ M, the actual concentration of nerolidol interacting with *L.*  
313 *fermentum* in MRS is significantly lower than 50  $\mu$ M during the whole experiment, thus  
314 explaining the ability of the inoculated bacteria to proliferate (data not shown). However, at 200  
315  $\mu$ M the concentration of nerolidol in the culture medium should be approximately 100  $\mu$ M after 8  
316 to 10 h of incubation, which could explain the rapid drop in the bacterial concentration (Fig. 3).  
317 The slow release and the photo-protection of nerolidol provided by the encapsulation systems  
318 [33] would prevent and limit the proliferation of spoilage bacteria in food products.

#### 319 4.3. Relationship between the antibacterial activity of terpenic and phenolic compounds and their 320 structural and physicochemical parameters

321 Various parameters may modulate the activity of antibacterial agents, including the drug  
322 hydrophobicity, the presence of functional groups, the bacterial cell envelope characteristics, and  
323 the incubation conditions (for instance, aeration).

##### 324 4.3.1. The influence of hydrophobicity on the antibacterial activity of molecules

325 It has been reported that molecules with high hydrophobicity exhibit a high affinity to the  
326 lipophilic structures in the target microorganisms, such as the bacterial membrane [55].  
327 Therefore, they may disrupt the membrane integrity, thus affecting membrane permeability and  
328 enzymes activity [55]. However, many exceptions could be revealed from this work, which  
329 suggests that the hydrophobicity is not always the key parameter governing the antibacterial



330 activity; and some structural features could be taken into consideration when analyzing the  
331 structure-antibacterial activity relationship.

332 Nerolidol, which has shown the strongest antibacterial activity against *L. fermentum*, has a LogP  
333 value (5.33-5.36) considerably higher than that of the other tested molecules, except  $\beta$ -  
334 caryophyllene (6.87) (Table 1). The latter, showed a modest antibacterial activity against *L.*  
335 *fermentum* as it lost its bactericidal potential at 500  $\mu$ M (Table 1). In fact,  $\beta$ -caryophyllene have  
336 been found to exhibit a weak antibacterial activity against different Gram-positive and Gram-  
337 negative bacteria [56].

338 On the other hand, the antibacterial potency of the compounds exhibiting an antibacterial activity  
339 at a concentration lower than 500  $\mu$ M was influenced by hydrophobicity (Table 1). Indeed,  $\beta$ -  
340 pinene showed a higher antibacterial activity than the less hydrophobic molecules, bornyl acetate  
341 and linalyl acetate (Table 1). The acetylation of linalool and borneol increased their  
342 hydrophobicity and their antibacterial activity (Table 1). Indeed, bornyl acetate and linalyl  
343 acetate retained their total anti-proliferative activity at 250  $\mu$ M, whereas borneol and linalool lost  
344 partially and totally the activity at 500  $\mu$ M, respectively (Table 1). Similarly, Knobloch et al.  
345 [57] reported a higher inhibition of H<sup>+</sup>-translocation by linalyl acetate, compared to linalool.  
346 Also, Dorman and Deans [58] observed a higher antibacterial activity of bornyl acetate compared  
347 to borneol against a wide range of bacterial strains, among which *Lactobacillus plantarum*.  
348 Moreover, phenolic compounds with LogP values close to or lower than 2, such as anisole (2.11)  
349 and *o*-cresol (1.95-1.98), showed no or a weak antibacterial activity against *L. fermentum* (Table  
350 2).

351 Acyclic monoterpenoids showed an increased antibacterial activity with the increment of their  
352 LogP value. Indeed, linalyl acetate showed a higher antibacterial activity than geraniol, which

353 was more potent than linalool (Table 1). This was also observed for bicyclic monoterpenes, as  
354 borneol and camphor had the lowest LogP values, and showed the weakest antibacterial activities  
355 (Table 1). However, despite  $\beta$ -pinene not being the most hydrophobic compound in the bicyclic  
356 monoterpenes chemical class, it exhibited the strongest antibacterial activity (Table 1).

357 For monocyclic monoterpenes, limonene,  $\alpha$ -phelladrene, and  $\gamma$ -terpinene, having higher LogP  
358 values than menthol, showed a stronger antibacterial activity (Table 1).  $\alpha$ -Terpinene was found  
359 to be an exception as it showed the weakest antibacterial potency, despite a high LogP value  
360 (Table 1).

#### 361 4.3.2. Structure-activity analysis of terpenic compounds

362 Different studies have demonstrated the role of terpenoid functional groups in the antimicrobial  
363 activity. Carvacrol showed a better antimicrobial activity compared to its derivatives, carvacrol  
364 methyl ether and *p*-cymene, which lack the hydroxyl group [58]. On the other hand, Kotan et al.  
365 [59] reported a better antibacterial activity for alcohol derivatives of oxygenated monoterpenes,  
366 when compared to ketone and acetate derivatives.

367 The weak antibacterial activity of  $\beta$ -caryophyllene, despite its high hydrophobicity, may be due  
368 to the absence of a hydrophilic functional group in the chemical structure of the molecule. The  
369 combination of a lipophilic character of the skeleton and the presence of a hydrophilic functional  
370 group was found to be important for the antimicrobial activity of essential oils components [60].

371 The structural features of nerolidol are in line with the previous findings. Indeed, the  
372 antibacterial activity of aliphatic terpene alcohols was demonstrated to be dependent on the  
373 hydrophobic chain length starting from the carbon connected to the hydroxyl group [38,61]. In  
374 fact, farnesol ( $C_{12}$ ) exhibited a stronger antibacterial activity than nerolidol ( $C_{10}$ ) followed by  
375 geraniol ( $C_8$ ), whereas linalool ( $C_6$ ) showed no antibacterial activity against *Staphylococcus*

376 *aureus* [61]. Similarly, Togashi et al. [38] reported a very weak antibacterial activity of geraniol  
377 and linalool against *S. aureus*. Additionally, farnesol (C<sub>12</sub>), nerolidol (C<sub>10</sub>), and plaunotol (C<sub>11</sub>),  
378 showed a strong antibacterial activity against *S. aureus*, in that order [38,62]. Also, no or weak  
379 antibacterial activity against *S. aureus* was reported for alcohols with chains containing more  
380 than 12 carbon atoms like farnesylacetol (C<sub>14</sub>) [61], geranylgeraniol, and phytol (C<sub>16</sub>) [38].  
381 Therefore, to exhibit a potent antibacterial effect, the authors suggested that the chain, starting  
382 from the hydroxyl group, should contain from 10 to 12 carbon atoms [38], or less than 12 carbon  
383 atoms but as close to 12 as possible [61]. Although the previous studies were conducted on *S.*  
384 *aureus*, our study supports the pattern of the antibacterial activity of terpene alcohols against *L.*  
385 *fermentum*. Indeed, nerolidol (C<sub>10</sub>; sesquiterpene) exhibited a stronger antibacterial activity than  
386 geraniol (C<sub>8</sub>; monoterpene), the latter being more potent than linalool (C<sub>6</sub>; monoterpene) (Table  
387 1).

#### 388 4.3.3. Structure-activity analysis of phenolic compounds

389 The importance of the propenyl side chain was noted among the tested phenolic compounds, as  
390 eugenol, *trans*-anethole, isoeugenol, and estragole, showed a significantly higher antibacterial  
391 activity against *L. fermentum* compared to anisole (Table 2). The propenyl side chain is absent in  
392 anisole compared to eugenol, *trans*-anethole, isoeugenol, and estragole (Fig. 2), which could be  
393 the reason for the weaker antibacterial activity of anisole.

394 On the other hand, eugenol showed the highest antibacterial activity among the tested phenolic  
395 compounds against the Gram-positive bacterium *L. fermentum*. However, Gharib et al. [63]  
396 reported a higher antibacterial activity for anethole and estragole compared to eugenol, against  
397 the Gram-negative bacterium *Escherichi coli*. Indeed, the hydrophobicity of the compound  
398 seemed to play a role in the potency of the antibacterial activity against *E. coli*, as anethole and

399 estragole have a higher hydrophobicity than eugenol [63] (Table 2). Whereas, the hydrophobicity  
400 of phenylpropenes did not seem to influence their antibacterial activity against the Gram-positive  
401 bacterium, *L. fermentum*. Therefore, our results strongly suggest the presence of other factors  
402 that modulate the antibacterial activity of phenylpropenes against *L. fermentum*. The higher  
403 antibacterial activity of eugenol compared to its ester, eugenyl acetate (Table 2), highlights the  
404 importance of the hydroxyl group in the phenolic structure (Fig. 2). Additionally, the absence of  
405 the hydroxyl group in *trans*-anethole and estragole structure (Fig. 2) could explain the lower  
406 antibacterial activity against *L. fermentum*, compared to eugenol (Table 2). Indeed, the hydroxyl  
407 group of eugenol and isoeugenol has been found to reinforce the lipid membrane-fluidizing  
408 effect, compared to anethole and estragole [63]. Also, the bacterial membrane characteristics  
409 play a crucial role in the differential bacterial susceptibility to a given antibacterial agent [64–  
410 66]. In fact, eugenol has previously shown lower MIC values against the Gram-positive bacteria  
411 *Staphylococcus aureus* (MIC= 2.5 mg/l) and *Bacillus subtilis* (MIC= 1.25 mg/l), compared to *E.*  
412 *coli* (MIC= 5 mg/l) [67].

413 Both ferulic acid and *p*-coumaric acid exhibited a weak anti-proliferative activity against *L.*  
414 *fermentum* in MRS broth (pH ~6.2) (Table 2). The antibacterial activity of hydroxycinnamic  
415 acids depends on pH, which controls the concentration of their undissociated form. The  
416 undissociated form can easily penetrate the cytoplasmic membrane of the bacterium [68]. The  
417 pKa values of ferulic acid and *p*-coumaric acid are between 4 and 5, thus a greater proportion of  
418 their dissociated forms is found at the pH of the culture medium (~6.2). In fact, the antibacterial  
419 activity of ferulic acid and *p*-coumaric acid was found to increase in culture media at pH values  
420 below 6 [69]. Moreover, *L. fermentum* is able to metabolize ferulic acid and coumaric acid into

421 less potent metabolites, such as phloretic acid, p-vinyl phenol, or dihydroferulic acid [69]. This  
422 also may explain the weak antibacterial activity of both acids against *L. fermentum*.

## 423 **5. Conclusion**

424 In this study, 28 terpenic and phenolic compounds were screened against *L. fermentum*, an  
425 ubiquitously present bacterium which could cause spoilage of different food products. Among  
426 the tested compounds, nerolidol exhibited the strongest antibacterial activity marked by the low  
427 MIC and MBC values. Moreover, a total bacterial kill was obtained within 16 h in presence of  
428 nerolidol (50  $\mu$ M). The antibacterial activity of nerolidol was dependent on different factors,  
429 including the hydrophobicity of the compound, as well as the position of the hydroxyl group.  
430 Compared to nerolidol solubilized in DMSO, HP- $\beta$ -CD/Ner inclusion complex exhibited 4 fold  
431 higher MIC and MBC values and a more rapid bactericidal activity. Indeed, HP- $\beta$ -CD/Ner  
432 inclusion complex was proven effective against *L. fermentum* in culture medium. The high  
433 demand for the replacement of synthetic food additives by natural molecules encourages the  
434 application of nerolidol in food. Moreover, due to the physico-chemical limitations of the  
435 application of natural bioactive components in food, the use of encapsulation systems has  
436 recently become a widely investigated novel approach for the delivery of bioactive compounds in  
437 food products. Therefore, further studies could be realized to investigate the antibacterial activity  
438 of free and encapsulated nerolidol in different food products including fruit juices, alcoholic  
439 beverages, and milk products. This evaluation would lead to a better understanding of the  
440 antibacterial potency of natural molecules such as nerolidol, under their free and complexed  
441 form, in various types of food matrices and under various conditions.

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444 **Conflict of Interest**

445 The authors declare no conflict of interest.

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450 **References**

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### 719 **Figure legends**

720 **Fig.1.** Chemical structure of terpenic compounds.

721 **Fig. 2.** Chemical structure of phenolic compounds.

722 **Fig. 3.** Nerolidol (50  $\mu$ M) and HP- $\beta$ -CD/Ner complex (200  $\mu$ M Ner) time-kill analysis against *L.*  
723 *fermentum* at 37 °C under anaerobic conditions.

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738 **Table 1: Bactericidal and anti-proliferative activity of terpenes against *L. fermentum*.**

	Borneol	Bornyl acetate	Camphene	Camphor	$\beta$ -Caryophyllene	<i>p</i> -Cymene	Geraniol	Limonene	Linalool	Linalyl acetate	Menthol	Nerolidol	$\alpha$ -Phellandrene	$\alpha$ -Pinene	$\beta$ -Pinene	$\alpha$ -Terpinene	$\gamma$ -Terpinene
<b>LogP</b>	3.01 [70]	3.86 [71]	4.22 [70]	2.74 [70]	6.87 [72]	4.1 [73]	3.56 [70]	4.58 [74] 4.23 [77] 4.57 [75] 4.38 [70] 4.83 [79]	2.97 [75]	3.93 [70]	3.4 [70] 3.38 [78]	5.36 [74]	4.55 [76]	4.83 [75] 4.49 [76] 4.48 [70]	4.16 [70] 4.42 [76]	4.25 [70]	4.5 [75] 4.36 [70]
<b>3500 <math>\mu</math>M Bacterial kill (%)</b>	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0	100
<b>Inhibition (%)</b>	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	89.1 $\pm$ 0.5	100
<b>500 <math>\mu</math>M Bacterial kill (%)</b>	0	100	0	0	0	0	0	0	0	100	0	100	0	0	100	ND	0

Inhibition (%)	28.5 ± 1.8	100	100	0	89 ± 2.4	100	80.9 ± 1.4	100	0	100	75.7 ± 4.4	100	100	100	100	ND	100
<b>250 μM</b> Bacterial kill (%)	ND	0	ND	ND	ND	ND	ND	ND	ND	0	ND	100	ND	ND	0	ND	ND
Inhibition (%)	ND	100	ND	ND	ND	ND	ND	ND	ND	100	ND	100	ND	ND	100	ND	ND
<b>100 μM</b> Bacterial kill (%)	ND	0	ND	ND	ND	ND	ND	ND	ND	0	ND	100	ND	ND	0	ND	ND
Inhibition (%)	ND	0	ND	ND	ND	ND	ND	ND	ND	0	ND	100	ND	ND	91.4 ± 0.8	ND	ND

739 ND: Not determined

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744 **Table 2: Bactericidal and anti-proliferative activity of phenolic compounds against *L.***  
 745 ***fermentum*.**

	<i>trans</i> -Anethole	Anisole	<i>p</i> -Coumaric acid	<i>o</i> -Cresol	Estragole	Eugenol	Eugenyl acetate	<i>trans</i> -Ferulic acid	Isoeugenol	Quercetin	Resveratrol
<b>LogP</b>	3.31[80]			1.95[73]	3.13[80]	2.40[83] 2.45[72]		1.51[73]			3.10 [85]
	3.0961[82]	2.11[81]	1.43[82]	1.975[86]	2.818[82]	2.29[87] 2.73[78] 2.99[70]	2.9[72]	1.249[82]	3.04[70]	1.82 [84]	3.06 [88]
<b>3500 <math>\mu</math>M</b>											
Bacterial kill (%)	0	0	0	0	0	100	95 $\pm$ 0.47	0	0	0	0
Inhibition (%)	91.4 $\pm$ 1.80	16.1 $\pm$ 0.92	21.9 $\pm$ 0.94	0	84.4 $\pm$ 0.13	100	100	17.3 $\pm$ 0.14	89.3 $\pm$ 0.09	0	0
<b>500 <math>\mu</math>M</b>											
Bacterial kill (%)	ND	ND	ND	ND	ND	0	0	ND	ND	ND	ND
Inhibition (%)	ND	ND	ND	ND	ND	0	0	ND	ND	ND	ND

746 ND: Not determined

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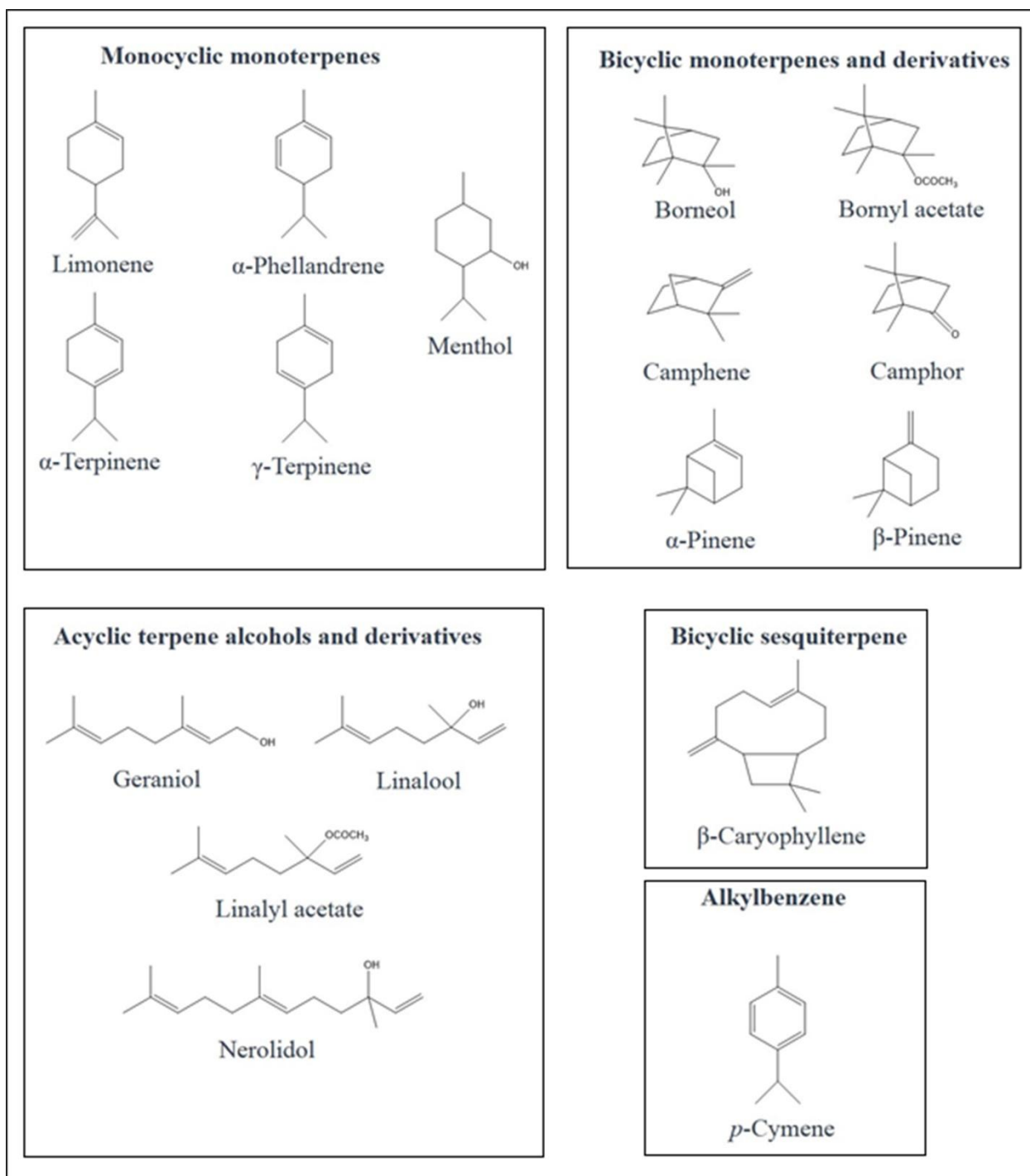
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756 **Table 3: MIC values of common antibiotics against *L. fermentum*.**

<b>Antibiotic</b>	<b>MIC (mg/l)</b>	<b>MIC (<math>\mu</math>M)</b>	<b>Reference</b>
Clindamycin	>64	>150.60	[46]
Chloramphenicol	4 <sup>a</sup>	12.38	[45]
Erythromycin	>128 - 256	>174.40 - 348.81	[46]
Gentamicin	4 - 8	8.38 - 16.75	[44]
Kanamycin	64	132.10	[48]
Metronidazole	>40	>233.7	[49]
N-alkyl dimethylbenzyl ammonium chloride	8	25.11	[47]
Nitrofurantoin	15	62.98	[49]
Novobiocin	>16	>26.12	[46]
Polymixin B	64	49.17	[48]
	16 <sup>a</sup>	27.51	[45]
Streptomycin	16	27.51	[48]
	8 - 128	13.76 - 220.09	[44]
	>128	>220.09	[46]
Teicoplanin	>256 <sup>a</sup>	>134.19-163.65	[45]
Tobramycin	>128	>273.79	[46]
Vancomycin	96	66.24	[48]
	>256 <sup>a</sup>	>176.64	[45]

757 <sup>a</sup>Values of MIC<sub>90</sub>

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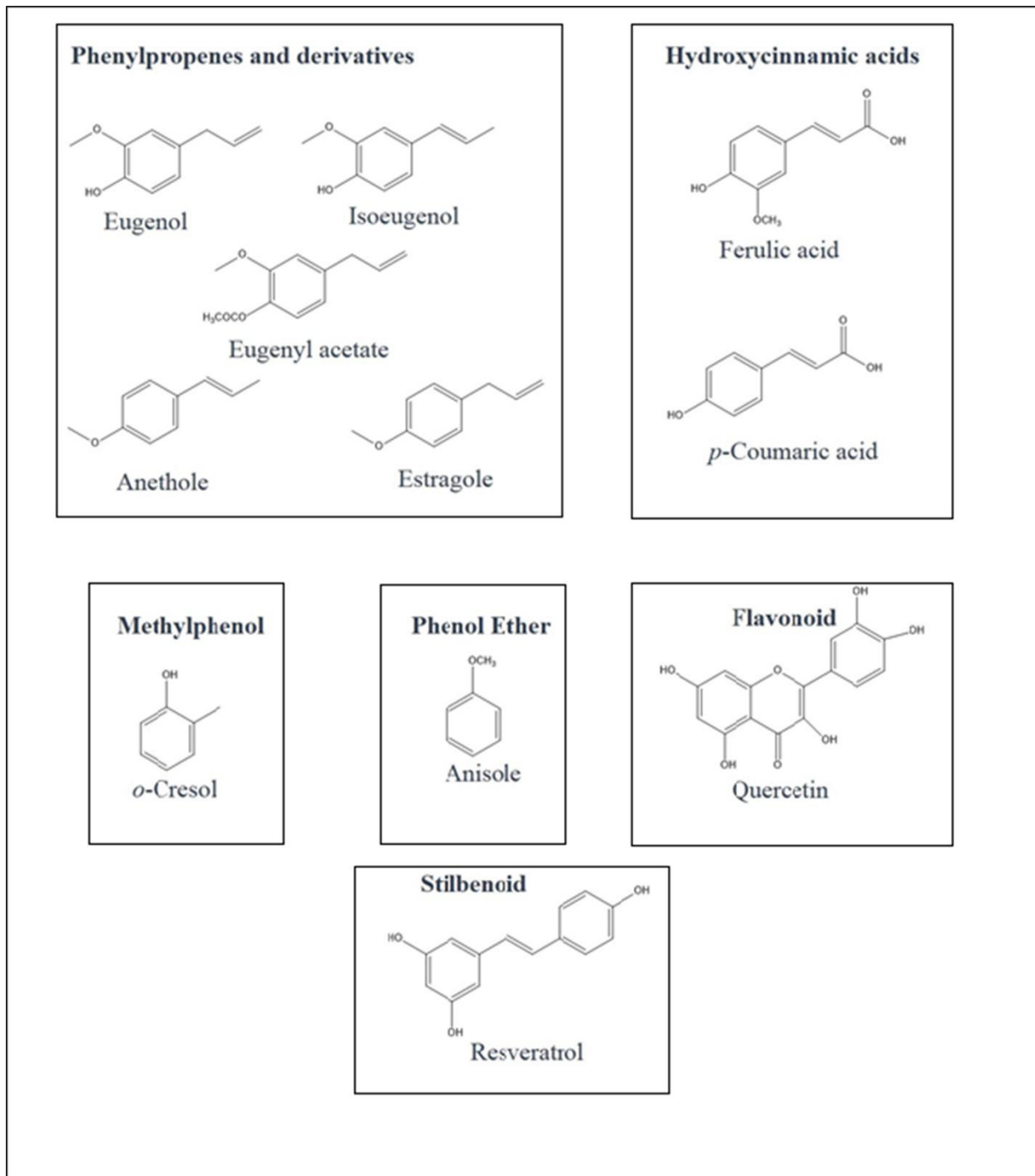


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760 **Fig. 1**

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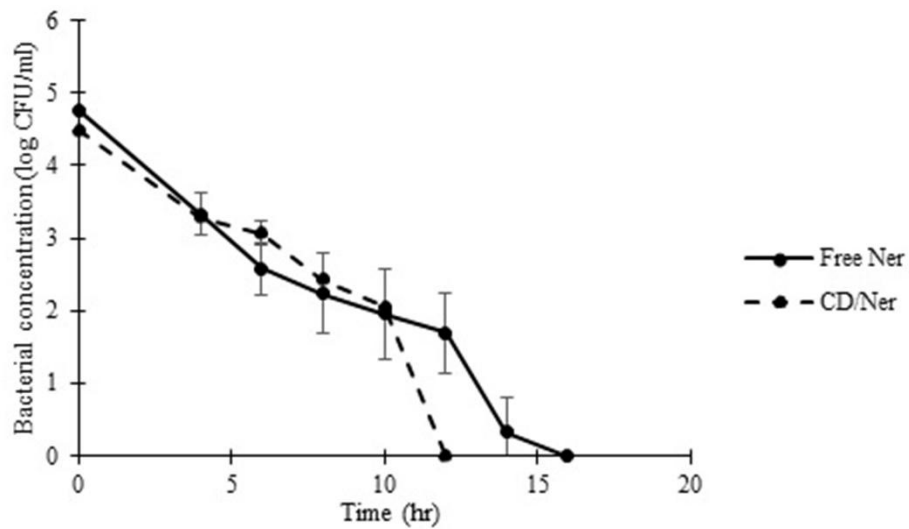
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764 **Fig. 2**

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768 **Fig. 3**

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