**In vitro** fermentation of grape seed flavan-3-ol fractions by human faecal microbiota: changes in microbial groups and phenolic metabolites

Carolina Cueva¹, Fernando Sánchez-Patán¹, María Monagas¹, Gemma E. Walton², Glenn R. Gibson², Pedro J. Martín-Alvarez¹, Begona Bartolome¹ & M. Victoria Moreno-Arribas¹

¹Instituto de Investigación en Ciencias de la Alimentación (CIAL), CSIC-UAM, Madrid, Spain; and ²Food Microbial Sciences Unit, School of Chemistry, Food and Pharmacy, University of Reading, Reading, UK

**Correspondence:** M. Victoria Moreno-Arribas, Instituto de Investigación en Ciencias de la Alimentación (CIAL), CSIC-UAM, C/Nicolás Cabrera 9, Campus de Cantoblanco, 28049 Madrid, Spain. Tel.: +0034 910017902; fax: +0034 910017905; e-mail: victoria.moreno@csic.es

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**Abstract**

With the aim of investigating the potential of flavan-3-ols to influence the growth of intestinal bacterial groups, we have carried out the *in vitro* fermentation, with human faecal microbiota, of two purified fractions from grape seed extract (GSE): GSE-M (70% monomers and 28% procyanidins) and GSE-O (21% monomers and 78% procyanidins). Samples were collected at 0, 5, 10, 24, 30 and 48 h of fermentation for bacterial enumeration by fluorescent *in situ* hybridization and for analysis of phenolic metabolites. Both GSE-M and GSE-O fractions promoted growth of *Lactobacillus/Enterococcus* and decrease in the *Clostridium histolyticum* group during fermentation, although the effects were only statistically significant with GSE-M for *Lactobacillus/Enterococcus* (at 5 and 10 h of fermentation) and GSE-O for *C. histolyticum* (at 10 h of fermentation). Main changes in polyphenol catabolism also occurred during the first 10 h of fermentation; however, no significant correlation coefficients (*P* > 0.05) were found between changes in microbial populations and precursor flavan-3-ols or microbial metabolites. Together, these data suggest that the flavan-3-ol profile of a particular food source could affect the microbiota composition and its catabolic activity, inducing changes that could in turn affect the bioavailability and potential bioactivity of these compounds.

**Introduction**

Polyphenols are plant-derived secondary metabolites abundantly present in foods such as fruits, tea, coffee, wine, chocolate and, to a lesser extent, in some vegetables, cereals and legume seeds. Polyphenol consumption has been associated with beneficial protective effects against cardiovascular disease, cancer and gut health (Crozier *et al.*, 2009).

Flavan-3-ol monomers, as well as oligomeric and polymeric forms (also known as condensed tannins or proanthocyanidins), are among the most abundant and bioactive dietary polyphenols. For example, in the Spanish diet, the mean daily intake of polyphenols was estimated between 2590 and 3016 mg per person per day, 450 mg day⁻¹ corresponding to highly polymerized proanthocyanidins (Saura-Calixto *et al.*, 2007). *In vivo* health effects of flavan-3-ols may be limited because of their recognition as xenobiotics by the human body, which limit their bioavailability (Selma *et al.*, 2009). Besides, flavan-3-ol chemical structure features such as the degree of polymerization (DP) affects bioavailability: monomeric flavan-3-ols are readily absorbed in the small intestine, whereas oligomeric and polymeric forms reach the colon where they can be transformed by the resident microbiota into metabolites that could be even more bioactive than their precursors (Monagas *et al.*, 2010). In the last few years, microbe-derived phenolic metabolites have been reported to exert beneficial health effects, such as antioxidant activities (Grimm *et al.*, 2004), antiproliferative actions and cytotoxicity (Tanaka *et al.*, 1993), antiinflammatory effects (Larrosa *et al.*, 2009) and antithrombotic activities (Rechner & Kroner, 2005), as well as effects on the intestinal microbiota (Lee *et al.*, 2006).

The large intestine is by far the most colonized region of the digestive tract, with a total population of...
$10^{11}-10^{12}$ CFU mL$^{-1}$ of contents with more than 500 bacterial species, of which over 99% are anaerobic (Gibson & Roberfroid, 1995). The balance among human gut microbiota has been linked to both beneficial and harmful effects in the host. Beneficial bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. have been observed to contribute to human health at different levels (Saulnier et al., 2009). However, there are other bacterial species associated with negative implications, such as *Clostridium histolyticum*, a group that is considered to increase risk for colorectal cancer (Hambly et al., 1997).

There are few studies investigating the effect of flavan-3-ols on the composition and activity of the human gut microbiota (Hervert-Hernandez & Goñi, 2011). In vitro fermentation studies with faecal microbiota have revealed that (+)-catechin and tea (epi)gallocatechins were able to inhibit bacteria groups capable of exerting harmful or pathogenic effects (e.g. *Clostridium perfringens*, *Clostridium difficile*, *C. histolyticum*, *Bacteroides* spp.) without significantly affecting, or even moderately encouraging, the growth of potentially beneficial bacteria (Cristóbal et al., 2009). Another intervention study showed that administration of grape seed proanthocyanidin-rich extracts induced a reduction in faecal counts for *C. perfringens* and other putrefactive bacteria groups but increases in the counts of *Bifidobacterium* spp., as well as increases in organic acids which can reduce faecal pH (Goto et al., 1999). Another intervention study showed that administration of grape seed proanthocyanidin-rich extracts induced a reduction in levels of putrefactive products in the intestine, which may be linked to a slight increase in the number of *Bifidobacterium* and decrease in *Enterobacteriaceae* (Yamakoshi et al., 2001). A recent intervention study comparing the effects between consumption of high- and low-cocoa flavan-3-ol diets has also shown significant increases in faecal bifidobacterial and lactobacilli populations but decreases in clostridia counts, with parallel reductions in plasma triacylglycerol and C-reactive protein (Tzounis et al., 2011). All these data suggest that dietary flavan-3-ols may modulate gut bacterial populations, contributing to the improvement of host health.

Our experimental hypothesis was that the flavan-3-ols could influence the growth and metabolism of intestinal bacteria. Therefore, the aim of the current study was to investigate the potential of two grape seed flavan-3-ol fractions to influence the growth of bacterial groups in a pH-controlled, stirred, batch-culture fermentation system that is reflective of the environmental conditions located in the distal region of the human large intestine (Tzounis et al., 2011). In addition, disappearance of grape seed polyphenols and formation of microbe-derived metabolites were monitored alongside the fermentations in an attempt to relate the changes in microbial groups to the presence of phenolic metabolites.

### Materials and methods

#### Chemicals

Standards of phenolic compounds were purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO), Phytolab (Vestenbergsgreuth, Germany) or Extrasynthèse (Genay, France). Unless otherwise stated, general chemicals and reagents were obtained from Sigma-Aldrich Co. Ltd. (Poole, Dorset, UK) or Fisher (Loughborough, Leics, UK). Bacteriological growth media supplements were obtained from Oxoid Ltd. (Basingstoke, Hants, UK). Rafúlose P95 fructooligosaccharides were purchased from Orafti (Tienen, Belgium). All the oligonucleotide probes used for fluorescent in situ hybridization (FISH) were commercially synthesized and labelled with the fluorescent dye Cy3 by MWG-Biotech Ltd. (Milton Keynes, Bucks, UK). Sterilization of media and instruments was achieved by autoclaving at 121 °C for 15 min.

#### Flavan-3-ol fractions

Two flavan-3-ols preparations were kindly provided by Dr. Piriou (Les Dérives Resiniques & Terpéniques, S.A., France). They were purified from the Vitalflavan® extract obtained from grape seeds (Les Dérives Resiniques & Terpéniques, S.A.).

#### Faecal batch-culture fermentation

Three independent fermentation experiments were carried out using faeces from different healthy volunteers, who had not ingested antibiotics for at least 6 months before the study. Briefly, 300-mL glass fermentation vessels were filled with 135 mL of prereduced sterile medium (Tzounis et al., 2008). The medium was adjusted to pH 7.0 and continuously sparged with O$_2$-free N$_2$ overnight. The pH was maintained at 6.8 and the temperature at 37 °C in order to mimic conditions located in the distal region of the human large intestine. Vessels were inoculated with 15 mL faecal slurry (10% (w/v)). Then, grape seed extract (GSE)-M and GSE-O were added to separate stirring batch-culture vessels containing faecal slurry at the final concentration of 600 mg L$^{-1}$. This concentration was selected taking into account the flavan-3-ol richness of the extracts and that a mean daily intake of 450 mg of flavan-3-ols per day would lead to an approximate gastrointestinal concentration between 300 and 450 mg L$^{-1}$ if the stomach volume was assumed to be between 1 and...
1.5 L (Tzounis et al., 2011). Batch cultures were run under anaerobic conditions for a period of 48 h during which samples were collected at six time points (0, 5, 10, 24, 30 and 48 h) for FISH and phenolic analyses. For this latter analysis, samples were stored at −70 °C until required.

Incubations of the faecal slurry with a known prebiotic compound (Raftilose P95, 1% w/v) as positive control, and without any addition (negative control), were carried out for each fermentation experiment. Also, incubations of GSE-M and GSE-O without faecal slurry inoculation (blanks) were run under the same conditions.

**Bacterial enumeration using FISH**

Changes in human faecal bacterial populations were assessed by FISH with 16S rRNA probes as described by Tzounis et al. (2011). These 16S rRNA probes, specific for predominant classes of the gut microbiota, were commercially synthesized and labelled with the fluorescent dye Cy3. The probes used were Bif164 (Manz et al., 1996), Lab158 (Harmsen et al., 1999), Chis150 (Franks et al., 1998), Bac303 (Manz et al., 1996), EUBmix (Daims et al., 1999), specific for Bifidobacterium spp., Lactobacillus spp., Enterococcus spp., and members of the domain Bacteria, respectively.

To determine changes in bacterial populations between treatments, we used an ‘index of specific bacteria’ (ISB). The ISB was calculated using the following equation:

\[
ISB = [(N_s (T_1) - N_s (T_0)) - (N_c (T_1) - N_c (T_0))]
\]

where \(N_s\) and \(N_c\) are the number log10 of specific bacteria in a specific test sample, \(N_s (T_0)\) and \(N_c (T_0)\) are the number log10 of specific bacteria in the negative control (medium+faecal slurry), \(T_1\) is a specific time point and \(T_0\) is the 0 h time point.

**Analysis of phenolic metabolites**

A previously developed UPLC-ESI-TQ MS method for the analysis of food flavan-3-ols and their metabolites (Sánchez-Patán et al., 2011) was applied to analysis of microbe-derived metabolites in the batch culture vessels. The equipment consisted of an UPLC system coupled to a photodiode array detector and an Acquity TQ tandem quadrupole mass spectrometer (UPLC-PAD-ESI-TQ MS) (Waters, Milford, MA, USA). Separation was performed on a Waters® BEH C18 column (2.1 × 100 mm; 1.7 μm) at 40 °C. A gradient composed of solvent A – water : acetic acid (98 : 2, v/v) and B-acetonitrile : acetic acid (98 : 2, v/v) was applied at flow rate of 0.5 mL min⁻¹ as follows: 0–1.5 min: 0.1% B, 1.5–11.17 min: 0.1–16.3% B, 11.17–11.5 min: 16.3–18.4% B, 11.5–14 min: 18.4% B, 14–14.1 min: 18.4–99.9% B, 14.1–15.5 min: 99.9% B, 15.5–15.6 min: 0.1% B, 15.6–18 min: 0.1%. The ESI parameter were as follows: capillary voltage, 3 kV; source temperature, 130 °C; desolvation temperature, 400 °C; desolvation gas (N₂) flow rate, 750 L h⁻¹; cone gas (N₂) flow rate, 60 L h⁻¹. The ESI was operated in negative mode. The Multiple reaction monitoring (MRM) transitions used for detection of flavan-3-ols were as follows: (+)-catechin and (−)-epicatechin (289/245), (+)-epicatechin-3-O-gallate (441/289), procyanidins B1, B2, B3 and B4 (577/289), procyanidins C1 and T2 (865/577) and B2- and B2’-3-O-gallates (729/577) (Sánchez-Patán et al., 2012). For detection of different structures of phenolic metabolites, the MRM transitions used were as follows: for phenyl-γ-valerolactone derivatives, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (207/163), 5-(3′-hydroxyphenyl)-γ-valerolactone (191/147) and γ-valerolactone (101/55); for 4-hydroxy-5-(phenyl)valeric acid derivatives, 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid (225/163), 4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid (209/147) and 4-hydroxy-5-(phenyl)valeric acid (193/175); for phenylpropionic acids, 3-(3,4-dihydroxyphenyl)-propionic acid (181/137), 3-(3- and 3-(4-hydroxyphenyl)-propionic acids (165/121) and phenylpropionic acid (149/105); for phenylacetic acids, 3,4-dihydroxyphenylacetic acid (167/123), 3- and 4-hydroxyphenylacetic acids (151/107) and phenylactic acid (135/91); and for benzoic acids and others, gallic acid (169/125), catechol/pyrocatechol, 4-hydroxymandelic acid (167/123), 4-hydroxybenzoic acid (137/93) and benzoic acid (121/77). In the absence of commercial standards, quantification of procyanidin B2 and B3 was carried out through the calibration curve of procyanidin B1. Procyanidins B1-3-O-gallate, B2-3-O-gallate and B2’-3-O-gallate were quantified using the (−)-epicatechin-3-O-gallate curve. Phenyl-γ-valerolactones derivatives were quantified as (−)-epicatechin and γ-valerolactone using its calibration curve. 4-Hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric and 4-hydroxy-5-(3′-hydroxyphenyl)-valeric acids were quantified using the calibration curves of 3-(3,4-di hydroxyphenyl)-propionic and 3-(3-hydroxyphenyl)-propionic acids, respectively. Data acquisition and processing was carried out using MASSLYNX 4.1 software.

Before injection, samples were defrosted, centrifuged (14 926 g, 20 °C, 10 min) and filtered through a 0.22-μm PVDF filter (Teknokroma, Barcelona, Spain), finally diluted (1 : 1, v/v) with a mixture of water/acetonitrile (6 : 4, v/v), and 2 μL of the diluted sample was injected onto the column.

**Statistical analysis**

The statistical methods used for the data analysis were repeated measures ANOVA to test jointly the effects of the two factors: time as within-subjects factor, with five levels
(0, 10, 24, 30 and 48 h), and fraction (GSE-M and GSE-O), like a categorical factor, on concentration of phenolic compounds, and two-sample t-test to assess significant differences between the two fractions at the same time. A value of $P = 0.05$ was fixed for the level of significance of the test. Finally, the relationship between changes in microbial populations and precursor flavan-3-ols or microbial metabolites was assessed using correlation analysis. All the statistical analyses were carried out using the STATISTICA program for Window, version 7.1 (StatSoft. Inc. 1984–2006, www.statsoft.com).

**Results**

**Effect of grape seed flavan-3-ol fractions on human faecal bacteria**

The GSE-M and GSE-O fractions were clearly differentiated by their flavan-3-ol profile (Supporting Information, Fig. S1). Main phenolic compounds present in both fractions were gallic acid, (+)-catechin and (-)-epicatechin, (-)-epigallocatechin-3-O-gallate, dimeric procyanidins B1, B2, B3, B4, galloylated dimeric procyanidins B2-3-O-gallate, B2-3′-O-gallate and B1-3-O-gallate, and trimeric procyanidins C1 and T2. The GSE-M contained a higher concentration of the monomeric flavan-3-ols (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-O-gallate than the GSE-O fraction, whereas procyanidin dimer B1, B2, B3 and B4 and trimer C1 were presented in higher concentration in the GSE-O fraction than in the GSE-M fraction. However, the procyanidin gallates were more abundant in the GSE-M fraction. The total phenolic content was 414 mg g$^{-1}$ for GSE-M (70% flavan-3-ol monomers and 28% procyanidins) and 279 mg g$^{-1}$ for GSE-O (21% flavan-3-ol monomers and 78% procyanidins) (Table 1), indicating that there was an important portion of uncharacterized material in both fractions.

From each of the three fermentation experiments using faecal samples from different donors, bacterial populations in the GSE-containing vessels, as well as in the negative (medium+faecal slurry) and positive (medium+faecal slurry+prebiotic) control vessels, were assessed at 0, 5, 10, 24, 30 and 48 h by FISH. This method has been used successfully to enumerate different bacterial groups and their changes over time in complex habitats such as the gut (Tzounis et al., 2008; Molan et al., 2010).

From the results of the FISH analysis, changes in bacterial groups were calculated as ISB, that is, changes relative to the negative control for each fermentation experiment (Fig. 1). Among the bacterial groups studied (Bifidobacterium spp., Lactobacillus/Enterococcus spp., C. histolyticum group, Bacteroides spp. and members of the domain **Bacteria**), addition of GSE fractions to the faecal fermentation mixtures clearly affected the growth of *C. histolyticum* group (Chis150 probe) and *Lactobacillus/Enterococcus* (Lab158 probe). Both GSE-M and GSE-O fractions caused a decrease in the growth of the *C. histolyticum* group from 5 h (GSE-M) or 10 h (GSE-O) to 48 h, although significant differences were only observed for GSE-O at 10 h of fermentation. An increase in the *Lactobacillus/Enterococcus* population was observed at first stages of fermentation (5–10 h) when both GSE-M and GSE-O were added to the faecal fermentation mixture, although only significant values were found for GSE-M. No other distinguished changes in the microflora were observed in the GSE-added fermentations. On the other hand, the positive control (prebiotic added) showed increased populations in *Bifidobacterium* spp. (Bif164 probe) (significant increase at all the times studied), *Lactobacillus/Enterococcus* spp. (significant increase at 5 h), *C. histolyticum* group (significant increase at 5 h) and *Bacteroides* spp. (Bac303 probe) (significant increases at 10, 24 and 48 h).

### Table 1. Concentration (mg g$^{-1}$) of individual phenolic compounds in the GSEs used in this study (GSE-O and GSE-M)

<table>
<thead>
<tr>
<th>No.</th>
<th>Phenolic compound</th>
<th>GSE-O Mean ± SD</th>
<th>GSE-M Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gallic acid</td>
<td>5.49 ± 0.04</td>
<td>8.00 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>Procyanidin B3</td>
<td>32.81 ± 0.35</td>
<td>20.90 ± 0.05</td>
</tr>
<tr>
<td>3</td>
<td>Procyanidin B1</td>
<td>55.34 ± 0.51</td>
<td>20.95 ± 0.58</td>
</tr>
<tr>
<td>4</td>
<td>(+)-Catechin</td>
<td>25.60 ± 1.09</td>
<td>135.48 ± 0.80</td>
</tr>
<tr>
<td>5</td>
<td>Procyanidin T2</td>
<td>13.92 ± 0.19</td>
<td>2.04 ± 0.09</td>
</tr>
<tr>
<td>6</td>
<td>Procyanidin B4</td>
<td>28.41 ± 0.06</td>
<td>21.23 ± 0.23</td>
</tr>
<tr>
<td>7</td>
<td>Procyanidin B2</td>
<td>61.24 ± 0.70</td>
<td>42.42 ± 0.91</td>
</tr>
<tr>
<td>8</td>
<td>B1-3-O-gallate</td>
<td>0.13 ± 0.01</td>
<td>0.13 ± 0.00</td>
</tr>
<tr>
<td>9</td>
<td>(-)-Epicatechin</td>
<td>30.21 ± 0.60</td>
<td>135.10 ± 1.37</td>
</tr>
<tr>
<td>10</td>
<td>B2-3-O-gallate</td>
<td>1.26 ± 0.03</td>
<td>3.27 ± 0.18</td>
</tr>
<tr>
<td>11</td>
<td>Procyanidin C1</td>
<td>20.99 ± 0.58</td>
<td>3.37 ± 0.34</td>
</tr>
<tr>
<td>12</td>
<td>B2-3′-O-gallate</td>
<td>1.23 ± 0.03</td>
<td>2.23 ± 0.08</td>
</tr>
<tr>
<td>13</td>
<td>(-)-Epicatechin-3-O-gallate</td>
<td>1.42 ± 0.07</td>
<td>19.07 ± 0.01</td>
</tr>
</tbody>
</table>

**Microbial transformations of grape seed flavan-3-ol fractions**

Changes in precursor phenolic compounds during fermentations as well as formation of microbial phenolic metabolites were monitored by MRM mode. Among phenolic metabolites, a wide range of compounds, including phenyl-γ-valerolactones and phenylvaleric acid derivatives, hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinamic, hydroxybenzoic and hydroxymandelic acids, as well as simple phenols, were targeted. As an example and for one of the faecal fermentations of GSE-M, Fig. S2 shows the MS response of precursor phenolic compounds: (+)-catechin, (-)-epicatechin, (-)-epigallocatechin
gallate, dimeric procyanidins B3, B1, B2 and B4 and galloylated dimeric procyanidins, at the different times of sample collection (0, 5, 10, 24, 30 and 48 h). However, to better show changes in the concentration of phenolic precursors as well as of microbial phenolic metabolites for both GSE-M and GSE-O fractions during the 48 h of fermentation, concentration values at time zero were subtracted from concentration values at 5, 10, 24, 30 and 48 h (changes relative to initial time) for each fermentation experiment, and mean concentration values (n = 3 fermentations) calculated for both fractions. Therefore, negative values in the concentration Y-axis indicate degradation of flavan-3-ol precursors (Figs 2 and 3), whereas positive values indicate formation of phenolic metabolites (Figs 4–6).

Changes in precursor phenolic compounds

Changes in flavan-3-ol concentration relative to initial time, observed during faecal fermentation, differed according to the grape seed polyphenol fraction used, GSE-M or GSE-O (Figs 2 and 3). Greater degradation (lower concentration relative to the initial time) was observed for GSE-M for those compounds presented in higher concentration in relation to GSE-O (Table 1), that is, (+)-catechin (Fig. 2a), (−)-epicatechin (Fig. 2b), (−)-epicatechin-3-O-gallate (Fig. 2c) and procyanidin gallates (Fig. 3e and F). Significant differences between both fractions were observed in all cases, except for (+)-catechin and (−)-epicatechin because of the large inter-individual variations. In any case, catabolism of these compounds was completed by 10–24 h of fermentation.

On the other hand, the concentration relative to initial time for C1 and T2 was significantly lower for GSE-O in comparison with GSE-M at almost all the collection times (Fig. 3g and h), indicating greater degradation of compounds that were present in higher content in the GSE-O fraction (Table 1). Complete degradation of C1 and T2 was attained at 10 h for GSE-M and at 24 h for GSE-O. For dimeric procyanidins B1-B4 (Fig. 3a–d), no significant differences in the concentration relative to initial time were observed between GSE-M and GSE-O for any of the times collected, probably because differences in the content of these compounds between fractions were not important, at least in comparison with procyanidin trimers (Table 1). All procyanidin dimers were completely
degraded by 24 h of fermentation in both fractions. In the case of B1, a transient increase in concentration was observed after 10 h of fermentation (Fig. 3a), a fact that has been previously reported to occur in other studies using the same batch culture fermentation system (Tzounis et al., 2008).

**Formation of phenolic microbial metabolites**

Among phenyl-γ-valerolactones and phenylvaleric acid derivatives, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acids were detected during faecal fermentations, both showing nonsignificant differences in the concentration relative to initial time between GSE-M and GSE-O fractions, for any of the times collected (Fig. 4a and b). In the case of GSE-M, changes in the concentration of both compounds were larger and registered later (24 h) than for GSE-O (10 h). This was similar to their monohydroxylated forms, 5-(3′-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3′-dihydroxyphenyl)-valeric acids (Fig. 4c and d). For GSE-M, both forms showed a progressive change in concentration from 10 to 48 h, whereas for GSE-O the peak change in concentration for both compounds was registered at 10 h, declining afterwards. However, this difference in evolution trend in function of the GSE was not observed for the nonhydroxylated metabolite 4-hydroxy-5-(phenyl)-valeric acid which showed a progressive change in concentration from 10 to 48 h, the changes being slightly larger for GSE-O, although not significantly different (Fig. 4f). In the case of phenyl-γ-valerolactones derivatives, the corresponding nonhydroxylated form was not detected but instead the nonphenyl form γ-valerolactone, which was particularly detected in the case of GSE-M (Fig. 4e).

Among hydroxyphenylpropionic acids, 3-(3,4-dihydroxyphenyl)-propionic acid registered an increase in its concentration relative to initial time during the time course of the fermentation of both GSE-O and GSE-M, attaining larger changes in the case of GSE-M, although not significantly different between them (Fig. 5a). Subsequent dehydroxylated acids, 3- and 4-hydroxyphenylpropionic acids also showed nonsignificant differences in their concentration during the fermentation of GSE-M and GSE-O (Fig. 5c and e). Larger changes are produced for GSE-O, especially for 4-hydroxyphenylpropionic acid. This effect was more pronounced for the nonhydroxylated form (phenylpropionic acid), for which statistically significant differences were found between both fractions in favour of GSE-O (Fig. 5g).

Interestingly, the profile of 3,4-dihydroxyphenylacetic acid revealed an early increase in concentration at 10 h for GSE-O but not for GSE-M, which remained practically constant during fermentations (Fig. 5b). Similar results were found for its 3-monohydroxylated form (3-hydroxyphenylacetic acid), for which again no significant changes
Fig. 3. Changes, relative to initial time, of dimeric and trimeric procyanidins during faecal fermentation of GSE-M and GSE-O. (a) Procyanidin B₁; (b) procyanidin B₂; (c) procyanidin B₃; (d) procyanidin B₄; (e) procyanidin B₂-3-O-gallate; (f) procyanidin B₂-3′-O-gallate; (g) procyanidin C₁ and (h) procyanidin T₂. Results are expressed as amount (µg mL⁻¹) in batch culture medium. Results are expressed as amount (µg mL⁻¹) in batch culture medium. Values are means and 95% confidence intervals (n = 3). *Significant differences (P < 0.05) between GSE-M and GSE-O at the same time.
in concentration were found during the fermentation of GSE-M and GSE-O (Fig. 5d). However, this was not the case for the 4-monohydroxylated (4-hydroxyphenylacetic acid) and nonhydroxylated forms (phenylacetic acid) which progressively showed larger changes in concentration during the fermentation of both extracts (Fig. 5f and h). Although no significant differences were observed among fractions for these compounds, changes registered in 4-hydroxyphenylacetic acid are higher for GSE-O than for GSE-M. However, for phenylacetic acid, the evolution profile was completely similar for both fractions.

Gallic acid, initially presented in the GSE fractions, showed a progressive decline in concentration during the fermentation of GSE-M (Fig. 6a). Gallic acid may also arise from the release of the gallic acid moiety from...
Fig. 5. Changes, relative to initial time, of phenylpropionic and phenylacetic acid derivatives during faecal fermentation of GSE-M and GSE-O.
(a) 3-(3,4-Dihydroxyphenyl)-propionic acid; (b) 3,4-dihydroxyphenylacetic acid; (c) 3-(3-hydroxyphenyl)-propionic acid; (d) 3-hydroxyphenylacetic acid; (e) 3-(4-hydroxyphenyl)-propionic acid; (f) 4-hydroxyphenylacetic acid; (g) phenylpropionic acid; (h) phenylacetic acid. Results are expressed as amount (µg mL⁻¹) in batch culture medium. Values are means and 95% confidence intervals (n = 3). *Significant differences (P < 0.05) between GSE-M and GSE-O at the same time.
Galloylated flavan-3-ols by microbial esterase activity (Gross et al., 2010; Roowi et al., 2010). However, only a slight accumulation of this compound was observed during the fermentation of GSE-O (Fig. 6a). These changes were also in accordance with the concurrent formation of catechol/pyrocatechol, arising from dehydroxylation of gallic acid, in particular during the fermentation of GSE-M (Fig. 6b). None of the compounds showed significant differences in their concentration relative to initial time between both GSE-M and GSE-O fractions.

In contrast to the profile of gallic acid, other phenolic acids such as 4-hydroxybenzoic and benzoic acids (Fig. 6c and d), which are considered to arise from the β-oxidation of phenylpropionic acid derivatives, showed a progressive increase in concentration. In any case, it is of note that no significant differences in concentration were observed among fractions.

**Discussion**

Human health effects of polyphenols depend on their bioavailability; thus, while monomeric flavan-3-ols are readily absorbed in small intestine, proanthocyanidins reach the colon where they can be metabolized by the intestinal microbiota into phenolic metabolites, some of which could have higher biological activity than their parent compounds (Monagas et al., 2010). In the present study, we assessed the microbial modulatory capacity and catabolism of two grape seed fractions enriched in flavan-3-ol monomers (GSE-M) or oligomers (GSE-O), by performing in vitro fermentations with human bacterial populations representative of the distal part of the human large intestine (faecal samples) under anaerobic conditions. Both GSE-M and GSE-O fractions (600 mg L⁻¹) lead to similar changes in the growth of specific bacterial populations, although significant differences were only observed for GSE-M at 5 h (increase in the growth of *Lactobacillus/Enterococcus*) and for GSE-O at 10 h of fermentation (decrease in the growth of *C. histolyticum*).

No significant changes were noticed for other groups such as *Bifidobacterium* spp., *Bacteroides* spp. and members of the domain *Bacteria*. Using the same in vitro fermentation model, (+)-catechin (150 mg L⁻¹) was found to induce a significant decrease in the growth of *C. histolyticum* and an increase in the growth of *Bifidobacterium* spp., *C. coccoides–E. rectale* and *E. coli*, although (−)-epicatechin only produced a significant increase on the *C. coccoides–E. rectale* population (Tzounis et al., 2008). Thus, when the in vitro fermentation model was fed with a flavanol-enriched cocoa extract (1000 mg L⁻¹), a significant decrease was also observed in the growth of the *C. histolyticum* group, together with an increase in the growth of *Lactobacillus* spp. and *Bifidobacterium* spp. (Tzounis et al., 2011). Therefore, the results of this study are in accordance with previous reports, with the exception of the
increase in Bifobacterium spp. group induced by flavan-3-ols that was not observed in this study.

Selective inhibitory of flavan-3-ols against the growth of various Clostridium spp. as well as enhancement of the growth of Lactobacillus spp. has been reported in pure culture (Ahn & Stiles, 1990). However, evidence related to the in vivo effects of polyphenols on the intestinal microbiota is scarce. First studies conducted in both humans and animals (pigs and chickens) have revealed an increase in Lactobacillus spp. and a decrease in Enterobacteriaceae following administration of monomeric flavan-3-ols from green tea (Goto et al., 1999). Smith & Mackie (2004) later showed that rats fed a tannin-rich diet significantly decreased the Clostridium leptum cluster and increased the growth of Bacteroides group. Similarly, Dolara et al. (2005) found that rats fed with red wine polyphenols had significantly lower levels of Clostridium spp. and higher levels of Lactobacillus spp. More recently, a significant increase in the numbers of bifidobacteria and/or lactobacilli, together with a significant decrease in the numbers of Bacteroides spp. and Clostridium spp., have been reported in rat fed blackcurrant extract powder (Molan et al., 2010). Viveros et al. (2011) also found that birds fed grape pomace concentrate and GSE had higher populations of E. coli, Lactobacillus spp., Enterococcus spp. and Clostridium spp. in the caecal digesta than the control group. Recently, changes in microbial population were found after the ingestion of tea polyphenols by humans, including Actinobacteria and Clostridium clusters (van Duynhoven et al., 2010).

From data obtained in the literature and our study, it is evident that flavanols can exert an antimicrobial effect on certain bacterial groups, such as C. histolyticum group. Among the mechanisms proposed to explain this effect could be the binding of polyphenols to bacterial cell causing disturbance of membrane function and consequently inhibiting cell growth (Sirk et al., 2009) and the formation of polyphenols–metal ions complex which would lead to iron deficiency in the gut and could therefore affect susceptible bacterial populations (Smith et al., 2005). Other proposed mechanisms are DNA gyrase inhibition (Cushnie & Lamb, 2005), enzyme inhibition (Navarro-Martinez et al., 2005), reactive oxygen generation (Arakawa et al., 2004) and inhibition of virulence factors (Evensen & Braun, 2009).

From a health perspective, the present results demonstrate that GSEs and/or their microbe-derived metabolites could benefit the host by inhibiting pathogen growth and regulating, or increasing, commensal bacteria. Increasing lactobacilli is associated with beneficial effects in the gut due to the production of organic acids which help prevent colonization of the gut epithelial layer by pathogenic bacteria (Saulnier et al., 2009). In contrast, bacteria belonging to C. histolyticum group have a high β-glucuronidase activity which is considered to increase risk for colorectal cancer (Hambly et al., 1997).

Concomitant with the changes seen in bacterial groups, the degradation of flavan-3-ols precursors together with the formation of numerous microbial phenolic metabolites was determined. Despite the large inter-individual differences observed among volunteers, the flavan-3-ol profile of the GSE fractions seemed to affect the degradation extent of flavan-3-ol precursors themselves. GSE-M favour greater degradation than GSE-O, independent of the initial flavan-3-ol composition. This fact was particularly significant for galloylated flavan-3-ols, which were higher in the GSE-M, indicating specificity of the tannase activity of the faecal microbiota. It is of note that despite effects on the degradation extent, most precursor flavan-3-ols attained completed degradation during the first 24 h of fermentation.

The largest changes in precursor flavan-3-ols and in the bacterial groups were observed during the first 10 h of fermentation. The significant inhibitory effects of GSE-O on C. histolyticum in comparison with growth-promoting effects of GSE-M on Lactobacillus/Enterococcus also seem to be in agreement with the flavan-3-ol composition of the extracts, as a higher inhibitory activity has been reported for flavan-3-ols having a DP of 3 than for monomers (Mayer et al., 2008). Changes in bacterial composition may have led to alterations of microbial activity, affecting the catabolism of flavan-3-ols that could explain changes observed in degradation extent between the GSE-M and GSE-O.

Considering the formation of phenolic microbial metabolites, a clear influence of fraction composition was also observed during the initial stages of microbial metabolism as the formation of the di- and monohydroxylated forms of both phenyl-γ-valerolactone and phenylvaleric acid followed the same pattern in each of the fractions. Although no significant differences were observed between the GSE-M and GSE-O, the former fraction favoured formation of the dihydroxylated forms of both metabolites, whereas that of the monohydroxylated forms was promoted by the GSE-O, particularly during the initial hours of fermentation. Further metabolism to the nonhydroxylated form also is initially favoured by GSE-O. These findings suggest that monomeric flavan-3-ols, abundant in the GSE-M, favoured the formation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and its possible chemical equilibrium with 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid. Using human microbiota, differences in the formation rate of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid were found depending on original structure of...
of the flavan-3-ol precursor (monomer or dimer) (Stoupi et al., 2010). Moreover, some additional metabolites only arising from dimeric procyanidins were tentatively identified (Stoupi et al., 2010). In contrast, recent studies using rat microbiota have revealed that dimeric procyanidins rendered fewer metabolites than that of monomers (Serra et al., 2011).

The influence of the fraction flavan-3-ol profile was also seen during subsequent stages of the catabolism. The metabolite 3-(3,4-dihydroxyphenyl)-propionic acid also is favoured by the GSE-M, but this effect was progressively lost from the conversion of 3-(3,4-dihydroxyphenyl)-propionic acid to 3-(4-hydroxyphenyl)-propionic acid, and especially to phenylpropionic acid, for which the GSE-O promoted significant changes concentration in comparison with GSE-M.

However, in the case of 3,4-dihydroxyphenylacetic acid, the largest changes in concentration were observed during the fermentation of GSE-O. These findings seem to be in line with those of Appeldoorn et al. (2009) who proposed that the formation of 3,4-dihydroxyphenylacetic acid could exclusively arise from the catabolism of the top unit of dimeric procyanidins. This also seemed to be the case in the monohydroxylated form 3-hydroxyphenylacetic acid, but not of 4-hydroxyphenylacetic acid and phenylacetic acid, for which larger changes in concentration were observed during fermentation of both fractions. However, it is of note that these metabolites, in particular the latter one, was also detected in the fermentation of the culture media alone (without phenolic incubation), contributing to the marked concentration changes observed (data not shown). Both metabolites have been reported to be formed from the metabolism of certain aminoacids, such as tyrosine and phenylalanine, respectively (Curtius et al., 1976).

Together, these findings suggest that different pathways exist for the catabolism of monomeric and oligomeric flavan-3-ols, as proposed in previous in vitro fermentation studies (Tzounis et al., 2008; Appeldoorn et al., 2009; Serra et al., 2011). Metabolites arising during the initial stages of metabolism were affected by the fraction composition, but significant differences only were observed in galloylated flavan-3-ols and phenylpropionic acid. When rats were fed with different diets containing monomeric, dimeric or oligomeric flavan-3-ols, the urinary excretion of microbial metabolites was also found to vary according to the flavan-3-ols source (Gonthier et al., 2003).

However, no significant correlation coefficients (P > 0.05) were found between changes in microbial population and changes in precursor flavan-3-ols and/or microbial metabolites during the fermentation of the two fractions (data not shown). In any case, the period for the most important changes in microbiota (5–10 h of fermentation) coincided with the maximum changes in both, precursors and phenolic metabolites (i.e. phenyl-γ-valerolactones and phenylvaleric acid derivatives). Recently, some authors have found correlation between changes in microbial population and microbial metabolites, but only after a prolonged exposure to polyphenols either in vitro (using a continuous gut model) or in vivo (long-term feeding studies in humans and animals) (Li et al., 2008; van Duynhoven et al., 2010). Therefore, besides the large inter-individual variations observed among volunteers, short-term exposure (48 h) to the GSE fractions could partly explain the lack of correlation found in our study. As pointed out by Kemperman et al. (2010), a prolonged exposure to polyphenols might be needed to induce polyphenol-resistance and bioconversion genes on the microbiota. This occurrence of this process may be needed to find correlation with metabolite data.

In conclusion, this study has demonstrated that, in vitro experiments, GSEs of different flavan-3-ol composition could promote the growth of potentially beneficial bacteria (Lactobacillus sp) and lower the counts of undesirable bacteria such as clostridia. Both fractions seemed to exert similar effects on the different microbial groups in spite of their differences in composition (monomers vs. oligomers). The extent of the microbial catabolism of flavan-3-ols, as measured by the decrease in the concentration of precursor flavan-3-ols and formation of phenolic microbial metabolites, also is affected by the fraction composition during the initial phases of the fermentation, but differences were not significant between the fractions, with the exception of galloylated flavan-3-ols and phenylpropionic acid. In addition, the formation of microbial metabolites revealed the possibility of the existence of different bioconversion pathways for the catabolism of monomeric and oligomeric flavan-3-ols. Despite that changes evidencing the polyphenol–microbiota interaction coincided in time (first 10 h of fermentation), no statistical correlation was found between changes in microbial population and changes in precursor flavan-3-ol or microbial metabolites, probably because of the short exposure period (48 h) and large inter-individual differences observed among volunteers. These findings suggest that the flavan-3-ol profile of a particular food source could affect the microbiota composition and its catabolic activity, inducing changes that could in turn affect the bioavailability and potential bioactivity of these compounds.

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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Comparative chromatograms (280 nm) of phenolic compounds from GSE-O (grape seed extract-oligomeric) and GSE-M (grape seed extract-monomeric) fractions.

**Fig. S2.** Changes in MS response of precursor phenolic compounds during faecal fermentation (0, 5, 10, 24, 30 and 48 h) of the grape seed extract GSE-M. MRM chromatograms of (+)-catechin, (−)-epicatechin, (−)-epigallocatechin gallate, dimeric procyanidins B3, B1, B2 and B4, galloylated dimeric procyanidins B2-3′-O-gallate and B2-3″-O-gallate, and trimeric procyanidins C1 and T2.