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## Phenolic compounds can delay the oxidation of polyunsaturated fatty acids and the growth of *Listeria monocytogenes*: structure-activity relationships

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1 **Phenolic compounds can delay the oxidation of polyunsaturated fatty**  
2 **acids and the growth of *Listeria monocytogenes*: structure-activity**  
3 **relationships**

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## ABSTRACT

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BACKGROUND: Phenolic compounds present a potential solution to ensuring food quality and safety. Indeed, they can limit oxidation reactions and bacterial growth in food products. Although their antioxidant mechanisms of action are well known, their antibacterial ones are less well understood, especially in light of their chemical structures. The aim of this study was to first quantify both aspects of a series of natural phenolic compounds and then link these activities to their chemical structure.

RESULTS: We evaluated antioxidant activity by measuring the capacity of phenolic compounds to delay free linoleic acid oxidation caused by the action of a hydrophilic azo-radical initiator (AAPH). We evaluated antibacterial activity by measuring the growth inhibition of *Listeria monocytogenes* and determining the non-inhibitory and minimum inhibitory concentrations for each compound. Compounds with *ortho*-diphenolic structures were the best antioxidants, whereas those belonging to the simple phenol category were the best antibacterial compounds.

CONCLUSION: The physico-chemical properties of the compounds influenced both activities, but not in the same way. The chemical environment of the phenolic group and the presence of delocalization structures are the most important parameters for antioxidant activity, whereas the partition coefficient logP is one of the most important factors involved in antibacterial activity.

## KEYWORDS

phenolic compounds, antioxidant, AAPH, antibacterial, *Listeria monocytogenes*, structure-activity

## INTRODUCTION

34

35 Over the last decade, the fatty acids' profile of food products has been modified to respect  
36 nutritional recommendations. The proportion of saturated fatty acids has considerably  
37 decreased in favor of monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids,  
38 in particular those of the omega-3 family.<sup>1</sup> However, these changes can limit food quality  
39 and safety. Indeed, the increase in the number of double bonds in fatty acids in food  
40 products leads to their higher oxidability during processing and storage. Oxidation  
41 reactions produce off-flavors due to the generation of volatile compounds, decrease  
42 nutritional quality by the partial loss of essential fatty acids and co-oxidation of vitamins,  
43 and increase the chemical risk by the generation of potentially toxic compounds during  
44 heating (cyclic monomers, polymers, furans, 4-hydroxynonenal).<sup>2,3</sup> Moreover, recent  
45 studies have shown that the growth of pathogenic Gram-positive microorganisms, such  
46 as *Staphylococcus aureus* or *Bacillus cereus*, may be favored in the presence of  
47 exogenous unsaturated fatty acids (UFA). The mechanisms are not fully understood, but  
48 it has been reported that these bacteria can incorporate exogenous UFA in their  
49 membranes to regulate their fluidity when fatty acid synthesis is blocked by an FSAII  
50 inhibitor<sup>4</sup> or impaired by low temperatures.<sup>5</sup> Under these conditions, pathogenic  
51 microorganisms can grow and reach infectious doses faster than expected, causing  
52 potential food safety issues in food products.

53 One solution to limit these phenomena is to use molecules with both antioxidant and  
54 antibacterial properties, such as phenolic compounds, which are widely found in plants  
55 or present in by-products from agro-industry. Several studies have reported the  
56 antioxidant and antibacterial properties of phenolic compounds. The antioxidant  
57 mechanisms of phenolic compounds are quite well understood and are based on the ability  
58 of phenolic groups to release an electron or a hydrogen to a lipid radical. The phenolic

59 radical formed is more stable than the lipid radical, due to the presence of delocalization  
60 structures.<sup>6</sup> Fewer studies have focused on the antibacterial activity of phenolic  
61 compounds. Solely the mechanism of action of simple phenols is well described. They  
62 act by partitioning of the molecule into the membrane phospholipid layers, inducing the  
63 leakage of cell components and bacterial death.<sup>7</sup> The antibacterial activity of several  
64 phenolic acids and flavonoids has already been reported, but their mechanism of action  
65 is not fully understood.<sup>8</sup>

66 We aimed to quantify both antioxidant and antibacterial activities of a series of natural  
67 phenolic compounds and investigate structure-activity relationships to determine their  
68 potential to ensure food quality and safety. Molecules were selected based on their  
69 antioxidant and/or antibacterial activity reported in published studies<sup>6,7,9</sup> and their  
70 structures, which covered a broad range of chemical families, making it possible to study  
71 the role of specific chemical groups in their effectiveness. *Listeria monocytogenes* was  
72 used as the bacterial model because it is a ubiquitous Gram-positive pathogen which is  
73 widespread in the environment and a potential contaminant at all stages of the food  
74 chain.<sup>10</sup> We tested the ability to delay oxidation with linoleic acid, because it is highly  
75 reactive and representative of PUFAs found in most food products.

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## EXPERIMENTAL

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### CHEMICAL REAGENTS

79

2,2'-azobis(2-amidinopropane) dichloride (AAPH), linoleic acid (purity > 98%), sodium  
80 dihydrogen phosphate dihydrate, sodium phosphate dibasic dodecahydrate, and sodium  
81 borate were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Absolute  
82 ethanol was purchased from VWR (Fontenay-sous-Bois, France), boric acid from Prolabo  
83 (Fontenay-sous-Bois, France), 1 mol.L<sup>-1</sup> sodium hydroxide and acetone from Carlo Erba

84 (Fontenay-aux-Roses, France), and Tween 20 from Sigma-Aldrich (St Quentin Fallavier,  
85 France).

86

## 87 BACTERIAL STRAIN AND GROWTH CONDITIONS

88 The strain used in this study was *Listeria monocytogenes* CNL 895805, serotype ½,  
89 isolated from sheep's brain. It was graciously provided by P. Velge (INRA, Nouzilly).<sup>11</sup>

90 The strain, stored in cryovials at -80°C, was resuscitated in two successive subcultures in  
91 tryptic soy broth (TSB, Biomérieux, France) at 30°C before each experiment.

92

## 93 PHENOLIC COMPOUNDS

94 *p*-hydroxybenzoic acid, protocatechuic acid, vanillic acid, gallic acid, *p*-coumaric acid,  
95 caffeic acid, ferulic acid, chlorogenic acid, thymol,  $\alpha$ -tocopherol, (-)-epicatechin,  
96 naringenin, kaempferol, and quercetin were purchased from Sigma-Aldrich (St Quentin  
97 Fallavier, France). Eugenol was purchased from Janssen (Beerse, Belgium), carvacrol  
98 from Fluka (Buchs, Switzerland), and eriodictyol, apigenin, luteolin, and rutin from  
99 Extrasynthèse (Genay, France). Chemical structures are shown Figure 1. The partition  
100 coefficients (logPs) of the compounds were found on the ChemSpider website. The  
101 predicted logP<sub>o/w</sub> from ACD/Labs Percepta Platform PhysChem Module were used.

102 For the evaluation of antioxidant activity, stock solutions of phenolic compounds were  
103 prepared by dissolution in absolute ethanol. For the evaluation of antibacterial activity,  
104 phenolic compounds were prepared according to various protocols we elaborated,  
105 depending on their solubility in the culture medium. *p*-hydroxybenzoic, protocatechuic,  
106 gallic, and chlorogenic acid powders were directly dissolved in the culture medium.  
107 Vanillic, *p*-coumaric and ferulic acids, carvacrol, thymol, and eugenol were first  
108 dissolved in acetone, which was evaporated under nitrogen flow after addition to the

109 culture medium. Caffeic acid, (-)-epicatechin, naringenin, and eriodictyol were first  
110 dissolved in acetone/distilled water (80/20 (v/v)) and the acetone evaporated under  
111 nitrogen flow. Bacterial growth controls were carried out to ensure the absence of  
112 inhibitory activity of trace amounts of acetone after evaporation.

113

#### 114 EVALUATION OF ANTIOXIDANT ACTIVITY

115 Antioxidant activity was evaluated according to a method previously described by Peyrat-  
116 Maillard *et al.*<sup>12</sup> with minor modifications. The test involves a multiphasic system and  
117 consists of measuring the capacity of phenolic compounds to delay the oxidation of  
118 linoleic acid. Conjugated diene hydroperoxides were generated by the action of a  
119 hydrophilic diazo initiator (AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride) on  
120 free linoleic acid and were followed by spectrophotometry at 234 nm<sup>13</sup> with a double-  
121 beam spectrophotometer (Spectrophotometer Specord 210 Plus, Analytik Jena,  
122 Germany).

123 A mixture containing 2.81 mL phosphate buffer (0.05 mol.L<sup>-1</sup>, pH 7.4), 30 µL substrate  
124 solution (0.016 mol.L<sup>-1</sup> 98% linoleic acid, 4.89 mmol.L<sup>-1</sup> Tween 20, 20 mmol.L<sup>-1</sup> sodium  
125 hydroxide in borate buffer (0.05 mol.L<sup>-1</sup>, pH 9.0)), and 10 µL freshly prepared ethanolic  
126 solution of the phenolic compound were added in a spectrophotometric cuvette and  
127 incubated at 37°C for 5 min. At least five concentrations of antioxidant were tested from  
128 0 to 9 µmol.L<sup>-1</sup> for (-)-epicatechin, to 10 µmol.L<sup>-1</sup> for eriodictyol, apigenin, luteolin,  
129 kaempferol, quercetin, and rutin, 12 µmol.L<sup>-1</sup> for caffeic acid and chlorogenic acid, 20  
130 µmol.L<sup>-1</sup> for protocatechuic acid, ferulic acid, and α-tocopherol, 30 µmol.L<sup>-1</sup> for eugenol  
131 and naringenin, 35 µmol.L<sup>-1</sup> for gallic acid, 40 µmol.L<sup>-1</sup> for vanillic acid, 50 µmol.L<sup>-1</sup> for  
132 *p*-hydroxybenzoic acid and *p*-coumaric acid, and 70 µmol.L<sup>-1</sup> for carvacrol and thymol.  
133 The maximum concentrations tested were chosen to reach equivalent antioxidant

134 effectiveness between compounds or their solubility threshold. At least three independent  
135 experiments were conducted for each phenolic compound. Then, 150  $\mu\text{L}$  AAPH solution  
136 ( $0.04 \text{ mol.L}^{-1}$  in phosphate buffer  $0.05 \text{ mol.L}^{-1}$ , pH 7.4) was added. The absorbance was  
137 measured at 234 nm for at least 5 h vs a control containing the same solution, except the  
138 substrate, which was replaced by 30  $\mu\text{L}$  borate buffer ( $0.05 \text{ mol.L}^{-1}$ , pH 9.0).

139 The antioxidant potential of a phenolic compound is defined by its effective concentration  
140 to delay oxidation for one hour ( $\text{EC}_{1\text{h}}$ ), relative to a control without antioxidant, and is  
141 expressed in  $\mu\text{mol.L}^{-1}.\text{h}^{-1}$ : the lower this value, the stronger the antioxidant. The  $\text{EC}_{1\text{h}}$  was  
142 determined by first plotting the concentration of conjugated dienes ( $C=A_{234\text{nm}}/(l.\epsilon)$ , with  
143  $l = 1 \text{ cm}$  and  $\epsilon = 27\,000 \text{ L.mol}^{-1}.\text{cm}^{-1}$ )<sup>13</sup> as a function of time for several concentrations of  
144 a given phenolic compound. Then, the time to reach a given concentration of conjugated  
145 dienes (arbitrarily chosen to be  $18.5 \mu\text{mol.L}^{-1}$ ) was determined ( $t_{\text{Ci}}$ ) for each concentration  
146 of antioxidant. The difference between this time ( $t_{\text{Ci}}$ ) and the time necessary to reach the  
147 same concentration with the control ( $t_{\text{C0}}$ ) was calculated ( $\Delta t_{\text{Ci}} = t_{\text{Ci}} - t_{\text{C0}}$ ).  $\Delta t_{\text{Ci}}$  was then  
148 plotted as a linear function of the concentration of the antioxidant and the slope calculated.  
149 The effective concentration to delay oxidation for one hour ( $\text{EC}_{1\text{h}}$ ) corresponds to the  
150 inverse of this slope. For statistical purposes, the delayed oxidation times ( $\Delta t_{\text{Ci}} = t_{\text{Ci}} - t_{\text{C0}}$ )  
151 were directly used. An analysis of covariance (ANCOVA) between the concentration-  
152 dependent effect of the phenolic compound and a phenolic compound\*concentration  
153 interaction was applied using XLSTAT 18.06 (Addinsoft, Paris, France). If significant  
154 effects were revealed ( $p < 0.05$ ), an estimated mean for the delayed oxidation time was  
155 calculated and compared using the Newman Keuls multiple comparison test (confidence  
156 interval 95%) to define groups of phenolic compounds with similar antioxidant behavior.

157

158 EVALUATION OF ANTIBACTERIAL ACTIVITY



159 Antibacterial activity was evaluated by growth inhibition of *L. monocytogenes* in tryptic  
160 soy broth (TSB) medium using a method previously described with minor  
161 modifications.<sup>14</sup> Bacterial growth was followed in an automatic spectrophotometer  
162 (Bioscreen C, Labsystems, Helsinki, Finland) by measuring Optical Density (OD) at 600  
163 nm for 72 h in two 100-well microplates. Two hundred microliters of TSB pH 7.2, with  
164 or without phenolic compounds, was added to each well. The final concentrations of the  
165 phenolic compounds in the wells varied from 0 to 2.5 mmol.L<sup>-1</sup> for carvacrol and thymol,  
166 to 2.6 mmol.L<sup>-1</sup> for eriodictyol, 2.7 mmol.L<sup>-1</sup> for naringenin, 8 mmol.L<sup>-1</sup> for eugenol, 9.4  
167 mmol.L<sup>-1</sup> for (-)-epicatechin, 20 mmol.L<sup>-1</sup> for *p*-coumaric acid, 30 mmol.L<sup>-1</sup> for *p*-  
168 hydroxybenzoic, protocatechuic, vanillic, caffeic, and ferulic acids, 35 mmol.L<sup>-1</sup> for  
169 chlorogenic acid, and 36 mmol.L<sup>-1</sup> for gallic acid. The maximum concentrations tested  
170 were chosen to obtain total growth inhibition or the solubility threshold. Five to 40  
171 concentrations, prepared from at least two different solutions, were tested per compound.  
172 Each well was inoculated with a standardized inoculum at 1% (v/v from the second  
173 subculture: approximately 10<sup>6</sup> CFU.mL<sup>-1</sup>) and the microplates incubated at 30°C with  
174 slow and continuous shaking. At least two growth curves were acquired for each  
175 concentration. The pH values of the TSB after the addition of various concentrations of  
176 phenolic compounds were measured with a pH-meter (SI Analytics lab 870, Mainz,  
177 Germany) in an independent experiment. The inhibitory activity is described by the  
178 minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC). The  
179 MIC is the concentration at which no bacterial growth is recorded and the NIC that below  
180 which the compound has no inhibitory activity. These values are expressed in mmol.L<sup>-1</sup>:  
181 the lower the MIC, the stronger the antibacterial effect. These values were obtained after  
182 two modeling steps. First, the maximum specific growth rates ( $\mu_{\max}$ ) were estimated from  
183 the growth kinetics by fitting the modified Gompertz model.<sup>14</sup> Second, the MIC and NIC

184 were determined for each phenolic compound by plotting growth rates transformed by  
185 square-root as a function of the concentration and modeling  $\mu_{\max}$  with the Lambert  
186 Pearson model.<sup>15</sup> The solver of Microsoft Excel<sup>®</sup> (2013) was used to minimize the sum  
187 of squares and allowed estimation of the model parameters. The standard deviations (SD)  
188 of model parameters and sums of squares were calculated with SolverAid, a  
189 complementary macro.<sup>16</sup>

190

## 191 RESULTS AND DISCUSSION

### 192 CHARACTERIZATION OF ANTIOXIDANT BEHAVIOR

193 We evaluated the antioxidant activity of the selected phenolic compounds by measuring  
194 their capacity to delay free linoleic acid oxidation (Table 1). Many of the tested molecules  
195 had similar antioxidant behavior to that of the well-known food antioxidant  $\alpha$ -tocopherol,  
196 which also has a phenolic group. Phenolic compounds belonging to flavan-3-ols,  
197 flavonols, and hydroxycinnamic derivatives, with an *ortho*-diphenolic structure, had the  
198 highest antioxidant potential. The results highlight the strong relationship between  
199 chemical structure and antioxidant activity. The model obtained by ANCOVA was  
200 significant ( $F_{39,225} = 331.3$ ,  $p < 0.0001$ ). The concentration effect was significant ( $p <$   
201  $0.0001$ ), with the delayed oxidation time ( $\Delta t_{Ci} = t_{Ci} - t_{C0}$ ) increasing proportionally with  
202 the concentration of the compound, whereas the phenol effect was not significant ( $p =$   
203  $0.310$ ), the intercept being equal for all phenolic compounds. The interaction effect of the  
204 phenolic compound\*concentration was also significant: the slope of the regression curve  
205 was associated with the nature of the phenolic compound.

206 We observed three different kinetic behaviors when the production of conjugated dienes  
207 was measured during linoleic acid oxidation induced by the azo-initiator AAPH.  
208 Kaempferol, vanillic acid, and protocatechuic acid production illustrate the three kinetic

209 behaviors (Figures 2a, 2b and 2c), each including various phenolic families. Several  
210 studies have reported such kinetic behaviors, but the corresponding mechanisms have not  
211 been completely elucidated.<sup>17-19</sup> The first group of antioxidants belonged to chain  
212 breakers<sup>19</sup> and included kaempferol (Figure 2a), caffeic acid, chlorogenic acid, gallic  
213 acid,  $\alpha$ -tocopherol, luteolin, quercetin, and rutin. The second group of antioxidants,  
214 considered to be retarders,<sup>19</sup> was composed of vanillic acid (Figure 2b), *p*-coumaric acid,  
215 *p*-hydroxybenzoic acid, apigenin, carvacrol, naringenin, and thymol. The third group of  
216 antioxidants, including protocatechuic acid (Figure 2c), ferulic acid, (-)-epicatechin,  
217 eriodictyol, and eugenol, showed intermediate behavior. Chain-breakers induce a lag-  
218 time phase, during which the lipidic substrate is not oxidized until complete consumption  
219 of the antioxidant, but is then oxidized with a peroxidation rate reaching the same value  
220 as the control without antioxidant.<sup>17</sup> Conversely, retarders reduce the peroxidation rate<sup>18</sup>  
221 without inducing a distinct lag-phase (Figure 2b) and, according to our study, need to be  
222 used at higher concentrations to preserve linoleic acid from oxidation. Indeed, phenolic  
223 compounds that act as retarders all showed a lower antioxidant potential, with  $EC_{1h} > 8$   
224  $\mu\text{mol.L}^{-1}$ .

225 Kinetic models have been presented in the literature<sup>17,19</sup> to help explain these two types  
226 of behavior. According to Foti<sup>17</sup>, an antioxidant should show effective chain-breaker  
227 behavior if lipid peroxy radicals  $LOO^{\bullet}$  react faster with antioxidants AH ( $LOO^{\bullet} + AH \rightarrow$   
228  $LOOH + A^{\bullet}$ ) than unsaturated fatty acids L'H ( $LOO^{\bullet} + L'H \rightarrow LOOH + L'^{\bullet}$ ), suggesting  
229 a higher kinetic constant for the first reaction and thus implying a lag period.<sup>17</sup> For  
230 retarders, kinetic constants should be similar, leading to the absence of a lag phase, since  
231 the antioxidant is slowly consumed. In reaction systems involving AAPH, Roche *et al.*<sup>19</sup>  
232 suggested that, in addition to inhibiting propagation by scavenging lipid radicals  $LOO^{\bullet}$ ,  
233 antioxidants should also be able to scavenge the azo-initiator radicals  $ROO^{\bullet}$  ( $ROO^{\bullet} + AH$

234  $\rightarrow \text{ROOH} + \text{A}^{\bullet}$ ). Thus, antioxidants should inhibit the initiation step of lipid oxidation  
235 ( $\text{ROO}^{\bullet} + \text{LH} + \text{O}_2 \rightarrow \text{ROOH} + \text{LOO}^{\bullet}$ ).<sup>19</sup> Phenolic compounds which efficiently inhibit  
236 both initiation and propagation of oxidation are described as chain-breakers; their kinetic  
237 curves show a lag phase until complete consumption of the antioxidant.<sup>19</sup> In contrast,  
238 compounds which efficiently inhibit initiation, but not propagation, are described as  
239 retarders.<sup>19</sup> Their kinetics do not show a lag phase and they essentially scavenge azo-  
240 initiator radicals.<sup>19</sup>

241 According to Foti<sup>17</sup>, the values of the kinetic constant of the reaction  $\text{LOO}^{\bullet} + \text{AH} \rightarrow$   
242  $\text{LOOH} + \text{A}^{\bullet}$  should be linked to specific chemical characteristics of the antioxidant  
243 molecules, such as: the bond dissociation energy (BDE) of the phenolic group; the steric  
244 hindrance effect of chemical structures near the phenolic group of phenolic antioxidants;  
245 and the presence of specific chemical structures, including catechol groups. Indeed, Liu  
246 and Wu<sup>20</sup> demonstrated that the spin-densities of the O<sup>•</sup> atom in antioxidant radicals could  
247 provide an explanation for the difference of the antioxidant effects among various  
248 compounds with small structural differences. Thus, chemical structure plays an important  
249 role in antioxidant activity. The kinetic constant values can also be linked to the location  
250 of the phenolic compound in a multiphasic system. In the AAPH system, as in the present  
251 study, lipid radicals are located in micelles of linoleic acid, whereas azo-initiator radicals  
252 are solubilized in the aqueous phase, thus adding an additional parameter to be taken into  
253 account.<sup>9,19</sup> These studies highlight the lack of a well-defined classification between  
254 chain-breakers and retarders. The differences in the shapes of the curves may not only be  
255 due to differences in the mechanism of action of the antioxidant, but also the kinetic  
256 constants of a complex set of reactions occurring in multiphasic systems in which  
257 partitioning of the compounds must be taken into account.

258

259 CHARACTERIZATION OF THE ANTIBACTERIAL BEHAVIOR

260 We evaluated the inhibitory activity of the phenolic compounds against the growth of  
261 *L. monocytogenes* through the modeling of growth curves and determination of two  
262 characteristic parameters for each compound, the MIC and NIC (Table 1). The method  
263 used here offers two advantages relative to semi-quantitative determination of the MIC:  
264 a standard deviation is associated to the MIC value and a complementary parameter (the  
265 NIC) is provided to better characterize the activity.

266 Simple phenols had the highest inhibitory effect, as previously described.<sup>7</sup> Flavonoids  
267 also showed high inhibitory activity (naringenin) when their level of solubility allowed  
268 performance of the assay. MICs of flavonoids for *L. monocytogenes* and other Gram-  
269 positive bacteria have been estimated in the literature, but the results are inconsistent.<sup>21–</sup>  
270 <sup>23</sup>

271 Although less effective than phenols, phenolic acids also showed good activity (Table 1).  
272 The most effective were *p*-coumaric and ferulic acids. The least effective were  
273 chlorogenic and gallic acids. MICs obtained in the present study for several phenolic acids  
274 were of the same order of magnitude as those obtained against *L. monocytogenes* in two  
275 other studies.<sup>24,25</sup>

276 The NIC provides additional useful information concerning the activity of the phenolic  
277 compound (Table 1 and Figure 3). Simple phenols and flavonoids were effective at very  
278 low concentrations, as shown for eugenol and naringenin (Figure 3). Other compounds  
279 were effective from a NIC that was slightly higher, as demonstrated for *p*-coumaric,  
280 ferulic (Figure 3), or vanillic acid. Finally, the NIC was much higher for many phenolic  
281 acids, including *p*-hydroxybenzoic acid and protocatechuic acid (Figure 3), as well as  
282 gallic, caffeic, and chlorogenic acids. These behaviors show that some compounds were

283 active from the lowest tested concentration, whereas the concentration of others needed  
284 to reach a certain threshold before reducing the bacterial growth rate.

285 The mechanism of action of simple phenols has been very-well described.<sup>7</sup> The most  
286 studied simple phenols are eugenol, thymol, and carvacrol, which are found in aromatic  
287 plants and recovered in high concentrations in essential oils and hydrosols.<sup>7,26,27</sup> These  
288 compounds intercalate into the phospholipid layers of the bacterial membrane and disturb  
289 the van der Waals interactions between the lipid acyl chains, leading to the disruption of  
290 phospholipid packing and membrane integrity.<sup>7</sup> Consequently, ion gradients are disrupted  
291 and vital constituents, such as ions and macromolecules, are released, leading to bacterial  
292 death.<sup>28-30</sup> Some specific values for lipophilicity parameters have been suggested in the  
293 literature to be associated with antibacterial activity<sup>30</sup>: a compound with a partition  
294 coefficient in octanol-water ( $\log P_{o/w}$ ) above 3 can deeply partition into membranes,  
295 whereas a  $\log P_{o/w}$  above 4 does not always result in higher antibacterial effectivity.  
296 Indeed, the ranking of antibacterial efficacy may be related to  $\log P$  (the  $\log P$  of carvacrol,  
297 thymol, and eugenol are 3.28, 3.28, and 2.20, respectively). The NIC threshold also  
298 appeared to be quite low for these three compounds (Figure 3), reflecting intercalation  
299 into the membrane at low concentrations.

300 The mechanisms of action for the antibacterial properties of flavonoids are not yet fully  
301 understood. However, as naringenin has a  $\log P$  similar to that of carvacrol and thymol  
302 (3.19), it is possible that naringenin has an antibacterial action similar to that of simple  
303 phenols, in accordance with the results of Tsuchiya *et al.*<sup>22</sup> Additionally, other  
304 mechanisms of action for flavonoids, including inhibition of nucleic acid synthesis and  
305 energy metabolism, have also been reviewed.<sup>31</sup> For these compounds, small NICs reflect  
306 high activity at the lowest concentration (Figure 3).

307 Antibacterial mechanisms of phenolic acids are not fully understood due to their complex  
308 multifactorial activity. First, the decrease in pH due to the introduction of the phenolic  
309 compound into the medium can have a simple and direct impact on growth. Moreover,  
310 organic acids can act by a very well-known antibacterial mechanism through the  
311 penetration of their non-dissociated form into the cell and acidification of the cytoplasm,  
312 leading to  $K^+$  efflux and cell death. The ionization rate of the molecule is fundamental  
313 and depends on both pH and the pKa: for the same pH, the higher the pKa, the higher the  
314 proportion of the non-dissociated form. The partition coefficients of phenolic acids may  
315 also contribute to their antibacterial activity, as some logPs can be as high as 2.43 for *p*-  
316 coumaric acid. Gallic and ferulic acids have been reported to irreversibly change the  
317 properties of bacterial membranes.<sup>32</sup> Regarding the five phenolic acids with a high NIC  
318 (as shown for *p*-hydroxybenzoic and protocatechuic acids in Figure 3), the effective  
319 concentrations appeared to correlate with a pH below 6 when dissociation of acids had  
320 probably overcome the buffering capacity of the medium. In contrast, the three phenolic  
321 acids with a very low NIC were effective at pH > 6 (as shown for *p*-coumaric and ferulic  
322 acids in Figure 3), probably reflecting a mechanism of action that is less dependent on  
323 acid dissociation.

324 The possibility that phenolic acids may act as antibacterial compounds through combined  
325 mechanisms is supported by several studies.<sup>33,34</sup> Ramos-Nino *et al.*<sup>34</sup> could predict the  
326 antibacterial behavior of the phenolic acid as a function of the lipophilicity parameter  
327 (logK), as well as the effect of the acid dissociation constant (pKa). However, the logP  
328 cannot explain all the differences we observed for phenolic acids; *p*-coumaric and ferulic  
329 acids have different partition coefficients (2.43 and 1.64 respectively) and a similar pKa,  
330 but had similar antibacterial activity.

331 Further studies are needed to exhaustively explore the impact of extracellular pH,  
332 undissociated acid activity, and logP on the complex antibacterial mechanism of action  
333 of phenolic acids.

334

#### 335 STRUCTURE-ACTIVITY RELATIONSHIPS

336 We assessed the antioxidant and antibacterial activities of phenolic compounds with  
337 various structures (Figure 1, Table 1), allowing us to describe some structure-activity  
338 relationships for both types of activities.

339 The formation of catechol groups by adding a second hydroxyl group in the *ortho*-position  
340 on the aromatic ring of phenolic acids (caffeic acid vs *p*-coumaric acid, protocatechuic  
341 acid vs *p*-hydroxybenzoic acid) or the B ring of flavonoids (eriodictyol vs naringenin,  
342 luteolin vs apigenin, quercetin vs kaempferol) significantly improved the antioxidant  
343 potential. Such higher antioxidant activity is due to stabilization by the catechol groups  
344 of their corresponding radicals by intramolecular hydrogen bonding or the regeneration  
345 of a catechol group, in parallel with quinone formation, from two catechol radicals.<sup>12,35–</sup>

346 <sup>41</sup> In contrast, adding a third hydroxyl group (gallic acid vs protocatechuic acid) slightly  
347 decreased the antioxidant potential, which may have been due to higher steric hindrance.

348 Concerning antibacterial activity, adding a second or third hydroxyl group to the phenolic  
349 ring of phenolic acids increased the MIC. This has also been reported for several bacteria,  
350 including *Bacillus cereus*<sup>25,42–44</sup>, although it cannot be generalized for all strains or  
351 species. Such addition of phenolic functions tended to decrease the partition coefficient  
352 (e.g. 1.42 for caffeic acid vs 2.43 for *p*-coumaric acid), suggesting that the compound may  
353 be less easily partitioned into the lipophilic bacterial membrane, thus having lower  
354 antibacterial activity.<sup>34</sup>



355 We obtained similar results in our study for flavonoids, for which eriodictyol (flavanone  
356 with a catechol group on the B ring), appeared to be less effective against  
357 *L. monocytogenes* than naringenin, its corresponding molecule with only one OH group  
358 (Table 1). This result supports the assumption that the decrease in lipophilicity induced  
359 by the addition of a hydroxyl group hinders the correct intercalation of the compound in  
360 the membrane (the logP of eriodictyol is 2.59 vs 3.19 for naringenin), as for phenolic  
361 acids. However, further studies are necessary to evaluate the impact of hydroxylation of  
362 the B ring on the antibacterial activity of flavonoids,<sup>31,45</sup> as contradictory results have  
363 been reported in the literature.<sup>23</sup>

364 The presence of a methoxyl group in the *ortho* position of a phenolic group (vanillic acid  
365 vs *p*-hydroxybenzoic acid and ferulic acid vs *p*-coumaric acid) significantly increased  
366 antioxidant activity, as *ortho* substitution with the electron donor methoxyl group  
367 increases the stability of the produced radical.<sup>20,38,46</sup> This had a small and variable effect  
368 on antibacterial activity, as already shown for cinnamic acids in the literature.<sup>25,44</sup>

369 Esterification of caffeic acid by quinic acid, leading to chlorogenic acid, did not have any  
370 significant influence on the antioxidant potential, because the catechol group is still  
371 available. However, it resulted in an increase in the MIC, as reported in the  
372 literature.<sup>12,25,47</sup> The addition of quinic acid decreased the partition coefficient (from 1.42  
373 to -0.36) and increased steric hindrance, probably conferring lower penetration of the  
374 compound into the membrane.

375 The presence of alkyl groups in the *ortho* or *para* position of the phenolic group (carvacrol  
376 and thymol) did not have any significant influence on the antioxidant potential,  
377 confirming published data.<sup>42</sup> These two isomers have generally similar activities on many  
378 species, but some strains or species may be more sensitive to carvacrol, as was  
379 *L. monocytogenes* in the present study.<sup>42</sup>

380 The addition of a cinnamic group (caffeic acid *vs* protocatechuic acid and ferulic acid *vs*  
381 vanillic acid) to the aromatic ring significantly increased both antioxidant and  
382 antibacterial activities. The capacity to stabilize the phenoxyl radical by resonance had a  
383 large effect on the antioxidant activity.<sup>12,36-38,48</sup> The increase in antibacterial activity may  
384 be related to the increased partition coefficient, due to the addition of a cinnamic  
385 group.<sup>24,44</sup>

386 For flavonoids, any chemical modifications that lead to higher resonance of the  
387 molecules, thus improving the stability of the phenoxyl radicals, increased the antioxidant  
388 activity, similar to the addition of a cinnamic group on phenolic acids<sup>12,46,48-50</sup>; the  
389 presence of a double bond in the 2,3-position of the C ring (luteolin *vs* eriodictyol), the  
390 addition and/or availability of a hydroxyl group in the 3-position, in combination with the  
391 double bond in the 2,3 position of the C ring (kaempferol *vs* apigenin, quercetin *vs* luteolin  
392 or rutin), or the presence of a ketone in the 4-position conjugated with the double bond in  
393 the 2,3 position of the C ring (quercetin *vs* epicatechin) (Table 1). The impact of this type  
394 of chemical structure in flavonoids on antibacterial activity could not be tested due to  
395 solubility issues.

396

397

## CONCLUSIONS

398 The chemical structure of phenolic compounds may have an opposite impact on  
399 antioxidant and antibacterial activities. These differences are attributable to completely  
400 different mechanisms of action between the two activities. The two main structural  
401 parameters that improve antioxidant activity are the chemical environment of the phenolic  
402 group and the presence of delocalization structures.

403 The partition coefficient may be one of the main parameters that influence antibacterial  
404 activity; in many cases, the higher the logP, the higher the bacterial growth inhibition.

405 This is in accordance with the antibacterial activity being driven by a high lipophilicity  
406 parameter for optimized intercalation into the bacterial membrane. Nevertheless, our  
407 structure comparisons show that logP is not the only parameter to explain the compound's  
408 multifactorial antibacterial effectiveness. Finally, a series of phenolic compounds with  
409 both activities could be selected, based on these results, to conduct further studies in more  
410 complex food systems, including emulsions. The double activity of phenolic compounds  
411 can be applied in the food industry by using a mix of phenolic compounds, such as plant  
412 extracts or agro-industrial by-products, to take advantage of their complementary effects.

413

414

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417

418

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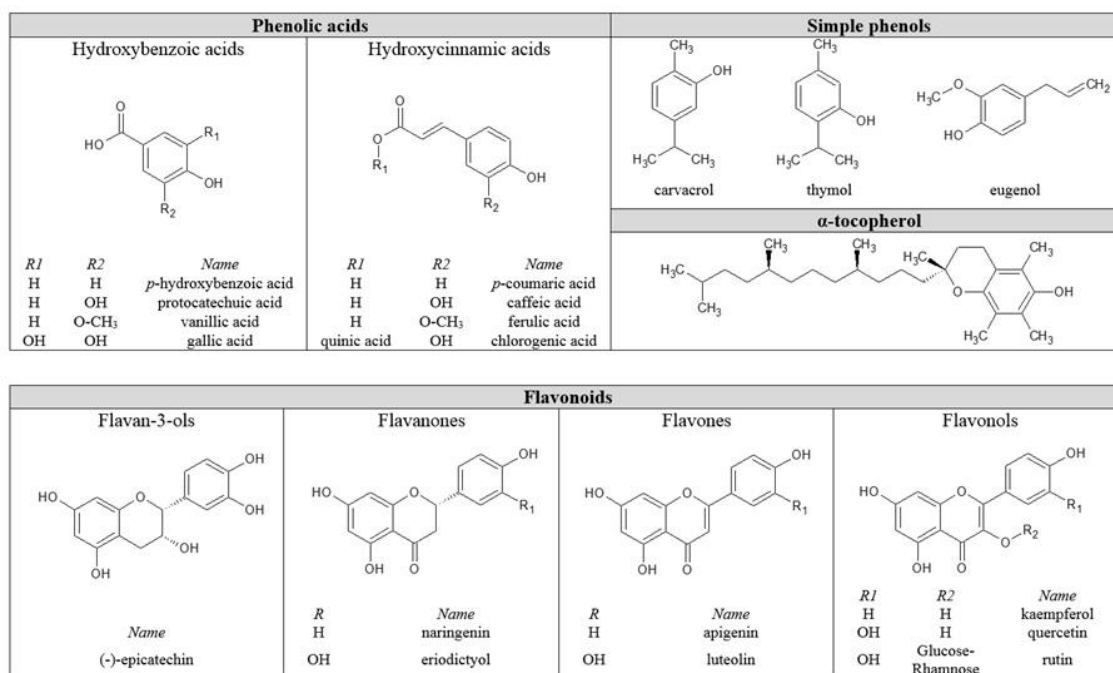
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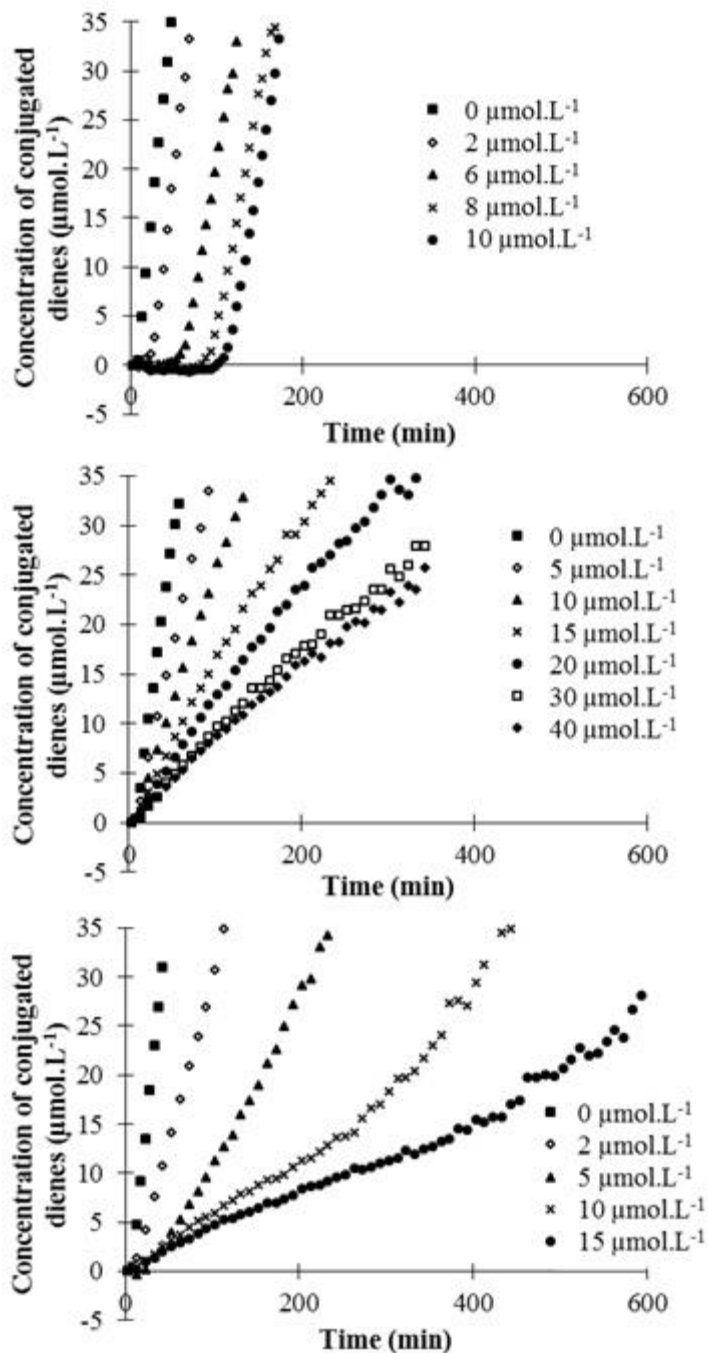


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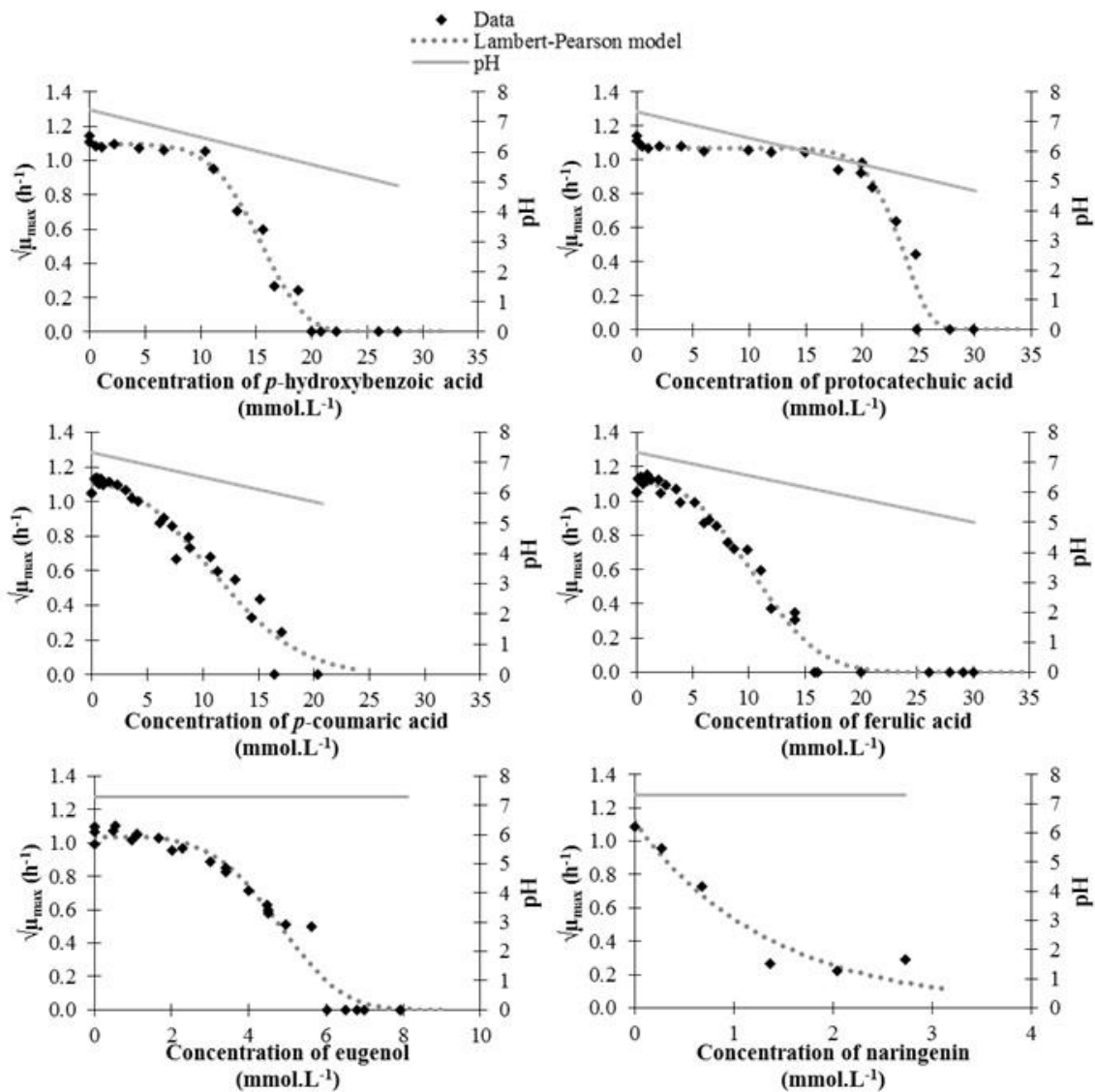
## FIGURES CAPTIONS



**Figure 1:** Chemical structures of the studied phenolic compounds.



**Figure 2:** Kinetics of linoleic acid oxidation in an AAPH system (followed by conjugated dienes measurement) in the presence of various concentrations of antioxidants with three different behaviors: kaempferol, representative of chain breakers (a), vanillic acid, representative of retarders (b), and protocatechuic acid, representative of compounds with an intermediate behavior (c).



**Figure 3:** Effect of increasing concentrations of phenolic compounds on the growth of *L. monocytogenes* (data fitted with the Lambert-Pearson model) and broth pH.

### TABLE CAPTION

**Table 1:** Antioxidant and antibacterial activities of phenolic compounds: effective concentration to delay oxidation and non-inhibitory and minimum inhibitory concentrations for *L. monocytogenes*.

For antioxidant activity, compounds are divided into groups (with letters) for which there is no significant difference in the estimated delay for oxidation, according to the Newman Keuls multiple comparison test (confidence interval 95%). For antibacterial activity, standard deviations are associated with NIC and MIC values. nd: not determined.

Phenolic compounds	Antioxidant activity		Antibacterial activity	
	EC <sub>1h</sub> ( $\mu\text{mol.L}^{-1}.\text{h}^{-1}$ )	Groups identified with Newman Keuls comparison	NIC $\pm$ SD ( $\text{mmol.L}^{-1}$ )	MIC $\pm$ SD ( $\text{mmol.L}^{-1}$ )
<b>Phenolic acids</b>				
caffeic acid	1.2	CD	13.76 $\pm$ 0.50	18.03 $\pm$ 1.21
chlorogenic acid	1.2	D	18.11 $\pm$ 0.81	33.44 $\pm$ 0.54
protocatechuic acid	2.0	F	19.50 $\pm$ 0.74	24.63 $\pm$ 0.32
ferulic acid	2.2	FG	5.04 $\pm$ 0.35	13.60 $\pm$ 0.31
gallic acid	3.8	H	22.55 $\pm$ 1.17	31.70 $\pm$ 0.60
vanillic acid	8.5	J	8.93 $\pm$ 0.50	21.30 $\pm$ 0.38
<i>p</i> -coumaric acid	28.5	K	4.61 $\pm$ 0.49	15.44 $\pm$ 0.54
<i>p</i> -hydroxybenzoic acid	239.6	K	10.27 $\pm$ 0.56	17.35 $\pm$ 0.29
<b>Simple phenols</b>				
eugenol	2.2	G	2.88 $\pm$ 0.20	5.62 $\pm$ 0.13
thymol	8.0	IJ	0.78 $\pm$ 0.04	1.31 $\pm$ 0.05
carvacrol	8.1	IJ	0.33 $\pm$ 0.10	0.95 $\pm$ 0.07
<b>Flavonoids</b>				
quercetin	0.8	A	nd	nd
(-)-epicatechin	1.0	B	2.34 $\pm$ 0.30	> 9.44
rutin	1.1	BC	nd	nd
luteolin	1.2	D	nd	nd
eriodictyol	1.5	E	0.23 $\pm$ 0.30	> 2.58
kaempferol	4.8	HI	nd	nd
apigenin	43.6	K	nd	nd
naringenin	267.7	K	0.12 $\pm$ 0.11	1.85 $\pm$ 0.38
$\alpha$ -tocopherol (referent)	2.4	G	nd	nd