

ORIGINAL ARTICLE

Effect of phenolic compounds on the growth of selected probiotic and pathogenic bacteria

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Significance and Impact of the Study: This study provides relevant information about the effects of phenolic compounds commonly present in fruit and vegetables on the growth of probiotic and pathogenic bacteria. The compounds selectively allowed the growth of probiotic lactobacilli (*Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus*) and inhibited pathogenic bacteria (*Escherichia coli* and *Salmonella Typhimurium*) at the same concentration (20 mmol l⁻¹). These findings can contribute to the formulation of nutraceutical products, such as synbiotics, that can restore or maintain an optimal composition of human microbiota, potentially improving the overall health of the consumer.

Keywords

antimicrobial, *E. coli*, Fruit, phenolic compounds, probiotic, *Salmonella*.

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Abstract

Fruit extracts from different tissues (pulp, seed and peel) have shown antimicrobial and prebiotic activities related to their phenolic profile, although structure-specific evaluations have not been reported yet. The effect of five phenolic compounds (catechin and gallic, vanillic, ferulic and protocatechuic acids) identified in different fruits, particularly in mango, was evaluated on the growth of two probiotic (*Lactobacillus rhamnosus* GG ATCC 53103 and *Lactobacillus acidophilus* NRRLB 4495) and two pathogenic (*Escherichia coli* 0157:H7 ATCC 43890 and *Salmonella enterica* serovar Typhimurium ATCC 14028) bacteria. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of phenolic acids ranged from 15–20 mmol l⁻¹ and 20–30 mmol l⁻¹ against *E. coli* and *S. Typhimurium*, respectively. For catechin, the MIC and MBC were 35 mmol l⁻¹ and >35 mmol l⁻¹ against *E. coli* and *S. Typhimurium*, respectively. The presence of catechin and gallic, protocatechuic and vanillic acids in MRS broth without dextrose allowed the growth of lactobacilli. Catechin combined with protocatechuic or vanillic acid mildly allowed the growth of both probiotics. In conclusion, phenolic compounds can selectively inhibit the growth of pathogenic bacteria without affecting the viability of probiotics.

Introduction

Currently, the consumption of fruits has increased due to concerns of living a healthier life. Mango is one of the most consumed tropical fruits worldwide due to its sensorial and nutritional properties (Kim *et al.* 2009). Mango cv. Ataulfo is a Mexican variety, and it has the highest phenolic compound (PC) content and antioxidant capacity (AOXC) among mango varieties (Manthey and Perkins-Veazie

2009). The major PC identified in mango pulp are chlorogenic (280–3010 mg kg⁻¹ dry weight, DW), gallic (946–987 mg kg⁻¹ DW), vanillic (169–244 mg kg⁻¹ DW), and protocatechuic (4.8–11 mg kg⁻¹ DW) acids (Palafox-Carlos *et al.* (2012). Ferulic acid, catechin and quercetin have also been identified (Abbasi *et al.* 2015). Phenolic compound are secondary metabolites involved in plant defence against pathogens, and are part of the antioxidant system (Bravo 1998; Naczk and Shahidi 2004). Phenolic

compound have well-documented benefits for human health, such as the prevention of cardiovascular diseases, many types of cancer and age-related illnesses (Velderrain-Rodríguez *et al.* 2014). These health effects are attributed to their AOXC and certain epigenetic mechanisms (Scalbert *et al.* 2005). However, most PC are found as conjugated glycosides or as part of high-complexity macromolecules (e.g. antioxidant dietary fiber), which are poorly absorbed in the small intestine (Manach *et al.* 2004). These compounds reach the colon, where they are de-glycosylated and metabolized by microbial enzymes (Velderrain-Rodríguez *et al.* 2014), and their metabolites are also described as modulators of human gut microbiota (HGM) (Cardona *et al.* 2013).

Human gut microbiota can be modified by certain dietary compounds, such as PC (Gullon *et al.* 2016; Zhang *et al.* 2016), which may act as prebiotics. Grapes, berries and other PC-rich fruits were effective at reducing detrimental bacteria, but they had a stimulatory effect on probiotics (Puupponen-Pimiä *et al.* 2001; Davis and Milner 2009; Hervert-Hernandez and Goni 2011). For example, pomegranate juice, which is rich in vanillic, ferulic and gallic acids, inhibited the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *S. Typhimurium* at 0.10 mg ml⁻¹ (Lantzouraki *et al.* 2015; Gullon *et al.* 2016). In addition, anthocyanins extracted from purple sweet potato induced the growth of *Lactobacillus/Enterococcus* spp. and *Bifidobacterium* spp., but inhibited the growth of *Bacteroides-Prevotella* spp. and *Clostridium histolyticum* (Zhang *et al.* 2016). Therefore, this study hypothesized that PC inhibit the growth of pathogenic bacteria, while they stimulate the growth of probiotics. The main goal was to evaluate the modulatory effect of fruit PC (catechin and gallic, vanillic, ferulic and protocatechuic acids) on the growth of two probiotic (*Lactobacillus rhamnosus* GG ATCC 53103 and *Lactobacillus acidophilus* NRRLB 4495) and two pathogenic (*Escherichia coli* 0157:H7 ATCC 43890 and *Salmonella enterica* serovar Typhimurium ATCC 14028) bacteria.

Results and discussion

Inhibitory effects of PC against bacterial growth

The MIC and MBC of phenolic acids against *E. coli* and *S. Typhimurium* showed values from 15 to 30 mmol l⁻¹ (Table 1), but catechin needed a higher concentration to achieve a similar effect (>35 mmol l⁻¹). Protocatechuic, ferulic and vanillic acids also inhibited the growth of *L. acidophilus* at a higher range of concentrations (20–30 mmol l⁻¹), as compared to the inhibitory doses needed for pathogenic bacteria. Merkl *et al.* (2010) reported a MIC for vanillic, ferulic and protocatechuic acids against *E. coli* that was similar to that of our study (20 mmol l⁻¹), however, they also observed higher antimicrobial action for PC butyl ester derivatives. The mechanism by which these mango PC exerted their antimicrobial activity against *E. coli* and *S. Typhimurium* is unknown, but it is well known that many PC are potent iron scavengers, and no availability of iron affects the growth of certain pathogenic bacteria by reduction in the ribonucleotide precursor of DNA (Smith *et al.* 2005; Lim *et al.* 2013).

The effect of PC on bacterial growth phases was measured, in order to explore their mechanism of action (Baranyi and Roberts 1994). On both *E. coli* and *S. Typhimurium*, an extension in lag phase, and decreases in the maximum growth rate (μ_{max} OD/h) and maximum growth (Y_{max}, OD) were observed (data not shown). Vanillic, ferulic and protocatechuic acids showed similar effectiveness against *E. coli*. However, gallic acid was more effective at decreasing its growth rate, as compared to catechin.

In a similar study, no effect of gallic acid or catechin at a higher concentration (100 mg ml⁻¹) on the growth of *L. acidophilus* CECT 903 was observed (Hervert-Hernández *et al.* 2009), however, grape seed extract had a strong stimulating effect. The PC effect on bacterial growth depends on the tested dose and cellular structure, including the cell membrane (Puupponen-Pimiä *et al.* 2001; Cushnie and Lamb 2011; Daglia 2012; Taylor 2013). Additionally, PC have the ability to bind to bacterial cell

Table 1 Growth inhibitory effects of phenolic compound (PC) on selected probiotic and pathogenic bacteria

	<i>E. coli</i>		<i>S. Typhimurium</i>		<i>L. rhamnosus</i>		<i>L. acidophilus</i>	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gallic acid (G)	20	30	20	30	>35	>35	>35	>35
Vanillic acid (V)	20	20	15	20	35	>35	25	>35
Protocatechuic acid (P)	20	20	15	25	>35	>35	30	>35
Ferulic acid (F)	20	25	20	25	25	>35	20	>35
Catechin (C)	35	>35	35	>35	>35	>35	>35	>35

Minimal inhibitory (MIC, mmol l⁻¹) or bactericidal (MBC, mmol l⁻¹) concentration; Bacteria: *Escherichia coli* O157:H7 ATCC 43890, *Salmonella enterica* serovar Typhimurium ATCC 14028, *Lactobacillus rhamnosus* GG ATCC 53103, *Lactobacillus acidophilus* NRRLB 4495.

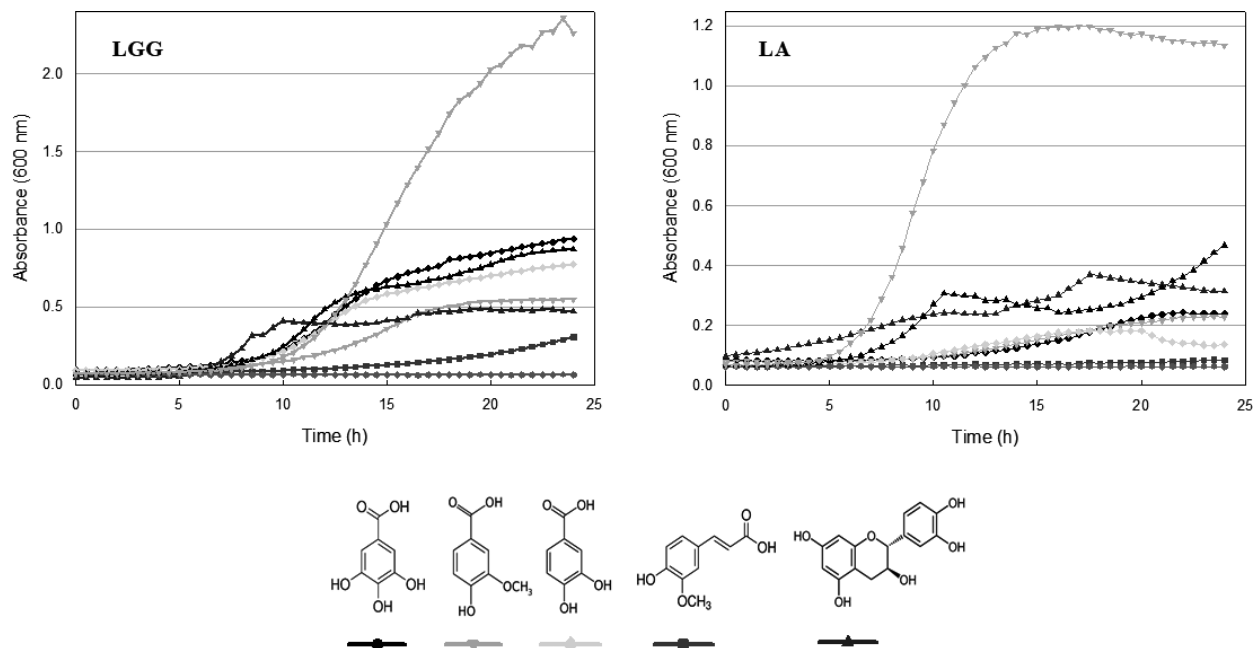


Figure 1 Effect of phenolic compound on probiotics. *Lactobacillus rhamnosus* GG ATCC 53103 (LGG), *Lactobacillus acidophilus* NRRLB 4495 (LA); Gallic (circle), vanillic (inverted triangle), protocatechuic (rhombus), and ferulic (square) acids, catechin (triangle); Controls: negative (gray circle), positive with (light gray triangle) and without (black triangle) dextrose. Phenolic acids were tested at 20 mmol l⁻¹ and catechin at 35 mmol l⁻¹.

membranes, and some of them can interact with lipids and proteins, altering membrane permeability (Kemperman *et al.* 2010). In addition, they can interfere with bacterial *quorum sensing* (Nazzaro *et al.* 2013; Yin *et al.* 2015).

Effects of individual PC on probiotic growth

Lactobacillus rhamnosus was able to grow at different rates (μ_{\max}) in both dextrose-supplemented (C1) and nonsupplemented (C2) MRS broths; *L. acidophilus* grew only in C1 media (Fig. 1, Table 2). Despite this result, the maximum population density of both probiotics in C2 did not reach C1 values (Table 2). Remarkably, no adverse effects of PC in C2 medium were observed for both probiotic bacteria, as compared to pure C1 medium (Fig. 1). Lactobacilli showed better adaptation to gallic, protocatechuic, vanillic acids and catechin than pathogenic bacteria, while ferulic acid did not promote the growth of the tested probiotics. Catechin and gallic acid reduced the lag phase of *L. acidophilus* and *L. rhamnosus*, improving the growth rate, as compared to C2 (Table 2). Goodness-of-fit adjustments showed a similar effect of PC (gallic, protocatechuic and vanillic acids) on lactobacilli growth, as compared to their growth kinetics in C2 broth.

Some bacteria isolated from HGM possess specific enzymes (e.g. α -rhamnosidase, β -galactosidase and β -

Table 2 Single and combined effects of phenolic compound (PC) on selected probiotic and pathogenic bacteria

Tx	<i>Lactobacillus rhamnosus</i> GG ATCC 53103			<i>Lactobacillus acidophilus</i> NRRLB 4495		
	Lag (h)	μ_{\max} (OD/h)	Y_{\max} (OD)	Lag (h)	μ_{\max} (OD/h)	Y_{\max} (OD)
C1	11.54 ^{ab}	0.263 ^a	2.24 ^a	6.43 ^a	0.191 ^a	1.17 ^a
C2	6.10 ^c	0.060 ^b	0.84 ^b	6.24	0.16	0.46
G	5.84 ^c	0.068 ^b	0.98 ^b	6.96 ^a	0.041 ^c	0.08 ^c
V	10.19 ^a	0.054 ^b	0.54 ^c	7.92 ^a	0.012 ^b	0.08 ^c
P	7.76 ^c	0.065 ^b	0.73 ^d	6.96 ^a	0.012 ^b	0.07 ^c
F	13.65 ^b	0.019 ^c	0.30 ^e	10.62 ^b	0.001 ^d	0.07 ^c
C	5.08 ^c	0.006 ^d	0.37 ^e	3.80 ^d	0.022 ^e	0.31 ^d
GP	9.49 ^a	0.044 ^b	0.62 ^c	NGD	NGD	NGD
GC	14.54 ^d	0.040 ^b	0.70 ^d	NGD	NGD	NGD
VC	13.61 ^b	0.043 ^b	0.63 ^c	8.54 ^{ab}	0.040 ^c	0.50 ^b
PC	14.87 ^d	0.043 ^b	0.61 ^c	6.66 ^a	0.033 ^c	0.51 ^b

Tx: treatments. MRS control broth with (C1) or without (C2) dextrose. Catechin (C), ferulic (F), gallic (G), protocatechuic (P) and vanillic (V) acids were supplemented in C2 MRS broth. No growth detected (NGD). Lag: lag phase, μ_{\max} : growth rate, Y_{\max} : stationary phase.

glucuronidase) that can metabolize PC as carbon sources and can stimulate the efficient usage of alternative nutrients (García-Ruiz *et al.* 2008). Particularly, certain species of the genus *Lactobacillus* possess a gallate decarboxylase

that degrades gallic acid into pyrogallol (Jiménez *et al.* 2013), which in turn is degraded to *cis*-aconitate and enters the Krebs cycle. Gallic acid can also be degraded to oxaloacetate and pyruvate (Bhat *et al.* 1998). Other enzymes, such as esterases, hydrogenases, dehydrogenases, dehydroxylases, decarboxylase and isomerases, are responsible for breaking down PC structures into C3-carbon intermediaries (Selma *et al.* 2014).

Effects of combined PC on probiotic growth

Combined PC caused a lower growth, as compared to the individual compounds (Table 2). Catechin, when combined with protocatechuic or vanillic acid, allowed the growth of both probiotics. When gallic acid was combined with either protocatechuic acid or catechin, the combination only allowed the growth of *L. rhamnosus*. Studies on the effect of PC combinations with prebiotic or antimicrobial effects are scarce, for example, green tea extracts permitted the survival of the probiotic *Bifidobacterium animalis* B94 better than saline solution, maintaining the highest levels of viable cells (De Lacey *et al.* 2014). On the other hand, Tabasco *et al.* (2011) observed that a grape seed extract supplemented with catechin (25 mg ml⁻¹) and gallic acid (5.5 mg ml⁻¹) had an inhibitory effect on different species of *Lactobacillus*.

Dual PC combinations had an additive antimicrobial effect against *E. coli* and *S. Typhimurium*, which was characterized by an extended lag phase (data not shown). Our results are similar to those reported by Rodríguez Vaquero *et al.* (2011), who tested the same combinations as those used in this study against *Listeria monocytogenes*, and observed an additive antibacterial effect of combined PC. Several authors have evaluated the effects of extracts rich in PC on the growth of several pathogens, and they have demonstrated the antimicrobial benefits of applying a mixture of phenolics (Puupponen-Pimiä *et al.* 2005; Nohynek *et al.* 2006; Vega-Vega *et al.* 2013).

Among the possible impacts of this research, it is possible to speculate on (i) the effect of PC as modulators of the HGM, and (ii) their effect as modulators of pathogens and probiotics on fruit surfaces, acting like components of pathogen biocontrol. Most of these compounds are attached to the food matrix (dietary fibre) and are released upon ingestion by the action of gastric juices and human digestive enzymes, but mostly due to the action of intestinal microbiota (Manach *et al.* 2005; Marín *et al.* 2015). Microbial enzymes are capable of degrading complex carbohydrates and breaking different types of bonds, releasing a great amount of PC to the intestinal lumen (Possemiers *et al.* 2011). Assuming that both pathogens and probiotics can be found among the HGM, a synergy in the inhibition of pathogens can be achieved. In

addition, when combining PC and probiotics, which are both known as pathogen inhibitors, a more effective result could be achieved from disinfection. Probiotics are known as pathogen antagonists due to their production of antimicrobial compounds, including bacteriocins (Zhang *et al.* 2011). On the other hand, PC affects pathogen viability through changes in membrane permeability (Kemperman *et al.* 2010), decreasing the risk of contamination while allowing the growth of probiotics and promoting their benefits. In summary, PC can selectively inhibit the growth of pathogenic bacteria without affecting the viability of probiotics; the most effective compounds to achieve these results were protocatechuic and vanillic acids.

Materials and methods

Phenolic compounds

(+)-catechin and gallic, vanillic, ferulic and protocatechuic acids ($\geq 98.5\%$ purity) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Each PC dissolved in 0.5 ml of pure dimethyl sulfoxide (DMSO) was adjusted to a final volume of 10 ml in Man Rogosa and Sharpe (MRS) broth not supplemented with dextrose (C2) (Actero™ Lactobacilli MRS Broth W/O Dextrose, Foodcheck systems Inc., Calgary, AB, Canada), or in 10 ml of Mueller-Hinton (MH) broth (DIFCO laboratories/Becton Dickinson and Co, MD, EUA), to obtain stock concentrations of 0.6 mg ml⁻¹. All analyses were performed in triplicate.

Bacterial strains and growth conditions

Lactobacillus rhamnosus GG ATCC 53103 was obtained from American Type Culture Collection, and *L. acidophilus* NRRLB 4495 was obtained from Agricultural Research Service culture collection. An aliquot (100 μ l⁻¹) of each strain was activated in MRS broth supplemented with 5 g l⁻¹ of cysteine (BD Difco™, Becton, Dickinson and Company Sparks, MD, USA) at 37°C for 24 h, and was further streaked onto MRS agar at 37°C for 48 h. *Escherichia coli* 0157:H7 ATCC 43890 and *Salmonella enterica* serovar Typhimurium ATCC 14028 were activated in Mueller-Hinton (MH) broth (BD Difco™) at 37°C for 24 h. On the day of the experiment, each bacterial culture was diluted in sterile saline solution (8.5 g l⁻¹) to a final concentration of 1.5×10^8 colony forming units (CFU) ml⁻¹.

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The experiments were performed in MRS broth without dextrose (C2) for probiotics (*L. rhamnosus*, *L. acidophilus*)

and in Mueller-Hinton broth for both pathogenic bacteria (*E. coli* and *S. Typhimurium*) using a 96-well microplate (COSTAR). Each well was inoculated with 5 μ l of each bacterial suspension (1.5×10^8 CFU per ml) and 300 μ l of each PC fresh serial dilution (0, 15, 20, 25, 30 and 35 mmol l⁻¹); these concentrations were chosen based on the antibacterial effect of phenolic acids against *E. coli* and *S. Typhimurium*. Positive (bacteria + medium) and negative (medium) controls, and a blank (PC + medium) were included. Plates were incubated for 24 h at 37°C, and they were then evaluated for bacterial growth using a microplate reader (BMG Labtech Inc., Model Omega, Cary, NC, USA). For each PC, the lowest concentration with no visible bacterial growth (blank reading) was considered to be the MIC.

To confirm each MIC and to establish the MBC, 10 μ l of the following dilutions were inoculated into dishes with MRS (*L. rhamnosus*, *L. acidophilus*) or MacConkey (*E. coli*, *S. Typhimurium*) agar to evaluate microbial growth: 15 mmol l⁻¹, 35 mmol l⁻¹ and MIC-corresponding dilution for each PC/strain set. After 24 h of incubation at 37°C, the plates with no apparent CFU of surviving micro-organisms were determined. Each experiment was repeated at least three times.

Growth kinetic curves

The effect of PC was assayed in C2-MRS broth supplemented with individual PC ($n = 5$) or dual combinations ($n = 4$) at 20 mmol l⁻¹ phenolic acids and catechin at 35 mmol l⁻¹. *Lactobacillus acidophilus* and *L. rhamnosus* growth kinetics were monitored spectrophotometrically by recording the OD₆₀₀ variations in periods of 30 min at 37°C (Baranyi and Roberts 1994). Growth curves were fitted using DMFit ver. 2.1 Excel[®] add-in (www.ifr.ac.uk/safety/DMfit) to estimate the following parameters: specific growth rate ($\mu_{\max, OD}$), lag time (λ/h) and maximum population density at the stationary phase ($Y_{\max, OD}$).

Statistical analysis

A minimum of three replicate trials for each bacteria and control were performed. For all the experiments, a completely random design was followed. ANOVA was performed to estimate significant differences ($P \leq 0.05$) between treatments and Tukey-Kramer tests was applied. The NCSS statistical software (2007) was used for data analysis (Hintze 2007).

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Conflict of Interest

The authors have no conflict of interest to declare.

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