Food Microbiology 80 (2019) 18–24

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

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

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ARTICLE INFO

Keywords:
Phenolic
Antimicrobial
Gamma concept
Undissociated form
Dissociated form

ABSTRACT

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

1. Introduction

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

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

Organic acids act by a very well-known antimicrobial mechanism through the penetration of their undissociated form into the cell and acidification of the cytoplasm leading to cell death. Phenolic acids are of interest due to their natural plant-based origin, their presence in large quantities in byproducts of the fruit, wine and cereal industries and their demonstrated global antimicrobial activity. We previously evaluated the inhibitory activity of several phenolic acids against *Listeria monocytogenes* as a function of their total concentration (Pernin et al., 2018). However, very few studies have focused on deciphering the various effects of their inhibitory activity, which can be similar to those of organic acids and/or simple phenols (Sorrentino et al., 2018).

We reasoned that the total inhibitory activity is a combination of the following effects: *i)* decreased pH in the presence of phenolic acids

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https://doi.org/10.1016/j.fm.2018.12.010
Received 17 August 2018; Received in revised form 25 November 2018; Accepted 17 December 2018
Available online 24 December 2018

which directly inhibits growth, *ii)* the inhibitory effect of the undissociated acid form, and *iii)* the inhibitory effect of the dissociated acid form. Each acid form could potentially either intercalate into the phospholipid membrane or cross the membrane and decrease the intracellular pH and/or interact with cellular constituents.

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

2. Materials and methods

2.1. Chemical reagents

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

2.2. Bacterial strain

The strain used in this study was *Listeria monocytogenes* CNL 895805, serotype ½ a, isolated from sheep brain. It was graciously provided by P. Velge (INRA, Nouzilly) (Van Langendonck et al., 1998). Before each experiment, the strain, stored in cryovials at −80 °C, was resuscitated in two successive subcultures in tryptic soy broth (TSB, Biomérieux, France) at 30 °C.

2.3. Phenolic compounds

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

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

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

The growth of *L. monocytogenes* was followed in TSB containing one of the eight phenolic acids at various pHs. The phenolic acids were added at various concentrations in either neutral 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 is optimal for the growth of *L.monocytogenes* (Anses, 2011). pH 5.5 allows to increase the concentrations of undissociated form of acids without decreasing too much the growth rate of *L. monocytogenes*. After solubilization of the phenolic acid at the target concentration in TSB 7.2, the pH was adjusted to 7.2 (7.2a) with 1 mol/L sodium hydroxide or not adjusted (7.2na) (Pernin et al., 2018). Similarly, TSB 5.5 was adjusted to a pH of 5.5 (5.5a) or not adjusted (5.5na) after addition of the phenolic acid. The pH of each culture medium was measured with an SI Analytics lab 870 pH-meter (Mainz, Germany) in an independent experiment. Gallic, caffeic, and chlorogenic acids were not tested in TSB 7.2a because the medium became brown after pH adjustment, probably due to oxidation.

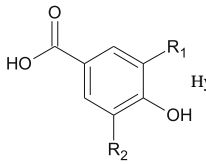
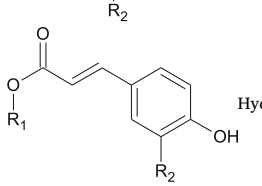
The final concentration of the phenolic compounds in TSB varied from 0 to at least 30 mmol/L. For some compounds, the minimal inhibitory concentration was not achieved due to solubility issues. Eight to 64 concentrations prepared from at least two different stock solutions were tested per compound for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

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

TSB at pH adjusted from 3 to 7 with 1 mol/L hydrochloric acid was prepared to evaluate the effect of pH on bacterial growth in the absence of the phenolic acids. In total, 15 pHs were tested in two independent experiments.

TSB of each series was inoculated at 1% (v/v from the second subculture; approximately 10^6 CFU/mL) with a standardized inoculum. Two hundred microliters of inoculated TSB from each series was added to the wells of 100-well microplates which were incubated at 30 °C with slow continuous shaking. Bacterial growth was followed in an

Table 1
Chemical structures and physico-chemical parameters of the eight studied phenolic acids.

Phenolic acid	R1	R2	Name	pKa (ChemIDPlus, PubChem)	logP of undissociated form, determined at pH = 1.7 (Chemicalize)	logP of dissociated form, determined at pH = 8.0 (Chemicalize)
 Hydroxybenzoic acids	H	H	*p*-hydroxybenzoic acid	4.54	1.33	−1.95
	H	OH	protocatechuic acid	4.26	1.02	−2.35
	H	O−CH_3	vanillic acid	4.51	1.17	−2.19
	OH	OH	gallic acid	4.40	0.72	−2.73
 Hydroxycinnamic acids	H	H	*p*-coumaric acid	4.64	1.83	−1.58
	H	OH	caffeic acid	4.62	1.53	−1.98
	H	O−CH_3	ferulic acid	4.58	1.67	−1.81
	quinic acid	OH	chlorogenic acid	2.66	−0.28	−3.79

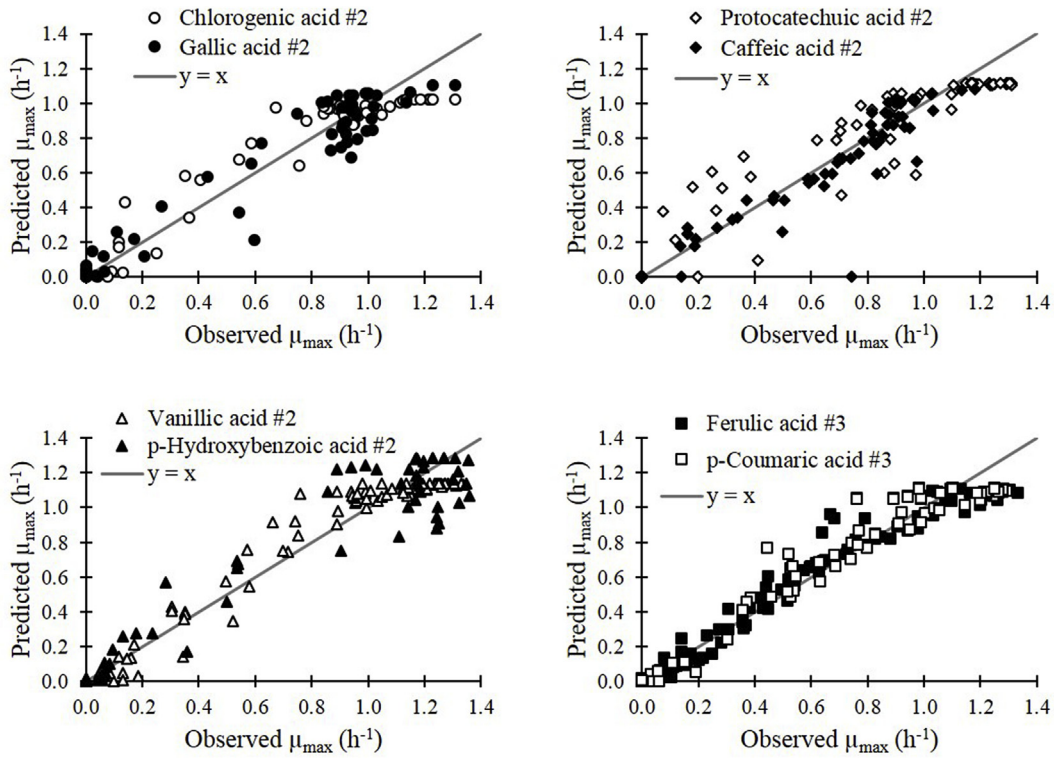


Fig. 1. Representation of the predicted μ_{max} as a function of the observed μ_{max} for the eight phenolic acids and their best fitting models (#3 for ferulic and *p*-coumaric acids, #2 for the others).

automatic spectrophotometer (Bioscreen C, Labsystems, Helsinki, Finland) by measuring the optical density (OD) at 600 nm for 72 h. In total, 1408 growth kinetics were acquired.

2.5. Determination of the growth rate

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

2.6. Modeling the effect of different acid forms on the growth rate

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

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

where:

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

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

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

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

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

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

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

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

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

Three different models are proposed.

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

$$\mu_{max} = \mu_{ref} \cdot \gamma(pH) \quad (5)$$

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

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

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 the parameters to estimate (eq. (7)).

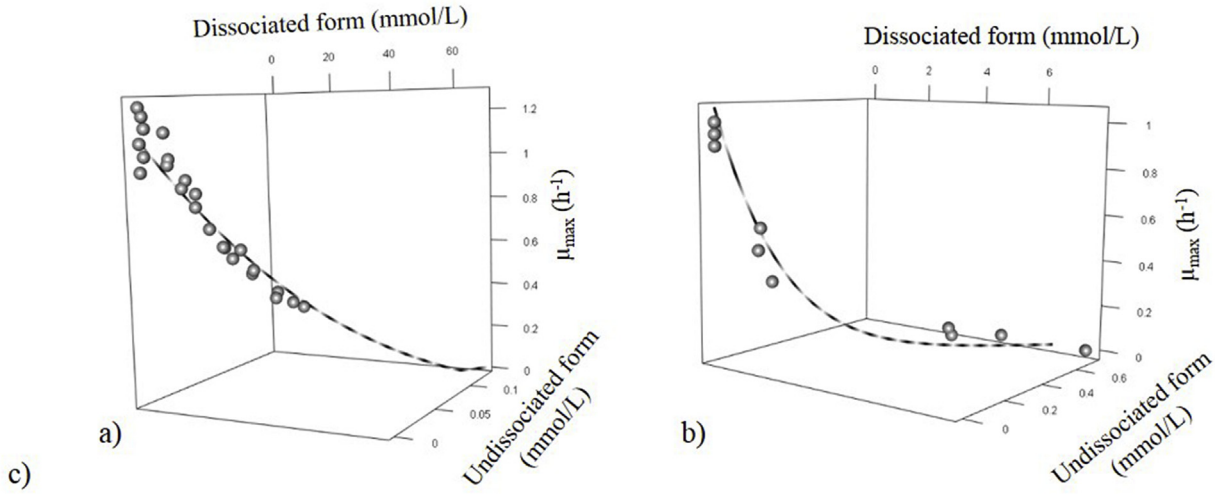
$$\mu_{max} = \mu_{ref} \cdot \gamma(pH) \cdot \gamma(A_u) \cdot \gamma(A_d) \quad (7)$$

2.7. Statistical approach

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

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

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



Total **ferulic acid** (mmol/L)	Undissociated acid form (mmol/L)	Dissociated acid form (mmol/L)	pH	Observed μ_{max} (h^{-1})	Reduction associated to undissociated form $\gamma(A_u)$	Reduction associated to dissociated form $\gamma(A_d)$	pH γ(pH)	Predicted μ_{max} (**model #3**) $\mu_{ref}\cdot\gamma(A_d)\cdot\gamma(A_u)\cdot\gamma$(pH)
0.00	0.00	0.00	7.3	1.25	1.00	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		0.82	0.88	0.84		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	5.6	0.99	1.00	1.00	0.96	1.06
0.91	0.08	0.83		0.45	0.57	0.99		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

Fig. 2. Fitted complete model #3 with an example of the partial dataset of ferulic acid (a) at pH 7.3 and (b) at pH 5.6 and (c) associated table of gamma values for the undissociated form, dissociated form and pH.

3. Results and discussion

3.1. Characterization of the models

Modeling with the Gamma concept approach (Lambert and Bidlas, 2007) allowed quantification of the relative importance of each impact factor. It is here difficult to show a graphical representation of the whole dataset of observed and predicted growth rates because there are four relevant impact factors: pH, A_{tot}, A_u, A_d. Thus, we show in Fig. 1 the predicted μ_{max} as a function of the observed μ_{max} for each phenolic acid. It illustrates that the chosen models fit well the datasets whatever the compound. Moreover, Fig. 2 shows how the models and the Gamma concept approach work. It gives more details about the fitness of model #3 applied to the ferulic acid dataset (Fig. 2a and b). The growth rates obtained with various concentrations are represented as a function of the concentrations of the undissociated and dissociated forms for two pHs (5.6 and 7.3). A given concentration of total acid at a given pH corresponds to a concentration of the undissociated and dissociated forms, together with an observed and a predicted μ_{max} (Fig. 2c). The model provides a value for the reduction of μ_{max} independently associated with the undissociated form, dissociated form, and pH. For example, when ferulic acid is introduced at 0.91 mmol/L in TSB adjusted to pH 5.6, the growth rate μ_{max} is divided by 1/0.57 (that is, 1/γ) due to the undissociated form, 1/0.99 due to the dissociated form, and 1/0.96 due to the decrease in pH, relative to the growth rate in the absence of the phenolic acid. In this example, the antimicrobial effect of the undissociated form is far more predominant than that of the dissociated form and pH (Fig. 2c). When ferulic acid is introduced at 30.28 mmol/L in TSB adjusted to pH 7.3, the growth rate μ_{max} is divided by 1/0.66 due to the undissociated form and 1/0.52 due to the dissociated form. The antimicrobial effect of the dissociated form is here almost the same as that of the undissociated form. The pH has no effect (γ = 1.00), as this pH is optimal for the growth of *L. monocytogenes*. Such tables, which set the three Gamma values for all concentrations of each phenolic acid help in understanding the relative impact of each factor in the inhibition of bacterial growth and are provided in Supplementary data.

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

In models # 2 and #3, a shape parameter describing the slope of the curve $\mu = f(A_u)$ at low concentrations is estimated for each phenolic

Table 2
Estimated parameters and BIC for the three models and the eight phenolic acids. Unshaded areas correspond to the best fitting model for a given phenolic acid.

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*
***p*-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*
***p*-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**

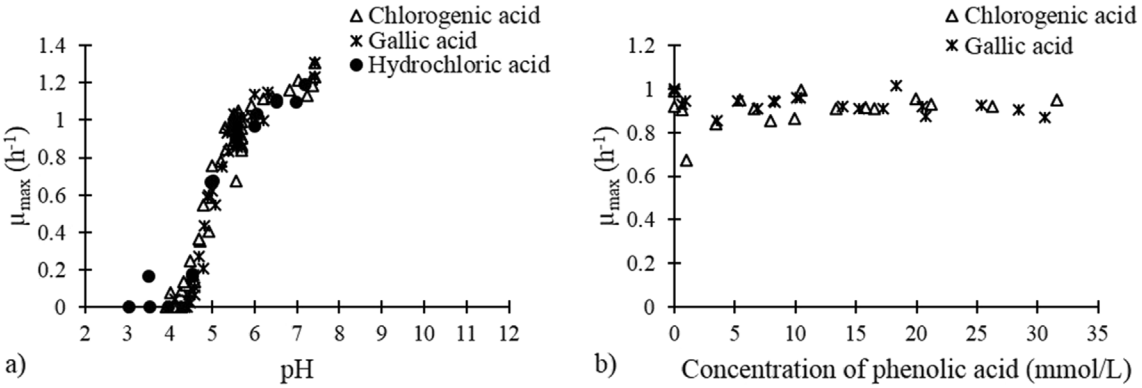


Fig. 3. *L. monocytogenes* growth rates (a) as a function of pH in the presence of various concentrations of chlorogenic and gallic acids compared to those at different pHs adjusted with hydrochloric acid and (b) as a function of the concentrations of chlorogenic and gallic acids at pH 5.5a.

acid (Table 2). It accounts for the potential non-linear shape of the decrease in μ when the concentration of the undissociated form increases. The implication of the α parameter value for each phenolic acid and its relation to the antimicrobial mechanism will be discussed in section 3.2.2.

To our knowledge, there is no other description in the literature of a model that takes into account the undissociated and dissociated forms in the inhibitory activity of phenolic acids. A very small number of studies have attempted to model the antimicrobial activity of phenolic acids, such as that of Ramos-Nino et al. (1996), which linked the inhibitory activity of phenolic acids with their logP and pKa. In contrast, more studies have attempted to model the antimicrobial activity of organic acids. They have used various types of models, such as principle component analysis (Hsiao and Siebert, 1999; Nakai and Siebert, 2003) or multivariate linear regression (George et al., 1996). However, only a few have studied the effect of the dissociated forms (Eklund, 1985, 1983; Presser et al., 1997). Eklund proposed a mathematical model to calculate the effect of the undissociated and dissociated forms of sorbic, benzoic, and propionic acid on several microorganisms (Eklund, 1985, 1983). In this model, the specific effect of each form was calculated from the measured MIC of the total form of the organic acid at different pHs (from pH 4.6 to 7.6).

The model of Presser et al. (1997) is closer to ours. These authors modelled growth rates of *Escherichia coli* as a function of pH and lactic acid concentration, taking into account a suboptimal pH term, an undissociated organic acid term, and a dissociated organic acid term. A shape parameter, α, for the undissociated acid term was added in our study, together with application of the parsimony principle, by combining the Gamma concept approach and calculation of the BIC.

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

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

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

Although the best-fitting model for chlorogenic acid and gallic acid was model #2 (Table 2), the calculated MIC_u are extremely high, above the solubility thresholds of these two compounds. At pH 5.5, 1766.72 mmol/L total chlorogenic acid and 637.06 mmol/L gallic acid would be needed to reach the MIC_u, at least 16-fold higher than that for protocatechuic acid. Thus, even if the undissociated forms of chlorogenic acid and gallic acid significantly decreased the growth rate, their effect was very small relative to that of other phenolic acids. In addition, the datasets of the bacterial growth rates obtained as a function of pH for various concentrations of chlorogenic and gallic acids clearly overlap to those obtained for hydrochloric acid (Fig. 3a). Moreover, at adjusted pH 5.5, increasing the concentration of chlorogenic or gallic acids did not have any visible impact on growth rates (Fig. 3b). Similarly, Wen et al. (2003) showed that chlorogenic acid had no antimicrobial activity on *L. monocytogenes* at pH 5.5 adjusted. Indeed, the mode of action for these two acids is mainly due to the decrease of extracellular pH.

In order to better illustrate the mechanism of action of these acids, we compared the growth rates in the presence of gallic acid to those in presence of vanillic acid. Addition of either acid to the culture medium to a total acid concentration of 10 mmol/L reduced the pH to

approximatively 4.6. However, according to the MIC_u and pKa values, $\gamma(pH) = 0.54$ and $\gamma(A_u) = 0.43$ for gallic acid and $\gamma(pH) = 0.58$ and $\gamma(A_u) = 0.00$ for vanillic acid (Supplementary data). The inhibitory effect of the undissociated form was significant for gallic acid but limited to a reduction by 1/0.43. In contrast, it was far more predominant for vanillic acid, as it completely inhibited growth.

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

Several hypotheses can be formulated to explain the lack of antimicrobial activity of the undissociated acid form of chlorogenic and gallic acids. First, their high steric hindrance, especially that of chlorogenic acid, could limit their penetration through the membrane even if Ramos-Nino et al. (1996) showed that this parameter was not relevant to describe the antimicrobial efficiency of hydroxybenzoic and hydroxycinnamic acids. Second, their low partition coefficient could limit their partition into the bacterial membrane (Table 1). However, organic acids are highly active while they have low partition coefficient (Hirshfield et al., 2003; Lu et al., 2011). In addition, several studies have demonstrated an antimicrobial effect of the undissociated form of chlorogenic acid and gallic acid at the membrane level (Borges et al., 2013; Lou et al., 2011). Gallic acid was shown to modify the membrane hydrophobicity and charge of *L. monocytogenes* and to cause local ruptures or pore formation in the cell membranes (Borges et al., 2013). Chlorogenic acid induces outer membrane modifications and loss of membrane integrity of *Streptococcus pneumoniae* and *Shigella dysenteriae* (Lou et al., 2011).

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

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

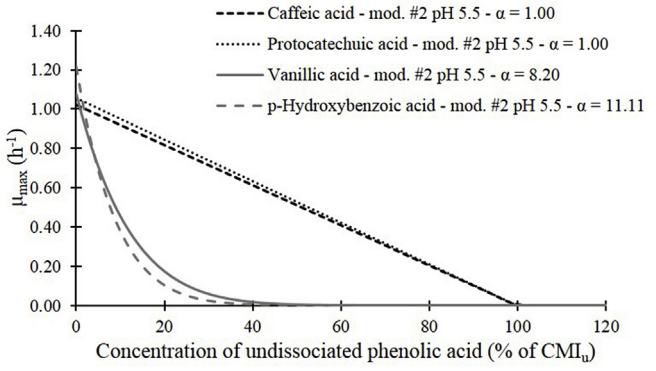


Fig. 4. *L. monocytogenes* growth rates as a function of the concentrations of undissociated acid at pH 5.5, for four phenolic acids with the best fitting model #2.

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

Model #3 best fits the *p*-coumaric acid and ferulic acid datasets. The antimicrobial effect of the undissociated form and decrease in the extracellular pH cannot explain the entire mode of action. The introduction of ferulic acid at 30.28 mmol/L in TSB adjusted to pH 7.3, divided 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 due to the dissociated form relative to that of the control (Fig. 2c). Similarly, introduction of *p*-coumaric acid at 31.55 mmol/L in TSB adjusted to pH 7.4 divided the growth rate by 1/0.78 due to the undissociated form and 1/0.57 due to the dissociated form relative to that of the control (Supplementary data). There are several examples for *p*-coumaric acid and ferulic acid in which $\gamma(A_d)$ was close to or even lower than $\gamma(A_u)$ (Supplementary data). Under specific conditions, the inhibitory effect of the dissociated form predominates over that of the undissociated form and pH. In contrast, $\gamma(A_d)$ never dropped below 1.00 for *p*-hydroxybenzoic, caffeic, or protocatechuic acids or below 0.93 for vanillic acid when model #3 was applied to other phenolic acids (Supplementary data). *p*-Coumaric and ferulic acids are therefore the most efficient antimicrobials at pH 7.2, relative to the other phenolic acids, due to their dissociated form which plays a significant role in decreasing the growth rate. Nevertheless, their MIC_d values calculated for pH 7.2 are very high, 72.63 and 63.67 mmol/L, respectively, which is above their solubility threshold. These results show that *p*-coumaric acid and ferulic acid can be efficient antimicrobial agents, even close to neutral pH, and this feature is important for the choice of compounds in several applications.

This is the first report of the inhibitory efficacy of the dissociated form of a phenolic acid. The MIC_d for *p*-coumaric acid and ferulic acid against *L. monocytogenes* are 10-times lower than the average order of magnitude found in the literature for organic acids on several bacteria: lactate (800–1250 mmol/L (Presser et al., 1997)), propionate (380–830 mmol/L (Eklund, 1985)), benzoate (90–200 mmol/L (Eklund, 1985)), and sorbate (50–400 mmol/L (Eklund, 1983)).

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

4. Conclusion

We present here an original modeling approach which allows a better understanding of the mechanism of action of eight phenolic acids. It quantifies the role of the three different antimicrobial factors (decrease in extracellular pH, the undissociated acid form, the dissociated acid form). The results allowed classification of these phenolic acids into three categories. First, chlorogenic acid and gallic acid

mainly inhibit the growth of *L. monocytogenes* through their ability to decrease extracellular pH, the molecules themselves having a low antimicrobial activity on bacterial growth. Second, caffeic acid, *p*-hydroxybenzoic acid, protocatechuic acid, and vanillic acid exert antimicrobial behavior mainly through the ability of their undissociated form to inhibit the growth of *L. monocytogenes*. Third, *p*-coumaric acid and ferulic acid similarly exert antimicrobial behavior through their undissociated form, but their dissociated form also shows significant antimicrobial activity. These results will help in choosing the most relevant antimicrobial compounds based on product characteristics, particularly pH.

Acknowledgments

The doctoral studies of Aurélia Pernin were financed by the French Government. The authors declare no competing financial interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.12.010.

Abbreviations used

MIC	minimal inhibitory concentration
BIC	Bayesian information criterion
TSB	trypic soy broth

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