Influence of *in vitro* gastrointestinal digestion of fruit juices enriched with pine bark extract on intestinal microflora

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**Abstract**

The selective antimicrobial effect of fruit juices enriched with pine bark extract (PBE) (0.5 g/L) has been studied before and after *in vitro* gastrointestinal digestion. PBE (a concentrate of water-soluble bioflavonoids, mainly including phenolic compounds) has been proven to have high stability to the digestion process. Pure phenolic compounds such as gallic acid had a high antimicrobial effect on *Staphylococcus aureus* and *Escherichia coli*, maintaining the lactic acid bacteria population (>100%). Otherwise, *E. coli* O157:H7 only grew 50% when PBE was added to the culture media, while a slight increase on the growth of lactobacilli and bifidobacteria was observed after exposition to the bark extract. Fresh fruit juices enriched with PBE showed the highest inhibitory effect on pathogenic intestinal bacterial growth, mainly *E. coli* and *Enterococcus faecalis*. The *in vitro* digestion process reduced the antibacterial effect of juices against most pathogenic bacteria in approximately 10%. However, the beneficial effect of fruit juices enriched with PBE (0.5 g/L) on gut microbiota is still considerable after digestion.

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**1. Introduction**

Phenolic compounds exert antioxidant properties and beneficial effects on human health that are widely recognised (Del Rio, Costa, Lean, & Crozier, 2010; Fraga, 2009; Gee & Johnson, 2001). These phytochemicals are abundantly present in our diet, and are mainly found in fruit, tea, coffee, and to a lesser extent, in other grains and vegetables, such as cereals and legumes (Manach, Scalbert, Morand, Remesy, & Jiménez, 2004). During the gastrointestinal digestion process, the stability and absorption of these compounds can be affected to different extents mainly depending on the lumen conditions and the food matrix (Frontela et al., 2011), resulting in a large proportion of dietary phenolics and their metabolites remaining unabsorbed, which can significantly affect the intestinal environment by modulation of the microbiota (Lee, Jenner, Low, & Lee, 2006; Parkar, Stevenson, & Skinner, 2008). Fruit juices represent a useful kind of foods as a way to satisfy the recommendation to eat more fruit and vegetables; however, juice processing can reduce the content of total phenolic compounds (Gil, Tomás-Barberán, Hess-Pierce, Holcroft, & Kader, 2000; Manach et al., 2004). Thus, supplementation with extracts containing phenolic components should improve the nutritional quality of processed fruit beverages. Pine (*Pinus pinaster* Ait.) bark extract (PBE), a concentrate of water-soluble polyphenols, has been proven to have strong antioxidant properties, which are mainly attributed to its phenolic constituents (Maimoona, Naeem, Saddige, & Jameel, 2011). Previous studies have demonstrated that, owing to its stability to the human *in vitro* gastrointestinal conditions, PBE could be considered a good source of phenolic compounds to enrich fruit juices (Frontela et al., 2011). Because intestinal microbiota exert a key activity on the host’s health, the study of the effect of different molecules and extracts, including phenolic compounds, on gut microbiota is worthy of attention (Bosscher, Breynaert, Pieters, & Hermans, 2009; Lee et al., 2006; Sousa et al., 2006). Therefore, the objective of the present study was to highlight the effect of fruit juices enriched with pine bark extract, as source of phenolic compounds, after an *in vitro* gastrointestinal digestion process on selected groups of human intestinal microbiota (pathogens, commensals and probiotics).

**2. Material and methods**

**2.1. Bacterial strain and culture conditions**

Ten common pathogenic, commensal and probiotic intestinal bacteria as representative human intestinal microflora were studied. The bacteria strain isolated from different sources in our laboratory were designed as NUTBRO collection. Facultative anaerobic pathogens included *Escherichia coli* O157:H7 (DSMZ 13526),
Staphylococcus aureus (NUTBRO collection; isolated from human nasal mucosa), and Enterobacter sakazakii (CECT 858); while commensal bacteria were Enterococcus faecalis (DSM220478) and E. coli (NUTBRO collection; isolated from human faeces). All of them were grown on nutrient Mueller–Hinton broth at 37 °C for 24 h. Another pathogen used was Listeria monocytogenes (NUTBRO collection; isolated from pork pâté), which was grown in a brain–heart infusion (BHI) broth at 37 °C for 24 h. In addition, the probiotics Lactobacillus gasseri (DSM 20077), Lactobacillus casei ssp. rhamnosus (ATCC6469), Bifidobacterium breve (ATCC15707) and Bifidobacterium longum (ATCC15700) were maintained on the Mann Rogosa and Sharpe (MRS) medium under anaerobic conditions at 37 °C for 48 h. The four probiotic bacteria were selected because their probiotic effect and benefits on human health have been widely demonstrated (Nova et al., 2007; Resenfeldt et al., 2003). To prepare the bacterial inoculums, 10⁶ CFU (0.5 McFarland scale) were taken in a sterile tube containing 2 mL 0.85% sodium chloride (NaCl). The bacterial inoculum was diluted at a ratio of 1:1000 in peptone water, achieving a final concentration of 10⁵ CFU/mL.

2.2. Chemicals

Enzymes and salt sedes were purchased from Sigma Chemical Co. (St. Louis, MO, USA): pepsin (porcine, catalogue No. P-7000), pancreatin (porcine, catalogue No. P-1750) and bile extract (porcine, catalogue No. B-8756). The pepsin solution was prepared by dissolving 0.2 g of pepsin in 5 mL of 0.1 mol/L hydrogen chloride (HCl). The pancreatin-bile extract solution was prepared by dissolving 0.05 g of pancreatin and 0.3 g of bile extract in 25 mL of 0.1 mol/L sodium bicarbonate (NaHCO₃). Dimethyl sulfoxide (DMSO) and reagents for high performance liquid chromatography (HPLC) analysis (acetonitrile and formic acid), were supplied by Merck KGaA, (Darmstadt, Germany). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Samples

Four different fruit juices were studied: (1) pineapple (Ananas comosus L.) juice; (2) red fruit juice, containing mainly water and a mixture of red grape (26%), cherry (2%), raspberry (1%), blackberry (0.6%) and blackcurrant (0.6%) concentrated juices; (3) pineapple juice with PBE (0.5 g/L); and (4) red fruit juice with PBE (0.5 g/L). Fruit juices were prepared at the pilot plant of Hero España S.A. (Alcantarilla, Murcia, Spain).

2.4. In vitro gastrointestinal digestion

The procedure was adapted from the method of Boato, Wortley, Liu, and Glahn (2002). The method briefly consists of two sequential steps: fruit juices were initially digested by pepsin/HCl (pH 2) for 1 h at 37 °C, followed by digestion with bile salts/pancreatin (pH 6.5) for 2 h at 37 °C. Control samples were run in parallel and consisted of an equivalent volume of purified water subjected to the same in vitro digestion process. The in vitro gastrointestinal digestion of all juices was carried out in triplicate.

2.5. Identification and quantification of phenolic compounds

Free phenolic acids in fruit juices, with or without PBE enrichment, were determined before and after in vitro gastrointestinal digestion. Immediately after digestion, aliquots of the digested juices were stabilised by acidification (pH 2.0), filtered (0.45 μm) and then injected onto an HPLC LiChroCART C18 column (150 mm × 3.9 mm, 5 μm; Waters, Milford, MA, USA). The HPLC analysis was performed on a Merck Hitachi liquid chromatograph (Darmstadt, Germany), which was equipped with an L-7100 pump, an L-7490 refraction index detector and an L-7350 column oven, according to the method described by Frontela et al. (2011). The wavelengths for the quantification of phenolic acids by diode array detection (DAD) were 280 nm for gallic acid, ferulic acid and taxifolin (dihydroquercetin), and 320 nm for chlorogenic acid and caffeic acid, according to the retention times of their corresponding standards. All processes were carried out in darkness.

2.6. Effect of fruit juices on intestinal bacteria growth

Antimicrobial activity of all samples on selected bacterial strains was measured in liquid cultures in a 96-well plate using micro-dilution assays. A 1% inoculum was inoculated into broth containing the phenolic compounds and juices (digested or non-digested). Absorbance at 600 nm was measured using a Synergy HT (BioTek Instrument, USA) spectrophotometer after 24 h of incubation at 37 °C. Fresh or digested fruit juices were tested at 2%, 4%, and 6%, and afforded pH values of 7–7.5 and osmolality values of 280–320 mOsm/L, which did not reduce the growth of tested bacteria. The pure phenolic compounds were dissolved in DMSO and studied at concentrations of 0.5, 0.25, 0.12, 0.06, 0.03 and 0.015 mg/ml (the maximum% of DMSO tested was 2.5%, which did not affect the growth of any bacteria studied).

Background wells were treated with equal volumes of growth medium as the sample wells but with no cells. The inhibitory effect of tested compounds and fruit juices was measured by comparing the absorbance of the control growth (bacteria grown in culture medium) with those obtained from cultures with phenolic compounds or juices (digested or non-digested). All measurements were repeated in three 96-well plates with six replicates.

2.7. Statistical analysis

Results were expressed as mean ± standard deviation from three independent determinations of each sample. Differences among samples were examined for statistical significance (p < 0.05) by one-way analysis for variance (ANOVA) and Student’s t-test to compare the values with an appropriate control.

3. Results

The contents of each phenolic compound in samples, before and after the in vitro digestion process, for each analysed fruit juice were determined by HPLC-DAD and are shown in Table 1. As can be seen, the two major compounds found in all samples were gallic acid and taxifolin, and in the case of non-enriched red fruit juice, chlorogenic acid (1.71 mg/100 mL and 1.77 mg/100 mL, digested or not, respectively) was also found to be an important phenolic compound. In the case of caffeic acid, among all tested samples, it was the minority compound only in non-enriched red fruit juice, digested or not. Table 1 also shows the stability of phenolic compounds after a simulated gastrointestinal digestion process of fruit juices. In this regard, it can be observed that in both pineapple and red fruit juice, chlorogenic acid and taxifolin were not significantly (p > 0.05) affected by digestion, whereas the levels of gallic acid (in pineapple juice), ferulic acid (in red fruit juice) and caffeic acid (in both non-enriched fruit juices) decreased significantly (p < 0.05) after the gastrointestinal digestion process. Surprisingly, the levels of ferulic acid in both pineapple and red fruit juice enriched with PBE and caffeic acid in pineapple juice with PBE, increased significantly (p < 0.05) after the digestion process. In all cases, the addition of PBE (0.5 g/L) increased the phenolic content compared with non-enriched fruit juices.
3.1. Effect of pure phenolic compounds on intestinal bacterial growth

The results obtained after comparing bacterial growth in the absence and presence of pure phenolic compounds can be found in Fig. 1 for pathogenic and commensal bacteria and in Fig. 2 for the probiotic strains of bifidobacteria and lactobacilli. Results are shown as percentages compared with bacteria grown in the culture media. DMSO showed no significant \((p < 0.05)\) effect on bacterial growth at the assayed concentrations and was used as control. As can be seen, the pathogenic and commensal bacteria tested reduced their growth with most of the phenolic compounds studied compared with the control, emphasising the effect of gallic acid on \(S. aureus\) and \(E. coli\) growth, which remained only 10% compared to control. \(E. sakazakii\) grew less than 50% after exposition to caffeic acid and ferulic acid; meanwhile, chlorogenic acid seems not to affect its growth. Taxifolin reduced the growth of \(E. coli\), remaining it below 50% compared with the control. The growth of pathogenic bacteria \(L. monocytogenes\) and \(E. faecalis\) was slightly reduced by all

### Table 1

HPLC-DAD analysis of phenolic compounds in fruit juices before and after in vitro gastrointestinal digestion. Values (mg/100 mL) are expressed as mean ± standard deviation \((n = 3)\).

<table>
<thead>
<tr>
<th>Phenolic compound</th>
<th>Pineapple juice</th>
<th>Digested pineapple juice</th>
<th>Red fruits juice</th>
<th>Digested red fruits juice</th>
<th>Pineapple juice + PBE</th>
<th>Digested pineapple juice + PBE</th>
<th>Red fruits juice + PBE</th>
<th>Digested red fruits juice + PBE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gallic acid</td>
<td>Chlorogenic acid</td>
<td>Caffeic acid</td>
<td>Ferulic acid</td>
<td>Taxifolin</td>
<td>Gallic acid</td>
<td>Chlorogenic acid</td>
<td>Caffeic acid</td>
</tr>
<tr>
<td>Pineapple juice</td>
<td>5.11 ± 0.09*</td>
<td>0.55 ± 0.02</td>
<td>1.14 ± 0.00*</td>
<td>0.38 ± 0.04</td>
<td>1.41 ± 0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digested pineapple juice</td>
<td>4.01 ± 0.15</td>
<td>0.51 ± 0.02</td>
<td>1.01 ± 0.00*</td>
<td>0.37 ± 0.03</td>
<td>1.37 ± 0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red fruits juice</td>
<td>4.46 ± 1.56</td>
<td>1.77 ± 0.25</td>
<td>1.27 ± 0.05*</td>
<td>1.31 ± 0.52*</td>
<td>1.67 ± 0.09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digested red fruits juice</td>
<td>4.51 ± 1.02</td>
<td>1.71 ± 0.07</td>
<td>1.13 ± 0.01</td>
<td>0.49 ± 0.06</td>
<td>1.42 ± 0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pineapple juice + PBE</td>
<td>6.79 ± 0.53</td>
<td>0.75 ± 0.00</td>
<td>1.51 ± 0.00</td>
<td>0.56 ± 0.01</td>
<td>1.93 ± 0.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digested pineapple juice + PBE</td>
<td>6.00 ± 0.54</td>
<td>0.83 ± 0.08</td>
<td>1.91 ± 0.00*</td>
<td>0.71 ± 0.01*</td>
<td>2.21 ± 0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red fruits juice + PBE</td>
<td>7.09 ± 0.34</td>
<td>2.62 ± 0.06</td>
<td>2.44 ± 0.04</td>
<td>1.64 ± 0.10</td>
<td>3.99 ± 0.09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digested red fruits juice + PBE</td>
<td>7.29 ± 0.09</td>
<td>2.89 ± 0.07</td>
<td>2.21 ± 0.25</td>
<td>2.13 ± 0.26*</td>
<td>4.11 ± 0.12</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Indicates, within each phenolic compound, significant \((p < 0.05)\) difference between the same undigested and digested juice.

![Fig. 1](image1.png)

**Fig. 1.** Effect of major PBE phenolics \((0.5 \text{ g/L})\) on 24 h growth/inhibition of various pathogenic bacteria. Data are percentage compared with control \((2.5\% \text{ DMSO in culture medium})\). Within each microorganism, * indicates statistical differences \((p < 0.05)\) between each phenolic compound and control.
the assessed phenolic compounds, obtaining an approximate growth of 80–85% and 65–70%, respectively. However, phenolic compounds did not seem to have activity against lactic acid bacteria and only taxifolin exert an inhibitory effect on the growth of *Lactobacillus casei* ssp. *rhamnosus* and *B. breve*, remaining their growth around 47%. It is worth noting that *L. gasseri* increased growth compared with the control in the presence of caffeic acid and gallic acid (30% and 21%, respectively).

Focusing on the effect of PBE (0.5 g/L), different inhibition percentages of pathogenic bacteria growth were observed, from *E. faecalis* which grew 85% to *E. coli* O157:H7 which only grew 45% compared to control, only the bark extract was able to inhibit this last enterohemorrhagic pathogen; meanwhile, the growth of *S. aureus* was not affected. On the contrary, the growth of *L. casei* ssp. *rhamnosus* was stimulated by this extract by 20% compared with the control.

### 3.2. Effect of fruit juices on intestinal bacterial growth

Fruit juices were assessed at 2%, 4% and 6% in growth medium. After checking that the first two concentrations did not show any significant effect on bacterial growth, the results corresponding to the effect of fruit juices (fresh and digested) at 6% on pathogenic, commensal and beneficial bacteria are shown in Tables 2 and 3, respectively. Overall, the effect of fruit juices on intestinal bacterial growth was enhanced when PBE was included as enrichment. Most of the studied pathogenic and commensal bacteria did not seem to significantly affect its growth when pineapple juice, digested or not, was added to the culture media. Only *E. coli* decreased its growth when digested non-enriched pineapple juice was added meanwhile enterohemorrhagic *E. coli* showed not to be affected by enriched pineapple juice after digestion. In general, when juices were enriched with PBE (0.5 g/L), an increase on the antibacterial effect on pathogenic bacteria was observed in the cases of *E. sakazakii* growing in the presence of digested pineapple juice and *E. coli* O157:H7 with non-digested pineapple juice. In the case of commensal bacteria, enriched fruit juices inhibited their growth in a higher percentage that non-enriched ones. Specifically, the growth of *E. coli* grown in the presence of digested red fruit juice; and *E. faecalis* in the presence of pineapple juice, digested or not, were reduced.

It should be noted that most of the pathogenic microorganisms studied were not affected by 6% pineapple juice, neither before nor after the digestion process. Independently of digestion, only *E. coli* significantly reduced (*p < 0.05*) its growth, remaining approximately at 50–55% after exposure to any juice, compared with the control (microorganisms grown in the culture media).

As can be observed, non-digested red fruit juice enriched with PBE induced the highest decrease in the growth of *S. aureus* (which grew only 55.8%) and *L. monocytogenes* (maintaining 87.80% of growth). In the case of commensal bacteria *E. coli* and *E. faecalis* showed a significant decrease (*p < 0.05*) in their growth after exposure to 6% fresh red fruit juice (with and without PBE added, 0.5 g/L), compare with the control. However, there were no significant differences between adding or not PBE.

When the effect of fruit juices on the growth of potential beneficial bacterial strains was studied (Table 3), a contrary effect was found when compared with entero-pathogenic microorganisms.
Overall, probiotic lactobacilli and bifidobacteria were unaffected by fresh or digested juices, as compared to the control, when fruit juices were added to the culture medium. Otherwise, red fruit juice enriched and not enriched with PBE (fresh or digested) at 6% in the culture media was shown to maintain the lactic acid bacteria population after exposure for 48 h, even increasing its growth compared with the probiotic bacteria grown in the culture media.

4. Discussion

Dietary phenolic compounds may influence bacterial population in the human gut because they can, selectively, modulate the growth of intestinal bacteria, pathogens and probiotics (Cueva et al., 2010; Tzounis et al., 2008). In the present study, the inhibitory effect of different pure phenolic compounds on selected intestinal bacterial species showed high variability depending on both the phenolic compound and the assessed microorganism. Most pathogenic bacteria showed the highest resistance to the inhibitory effect of pure phenolic compounds compared with commensal and probiotic microorganisms. In this regard, at the assayed concentration for phenolic compounds (0.5 g/L) including PBE, only L. monocytogenes showed a slight decrease in growth. The mixture of polyphenols (PBE) did not seem to exert a synergistic effect on microorganisms as could be expected (Karioti et al., 2011; Tafesh et al., 2011). A similar trend was observed for the other gram-positive microorganisms (E. faecalis and S. aureus) that could be due to differences in cell surfaces and membranes when exposed to different phenolic compounds. Similar results were found by Puupponen-Pimiä et al. (2001) for the effect of polyphenols from berries on gram-positive and gram-negative bacteria. In the case of S. aureus, only was susceptible to gallic acid at the concentration tested. This phenolic acid has been previously proven to be effective in the control of this bacteria strain in foods (Rúa et al., 2011); however, there are also some studies on human gut microbiota that report gallic acid to be more effective against gram-negative microorganisms (Requena et al., 2010). Otherwise, caffeic acid and ferulic acid showed inhibitory activity against E. sakazukii, while taxifolin and gallic acid significantly reduced the growth of the commensal bacteria E. coli (p < 0.05). Regarding the effect of isolated phenolic compounds or PBE (0.5 g/L) on acid-lactic bacteria and lactobacilli, only taxifolin reduced the bacterial growth, maintaining it around 50% in the case of the probiotic bacteria L. casei ssp. rhamnosus and B. breve; meanwhile, the other assessed phenolic compounds, including PBE, maintained the probiotic bacteria population above 85% compared with the control. This is probably because taxifolin exerts a different antimicrobial mechanism of action compared to the other tested phenolic compounds, being L. casei ssp. rhamnosus and B. breve especially sensitive to this compound. Puupponen-Pimiä et al. (2001) found that different phenolic extracts used at the same concentrations, as the present study, were not active against gram-positive lactic acid bacteria. Moreover, Lee et al. (2006) observed that probiotics, such as lactobacilli and bifidobacteria, are relatively unaffected in the presence of phenolic compounds. In this regard, certain flavonoids, i.e. taxifolin, have previously shown higher antimicrobial activity than other phenolic compounds (Delva & Goodrich-Schneider, 2013). After exposing the microorganisms to fresh fruit juices (6% v/v), the highest inhibitory effect on S. aureus, E. coli and E. faecalis was observed in samples containing a greater amount of taxifolin (red fruit juice enriched with PBE, ≈4 mg/100 mL) (Frontela et al., 2011). The gastrointestinal digestion process of the fruit juices used in the present study has been previously addressed, and showed a different behaviour depending on the phenolic compound; nevertheless, gallic acid and taxifolin were the major phenolic compounds found before and after digestion (Frontela et al., 2011). This fact could explain the differences found in bacterial growth between microorganisms exposed to different samples before and after in vitro digestion. A synergistic effect of phenolic compounds as antibacterial agents could be expected when microorganisms are exposed to fruit juices. In this regard, E. coli was highly susceptible and significantly reduced its growth after exposure to a low concentration of any fresh or digested fruit juices (both 6%) over 24 h. However, a significant reduction of the inhibitory effect of red fruit juice was observed when fresh and digested juices were compared, except for the enterohemorrhagic bacteria E. coli O157:H7. In the case of pineapple juice in which the phenolic...
content of fresh juice was near to that observed after digestion (Frontela et al., 2011), no differences were found for the antimicrobial activity on most of the assessed pathogenic bacteria. Phenolic compounds have been reported to selectively inhibit the growth of intestinal bacteria (Puupponen-Pimiä, Nohynek, Alakomi, & Oksman-Caldentey, 2005; Puupponen-Pimiä, Nohynek, Hartmann-Schmidlin et al., 2005). Related to this, Bialonska, Kasimsetty, Schrader, and Ferreira (2009) reported that pomegranate by-products, that are rich in polyphenols, did generally not affect probiotic lactobacilli and bifidobacteria, and this modulation of the gut bacteria population would be of great importance for the maintenance of human health. In our study, the four probiotic bacteria tested, slightly reduced their growth when they were exposed to pineapple juice independently if it was enriched or not, and digested or not. However, in the case of the fresh and digested red fruit juice (enriched or non enriched with PBE), an enhancement on the growth of lactobacilli and bifidobacteria was observed compared with pineapple juice. The high amount of phenolic compounds in red fruits would give a plausible explanation and agree with results found by Bialonska et al. (2009), without any effect on digestion worthy of mention regarding the prebiotic activity of juices enriched or not enriched with PBE (0.5 g/L).

Results of this study are in accordance with previous investigations relating the consumption of foods rich in polyphenols with the modulation of intestinal microbiota and a decreased risk of gut diseases (Lee et al., 2006; Puupponen-Pimiä et al., 2001).

5. Conclusion

The addition of a rich-phenolic extract to fruit juices may have the potential to modulate the gut microflora with a decrease of pathogenic bacteria, thereby maintaining the probiotic population. The gastrointestinal digestion of fruit juices whether enriched or not with PBE (0.5 g/L) seems not to significantly affect this selective antimicrobial activity. Studies focussing on the gut environment that are able to reproduce the effect of the diet and interactions between food components on the gut microbiota could provide valuable information for promoting gut health.

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References


