

Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria

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Abstract

Phenolic acids (benzoic, phenylacetic and phenylpropionic acids) are the most abundant phenolic structures found in fecal water. As an approach towards the exploration of their action in the gut, this paper reports the antimicrobial activity of thirteen phenolic acids towards *Escherichia coli*, *Lactobacillus* spp., *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. The growth of *E. coli* ATCC 25922 was inhibited by only four of the phenolic acids tested at a concentration of 1000 µg/mL, whereas pathogenic *E. coli* O157:H7 (CECT 5947) was susceptible to ten of them. The genetically manipulated *E. coli* *lpxC/tolC* strain was highly susceptible to phenolic acids. The growth of lactobacilli (*Lactobacillus paraplantarum* LCH7, *Lactobacillus plantarum* LCH17, *Lactobacillus fermentum* LPH1, *L. fermentum* CECT 5716, *Lactobacillus brevis* LCH23, and *Lactobacillus coryniformis* CECT 5711) and pathogens (*S. aureus* EP167 and *C. albicans* MY1055) was also inhibited by phenolic acids, but to varying extents. Only *P. aeruginosa* PAO1 was not susceptible to any of the phenolic compounds tested. Structure–activity relationships of phenolic acids and some of their diet precursors [(+)-catechin and (–)-epicatechin] were established, based on multivariate analysis of microbial activities. The antimicrobial properties of phenolic acids reported in this paper might be relevant in vivo. © 2010 Elsevier Masson SAS. All rights reserved.

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1. Introduction

Human gut microbiota plays key roles in multiple host functions, including protection against pathogenic organisms, immunomodulation, the production of essential nutrients and the degradation of xenobiotic compounds. Diet is a major factor determining the composition and evolution of the gut microbiota. In fact, the bacterial conversion of carbohydrates, proteins and non-nutritive compounds such as phenolic

compounds leads to the formation of a large number of compounds that may have beneficial or adverse effects on human health (Blaut and Clavel, 2007).

Phenolic compounds are one of the most diverse groups of secondary metabolites in edible plants. Although earlier studies were focused on their mutagenic and genotoxic activity, later epidemiological studies have indicated that phenolic compounds may play an important role in the prevention of several diseases common to Western societies. Phenolics are recognized as direct antioxidants, but they may also exhibit indirect antioxidant effects through the induction of endogenous protective enzymes, and beneficial regulatory effects on signalling pathways (Stevenson and Hurst, 2007). In

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general, phenolic compounds are poorly absorbed in the small intestine; it is estimated that 90–95% of dietary phenolics accumulate in the colon (Clifford, 2004). In the gut, phenolics may selectively suppress or stimulate the growth of some components of intestinal microbiota; consequently, they may influence bacterial population dynamics (Tzounis et al., 2008). However, interactions between phenolics and gastrointestinal bacteria are still poorly understood. Among the different classes of phenolic compounds, phenolic acids (i.e., benzoic, phenylacetic and phenylpropionic acids, Fig. 1) and other aromatic acids are the predominant structures found in fecal water (Jenner et al., 2005). Although some of these acids could originate directly from dietary or endogenous sources, most are thought to come from the microbial metabolism of other dietary phenolic compounds (mainly flavan-3-ols, flavonols, flavones and anthocyanins) (Aura, 2008). The fecal phenolic acid profile seems to vary widely, depending on the phenolic compounds ingested. For example, 3-hydroxyphenylpropionic and 3-hydroxybenzoic acids were the major phenolic acids detected in hydrolyzed rat urine samples after a diet supplementation with (+)-catechin, whereas rat diets supplemented with dimer B3 had higher excretion rates of 3,4-dihydroxyphenylacetic and 3-hydroxyphenylacetic acids (Gonthier et al., 2003). 4-Hydroxy-3-methoxyphenyl and 4-hydroxy-3-methoxybenzoic acids were the most abundant metabolites in hydrolyzed human urine samples after the intake of berry anthocyanins (Nurmi et al., 2009).

Recently, microbial-derived phenolic acids have been implicated in providing a variety of health benefits to the host, such as the inhibition of platelet aggregation (Rechner and Kroner, 2005) and antiproliferative activity in prostatic and tumoral cells (Gao et al., 2006). In addition, the antimicrobial activity of specific phenolic acids towards beneficial gut bacteria and pathogens has been assessed while evaluating the antimicrobial properties of pure phenolics and plant extracts. Si and co-workers (Si et al., 2006) focused on benzoic acids (benzoic and 2-hydroxybenzoic, 3-hydroxybenzoic, 4-hydroxybenzoic and 3-hydroxy-4-methoxybenzoic acids), and

Lee and co-workers (Lee et al., 2006) included phenylpropionic, 4-hydroxyphenylacetic and 4-hydroxypropionic acids. However, to our knowledge, no study has systematically evaluated the antimicrobial properties of phenolic acids of different ring substitutions, or saturated side chain.

The *Escherichia coli* species includes non-pathogenic, pathogenic and commensal bacterial strains that generally inhabit the normal human gut. In fact, *E. coli* strain Nissle 1917 (O6:K5:H1) is employed in an infant probiotic preparation; oral application of this strain to full-term and premature infants can reduce the number and incidence of infections, stimulate specific humoral and cellular responses and induce non-specific natural immunity (Cukrowska et al., 2002). In contrast, *E. coli* O157:H7 is a foodborne pathogen that causes enterohemorrhagic infection and, occasionally, kidney failure. *E. coli* strains are widely used in antimicrobial screening studies, in conjunction with other beneficial and pathogenic bacteria. Thus, lactobacilli have a long history of safety; they are ubiquitous in the diet and are found in the gastrointestinal tract soon after birth. Lactobacilli have received special attention as probiotics, based on their health-promoting capacities (Lebeer et al., 2008).

As an approach to exploring the action of phenolic metabolites in the gut, we have assayed the antimicrobial activity of a structural array of thirteen benzoic, phenylacetic and phenylpropionic acids (Fig. 1) towards strains of innocuous and pathogenic *E. coli* strains and other beneficial bacteria (*Lactobacillus* spp.) and pathogens (*Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*). The antimicrobial activities of phenolic acid metabolites have been compared to those of some of their dietary precursors (flavan-3-ol monomers and dimers). Finally, multivariate statistical analyses have been applied to confirm similarities and differences among phenolic acids based on their antimicrobial potency.

2. Materials and methods

2.1. Phenolic compounds

3-Hydroxybenzoic acid, 3,4-dihydroxybenzoic acid (protocatechuic acid), 3-hydroxyphenylacetic acid and (+)-catechin were obtained from Sigma (St. Louis, MO, USA); 4-hydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylpropionic acid, 4-hydroxy-3-methoxybenzoic acid (vanillic acid) and (–)-epicatechin from Fluka AG (Buchs, Switzerland); 4-hydroxyphenylacetic acid and 4-hydroxyphenylpropionic acid from Janssen Chimica (Beerse, Belgium); benzoic acid and phenylacetic acid from Aldrich (Steinheim, Germany); phenylpropionic acid from Extrasynthèse (Genay, France) and 3-hydroxyphenylpropionic acid from Alfa Aesar (Karlsruhe, Germany). Stock phenolic solutions (10 mg/mL) were prepared in 20% dimethyl sulfoxide (DMSO), except for benzoic and vanillic acids which were dissolved in 50% DMSO (20 mg/mL) to improve solubility. Serial-dilution solutions were prepared from the stock solutions: 5000, 2500, 1250 and 625 µg/mL for all the

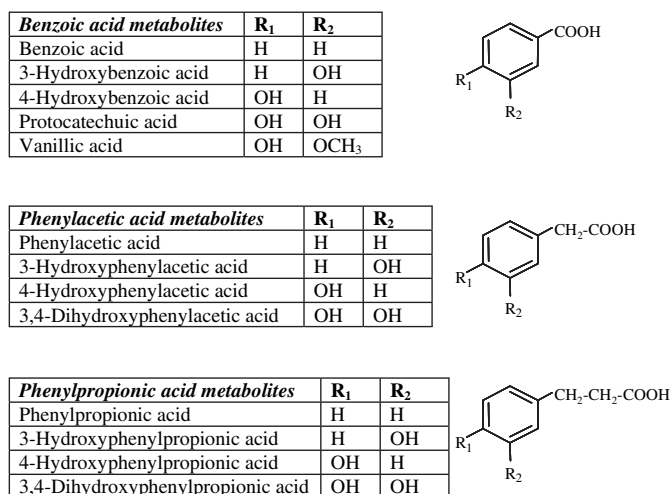


Fig. 1. Chemical structure of benzoic, phenylacetic and phenylpropionic acids.

phenolics, except for benzoic and vanillic acids prepared at 10 000, 5000, 2500 and 1250 µg/mL.

2.2. Microorganisms and inoculum preparation

A total of 12 microorganisms were included in this study. *E. coli* ATCC 25922 and *E. coli* O157:H7 (CECT 5947; virulence factor deleted) were obtained from the Spanish Type Culture Collection (CECT). *E. coli* *lpxC/tolC* (*lpxC* and *tolC* genes mutated which resulted in an outer-membrane-permeable and efflux-negative mutant) was from the Merck Culture Collection (Kodali et al., 2005). The *Lactobacillus* spp. strains were from the collection of the Department of Nutrition and Food and Science Technology, Universidad Complutense de Madrid (Madrid, Spain) and included: *Lactobacillus paraplantarum* LCH7, *Lactobacillus plantarum* LCH17, *Lactobacillus fermentum* LPH1, *L. fermentum* CECT 5716 and *Lactobacillus brevis* LCH23, which were isolated from the milk of healthy mothers (Jiménez et al., 2008; Martín et al., 2003), and *Lactobacillus coryniformis* CECT 5711 which produces reuterin and was originally isolated from goat's milk cheese (Martín et al., 2005b). Human pathogenic reference strains *C. albicans* MY1055, *S. aureus* MSSA (methicillin-susceptible *S. aureus*) EP167 (Novick, 1990) and *P. aeruginosa* PAO1 (Holloway et al., 1979) were from the Merck Culture Collection.

Inocula of each species were prepared by adding the contents of thawed cryovials to the relevant growth medium and incubating overnight. To determine the cfu/mL value, cells from the cryovials were serially diluted in saline solution (0.9%). Dilutions were spread onto agar medium and plates were incubated until colonies reached a suitable size (approximately 24 h). All determinations were made in duplicate. After estimating the value of cfu/mL, each thawed bacterial suspension was diluted in a growth medium to achieve an inoculum of 1×10^6 cfu/mL for the antimicrobial activity test. In the case of *C. albicans* MY1055, the inoculum preparation differed slightly. Frozen stocks were directly inoculated onto Sabouraud dextrose agar (SDA) plates to produce confluent growth at 35 °C for 24 h. Colonies were harvested from SDA plates and suspended in the appropriate growth medium (RPMI modified pH 7.0). The optical density at 660 nm (OD₆₆₀) was adjusted to 0.25 using the growth medium for dilution. This suspension was diluted 1:10 and kept on ice until used for the antimicrobial activity test. The concentration of this inoculum was 1.0×10^7 cfu/mL, a value 10-fold higher relative to the other microorganisms studied (Bills et al., 2009; Roemer et al., 2003; Vicente et al., 2009).

2.3. Antimicrobial activity assay

The assay used was previously described for the study of the antimicrobial properties of fungal and bacterial extracts against pathogens (Peláez et al., 2008; Vicente et al., 2009). Pipetting and mixing were performed in a TECAN AQUARIUS pipetting station (Tecan, Durham, USA). The plates used were microtiter 96-plates Costar 3370 (Corning,

NY, USA). First, 10 µL of the antibiotic solution (positive and negative controls), solvent (20% DMSO) or phenolic solutions were added to different wells of the microplate. In a second step, 90 µL of broth medium were added to the solvent wells, corresponding to the blank. Finally, 90 µL of the diluted strain were added to the remaining microplate wells, including the controls. The final assay volume was 100 µL. When the phenolic compounds were dissolved in 50% DMSO, the volumes were adjusted to a final assay volume of 200 µL to mitigate the negative effects of DMSO: 20 µL of positive or negative control solutions plus 180 µL of the diluted strain; 10 µL of the solvent (50% DMSO) plus 190 µL of the broth medium; and 10 µL of the phenolic solution plus 190 µL of the diluted strain. After dispensing the inoculum, the plates were read in a Tecan Ultra Evolution (Tecan, Durham, USA) spectrophotometer at 612 nm for T0 (Zero Time). Then the plates were incubated, without shaking, until the stationary phase was achieved. Before absorbance was read for TF (Final Time), plates were shaken in the Micromix (Thermo Fisher Scientific, Waltham, USA) to resuspend any possible precipitate. Tests were carried out in duplicate.

The inhibition percentage was calculated as

$$\% \text{Inhibition} = 1 - \frac{(\text{TF}_{\text{Sample}} - \text{T0}_{\text{Sample}}) - (\text{TF}_{\text{Blank}} - \text{T0}_{\text{Blank}})}{(\text{TF}_{\text{Growth}} - \text{T0}_{\text{Growth}}) - (\text{TF}_{\text{Blank}} - \text{T0}_{\text{Blank}})} \times 100$$

where T0_{Sample} and TF_{Sample} corresponded to the absorbance at 612 nm of the strain growth in the presence of the phenolic compound before and after incubation, respectively; T0_{Blank} and TF_{Blank} corresponded to the broth medium with solvent before and after incubation, respectively; and T0_{Growth} and TF_{Growth} to the strain growth in the absence of the phenolic compound before and after incubation, respectively. Inhibition values below 10% were considered negligible.

2.4. Statistical analysis

The statistical methods used for data analysis were: cluster analysis (CA) to determine the grouping of the studied phenolic compounds according to their antimicrobial activity; principal component analysis (PCA) from standardized variables, to examine the relationship between the analyzed variables and phenolic compounds; and analysis of variance (ANOVA) and Student–Newman–Keuls (SNK) test for means comparisons. Data were processed with the STATISTICA program for Windows, version 7.1 (StatSoft, Inc., 2005, www.statsoft.com.) and the SPSS program for Windows, version 15.0 (SPSS Inc. 1989–2006, www.spss.com).

3. Results and discussion

3.1. Antimicrobial activity against *E. coli* strains

The growth response (% inhibition) of the three strains of *E. coli* was measured in the presence of fecal phenolic acids

(Fig. 1) at several concentrations from 62.5 to 1000 $\mu\text{g/mL}$ (Fig. 2). This concentration range was selected on the basis of previous data reported in the literature for the inhibition of phenolic acids (Si et al., 2006; Lee et al., 2006). Susceptibility of *E. coli* to phenolic acids was strain-dependent. The growth of the non-pathogenic strain ATCC 25922 was only inhibited by four of the phenolic acids tested, while the pathogenic strain O157:H7 CECT 5947 was susceptible to ten of the thirteen phenolic acids studied (Fig. 2). The *E. coli* *lpxC/tolC* strain was highly susceptible to the presence of phenolic acids; its growth was inhibited by all the phenolic acids tested, at concentrations of 1000 $\mu\text{g/mL}$ or less (Fig. 2). Gram-negative

bacteria, such as *E. coli*, are characterized by an outer membrane that provides the cell with a hydrophilic surface that is able to exclude certain hydrophobic molecules, therefore imparting an intrinsic resistance of these bacteria to antimicrobial compounds (Perry et al., 2009). Small phenolic compounds, such as phenolic acids, would easily cross the membrane and exert their antimicrobial activity (Ikigai et al., 1993). The strain of *E. coli* *lpxC/tolC* has two mutated genes: the *lpxC* gene is an essential gene coding, UDP-3-O-acyl-N-acetylglucosamine deacetylase, which catalyzes the second step in lipid A biosynthesis, a component of the lipopolysaccharide (LPS) of Gram-negative bacterial membranes (Young

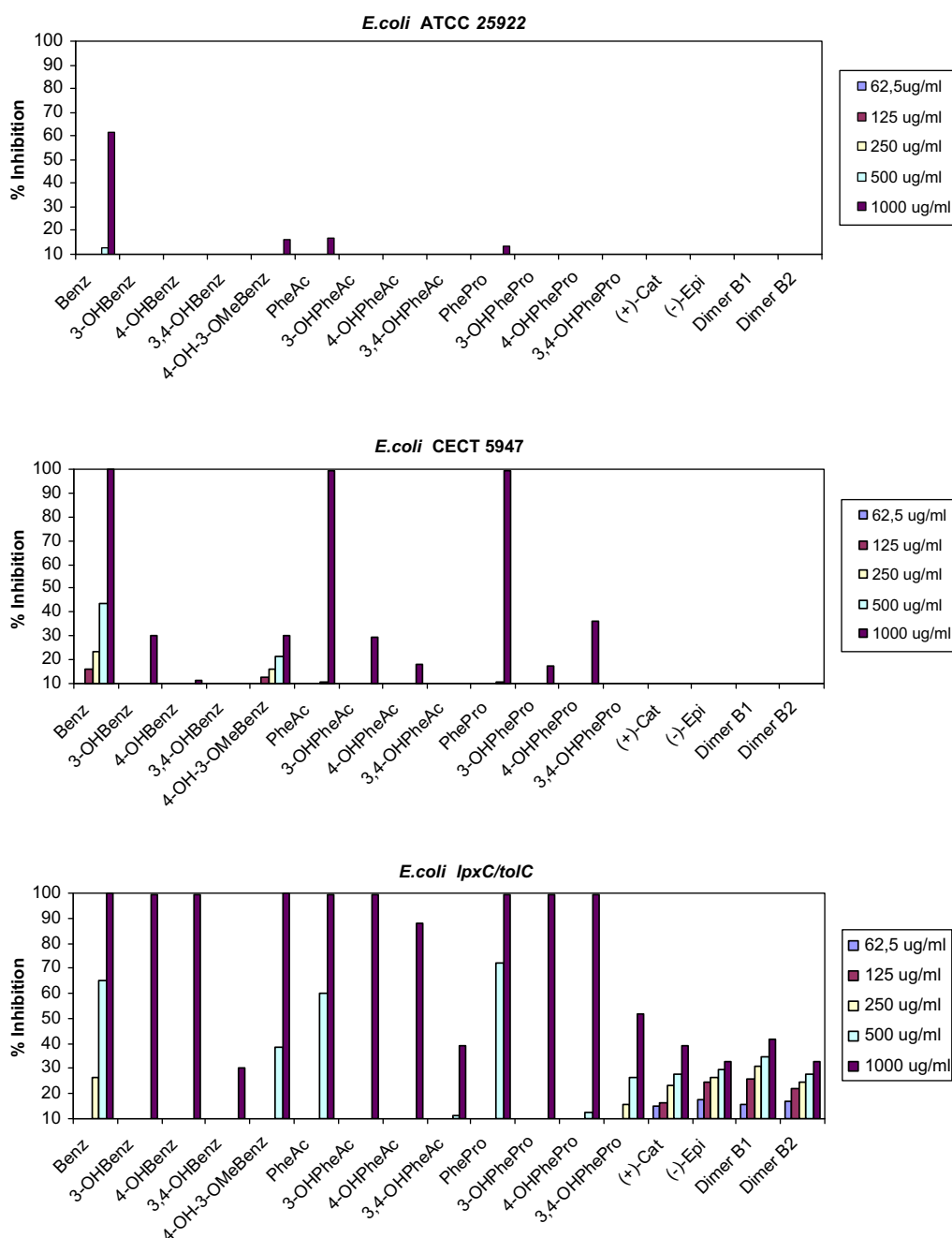


Fig. 2. Inhibition (%) of growth of *E. coli* strains by phenolic acids and flavan-3-ols.

et al., 1995); the product of the *tolC* gene is an outer-membrane protein (Koronakis et al., 2000), which interacts with a membrane fusion protein (AcrA) and an inner membrane protein (AcrB) forming the AcrAB–TolC complex (Tamura et al., 2005), which is involved in exporting a wide variety of drugs and toxic compounds out of the cell. These *lpxC* and *tolC* gene mutations may facilitate the antimicrobial action of phenolic acids against *E. coli*, but further studies with specific mutants should be carried out to confirm these results.

The antimicrobial effects of the phenolic acids against *E. coli* were structure-dependent (i.e., benzene ring substitutions and saturated side-chain length). Benzoic, phenylacetic and phenylpropionic acids were the most active compounds, reaching inhibitions of 100% for *E. coli* strains O157:H7 and *lpxC/tolC* at a concentration of 1000 µg/mL (Fig. 2). For both classes of benzoic and phenylacetic acids, *E. coli* was inhibited in the following order of potency: non-substituted >> 4-hydroxy-3-methoxy- > 3-hydroxy- > 4-hydroxy- > 3,4-dihydroxy-substituted acid (Fig. 2). For phenylpropionic acids, the order differed slightly: non-substituted >> 4-hydroxy- > 3-hydroxy- >> 3,4-dihydroxy-substituted acid (Fig. 2). With respect to the saturated side chain, the order of potency, for the same benzene ring substitution, was benzoic > phenylacetic > phenylpropionic acid for the strain ATCC 25922, although the relationship was not so evident for the other two *E. coli* strains (Fig. 2).

Some of these results agree with the few literature reports on the antimicrobial properties of phenolic acids against gut bacteria. A MIC (minimal inhibitory concentration) value of 2667 µg/mL for 3,4-dihydroxybenzoic acid for *E. coli* ATCC 25922 has been reported (Taguri et al., 2006), which means that inhibition would not be detected at the concentrations used in this study (62.5–1000 µg/mL). Other phenolic compounds, such as epigallocatechin-3-O-gallate, have also shown good activity against *E. coli* ATCC 25922 (MIC value = 533 µg/mL) (Taguri et al., 2006). For *E. coli* O157:H7, inhibition (≤50%) by benzoic, 3-hydroxybenzoic, and 3-hydroxy-4-methoxybenzoic acids has been reported (Si et al., 2006), but none by 4-hydroxybenzoic acid, all at a concentration of 500 µg/mL. For two pathogenic and one non-pathogenic *E. coli* strains, strong inhibition (>90%) by phenylpropionic, 4-hydroxyphenylacetic and 4-hydroxypropionic acids at concentrations of 1000 µg/mL has been observed (Lee et al., 2006).

Other phenolic compounds tested [(+)-catechin, (–)-epicatechin, B1 and B2] showed no inhibitory effects against ATCC 25922 and O157:H7 CECT 5947 strains, and were only slightly active against *E. coli lpxC/tolC* (Fig. 2). Therefore, phenolic acids seemed to show greater antimicrobial potency against *E. coli* strains than their corresponding dietary precursors such as the flavan-3-ol monomers and dimers tested in this study.

The antimicrobial activity of phenolic acids against *E. coli* was concentration-dependent (Fig. 2), as in other studies with other phenolic compounds (Juvent et al., 1994). The maximum concentration tested for phenolic acids (1000 µg/mL) was

around 3000–5000-fold higher than the concentrations of commercial antibiotics (ciprofloxacin or novobiocin) that led to similar inhibition percentages against *E. coli* strains under the same experimental conditions (results not shown). However, concentrations of 1000 µg/mL (8.1 mM for benzoic acid, 7.3 mM for phenylacetic acid and 6.6 mM for phenylpropionic acid) may be physiologically relevant in the gut, because they are in line with the estimation that the dilution of 500 mg of phenolics with the digestive bolus in the colon would give a local concentration of 3 mM (Scalbert and Williamson, 2000).

3.2. Antimicrobial activity against lactobacilli

The antimicrobial activity of phenolic acids was evaluated against *L. paraplantarum* LCH7, *L. plantarum* LCH17, *L. fermentum* LPH1, *L. fermentum* CECT 5716, *L. brevis* LCH23 and *L. coryniformis* CECT 5711 (Table 1). Among them, *L. paraplantarum* LCH7 was most susceptible to the action of phenolic acids (62.5–1000 µg/mL), while *L. fermentum* LPH1 was most resistant. The phenolic susceptibility of different strains from the same species (*L. fermentum* CECT 5716 and *L. fermentum* LPH1) changed slightly. 4-Hydroxybenzoic was the most active compound against all strains except for *L. coryniformis* CECT 5711, for which both 4-hydroxybenzoic and phenylpropionic were the most potent compounds, and for *L. fermentum* CECT 5716, for which 3-hydroxyphenylpropionic acid was the most potent (Table 1).

Unlike *E. coli* (Gram-negative), lactobacilli (Gram-positive) are enclosed in a plasma membrane covered by a thick peptidoglycan wall and lack an outer membrane. The number and position of substitutions in the phenolic acids' benzene ring influenced their antimicrobial potential against lactobacilli, but in a different manner from that in the *E. coli* species. For benzoic acids, the order of potency was: 4-hydroxy- > 3-hydroxy- > non-substituted > 4-hydroxy-3-methoxy- > 3,4-dihydroxy-substituted acids, except for *L. coryniformis* CECT 5711 (4-hydroxy- > non-substituted > 3-hydroxy > 4-hydroxy-3-methoxy-substituted acids) (Table 1). For phenylacetic acids, growth inhibition of lactobacilli was on the order of non-substituted > 3-hydroxy- > 4-hydroxy- > 3,4-dihydroxy-substituted acids. For phenylpropionic acids, growth inhibition was as follows: non-substituted > 4-hydroxy- > 3-hydroxy > 3,4-dihydroxy-substituted acids, except for *L. fermentum* CECT 5716 (3-hydroxy > non-substituted >> 4-hydroxy- and 3,4-dihydroxy-substituted acids) and *L. plantarum* LCH17 (non-substituted > 3-hydroxy- > 4-hydroxy- >> 3,4-dihydroxy-substituted acids) (Table 1). The relationship of the saturated side chain to the antimicrobial potential against lactobacilli was unclear and differed from that seen for *E. coli* ATCC 25922 (see above).

Although the antimicrobial activity of phenolic acids against these lactobacilli species has not been previously reported, others have observed a certain inhibition for *L. casei* by phenylpropionic (62%), 4-hydroxyphenylacetic (19%) and 4-hydroxypropionic (19%) acids at a concentration of 1000 µg/mL (Lee et al., 2006). However, *L. rhamnosus* GG

Table 1
Inhibition (%) of the growth of different beneficial bacteria and pathogens by phenolic acids and flavan-3-ols.

Compound	Final conc. (µg/mL)	<i>L. paraplantarum</i> LCH7	<i>L. plantarum</i> LCH17	<i>L. fermentun</i> LPH1	<i>L. fermentun</i> CECT 5716	<i>L. brevis</i> LCH23	<i>L. coryniformis</i> CECT 5711	<i>C. albicans</i> MY1055	<i>S. aureus</i> EP167	<i>P. aeruginosa</i> PA01
Benzoic acid	62.5	—	—	—	—	—	—	—	—	—
	125	—	—	—	—	—	—	—	—	—
	250	—	—	—	—	—	23.43	—	—	—
	500	15.55	—	—	—	—	37.41	—	17.41	—
	1000	93.94	10.52	—	10.52	—	63.70	15.99	94.35	—
3-Hydroxybenzoic acid	62.5	—	—	—	—	—	—	—	—	—
	125	—	—	—	—	—	—	—	—	—
	250	19.78	—	—	—	—	—	—	—	—
	500	80.59	11.26	—	—	—	25.40	—	18.58	—
	1000	94.35	42.28	—	—	—	45.45	—	90.54	—
4-Hydroxybenzoic acid	62.5	33.79	—	—	—	—	23.84	—	—	—
	125	55.64	—	—	—	—	25.31	—	—	—
	250	91.77	14.44	—	—	—	37.35	—	—	—
	500	95.03	30.88	—	—	—	54.40	—	33.13	—
	1000	96.10	68.68	17.93	20.08	35.06	71.26	—	97.20	—
3,4-Dihydroxybenzoic acid	62.5	—	—	—	—	—	—	—	—	—
	125	—	—	—	—	—	—	—	—	—
	250	16.85	—	—	—	—	—	—	—	—
	500	31.09	—	—	—	—	—	—	—	—
	1000	29.65	—	—	—	—	—	—	55.60	—
4-Hydroxy-3-methoxybenzoic acid	62.5	—	—	—	—	—	—	—	—	—
	125	—	—	—	—	—	—	—	—	—
	250	—	—	—	—	—	12.76	—	—	—
	500	—	—	—	—	—	16.54	—	—	—
	1000	89.86	—	—	—	—	31.83	—	71.89	—
Phenylacetic acid	62.5	—	—	—	—	—	—	—	—	—
	125	—	—	—	—	—	—	—	—	—
	250	—	—	—	—	—	—	—	—	—
	500	66.98	—	—	—	—	24.41	—	—	—
	1000	93.84	27.14	—	—	—	53.09	—	72.78	—
3-Hydroxyphenylacetic acid	62.5	—	—	—	—	—	—	—	—	—
	125	—	—	—	—	—	—	—	—	—
	250	—	—	—	—	—	—	—	—	—
	500	71.64	—	—	—	—	—	—	—	—
	1000	93.61	24.28	—	—	—	28.07	—	70.40	—
4-Hydroxyphenylacetic acid	62.5	—	—	—	—	—	—	—	—	—
	125	—	—	—	—	—	—	—	—	—
	250	—	—	—	—	—	—	—	—	—
	500	73.86	—	—	—	—	—	—	—	—
	1000	92.94	13.40	—	—	—	21.09	—	65.75	—

(continued on next page)

Table 1 (continued)

Compound	Final conc. ($\mu\text{g/mL}$)	<i>L. paraplantarum</i> LCH7	<i>L. plantarum</i> LCH17	<i>L. fermentum</i> LPH1	<i>L. fermentum</i> CECT 5716	<i>L. brevis</i> LCH23	<i>L. coryniformis</i> CECT 5711	<i>C. albicans</i> MY1055	<i>S. aureus</i> EP167	<i>P. aeruginosa</i> PA01
3,4-Dihydroxyphenylacetic acid	62.5	—	—	—	—	—	—	—	—	—
	125	—	—	—	—	—	—	—	—	—
	250	—	—	—	—	—	—	—	—	—
	500	—	—	—	—	—	—	—	—	—
	1000	89.22	—	—	—	—	—	—	33.53	—
Phenylpropionic acid	62.5	—	—	—	—	—	—	—	—	—
	125	—	—	—	—	—	—	—	—	—
	250	29.47	—	—	—	—	22.29	—	—	—
	500	92.70	—	—	—	—	51.45	15.13	—	—
	1000	96.46	30.02	—	12.03	14.14	86.19	29.40	89.54	—
3-Hydroxyphenylpropionic acid	62.5	—	—	—	—	—	—	—	—	—
	125	—	—	—	—	—	—	—	—	—
	250	—	—	—	—	—	—	—	—	—
	500	41.74	—	—	—	—	12.57	—	—	—
	1000	92.99	27.88	—	43.60	—	31.02	—	67.21	—
4-Hydroxyphenylpropionic acid	62.5	—	—	—	—	—	23.80	—	—	—
	125	—	—	—	—	—	22.82	—	—	—
	250	14.88	—	—	—	—	27.27	—	—	—
	500	86.09	—	—	—	—	37.92	—	—	—
	1000	94.58	15.61	—	—	—	56.29	—	69.86	—
3,4-Dihydroxyphenylpropionic acid	62.5	—	—	—	—	—	—	—	—	—
	125	—	—	—	—	—	—	—	—	—
	250	—	—	—	—	—	—	—	—	—
	500	—	—	—	—	—	—	—	—	—
	1000	32.99	—	—	—	—	—	—	40.73	—
(+)–Catechin	62.5	—	—	—	—	—	—	—	—	—
	125	11.67	—	—	—	—	—	—	—	—
	250	30.15	—	—	—	—	—	—	—	—
	500	44.08	—	—	—	—	—	—	—	—
	1000	59.95	—	—	—	—	—	—	—	—
(–)–Epicatechin	62.5	—	—	—	—	—	—	—	—	—
	125	—	—	—	—	—	—	—	—	—
	250	27.06	—	—	—	—	—	—	—	—
	500	51.11	—	—	—	—	—	—	—	—
	1000	64.83	—	—	—	—	—	—	—	—

ATCC 53103 was more resistant (<10% of inhibition) to the action of the same acids (Lee et al., 2006).

Flavan-3-ol monomers [(+)-catechin, (–)-epicatechin] were only active against *L. paraplantarum* LCH7 at a concentration up to 125 µg/mL for (+)-catechin and 250 µg/mL for (–)-epicatechin (Table 1). As seen for *E. coli* strains, phenolic acids seemed to be more antimicrobial against lactobacilli species than their corresponding dietary precursors.

The antimicrobial effects of phenolic acids against lactobacilli may be relevant in vivo, as it has been suggested that *L. brevis* LCH23, *L. fermentum* CECT 5716, *L. fermentum* LPH1, *L. paraplantarum* LCH7 and *L. plantarum* LCH17 could have an intestinal origin, even though they were isolated from human milk (Martín et al., 2003). In addition, some of the lactobacilli strains studied herein have a proven probiotic character (Martín et al., 2005a). Among them, *L. coryniformis* CECT 5711 is a reuterin-producing strain currently commercialized in dairy products (Lara-Villoslada et al., 2007). Our results indicate that high concentrations of phenolic acids (1000 µg/mL) may limit the in vivo viability of the lactobacilli tested. However, more studies are required to elucidate the influence of phenolic acids on the survival and probiotic activities of lactobacilli in complex ecosystems such as the gut.

3.3. Antimicrobial activity against pathogens

Among the pathogens tested, the Gram-positive bacteria *S. aureus* EP167 strain was most susceptible; all phenolic acids inhibited its growth, at least, at a concentration of 1000 µg/mL (Table 1). Growth of the yeast *C. albicans* MY1055 was only marginally inhibited by benzoic acid (16% at a concentration of 1000 µg/mL) and phenylpropionic acid (29% at a concentration of 1000 µg/mL). Phenolic acids failed to inhibit growth of the Gram-negative bacterium *P. aeruginosa* PAO1 at any concentration tested (Table 1).

In the case of *S. aureus* EP167, antimicrobial activity was in the following order: 4-hydroxy- > non-substituted > 3-hydroxy- > 4-hydroxy-3-methoxy- > 3,4-dihydroxy-substituted acid for benzoic acids. For phenylacetic acids, growth inhibition of *S. aureus* EP167 was in the following order: non-substituted > 3-hydroxy- > 4-hydroxy- > 3,4-dihydroxy-substituted acids, whereas for phenylpropionic acids the order was: non-substituted > 4-hydroxy- > 3-hydroxy > 3,4-dihydroxy-substituted acids (Table 1), which is the same pattern observed for lactobacilli species (see above) which are also Gram-positive bacteria. In relation to the saturated side chain, benzoic acids were more active than their corresponding phenylacetic and phenylpropionic acids for the different substitutions in the benzene ring (non-substituted, 3-hydroxy-, 4-hydroxy-, and 3,4-dihydroxy-substituted acids) (Table 1).

Previous studies on *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 have shown that the susceptibility of the two species differed greatly towards 3,4-dihydroxybenzoic acid (MIC values of 667 and 2133 µg/mL, respectively) (Taguri et al., 2006), which agrees with our results. However, others reported almost 100% inhibition of the growth of

S. aureus NCTC 7447 and *P. aeruginosa* (hospital isolate) by phenylpropionic, 4-hydroxyphenylacetic and 4-hydroxypropionic acids at a concentration of 1000 µg/mL (Lee et al., 2006). Other phenolic compounds, such as epigallocatechin-3-O-gallate, have also shown good activity against these two same strains (MIC value = 133 µg/mL for *S. aureus* ATCC 29213 and MIC value = 400 µg/mL for *P. aeruginosa* ATCC 27853) (Taguri et al., 2006).

As with the lactobacilli, (+)-catechin and (–)-epicatechin produced no growth inhibition of any of the pathogens tested at concentrations ≤ 1000 µg/mL (Table 1).

A possible mechanism to explain the antimicrobial action of phenolic acids against pathogens is hyperacidification at the plasma membrane interphase (Vattem et al., 2005) as a consequence of dissociation of phenolic acids (Choi and Gu, 2001). This hyperacidification would alter cell membrane potential, making it more permeable, as well as affecting the sodium–potassium ATPase pump implicated in ATP synthesis (Vattem et al., 2005). This mechanism would explain the differences in sensitivity towards phenolic acids among the pathogenic microorganisms *S. aureus* EP167, *P. aeruginosa* PAO1 and *C. albicans* MY1055. The Gram-positive bacterium *S. aureus* lacks an outer membrane, which would facilitate diffusion of the phenolic acids through the cell wall and intracellular acidification, as well as irreversible alterations in the sodium–potassium ATPase pump, therefore leading to cell death. The outer membrane of *P. aeruginosa* (Gram-negative) would act as a barrier to hyperacidification, and together with the transport system MexE–MexF–OprN (Köhler et al., 1999) that pumps toxins out of the cell, these features make this species resistant to the action of phenolic compounds. *C. albicans* has a cell wall consisting of glucans, chitin and proteins (Chaffin, 2008), providing a barrier against the action of phenolic compounds (Papadopoulos et al., 2005). In our study, *C. albicans* MY1055 was only inhibited by benzoic and phenylpropionic acids.

3.4. Statistical relationship among the compounds and microorganisms studied

In order to summarize the structure–activity relationships of phenolic compounds and their antimicrobial activity, CA was applied to the inhibition percent data corresponding to phenolic acids and (+)-catechin and (–)-epicatechin at a concentration of 1000 µg/mL for all microorganisms tested, except *P. aeruginosa*. Euclidean distance was taken as a measure of proximity between two samples and Ward's method was used as the linkage rule. The variables were previously standardized. Three distinct groups of compounds could be discriminated (Fig. 3). Group A consisted of phenolic acids with a hydroxyl substituent in position 3 or 4; group B included non-substituted acids (benzoic, phenylacetic and phenylpropionic acids); and group C included monomers of flavan-3-ols [(+)-catechin and (–)-epicatechin] together with 3,4-dihydroxy-substituted phenolic acids. Therefore, the antimicrobial properties of phenolic compounds were indeed grouped based on their chemical structure.

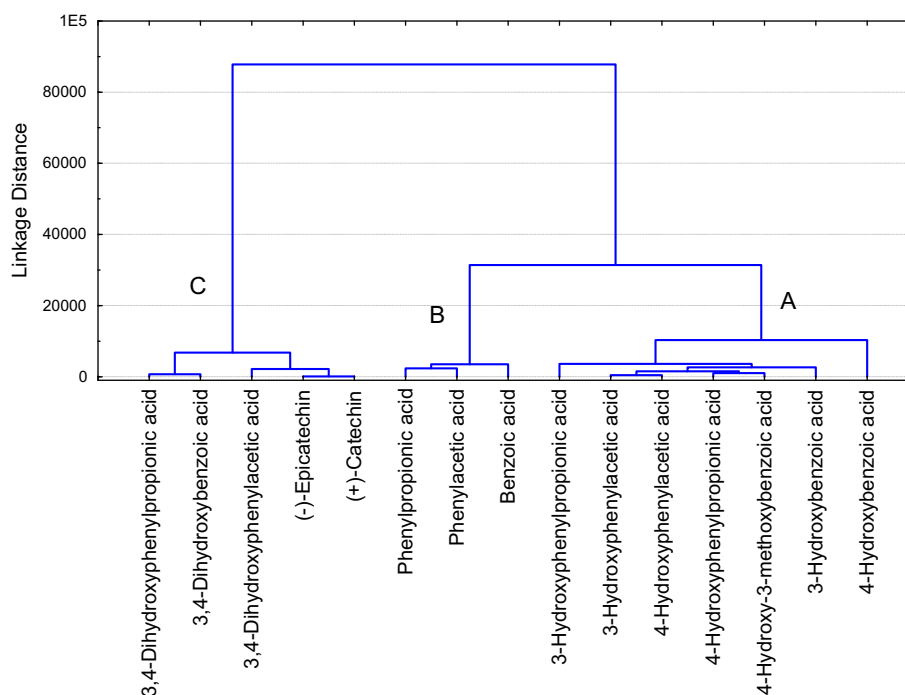


Fig. 3. Dendrogram and grouping of the phenolic compounds as a function of their antimicrobial activity.

To compare the antimicrobial activity of phenolics grouped by their chemical structure (groups A, B and C) against the different microorganisms, ANOVA and subsequently the SNK test were used. Significant differences ($p < 0.05$) were confirmed among the three groups for all antimicrobial activities studied except for *L. brevis* LCH23, *L. fermentum* CECT 5716 and *L. fermentum* LPH1 (results not shown). Within group A, the position of the hydroxyl substitution in the aromatic ring (3- or 4-) influenced the antimicrobial activity of the compounds, although this behavior varied depending on the type of microorganism (Fig. 2, Table 1). Within group B, the length of the saturated side-chain influenced the antimicrobial activity; thus, benzoic acid was more active against Gram-negative bacteria (three strains of *E. coli*), while phenylpropionic acid was more active against Gram-positive bacteria and the yeast *C. albicans* MY1055 (Fig. 2, Table 1). Compounds of group C only inhibited the most susceptible microorganisms: *E. coli* lpxC/TolC, *L. paraplantarum* LCH17, *L. coryniformis* CECT 5711 and *S. aureus* EP167 (Fig. 2, Table 1).

PCA was used to examine the relationship between the antimicrobial activities of the different microorganisms and phenolic compounds. Two principal components explained 72% of the total variation. The first principal component (PC1, 50.1% of the total variance) was negatively correlated with the inhibition percentage towards *L. coryniformis* CECT 5711 (−0.97), *E. coli* lpxC/TolC (−0.86), *S. aureus* EP167 (−0.87) and *L. paraplantarum* LCH17 (−0.77), and differentiated the three groups of phenolic compounds. The second principal component (PC2, 22.1% of the total variance) was negatively correlated with *L. fermentum* LPH1 (−0.79) and positively with *E. coli* ATCC 25922 (0.65) and *E. coli* CECT 5947 (0.64) and separated group B from the other two. The scores of the

phenolic compounds in groups A, B and C and the loadings of the antimicrobial activity against various microorganisms, were plotted as a biplot in the plane defined by the first two principal components (Fig. 4). Separation among the three groups of phenolic compounds was evident. The compounds of group B showed negative values for PC1, i.e., higher values of the variables negatively correlated with that component; those in group C showed positive values for PC1, and thus these compounds have low values of the variables that are negatively correlated with that component; and those in group A were located in the central plane (intermediate values of PC1 and PC2). This distribution of the compounds based on their chemical structures was similar to that observed in cluster analysis (Fig. 3). The biplot provided more information on variable values in samples by projection of the coordinates of the samples in the vectors corresponding to the variables; thus, the antimicrobial activities against the microorganisms *E. coli* ATCC 25922, *E. coli* O157:H7 and *C. albicans* MY1055 were higher for the three phenolic compounds in group B, and the antimicrobial activity against *L. fermentum* LPH1 was higher for 4-hydroxybenzoic acid (group A).

In conclusion, this paper reports that phenolic acids (benzoic, phenylacetic and phenylpropionic) originating from microbial degradation of different classes of dietary phenolic compounds can inhibit growth of intestinal bacteria and pathogens at concentrations that might be physiologically relevant. These colonic metabolites can have effects on the epithelium at the site of conversion and may also affect colonic microbiota locally. The antimicrobial effect of phenolic acids on *E. coli* growth was found to be strain-dependent. The pathogenic strain *E. coli* O157:H7 was susceptible to most of the phenolic acids tested at a concentration of at least 1000 µg/mL, while growth of the non-

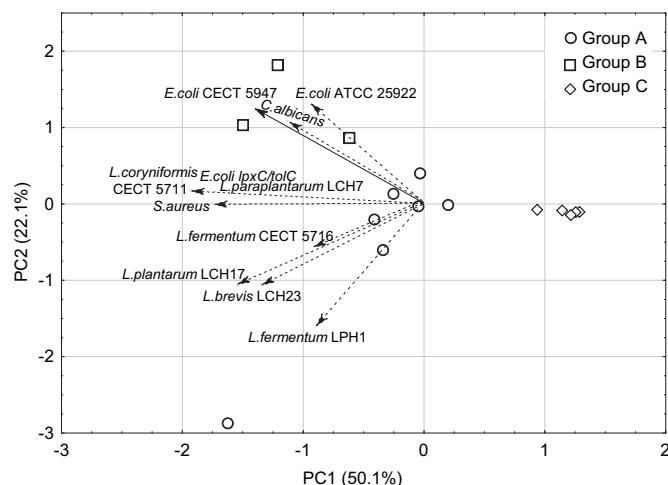


Fig. 4. Plot of the phenolic compounds in groups A (3-hydroxybenzoic, 4-hydroxybenzoic, 4-hydroxy-3-methoxybenzoic, 3-hydroxyphenylacetic, 4-hydroxyphenylacetic, 3-hydroxyphenylpropionic and 4-hydroxyphenylpropionic acids), B (benzoic, phenylacetic and phenylpropionic acids) and C [(+)-catechin, (–)-epicatechin, and 3,4-dihydroxybenzoic, 3,4-dihydroxyphenylacetic and 3,4-dihydroxyphenylpropionic acids], and the loadings of the microorganisms in the plane defined by the first two principal components.

pathogenic ATCC 25922 strain was only inhibited by the most active phenolic acids. However, lactobacilli strains of the same species (*L. fermentum* CECT 5716 and *L. fermentum* LPH1) showed similar susceptibility. In general, variations in antimicrobial activities among bacteria may reflect differences in cell surface structures between Gram-negative and Gram-positive species. *Lactobacillus* spp. and *S. aureus* (Gram-positive) appeared more susceptible to the action of phenolic acids than Gram-negative bacteria such as *E. coli* and *P. aeruginosa*. Mutations of the *lpxC* and *tolC* genes on *E. coli* seemed to amplify the phenolic acid antimicrobial mechanisms of action against Gram-negative species.

Also, the number and position of substitutions in the benzene ring of the phenolic acids and the saturated side-chain length influenced the antimicrobial potential of the phenolic acids against the different microorganisms, but in different ways. Phenolic acids seemed to show greater antimicrobial potency than their corresponding precursors such as the monomers (+)-catechin, (–)-epicatechin and dimers B1 and B2. Therefore, microbial transformations of dietary phenolics (flavonols, flavan-3-ols, flavones and anthocyanins) could lead to more potent microbial-inhibitory compounds (phenolic acids) that selectively influence intestinal bacteria species, and therefore could affect the diversity and metabolic activity of the intestinal microbiota, including the transformation of phenolics in the gut. Further studies taking into account the diversity and complexity of the human microbiota are required in order to confirm the potential microbiota-modulating effects of phenolic acids.

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