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Inhibitory activity of phenolic acids against *Listeria monocytogenes*: deciphering the mechanisms of action using three different models.

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12 **Abstract**

13 Phenolic compounds are well known for their antimicrobial activity. They may provide an
14 interesting solution to ensure food safety by preventing the growth of foodborne pathogens
15 while addressing the wishes of consumers for the use of natural preservatives in food and
16 favoring the reuse of agro-industry byproducts. However, their mechanism of action is still
17 not very well known. Here, we aimed to decipher the complex mechanism of action of eight
18 phenolic acids by decomposing their effects, such as the general effect of the decrease of
19 extracellular pH ($\gamma(\text{pH})$) and specific inhibitory effects of the undissociated ($\gamma(\text{A}_u)$) and
20 dissociated ($\gamma(\text{A}_d)$) forms. We thus developed three different models and applied them to a
21 dataset of *Listeria monocytogenes* growth rates experimentally obtained in the presence of
22 various concentrations of phenolic acids at several pHs. The model that best fits the dataset
23 was selected for each phenolic acid to explore the potential mechanisms. The results show
24 that the antimicrobial activity is mainly due to the effect of the undissociated forms, except
25 for chlorogenic and gallic acids, for which the antimicrobial activity is mainly due to a
26 decrease in extracellular pH. In addition, the dissociated forms of *p*-coumaric and ferulic acids
27 show significant inhibitory activity.

28

29 **Keywords**

30 phenolic, antimicrobial, Gamma concept, undissociated form, dissociated form

31

32 **Introduction**

33 *Listeria monocytogenes* is a ubiquitous, wide spread, highly environmentally resistant (soil,
34 lakes, rivers, etc.) soil bacterium that can contaminate food at all stages of the food chain
35 (Anses, 2011). It causes listeriosis, which affects humans and animals and occurs mainly as an
36 invasive form (Anses, 2011). Since the 90's, the prevalence of *L. monocytogenes* in many
37 food categories has been reduced due to improved control measures (Buchanan et al., 2017).
38 However, the rate of illness has remained constant over the last decade and recent outbreaks
39 have challenged control measures (Allerberger and Wagner, 2010; Buchanan et al., 2017).
40 Ready-to-eat food, meat, fish, and dairy products, as well as fruits and vegetables, are the
41 predominant vehicles involved in the main listeriosis outbreaks (Rodriguez-Lopez et al.,
42 2018).

43 The use of preservatives can help to inhibit microbial growth or inactivate pathogenic
44 bacteria. However, this approach is increasingly challenging due to the emergence of bacterial
45 resistance, mistrust of consumers towards chemical additives, and regulatory constraints that
46 reduce the list of protective ingredients. Thus, there is renewed interest in several families of
47 natural antimicrobials (Sorrentino et al., 2018; Weiss et al., 2015). Simple phenols, such as
48 eugenol, thymol, and carvacrol, are found in aromatic plants and recovered in high
49 concentrations in essential oils and hydrosols. They intercalate into the phospholipid layers of
50 the bacterial membrane and disturb the van der Waals interactions between the lipid acyl
51 chains, leading to the disruption of phospholipid packing and membrane integrity (Burt,
52 2004). Consequently, ion gradients are disrupted and vital constituents, such as ions and
53 macromolecules, are released, leading to bacterial death.

54 Organic acids act by a very well-known antimicrobial mechanism through the penetration of
55 their undissociated form into the cell and acidification of the cytoplasm leading to cell death.
56 Phenolic acids are of interest due to their natural plant-based origin, their presence in large

57 quantities in byproducts of the fruit, wine and cereal industries and their demonstrated global
58 antimicrobial activity. We previously evaluated the inhibitory activity of several phenolic
59 compounds against *Listeria monocytogenes* as a function of their total concentration (Pernin
60 et al., 2018). However, very few studies have focused on deciphering the various effects of
61 their inhibitory activity, which can be similar to those of organic acids and/or simple phenols
62 (Sorrentino et al., 2018).

63 We reasoned that the total inhibitory activity is a combination of the following effects: *i*)
64 decreased pH in the presence of phenolic acids which directly inhibits growth, *ii*) the
65 inhibitory effect of the undissociated acid form, and *iii*) the inhibitory effect of the dissociated
66 acid form. Each acid form could potentially either intercalate into the phospholipid membrane
67 or cross the membrane and decrease the intracellular pH and/or interact with cellular
68 constituents.

69 Here, we aimed to decipher the complex mechanism of action of phenolic acids by
70 decomposing the various inhibitory effects. We thus developed three different models using
71 the Gamma concept approach (Lambert and Bidlas, 2007; Zwietering et al., 1992). These
72 models aim to describe the proportion of inhibition due to the three potential effects. The
73 eight phenolic acids (*p*-hydroxybenzoic, protocatechuic, gallic, vanillic, *p*-coumaric, caffeic,
74 ferulic and chlorogenic acids) studied were chosen based on their efficiency in our previous
75 study (Pernin et al., 2018). The model that best fits the dataset was selected for each phenolic
76 acid to explore the potential mechanisms.

77

78 **2. Materials and methods**

79 ***2.1. Chemical reagents***

80 Hydrochloric acid (1 mol/L), sodium hydroxide (1 mol/L), and acetone were purchased from
81 Carlo Erba (Fontenay-aux-Roses, France).

82

83 **2.2. Bacterial strain**

84 The strain used in this study was *Listeria monocytogenes* CNL 895805, serotype 1/2 a, isolated
85 from sheep brain. It was graciously provided by P. Velge (INRA, Nouzilly) (Van
86 Langendonck et al., 1998). Before each experiment, the strain, stored in cryovials at -80°C,
87 was resuscitated in two successive subcultures in tryptic soy broth (TSB, Biomérieux, France)
88 at 30°C.

89

90 **2.3. Phenolic compounds**

91 *p*-Hydroxybenzoic acid, protocatechuic acid, vanillic acid, gallic acid, *p*-coumaric acid,
92 caffeic acid, ferulic acid, and chlorogenic acid were purchased from Sigma-Aldrich (St
93 Quentin Fallavier, France). The chemical structures and physicochemical parameters (pKa,
94 logP) are provided in Table 1.

95 Stock solutions of the phenolic compounds were prepared according to specific protocols
96 depending on their ability to be solubilized in the culture medium. *p*-Hydroxybenzoic,
97 protocatechuic, gallic, and chlorogenic acid powders were directly dissolved in the culture
98 medium. Vanillic, *p*-coumaric, and ferulic acids were first dissolved in acetone, which was
99 evaporated under nitrogen flow after addition to the culture medium. Caffeic acid was first
100 dissolved in acetone/distilled water (80/20 (v/v)) and the acetone evaporated under nitrogen
101 flow. Bacterial growth controls were carried out to ensure the absence of inhibitory activity of
102 trace acetone after evaporation.

103

104 **2.4. Growth in the presence of phenolic compounds at various pHs**

105 The growth of *L. monocytogenes* was followed in TSB containing one of the eight phenolic
106 acids at various pHs. The phenolic acids were added at various concentrations in either neutral

107 TSB pH 7.2 (7.1-7.4) or acidic TSB pH 5.5 (5.5-5.7). pH 7.2 is the native pH of TSB and it is
108 optimal for *L. monocytogenes* growth (Anses, 2011). pH 5.5 allows to increase the
109 concentrations of undissociated forms of acids without decreasing too much the growth rate of
110 *L. monocytogenes*. After solubilization of the phenolic acid at the target concentration in TSB
111 7.2, the pH was adjusted to 7.2 (7.2a) with 1 mol/L sodium hydroxide or not adjusted (7.2na)
112 (Pernin et al., 2018). Similarly, TSB 5.5 was adjusted to a pH of 5.5 (5.5a) or not adjusted
113 (5.5na). The pH of each culture medium was measured with an SI Analytics lab 870 pH-meter
114 (Mainz, Germany) in an independent experiment. Gallic, caffeic, and chlorogenic acids were
115 not tested in TSB 7.2a because the medium became brown after pH adjustment, probably due
116 to oxidation.

117 The final concentration of the phenolic compounds in TSB varied from 0 to at least 30
118 mmol/L. For some compounds, the minimal inhibitory concentration was not achieved due to
119 solubility issues. Eight to 64 concentrations prepared from at least two different stock
120 solutions were tested per compound for each series of experiments and a total of 47 to 146
121 assays were conducted for each compound.

122 The largest possible range of conditions was tested to allow the best adjustment of the models:
123 total acid concentration, concentration of undissociated or dissociated forms, and pH. Series
124 7.2na covered pH ranges from 7.2 to 3.2 (0 to 10 mmol/L of the undissociated form and 0 to
125 20 mmol/L of the dissociated form) and series 5.5na, 5.5 to 4.0 (0 to 15 mmol/L of the
126 undissociated form and 0 to 10 mmol/L of the dissociated form). Series 7.2a and 5.5a were set
127 to the pH of the initial TSB batch, around 7.2 and 5.5, respectively. The concentration of the
128 undissociated form was close to zero and that of the dissociated form from 0 to 30 mmol/L for
129 series 7.2a. The corresponding values for the series 5.5a were from 0 to 1.5 mmol/L and 0 to
130 25 mmol/L.

131 TSB at pH adjusted from 3 to 10 with 1 mol/L hydrochloric acid or 1 mol/L sodium
132 hydroxide solutions was prepared to evaluate the effect of pH on bacterial growth in the
133 absence of the phenolic acids. In total, 19 pHs were tested in two independent experiments.
134 TSB of each series was inoculated at 1% (v/v from the second subculture; approximately 10^6
135 CFU/mL) with a standardized inoculum. Two hundred microliters of inoculated TSB from
136 each series was added to the wells of 100-well microplates which were incubated at 30°C
137 with slow continuous shaking. Bacterial growth was followed in an automatic
138 spectrophotometer (Bioscreen C, Labsystems, Helsinki, Finland) by measuring the optical
139 density (OD) at 600 nm for 72 h. In total, 1408 growth kinetics were acquired.

140

141 ***2.5. Determination of the growth rate***

142 Maximum specific growth rates (μ_{max}) of *L. monocytogenes* were estimated from the growth
143 kinetics by fitting the modified Gompertz model (Guillier et al., 2007; Pernin et al., 2018).
144 The standard deviations of the model parameters and sum of squares were calculated using
145 the complementary macro SolverAid (de Levie, 2012).

146

147 ***2.5. Modelling the effect of different acid forms on the growth rate***

148 The effect of phenolic acids on the growth rate of *L. monocytogenes* was modelled using the
149 Gamma concept approach (Lambert and Bidlas, 2007; Zwietering et al., 1992). The effects of
150 pH and undissociated and dissociated forms of the acid were taken into account (eq. (1)).

$$151 \quad \mu_{max} = \mu_{ref} \cdot \gamma(pH) \cdot \gamma(A_u) \cdot \gamma(A_d) \quad \text{eq. (1)}$$

152 where:

153 μ_{ref} is the *L. monocytogenes* maximum specific growth rate at 30°C in TSB at optimal pH and
154 without phenolic acid and $\gamma(pH)$ describes the pH effect as described in eq. (2) (Presser et al.,
155 1997).

156
$$\gamma(pH) = 1 - \frac{10^{pH_{min}}}{10^{pH}} \quad \text{eq. (2)}$$

157 The pH_{min} value was set to 4.24 (Augustin et al., 2005).

158 $\gamma(A_u)$ describes the effect of the undissociated form as described in eq. (3) (Presser et al.,
159 1997).

160
$$\gamma(A_u) = \left(1 - \frac{A_{tot}}{MIC_u \cdot (1 + 10^{pH - pKa})}\right)^\alpha \quad \text{eq. (3)}$$

161 where MIC_u is the minimum inhibitory concentration of the undissociated form of the acid, α
162 is a shape parameter, and A_{tot} is the concentration of total acid.

163 $\gamma(A_d)$ describes the effect of the dissociated form as described in eq. (4) (Presser et al., 1997).

164
$$\gamma(A_d) = \left(1 - \frac{A_{tot}}{MIC_d \cdot (1 + 10^{pKa - pH})}\right) \quad \text{eq. (4)}$$

165 where MIC_d is the minimum inhibitory concentration of the dissociated form of the acid.

166

167 Three different models are proposed.

168 In model #1, pH is the only factor taken into account and μ_{ref} is the only parameter to estimate
169 (eq. (5)).

170
$$\mu_{max} = \mu_{ref} \cdot \gamma(pH) \quad \text{eq. (5)}$$

171 In model #2, pH and A_u are the factors taken into account and μ_{ref} , MIC_u and α are the
172 parameters to estimate (eq.(6)).

173
$$\mu_{max} = \mu_{ref} \cdot \gamma(pH) \cdot \gamma(A_u) \quad \text{eq. (6)}$$

174 In Model #3, pH, A_u and A_d are the factors taken into account and μ_{ref} , MIC_u , α and MIC_d are
175 the parameters to estimate (eq.(7)).

176
$$\mu_{max} = \mu_{ref} \cdot \gamma(pH) \cdot \gamma(A_u) \cdot \gamma(A_d) \quad \text{eq. (7)}$$

177

178 **2.6. Statistical approach**

179 The model parameters were fitted with Excel solver according to minimization of the residual
180 sum of square errors (RSS) for the tested mathematical model. The three models were
181 compared according to the Bayesian information criterion (BIC) (eq. (8)).

$$182 \quad BIC = n \cdot \ln\left(\frac{RSS}{n}\right) + k \cdot \ln(n) \quad \text{eq.(8)}$$

183 where n is the number of experimental points and k the number of parameters of the model.

184 **3. Results and Discussion**

185 **3.1. Characterization of the models**

186 Modeling with the Gamma concept approach (Lambert and Bidlas, 2007) allowed
187 quantification of the relative importance of each impact factor. It is here difficult to show a
188 graphical representation of the whole dataset of observed and predicted growth rates because
189 of there are four relevant impact factors: pH, A_{tot} , A_{u} , A_{d} . Thus, we show in Figure 1 the
190 predicted μ_{max} as a function of the observed μ_{max} for each phenolic acid. It illustrates that the
191 chosen models fit well the datasets whatever the compound. Moreover, Figure 2 shows how
192 the models and the Gamma concept approach work. It gives more details about the fitness of
193 model #3 applied to the ferulic acid dataset (Figures 2a and b). The growth rates obtained with
194 various concentrations are represented as a function of the concentrations of the undissociated
195 and dissociated forms for two pHs (5.6 and 7.3). A given concentration of total acid at a given
196 pH corresponds to a concentration of the undissociated and dissociated forms, together with
197 an observed and a predicted μ_{max} (Figure 2c). The model provides a value for the reduction of
198 μ_{max} independently associated with the undissociated form, dissociated form, and pH. For
199 example, when ferulic acid is introduced at 0.91 mmol/L in TSB adjusted to pH 5.6, the
200 growth rate μ_{max} is divided by 1/0.57 (that is, $1/\gamma$) due to the undissociated form, 1/0.99 due to
201 the dissociated form, and 1/0.96 due to the decrease in pH, relative to the growth rate in the
202 absence of the phenolic acid. In this example, the antimicrobial effect of the undissociated
203 form is far more predominant than that of the dissociated form and pH (Figure 2c). When
204 ferulic acid is introduced at 30.28 mmol/L in TSB adjusted to pH 7.3, the growth rate μ_{max} is
205 divided by 1/0.66 due to the undissociated form and 1/0.52 due to the dissociated form. The
206 antimicrobial effect of the dissociated form is here almost the same as that of the
207 undissociated form. The pH has no effect ($\gamma = 1.00$), as this pH is optimal for the growth of *L.*
208 *monocytogenes*. Such tables, which set the three Gamma values for all concentrations of each

209 phenolic acid help in understanding the relative impact of each factor in the inhibition of
210 bacterial growth and are provided in Supplementary data.

211 We compared the BICs obtained for each of the three models for each phenolic acid (Table
212 2): the lower the BIC, the better the model fits the dataset. The use of the Gamma concept
213 approach together with the calculated BIC allows adjustment of the number of parameters to
214 that which is most relevant. The parsimony principle (Ross and Dalgaard, 2004) states that
215 “models should have no more parameters than are required to describe the underlying
216 behavior studied. Too many parameters can lead to a model that fits the error in the data, *i.e.*
217 generates a model that is specific to a particular set of observations”. We thus deleted
218 parameters that did not improve the BIC.

219 In models # 2 and #3, a shape parameter describing the slope of the curve $\mu = f(A_u)$ at low
220 concentrations is estimated for each phenolic acid (Table 2). It accounts for the potential non-
221 linear shape of the decrease in μ when the concentration of the undissociated form increases.
222 The implication of the α parameter value for each phenolic acid and its relation to the
223 antimicrobial mechanism will be discussed in section 3.2.2.

224 To our knowledge, there is no other description in the literature of a model that takes into
225 account the undissociated and dissociated forms in the inhibitory activity of phenolic acids. A
226 very small number of studies have attempted to model the antimicrobial activity of phenolic
227 acids, such as that of Ramos-Nino *et al.*, (1996), which linked the inhibitory activity of
228 phenolic acids with their logP and pKa (Ramos-Nino *et al.*, 1996). In contrast, more studies
229 have attempted to model the antimicrobial activity of organic acids. They have used various
230 types of models, such as principle component analysis (Hsiao and Siebert, 1999; Nakai and
231 Siebert, 2003) or multivariate linear regression (George *et al.*, 1996). However, only a few
232 have studied the effect of the dissociated forms (Eklund, 1985, 1983; Presser *et al.*, 1997).
233 Eklund proposed a mathematical model to calculate the effect of the undissociated and

234 dissociated forms of sorbic, benzoic, and propionic acid on several microorganisms (Eklund,
235 1985, 1983). In this model, the specific effect of each form was calculated from the measured
236 MIC of the total form of the organic acid at different pHs (from pH 4.6 to 7.6).
237 The model of Presser *et al.* (1997) is closer to ours. These authors modeled growth rates of
238 *Escherichia coli* as a function of pH and lactic acid concentration, taking into account a
239 suboptimal pH term, an undissociated organic acid term, and a dissociated organic acid term
240 (Presser et al., 1997). A shape parameter, α , for the undissociated acid term was added in our
241 study, together with application of the parsimony principle, by combining the Gamma concept
242 approach and calculation of the BIC.

243

244 ***3.2. Hypothesis on the antimicrobial mechanism based on the best-fitting model***

245 Model #2 fits the best for caffeic, chlorogenic, gallic, *p*-hydroxybenzoic, protocatechuic, and
246 vanillic acids. Model #3 fits the best for *p*-coumaric acid and ferulic acid (Table 2). Model #1
247 never provided a best fit. Thus, considering only decreases in extracellular pH cannot
248 completely describe the dataset for any of the tested phenolic acids.

249

250 *3.2.1. Decreasing extracellular pH: the main antimicrobial mechanism of action for* 251 *chlorogenic and gallic acids*

252 Although the best-fitting model for chlorogenic acid and gallic acid was model #2 (Table 2),
253 the calculated MIC_u are extremely high, above the solubility thresholds of these two
254 compounds. At pH 5.5, 1,766.72 mmol/L total chlorogenic acid and 637.06 mmol/L gallic
255 acid would be needed to reach the MIC_u, at least 16-fold higher than that for protocatechuic
256 acid. Thus, even if the undissociated forms of chlorogenic acid and gallic acid significantly
257 decreased the growth rate, their effect was very small relative to that of other phenolic acids.
258 In addition, the datasets of the bacterial growth rates obtained as a function of pH for various

259 concentrations of chlorogenic and gallic acids clearly overlap to those obtained for
260 hydrochloric acid (Figure 3a). Moreover, at adjusted pH 5.5, increasing the concentration of
261 chlorogenic or gallic acids did not have any visible impact on growth rates (Figure 3b).
262 Similarly, Wen *et al.* (2003) showed that chlorogenic acid had no antimicrobial activity on *L.*
263 *monocytogenes* at pH 5.5 adjusted (Wen et al., 2003). Indeed, the mode of action for these
264 two acids is mainly due to the decrease of extracellular pH.

265 In order to better illustrate the mechanism of action of these acids, we compared the growth
266 rates in the presence of gallic acid to those in presence of vanillic acid. Addition of either acid
267 to the culture medium to a total acid concentration of 10 mmol/L reduced the pH to
268 approximately 4.6. However, according to the MIC_u and pKa values, $\gamma(pH) = 0.54$ and $\gamma(A_u)$
269 $= 0.43$ for gallic acid and $\gamma(pH) = 0.58$ and $\gamma(A_u) = 0.00$ for vanillic acid (Supplementary
270 data). The inhibitory effect of the undissociated form was significant for gallic acid but
271 limited to a reduction by $1/0.43$. In contrast, it was far more predominant for vanillic acid, as
272 it completely inhibited growth.

273 Most published studies do not separate the specific inhibitory effect due to the undissociated
274 form and the decrease of extracellular pH. However, chlorogenic acid and gallic acid have
275 been shown to be relatively ineffective antimicrobials among phenolic acids (Gutiérrez-
276 Larraínzar et al., 2012; Pernin et al., 2018; Saavedra et al., 2010; Sánchez-Maldonado et al.,
277 2011; Wen et al., 2003).

278 Several hypotheses can be formulated to explain the lack of antimicrobial activity of the
279 undissociated acid form of chlorogenic and gallic acids. First, their high steric hindrance,
280 especially that of chlorogenic acid, could limit their penetration through the membrane even if
281 Ramos-Nino *et al.* (1996) showed that this parameter was not relevant to describe the
282 antimicrobial efficiency of hydroxybenzoic and hydroxycinnamic acids. Second, their low
283 partition coefficient could limit their partition into the bacterial membrane (Table 1).

284 However, organic acids are highly active while they have low partition coefficient (Hirshfield
285 et al., 2003; Lu et al., 2011). In addition, several studies have demonstrated an antimicrobial
286 effect of the undissociated form of chlorogenic acid and gallic acid at the membrane level
287 (Borges et al., 2013; Lou et al., 2011). Gallic acid was shown to modify the membrane
288 hydrophobicity and charge of *L. monocytogenes* and to cause local ruptures or pore formation
289 in the cell membranes (Borges et al., 2013). Chlorogenic acid induces outer membrane
290 modifications and loss of membrane integrity of *Streptococcus pneumoniae* and *Shigella*
291 *dysenteriae* (Lou et al., 2011).

292

293 *3.2.2. The antimicrobial effect of the undissociated forms: the main antimicrobial mechanism*
294 *of action for caffeic, vanillic, protocatechuic and p-hydroxybenzoic acids.*

295 Most of the phenolic acids tested, namely caffeic acid, *p*-hydroxybenzoic acid, protocatechuic
296 acid, and vanillic acid, best fit with model #2, with realistic MIC_u values (Table 2). The
297 comparison of phenolic acids with different α shape parameters resulted in different profiles
298 (Figure 4). Some acids, such as protocatechuic acid and caffeic acid, display a linear
299 antimicrobial effect when concentration of undissociated form of phenolic acids increase ($\alpha =$
300 1). In contrast, phenolic compounds with $\alpha > 1$ have a steeper slope, as shown for vanillic acid
301 ($\alpha = 8.20$) and *p*-hydroxybenzoic acid ($\alpha = 11.11$) in model #2 represented at pH 5.5 (Figure
302 4). The higher the value of $\alpha (>1)$, the higher the effect of an additional molecule at low
303 concentrations. Compounds with $\alpha = 1$, such as caffeic and protocatechuic acids, have very
304 close chemical structures (2 OH groups on the phenolic ring; Table 1). Differences between α
305 could be due to differences in the ability of these phenolic acids to penetrate through or
306 accumulate in the bacterial membrane. This is the first report characterizing a shape parameter
307 α for the effect of undissociated forms of phenolic acids. This parameter can be a key factor in
308 choosing an antimicrobial agent that will be highly active at very low concentrations.

309

310 3.2.3. Phenolic acids with a dissociated form that is significantly antimicrobial

311 Model #3 best fits the *p*-coumaric acid and ferulic acid datasets. The antimicrobial effect of
312 the undissociated form and decrease in the extracellular pH cannot explain the entire mode of
313 action. The introduction of ferulic acid at 30.28 mmol/L in TSB adjusted to pH 7.3, divided
314 the growth rate μ_{\max} by 1/1.00 due to the pH, 1/0.66 due to the undissociated form, and 1/0.52
315 due to the dissociated form relative to that of the control (Figure 2c). Similarly, introduction
316 of *p*-coumaric acid at 31.55 mmol/L in TSB adjusted to pH 7.4 divided the growth rate by
317 1/0.78 due to the undissociated form and 1/0.57 due to the dissociated form relative to that of
318 the control (Supplementary data). There are several examples for *p*-coumaric acid and ferulic
319 acid in which $\gamma(A_d)$ was close to or even lower than $\gamma(A_u)$ (Supplementary data). Under
320 specific conditions, the inhibitory effect of the dissociated form predominates over that of the
321 undissociated form and pH. In contrast, $\gamma(A_d)$ never dropped below 1.00 for *p*-
322 hydroxybenzoic, caffeic, or protocatechuic acids or below 0.93 for vanillic acid when model
323 #3 was applied to other phenolic acids (Supplementary data). *p*-Coumaric and ferulic acids
324 are therefore the most efficient antimicrobials at pH 7.2, relative to the other phenolic acids,
325 due to their dissociated form which plays a significant role in decreasing the growth rate.

326 Nevertheless, their MIC_d values calculated for pH 7.2 are very high, 72.63 and 63.67 mmol/L,
327 respectively, which is above their solubility threshold. These results show that *p*-coumaric
328 acid and ferulic acid can be efficient antimicrobial agents, even at neutral pH, and this feature
329 is important for the choice of compounds in several applications.

330 This is the first report of the inhibitory efficacy of the dissociated form of a phenolic acid. The
331 MIC_d for *p*-coumaric acid and ferulic acid against *L. monocytogenes* are 10-times lower than
332 the average order of magnitude found in the literature for organic acids on several bacteria:
333 lactate (800-1250 mmol/L (Presser et al., 1997)), propionate (380-830 mmol/L (Eklund,

334 1985)), benzoate (90-200 mmol/L (Eklund, 1985)), and sorbate (50-400 mmol/L (Eklund,
335 1983)).

336 Only a few hypothesis has been previously proposed concerning the potential antimicrobial
337 mechanism of action of the dissociated form of organic acids such as effects on cell wall and
338 membrane, inhibition of substrate transport or of key enzymes in the case of sorbates (Sofos
339 et al., 1986). Interestingly, *p*-coumaric and ferulic acids share similarities in their chemical
340 structures. They both contain a cinnamic group between the phenolic ring and the carboxylic
341 function and have only one OH group on their phenolic ring (Table 1). Thus, it is possible that
342 specific chemical structures can interact with the bacterial membrane or specific receptors.
343 Moreover, the logPs of *p*-coumaric and ferulic acids at pH 8.0 (where ~ 100% of the acid is
344 dissociated) are the highest among phenolic compounds: -1.58 and -1.81, respectively (Table
345 1). Their « higher » partition coefficient may favor interaction with the bacterial membrane,
346 even if these values are far from the optimal logP values described for this activity (Ultee et
347 al., 2002). Further studies are needed to better understand the antimicrobial mechanism of
348 action of the dissociated forms of both organic and phenolic acids.

349

350 **Conclusion**

351 We present here an original modeling approach which allows a better understanding of the
352 mechanism of action of eight phenolic acids. It quantifies the role of the three different
353 antimicrobial factors (decrease in extracellular pH and the undissociated acid form and
354 dissociated acid form). The results allowed classification of these phenolic acids into three
355 categories. First, chlorogenic acid and gallic acid mainly inhibit the growth of *L.*
356 *monocytogenes* through their ability to decrease extracellular pH, the molecules themselves
357 having a low antimicrobial activity on bacterial growth. Second, caffeic acid, *p*-
358 hydroxybenzoic acid, protocatechuic acid, and vanillic acid exert antimicrobial behavior

359 mainly through the ability of their undissociated form to inhibit the growth of *L.*
360 *monocytogenes*. Third, *p*-coumaric acid and ferulic acid similarly exert antimicrobial behavior
361 through their undissociated form, but their dissociated form also shows significant
362 antimicrobial activity. These results will help in choosing the most relevant antimicrobial
363 compounds based on product characteristics, particularly pH.

364

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368

369 **Abbreviations used**

370 MIC = minimal inhibitory concentration

371 BIC = Bayesian information criterion

372 TSB = tryptic soy broth

373

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465 **Figure 1** – Representation of the predicted μ_{\max} as a function of the observed μ_{\max} for the eight
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468 and (b) at pH 5.6 and (c) associated table of gamma values for the undissociated form, dissociated
469 form and pH.

470 **Figure 3** – *L. monocytogenes* growth rates (a) as a function of pH in the presence of various
471 concentrations of chlorogenic and gallic acids compared to those at different pHs adjusted with
472 hydrochloric acid and (b) as a function of the concentrations of chlorogenic and gallic acids at pH 5.5a

473 **Figure 4** – *L. monocytogenes* growth rates as a function of the concentrations of undissociated acid at
474 pH 5.5, for four phenolic acids with the best fitting model #2.

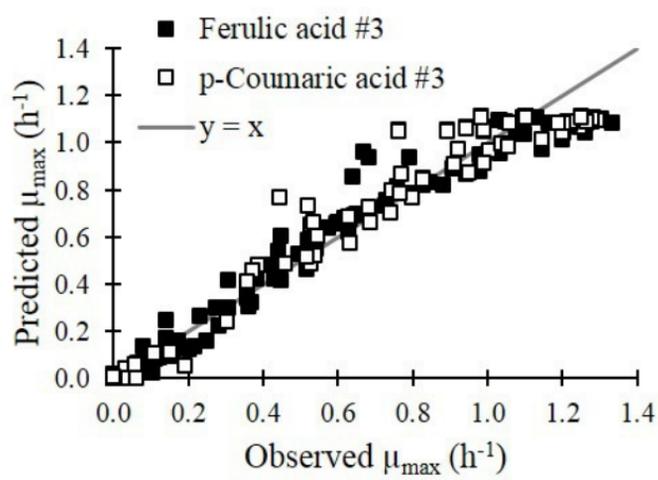
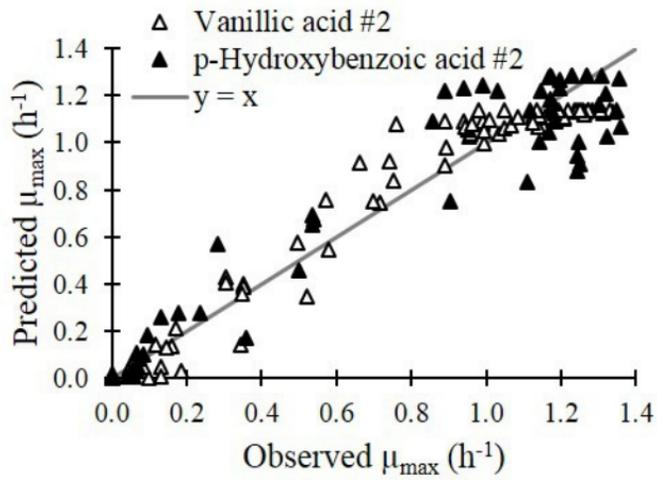
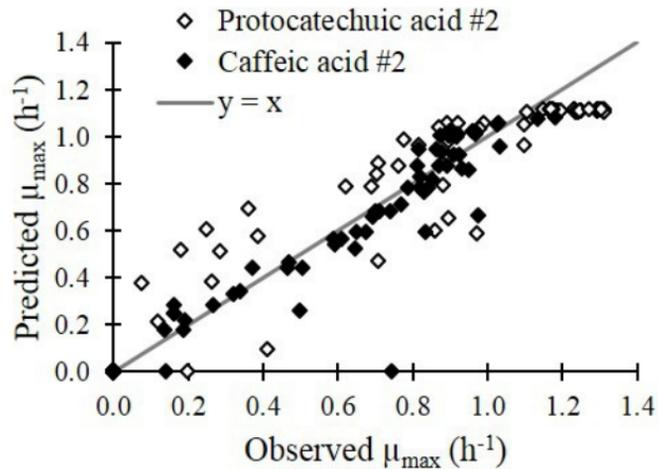
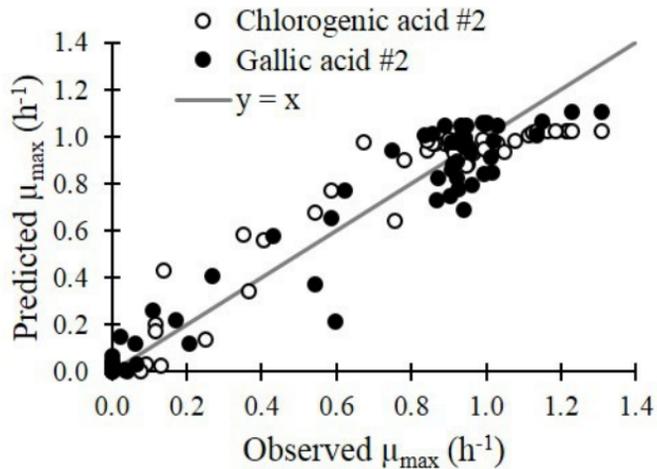
475 **List of tables**

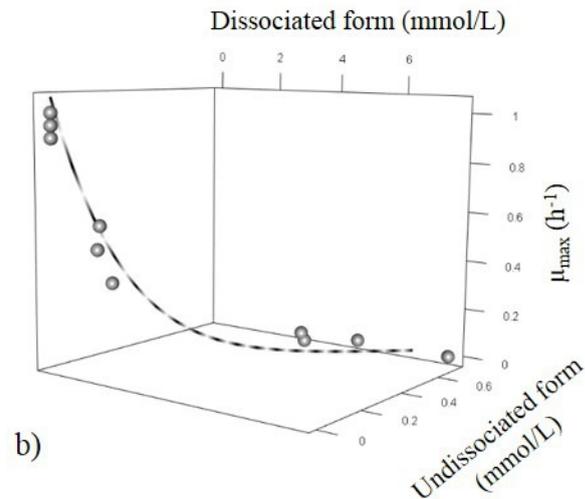
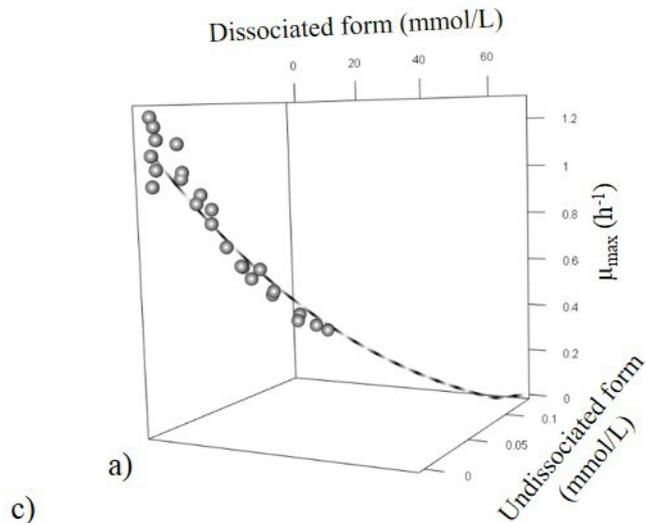
476 **Table 1** – Chemical structures and physico-chemical parameters of the eight studied phenolic acids

477 **Table 2** – Estimated parameters and BIC for the three models and the eight phenolic acids

478 *Unshaded areas correspond to the best fitting model for a given phenolic acid*

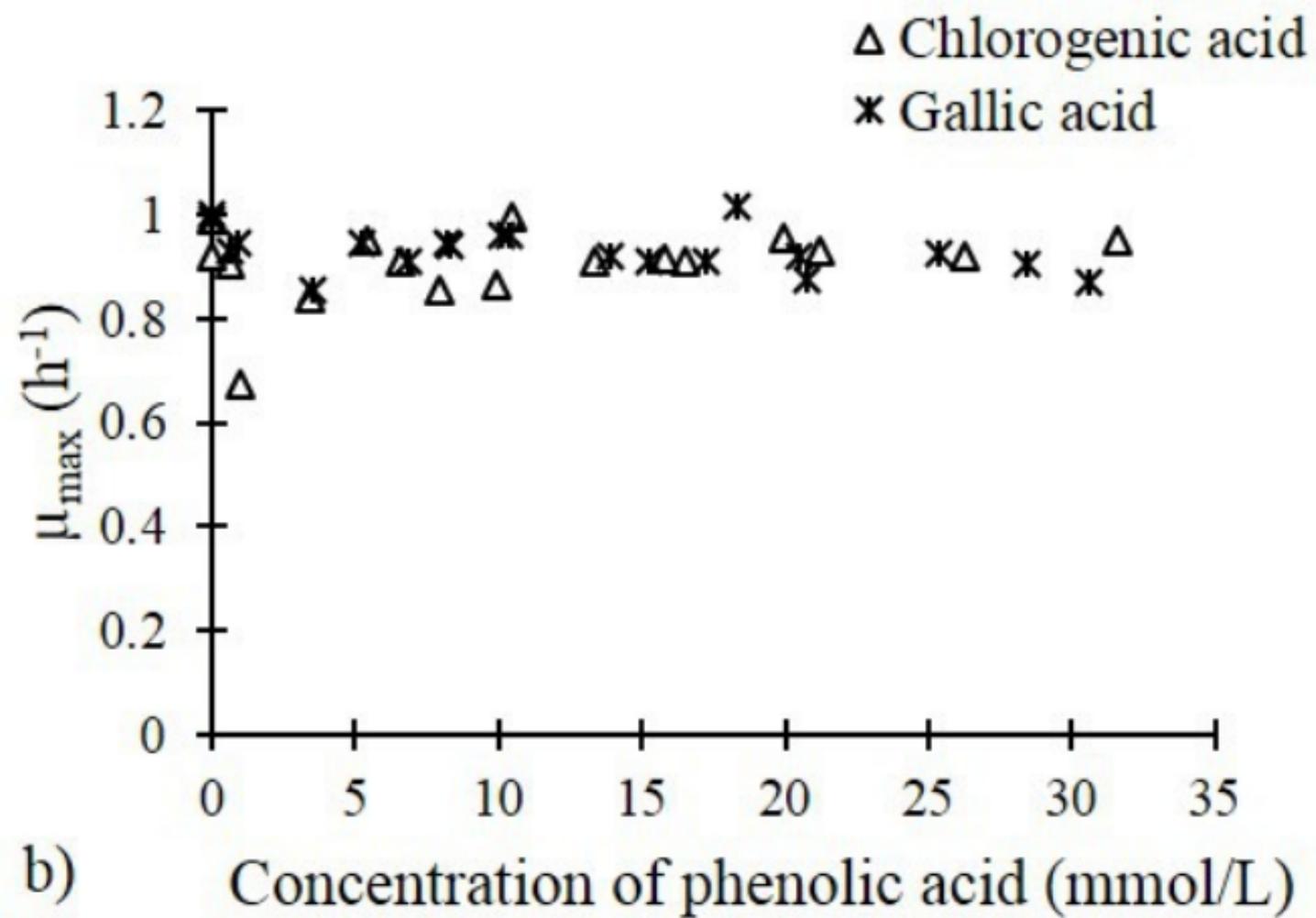
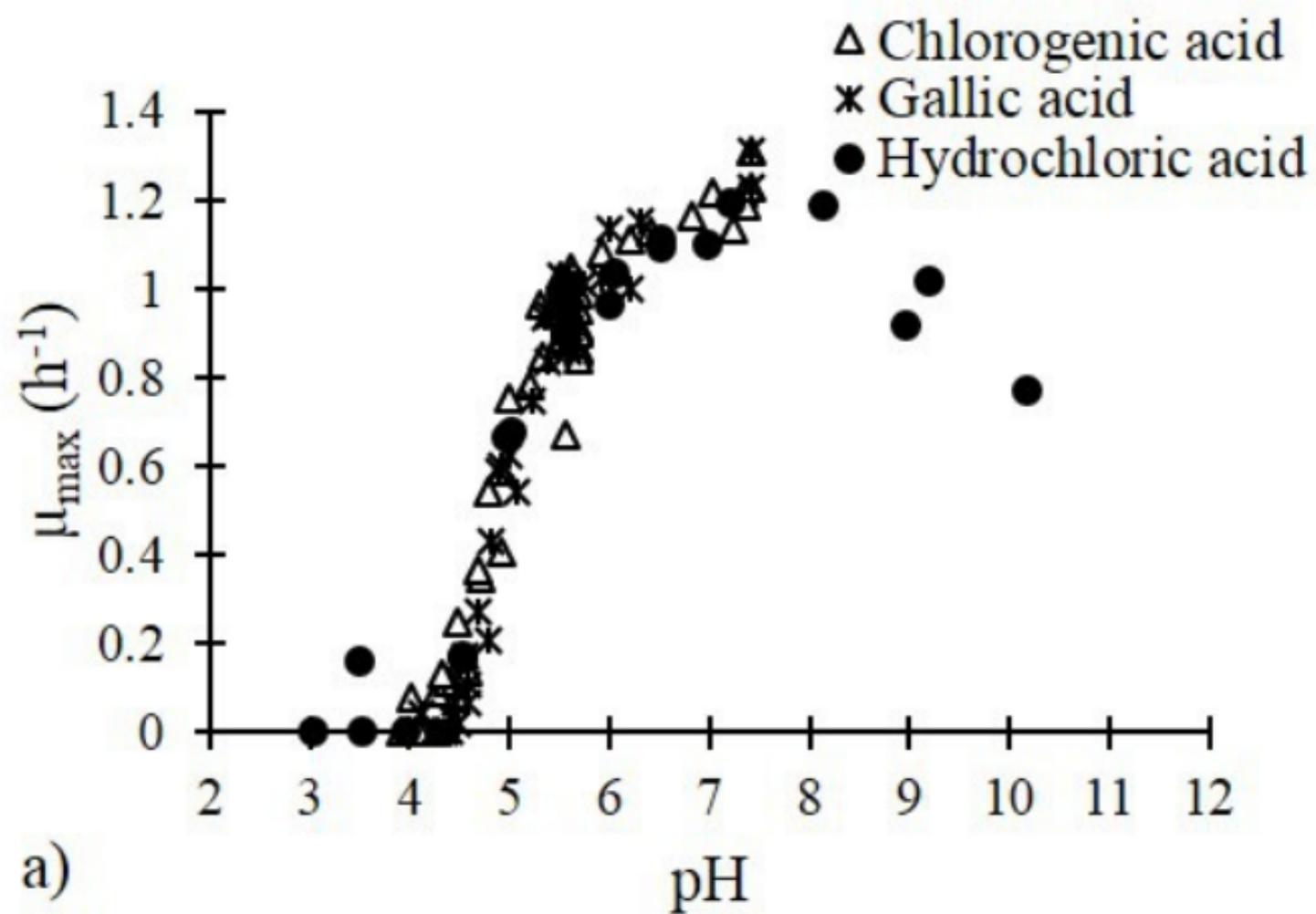
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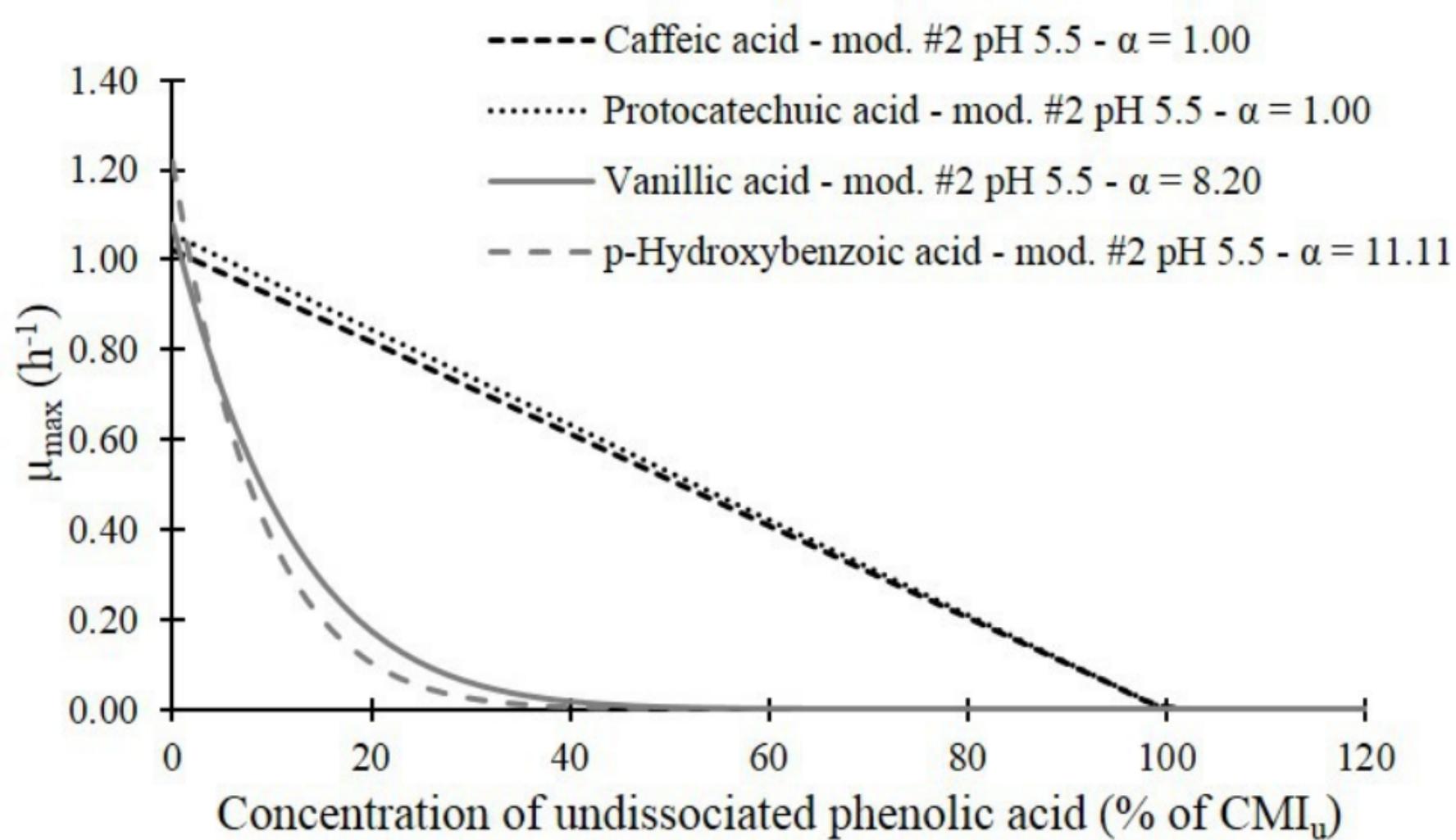


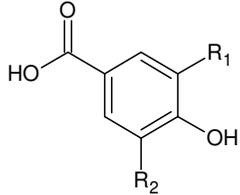
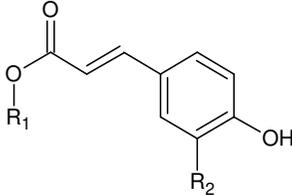


c)

Total ferulic acid (mmol/L)	Undissociated acid form (mmol/L)	Dissociated acid form (mmol/L)	pH	Observed μ_{\max} (h^{-1})	Reduction associated to			Predicted μ_{\max} (model #3) $\mu_{\text{ref}} \cdot \gamma(\text{A}_d) \cdot \gamma(\text{A}_u) \cdot \gamma(\text{pH})$
					undissociated form $\gamma(\text{A}_u)$	dissociated form $\gamma(\text{A}_d)$	pH $\gamma(\text{pH})$	
0.00	0.00	0.00		1.25	1.00	1.00		1.11
0.60	0.00	0.60		1.21	0.99	0.99		1.09
5.13	0.01	5.12		1.01	0.94	0.92		0.96
10.26	0.02	10.24	7.3	0.82	0.88	0.84	1.00	0.82
15.14	0.03	15.11		0.64	0.81	0.76		0.69
20.29	0.04	20.25		0.52	0.76	0.68		0.57
25.65	0.05	25.60		0.41	0.73	0.60		0.48
30.28	0.06	30.22		0.36	0.66	0.52		0.38
0.00	0.00	0.00		0.99	1.00	1.00		1.06
0.91	0.08	0.83	5.6	0.45	0.57	0.99	0.96	0.60
5.18	0.45	4.73		0.07	0.02	0.93		0.02
8.23	0.72	7.51		0.00	0.00	0.88		0.00



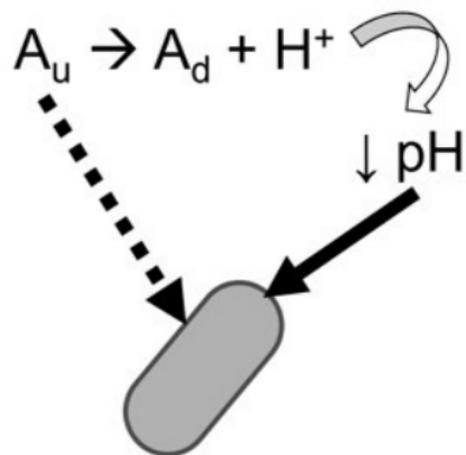


Phenolic acid	R1	R2	Name	pKa (ChemIDPlus, PubChem)	logP (ChemSpider)	logP of undissociated form, determined at pH=1.7 (Chemicalize)	logP of dissociated form, determined at pH=8.0 (Chemicalize)
Hydroxybenzoic acids 	H	H	<i>p</i> -hydroxybenzoic acid	4.54	1.42	1.33	-1.95
	H	OH	protocatechuic acid	4.26	1.16	1.02	-2.35
	H	O-CH ₃	vanillic acid	4.51	1.33	1.17	-2.19
	OH	OH	gallic acid	4.40	0.91	0.72	-2.73
Hydroxycinnamic acids 	H	H	<i>p</i> -coumaric acid	4.64	2.43	1.83	-1.58
	H	OH	caffeic acid	4.62	1.42	1.53	-1.98
	H	O-CH ₃	ferulic acid	4.58	1.64	1.67	-1.81
	quinic acid	OH	chlorogenic acid	2.66	-0.36	-0.28	-3.79

Phenolic acids	pH _{min}	Models										
		#1		#2				#3				
		μ_{ref}	BIC	μ_{ref}	α	MIC _u	BIC	μ_{ref}	α	MIC _u	MIC _d	BIC
Chlorogenic acid	4.24	0.95	- 228	1.03	6.34	2.55	- 241	1.04	6.33	2.55	6420.34	- 237
<i>p</i> -Coumaric acid		0.34	- 157	1.11	3.69	0.94	- 450	1.11	3.70	0.94	72.43	- 530
Caffeic acid		0.41	- 166	1.08	1.00	1.06	- 476	1.04	0.64	0.91	66 356.72	- 372
Gallic acid		0.84	- 173	1.10	9.39	46.88	- 230	1.11	9.35	47.10	1 000.00	- 225
Vanillic acid		0.48	- 177	1.14	8.20	3.24	- 552	1.16	7.70	3.09	424.09	- 549
Protocatechuic acid		0.76	- 169	1.12	1.00	2.12	- 309	1.18	4.21	5.07	20 718.84	- 297
<i>p</i> -Hydroxybenzoic acid		0.48	- 121	1.29	11.11	3.30	- 337	1.29	10.17	3.06	41 176.98	- 333
Ferulic acid		0.25	- 133	1.06	8.00	1.20	- 485	1.11	8.55	1.26	63.52	- 536

Mechanism 1

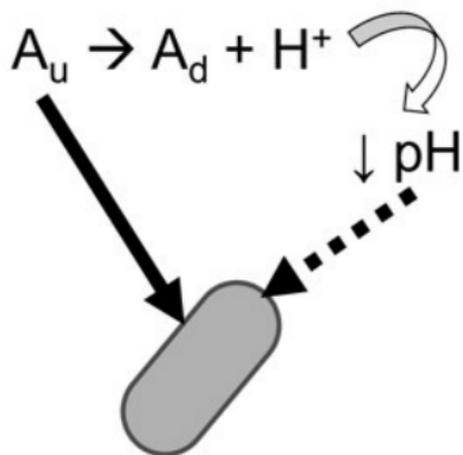
Decreasing
extracellular pH



Chlorogenic acid
Gallic acid

Mechanism 2

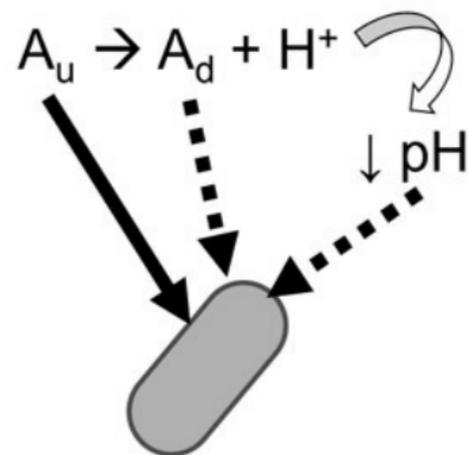
Antimicrobial
undissociated form



Protocatechuic acid
p-Hydroxybenzoic acid
Vanillic acid
Caffeic acid

Mechanism 3

Antimicrobial
undissociated
and dissociated forms



Ferulic acid
p-Coumaric acid