GUT MICROBIAL CATABOLISM OF GRAPE SEED FLAVAN-3-OLS BY HUMAN FAECAL MICROBIOTA. TARGETED ANALYSIS OF PRECURSOR COMPOUNDS, INTERMEDIATE METABOLITES AND END PRODUCTS

Fernando Sánchez-Patán[1], Carolina Cueva[1], Maria Monagas[1], Gemma E.Walton[2], Glenn R. Gibson[2], Pedro J. Martín-Álvarez[1], M. Victoria Moreno-Arribas[1], Begoña Bartolomé[1*]

[1]Instituto de Investigación en Ciencias de la Alimentación (CIAL). CSIC-UAM. C/ Nicolás Cabrera, 9. 28049. Madrid. Spain

[2]Food and Microbial Sciences Unit, School of Chemistry, Food and Pharmacy, University of Reading, Reading RG6 6AP, UK

* Corresponding author: bartolome@ifi.csic.es

Key words: 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, 4-hydroxy-5-(3',4'-hydroxyphenyl)-valeric acid, flavan-3-ols, grape seed extract, microbial catabolism.

Abstract

I*n vitro* batch culture fermentations were conducted with grape seed polyphenols and human faecal microbiota, in order to monitor both changes in precursor flavan-3-ols as well as the formation of microbial-derived metabolites. By the application of UPLC-DAD-ESI-TQ MS, monomers, dimeric and trimeric procyanidins, were shown to be degraded during the first 10 h of fermentation, with notable inter-individual differences being observed between fermentations. This period (10 h) also coincided with the maximum formation of intermediate metabolites such as 5-(3',4'-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3',4'-hydroxyphenyl)-valeric acid, and of several phenolic acids, including 3-(3,4-dihydroxyphenyl)-propionic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxymandelic acid, and gallic acid (5-10 h maximum formation). Later phases of the incubations (10-48 h) were characterized by the appearance of mono- and non-hydroxylated forms of previous metabolites by dehydroxylation reactions. Of particular interest was the detection of γ-valerolactone, which was seen for the first time as a metabolite from the microbial catabolism of flavan-3-ols. Changes registered during fermentation were finally summarized by a principal component analysis (PCA). Results revealed that 5-(3',4'-dihydroxyphenyl)-γ-valerolactone was a key metabolite in explaining inter-individual differences and delineating the rate and extent of the microbial catabolism of flavan-3-ols, which could finally affect absorption and bioactivity of these compounds.

INTRODUCTION

Over the last decade, a number of epidemiological and interventional studies have demonstrated that there may be an association between polyphenol consumption and human health (Arts, Jacobs Jr, Harnack, Gross & Folsom, 2001; Hertog, Feskens & Kromhout, 1997; Knekt et al., 2000). However, before exhibiting their activity in the human organism, polyphenols need to be bioavailable. Therefore, bioavailability is a key issue linking food polyphenols and human health. Many polyphenols present poor absorption in the small intestine but are metabolized by the colonic microbiota into simple phenolic compounds that could be further absorbed (Aura, 2008; Selma, Espín & Tomás-Barberán, 2009; Williamson & Clifford, 2010). A recent study in ileostomy subjects has estimated that around 70% of a green tea intake is present in ileal fluid (Stalmach, Mullen, Steiling, Williamson, Lean & Crozier, 2010), confirming that, in general, the amount of non-absorbable polyphenols reaching the colon is high and that microbial-derived phenolic metabolites must be partly responsible for phenolic-associated health benefits (Williamson et al., 2010). Among these non-absorbable polyphenols are flavan-3-ols, in particular their oligomeric and polymeric forms or proanthocyanidins, which are very abundant in wine, grape, cocoa, apple, and nut skins, among other sources (Manach, Scalbert, Morand, Rémésy & Jiménez, 2004). Bioavailability of flavan-3-ols is influenced by the degree of polymerization (DP). Monomeric flavan-3-ols are readily absorbed in the small intestine, dimeric procyanidins present low absorption, whereas oligomeric (DP ≥3) and polymeric forms require activities by the colonic microbiota in order to be absorbed and further metabolized (Déprez et al., 2000; Gonthier, Donovan, Texier, Felgines, Remesy & Scalbert, 2003b; Monagas et al., 2010). Moreover, the colonic microbiota also plays an

important role in the overall bioavailability of the absorbable-flavan-3-ol fraction, since these compounds also reach the colon by enterohepatic recirculation as phase II conjugates that are deconjugated and further catabolized by the microbiota. The colonic metabolism of flavan-3-ols is complex and despite enormous effort being devoted to this area in recent years, catabolic pathways are still under elucidation (Appeldoorn, Vincken, Aura, Hollman & Gruppen, 2009; Stoupi, Williamson, Drynan, Barron & Clifford, 2010) and the bacteria responsible have not been identified yet. Although many of the final microbial metabolites are common to those formed by the catabolism of other flavonoids, which mainly includes phenylacetic, phenylpropionic and benzoic acids of different degree of hydroxylation, intermediate metabolites such as 5-(3',4'-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3',4'-hydroxyphenyl)-valeric acid, are characteristics of the catabolism of flavan-3-ols but often overlooked. However, recent studies have indicated that their occurrence and residence time in humans deserves consideration for possible local or systemic effects (Garrido et al., 2010; Llorach et al., 2010). Although the bioactivity of microbial catabolites are still largely unknown, in particular in their actual conjugated form, evidence related to the antioxidant, anti-inflammatory and anti-proliferative effects of 5-(3,4-dihydroxyphenyl)-γ-valerolactone have been reported (Grimm, Schäfer & Hoögger, 2004; Lambert, Rice, Hong, Hou & Yang, 2005). Concerning microbial-derived phenolic acids, antioxidant, anti-thrombotic, anti-inflammatory and anti-proliferative activities, as well as inhibition of pathogenic bacteria and modulation of lipid metabolism, have been described (Monagas et al., 2010).

Studies on the microbial catabolism of grape or wine flavan-3-ols (Gross et al., 2010; Grün et al., 2008; van Dorsten, Grün, van Velzen, Jacobs, Draijer & van Duynhoven,

2009; Ward, Croft, Puddey & Hodgson, 2004) or individual monomeric flavan-3-ols and dimeric procyanidins (Appeldoorn et al., 2009; Kohri, Suzuki & Nanjo, 2003; Meselhy, Nakamura & Hattori, 1997; Roowi, Stalmach, Mullen, Lean, Edwards And & Crozier, 2010; Stoupi et al., 2010; Takagaki & Nanjo, 2010; van't Slot & Humpf, 2009), have been carried out. Most of these studies have monitored the formation of metabolites during the time-course of fermentation but rarely have concurrently studied disappearance of the original substrate or precursor compounds. The aim of the present work was to conduct *in vitro* batch fermentation experiments of a grape seed extract with human faecal microbiota in order to assess both changes in non-galloylated and galloylated monomeric, dimeric and trimeric precursor flavan-3-ols, as well as the formation of a wide range of intermediate and final microbial metabolites derived from flavan-3-ol catabolism.

MATERIALS AND METHODS

Materials

Standards of phenolic compounds were purchased from Sigma-Aldrich Chemical Co (St. Loius, MO), Phytolab (Vestenbergsgreuth, Germany) or Extrasynthèse (Genay, France). Vitaflavan® extract obtained from grape seeds was kindly provided by Les Dérives Resiniques & Terpéniques (DRT), S.A. (France). LC grade solvents were purchased from Lab-Science (Sowinskiego, Poland) or from Scharlau (Barcelona, Spain). Bacteriological growth media supplements were obtained from Oxoid Ltd (Basingstoke, Hants, UK). The remaining chemicals and reagents were obtained either from Sigma-Aldrich Co. Ltd (Poole, Dorset, UK) or Fisher (Loughborough, Leics, UK)

Faecal sample preparation

Faecal samples were collected from three different individuals. All volunteers were in good health and had not been given antibiotics for at least 6 months before the study. Samples were collected, on site, on the day of the experiment and were used immediately. Samples were diluted 1:10 (w/v) with anaerobic phosphate buffer (1 M; pH 7.2) and homogenized in a stomacher for 2 min. Resulting faecal slurries from each individual (i.e. faecal samples were not pooled) were used to inoculate batch-culture vessels.

Batch-culture fermentations

Batch-culture fermentation vessels (300 ml volume; one vessel per treatment group) were sterilized and filled with 135 ml basal nutrient medium (peptone water (2 g/l), yeast extract (2 g/l), NaCl (0.1 g/l), K_2HPO_4 (0.04 g/l), KH_2PO_4 (0.04 g/l), $NaHCO_3$ (2 g/l), $MgSO_4.7H_2O$ (0.01 g/l), $CaCl_2.6H_2O$ (0.01 g/l), Tween 80 (2 ml/l), hemin (50 mg/l), vitamin K (10 μl/l), L-cysteine (0.5 g/l), bile salts (0.5 g/l), resazurin (1 mg/l) and distilled water). The pH of the basal medium was adjusted to 7.0 and autoclaved before dispensing it to the vessels. Medium was then gassed overnight (15mL/min) with O_2-free N_2. Before the addition of faecal slurry samples, temperature of the basal nutrient medium was set to 37ºC by using a circulating water-bath and the pH was maintained at 6.8, using an Electrolab pH controller, in order to mimic conditions in the distal region of the human large intestine (anaerobic; 37ºC; pH about 6.8). Vessels were inoculated with 15 ml faecal slurry (1:10, w/v) and then Vitaflavan® grape seed extract (Table 1) was added at a concentration of 600 mg/l. 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) in sterile Eppendorf tubes (1.5 ml). Two different control

experiments were conducted: *a)* incubations of the faecal microbiota in medium, but lacking the grape seed extract, to monitor metabolites arising from basal metabolism; *b)* incubations of the grape seed extract in medium but without faecal microbiota, to monitor changes due to the non-microbial chemical transformation of precursor compounds of the substrate. Samples were stored at -70ºC until required for the analysis of phenolic compounds by UPLC-PAD-ESI-TQ MS. Before injection, samples were defrosted, centrifuged (14926 *g*, 20 ºC, 10 min) and filtered through a 0.22 µm PVDF filter (Teknokroma, Barcelona, Spain), and finally diluted (1:1, v/v) with a mixture of water/acetonitrile (6:4, v/v).

Analysis of phenolic precursors and microbial metabolites by UPLC-DAD-ESI-TQ MS

An UPLC system coupled to a Acquity PDA eλ photodiode array detector (DAD) and a Acquity TQD tandem quadrupole mass spectrometer equipped with Z-spray electrospray interfece (UPLC-DAD-ESI-TQ MS) (Waters, Milford, MA, USA) was used. Separation (2 µL) was performed on a Waters® BECH C18 column (2.1 x 100 mm; 1.7 µm) at 40ºC. 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 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% B. The DAD was operated in the 250-420 nm wavelength range at a 20 point/s rate and 1.2 nm resolution. The ESI parameters were: Capillary voltage, 3 kV; source temperature, 130 °C; desolvation temperature, 400 °C; desolvation gas (N_2) flow rate, 750 L/h; cone gas (N_2) flow rate, 60 L/h. The ESI was operated in negative mode, except for γ-valerolactone which was operated in positive

mode. For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound, and using external calibration curves. For microbial phenolic metabolites, MRM transitions were those described by Sánchez-Patán et al. (Sánchez-Patán, Monagas, Moreno-Arribas & Bartolomé, 2011). For phenolic precursors in the both grape seed extract and culture samples, the MS parameter optimization, MRM transitions, tested concentration range, and limits of detection and quantification are summarized in Table 2. MRM transitions of other metabolites studied were: 1-(3',4'-dihydroxyphenyl)-3-(2", 4", 6"-trihydroxyphenyl)-propan-2-ol (291/247); 1-(hydroxyphenyl)-3-(2", 4", 6"-trihydroxyphenyl)propan-2-ol (275/231); 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (207/163); 5-(3'-hydroxyphenyl)-γ-valerolactone (191/147); 5-(phenyl)-γ-valerolactone (175/147); γ-valerolactone (101/55); 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid (225/163); 4-hydroxy-5-(3'-hydroxyphenyl)-valeric acid (209/147); 4-hydroxy-5-(phenyl)-valeric acid (193/175). Quantification of procyanidin B2 and B3 was carried out in function of 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 on 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-dihydroxyphenyl)-propionic and 3-(3-hydroxyphenyl)-propionic acids, respectively. Table 2 reports limit of detection (LOD) and limit of quantification (LOQ) of the flavan-3-ol standards used. For phenolic acids and other related phenolic compounds, LOD and LOQ data under these analytical conditions are reported elsewhere (Sánchez-Patán et al., 2011). LOD and LOQ for γ-valerolactone were calculated as 0.035 and

0.100 μg/mL, respectively. Data acquisition and processing was carried out using MassLynx 4.1. software.

Statistical analysis

The statistical methods used for data processing were: simple regression to study the time-course degradation of one of the volunteers using a logarithmic-Y square root-X model (Y= exp (a + b* sqrt(X)) and the STATGRAPHICS Centurion XV program for Windows, version 15.2.00 (StatPoint Inc. 1982-2006, www.statgraphics.com); and principal component analysis (PCA), from standardized variables, to summarize changes in the concentration of both precursor flavan-3-ols and microbial phenolic metabolites resulting from the batch culture fermentation of the grape seed extract, with the STATISTICA program for Windows, version 7.1 (StatSoft. Inc. 1984–2006, www.statsoft.com).

RESULTS AND DISCUSSION

Changes in precursor phenolic compounds during faecal fermentation of the grape seed extract

Phenolic composition of the grape seed extract used in this study is summarized in Table 1. It was mainly composed of flavan-3-ols including: non-galloylated monomers (+)-catechin and (-)-epicatechin (42.2 %), (-)-epigallocatechin gallate (7.8 %), non-galloylated dimeric procyanidins B1, B2, B3, B4 (42.1 %), galloylated dimeric procyanidins B2-3-*O*-gallate and B2-3'-*O*-gallate (1.0 %), and trimeric procyanidins C1 and T2 (4.1%). Non-flavonoid compounds such as gallic acid accounted for 2.7 % of total individual phenolic compounds quantified.

In order to follow progress of the *in vitro* fermentations of the grape seed extract with faecal microbiota, both precursor phenolic compounds and microbial phenolic metabolites were targeted during the time course of different experiments. Changes in the flavan-3-ol profile originally present in the grape seed extract referred to flavan-3-ols monomers ((+)-catechin, (-)-epicatechin and (-)-epigallocatechin gallate) (Figure 1), dimeric procyanidins (B1, B2, B3, B4, B2-3-O-gallate and B2-3'-O-gallate), and trimeric procyanidins (C1 and T2) (Figure 2). During the fermentations, a progressive decline in the concentration of flavan-3-ols was observed, showing very large interindividual variation among volunteers. For non-galloylated monomers, (+)-catechin and (-)-epicatechin, volunteers 2 and 3 (V2 and V3) showed faster degradation rate than volunteer 1 (V1), showing almost complete degradation (≈ 92 %) of these substrates during the first 10 h of fermentation, whereas for V1 this was attained at 24 h (Figures 1A and 1B) . The slower degradation rate of V1 was also observed in the case of non-galloylated dimers B1, B2 and B3 and trimers C1 and T2, independently of the structural conformation (Figures 2A-D, 2G and 2H). However, similarity in the monomeric flavan-3-ol profile observed for V2 and V3 was not seen for these compounds, in particular at 5 h of fermentation, time at which V3 showed an unexpected increase in the level of each of these dimeric and trimeric precursors. A marked increase was also observed for procyanidin B4 for the three volunteers (V1 at 10 h, and V2 and V3 at 5 h). Transient increases in the concentration of trimeric and tetrameric procyanidins, during batch culture fermentations have also been described in previous studies performed with a cocoa flavan-3-ol extract (Tzounis, Rodriguez-Mateos, Vulevic, Gibson, Kwik-Uribe & Spencer). Despite these findings, procyanidins were practically completed degraded by the 10 h of fermentation, as observed for monomers.

Regarding galloylated flavan-3-ols, changes were observed in the degradation profile of volunteers in comparison to non-galloylated flavan-3-ols. For example, V1, who showed the lowest rate of degradation of non-galloylated flavan-3-ols, presented a fast degradation of both monomeric ((-)-epicatechin-3-*O*-gallate) and dimeric (B2-3-*O*-gallate and B2-3'-*O*-gallate) galloylated forms, and comparable to that observed for V2, whereas V3 was a rather slow biotransformer of these compounds. These findings suggest possible variations in the metabolic activity of the microbiota, particularly in esterase activity (i.e., tannase), among volunteers. This microbiota activity has been reported to occur during *in vitro* batch fermentations of green tea galloylated flavan-3-ols (Kohri et al., 2003; Meselhy et al., 1997; Roowi et al., 2010; Takagaki et al., 2010).

In summary, from the three volunteers, V2 was the only one that appeared to be a good biotransformer of both galloylated non-galloylated flavan-3-ols. In the case of monomers, the time-course degradation of this volunteer was satisfactorily adjusted to a logarithmic-Y square root-X model: (+)-catechin = $e^{2.52716 - 1.08351*\sqrt{time}}$ (with R^2=0.937 and standard error of the estimate= 0.7828); (-)-epicatechin = $e^{2.92131 - 1.26803*\sqrt{time}}$ (with R^2=0.971 and standard error of the estimate= 0.607436);

(-)-epicatechin-3-O-gallate = $e^{1.56667 - 1.2748*\sqrt{time}}$ (with R^2=0.902 and standard error of the estimate= 1.1706). Taking into consideration that the constant term of the equation (1.08 for (+)-catechin, 1.26 for (-)-epicatechin and 1.57 for (-)-epicatechin-3-*O*-gallate) is indicative of the rate of degradation, it seems that for V2, (+)-catechin is degraded slightly slower than (-)-epicatechin and its gallic acid ester, but there was no apparent influence of galloylation in the rate of degradation of monomeric flavan-3-ols.

Changes in microbial phenolic metabolites during faecal fermentation of the grape seed extract

The catabolic pathway of flavan-3-ols, which is still under elucidation, is very complex involving numerous reactions (hydrolysis, hydrogenation, α- and β-oxidation, dehydroxylation, demethoxylation, decarboxylation) that result in the formation of a wide range of phenolic and aromatic catabolites, many of which may not yet have been identified. A total of 47 potential phenolic metabolites including, hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, hydroxybenzoic and hydroxymandelic acids, as well as simple phenols, were targeted by UPLC-DAD-ESI-TQ MS during the time course of batch fermentations, as described by Sánchez-Patán et al. (Sánchez-Patán et al., 2011). In addition, characteristic metabolites exclusively derived from the catabolism of flavan-3-ols such as phenyl-γ-valerolactones and phenylvaleric acid derivatives, were also screened. From all targeted metabolites, methoxylated metabolites including 3-*O*- and 4-*O*-methyl gallic acids, 4-hydroxy-3-methoxy-phenylacetic acid, syringic, vanillic and ferulic acids, as well as other phenolic acids such as *p*-coumaric, caffeic and protocatechuic acids, were only detected at trace levels that were under the limits of quantification of our method (Sánchez-Patán et al., 2011).

Phenyl-γ-valerolactone and 4-hydroxy-5-(phenyl)-valeric acid derivatives

The first steps in the microbial degradation of flavan-3-ols involve reductive cleavage of the heterocyclic C-ring resulting in the formation of diphenylpropan-2-ols, which by further breakdown of the A-ring and lactonization results in phenylvalerolactones derivatives (Groenewoud & Hundt, 1986; Meselhy et al., 1997). As an intermediate metabolite, 1-(3',4'-dihydroxyphenyl)-3-(2", 4", 6"-trihydroxyphenyl)-propan-2-ol was

only detected at trace levels at 10 h of fermentation (data not shown). However, the formation of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone was clearly detected and started to be registered between 0-5 h of fermentation reaching a maximum concentration at 10 h (Figure 3A), time period which coincided with an almost total disappearance of precursor flavan-3-ols (Figure 1 and 2). Results from *in vitro* studies have shown that the time at which the maximum formation of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone occurs (T_{max}) is largely variable and could also be affected by the flavan-3-ol structure of the precursor compound and type of microbiota used (i.e, human or animal). Using human microbiota, Stoupi et al. (Stoupi et al., 2010) found that the maximum concentration of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone occurred at 12 h during the *in vitro* fermentation of procyanidin B2, although it was detected much later (24 h) in the case of (-)-epicatechin. For (-)-epicatechin-3-*O*-gallate, the maximum production of this metabolite also occurred around 24 h (Meselhy et al., 1997). Other authors have reported that the T_{max} of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone varied between 8-24 h during the *in vitro* fermentation for green tea catechins [(-)-epicatechin, (-)-epigallocatechin, or (-)-epigallocatechin-3-O-gallate] (Roowi et al., 2010). Using rat faecal microbiota, only the 3',5'-dihydroxylated form (i.e., 5-(3',5'-dihydroxyphenyl)-γ-valerolactone) was detected and found as a minor product reaching T_{max} at 69 h of fermentation (Takagaki et al., 2010).

Large inter-individual variations were again observed among volunteers but coinciding with the fact that V1, the slower biotransformer of flavan-3-ols, also presented the lowest accumulation of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone. On the other hand, V3, which exhibited a transient increase at 5 h in the level of dimeric and trimeric procyanidins (Figure 2), also produced the highest level of this metabolite. *In vivo*

studies have also reported large inter-individual variation in the urinary excretion of this metabolite after ingestion of tea (Li et al., 2000; Meng et al., 2002) and almond skin (Garrido et al., 2010; Llorach et al., 2010) polyphenols.

The presence of 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid, the open form of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, was also detected, and gave a very similar profile to that of the 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (Figure 3B). It has been proposed that these compounds could arise from the degradation of diphenylpropan-2-ols, concurrently with the phenylvalerolactone form (Kohri et al., 2003). Recently, other authors have proposed that an interconversion between both forms (5-(3',4'-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid) may also exist, but being largely displaced towards the formation of 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid (Stoupi et al., 2010). Metabolomic studies carried out with human urine samples after the intake of a flavan-3-ol-rich extract from almond skins also suggested that formation of these metabolites was largely interrelated (Llorach et al., 2010). Although this interconversion has not still been demonstrated to occur *in vivo*, recent studies have reported that the pH of extraction medium promotes chemical interconversion between both forms (Takagaki et al., 2010).

Due to large inter-individual differences observed among volunteers, it was difficult to establish the correct sequence of formation of 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid. On one hand, the similarity in profile with 5-(3',4'-dihydroxyphenyl)-γ-valerolactone observed for V1 and V2 suggests that 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid could be formed together with former metabolite. However, on the other hand, the profile presented by V3 showing a further

accumulation up to 24 h, suggests that the second pathway involving interconversion could also occur. In fact, three different pathways have been recently proposed for the catabolism of (-)-epigallocatechin-3-O-gallate by rat intestinal microflora (Takagaki et al., 2010). Considering these facts, large differences in the occurrence (as well as the T_{max}) of 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid have been reported varying with the structure of the flavan-3-ol precursor and type of microbiota, as in the case of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (Stoupi et al., 2010; Takagaki et al., 2010).

Dehydroxylation reactions, which preferentially take place at position C-4', occurred during later phase of the flavan-3-ol catabolism, leading to the formation 5-(3'-hydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3'-hydroxyphenyl)-valeric acid (Figure 3C and 3D, respectively). The trend of these two metabolites was very similar for each volunteer, showing a progressive increase up to 48 h of fermentation in the case of V1, and up to 30 h in the case of V3. However, for V2 the formation of both metabolites occurred earlier (up to 24 h) and markedly decreased afterwards. It is important to note change in the formation extent of these later metabolites shown by each volunteer in comparison to that of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone: V3 now showed a lower/similar formation rate than V1, indicating possible differences in the metabolic activity (i.e. dehydroxalases) of the microbiota among volunteers. In any case, these trends indicate that dehydroxylation reactions certainly occur after the formation of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid, and not from the flavan-3-ol compounds originally present in grape seed extract. This was further confirmed by the profile observed for 4-hydroxy-5-(phenyl)-valeric acid (Figure 3G), which showed a much slower accumulation during the time-course of fermentation in the case of the three volunteers.

However, the corresponding non-hydroxylated derivative of the lactone form, 5-(phenyl)-γ-valerolactone (Figure 3F), was not detected. Instead, of particular interest was the detection of the simple γ-valerolactone metabolite, which tended to accumulate in the last phases of microbial fermentation up to 24 h (for V1 and V3) or in keeping with the profile observed for 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (for V2), indicating that the breakdown of the phenyl moiety may also occur as a consequence of microbial catabolism. As far as we are aware, this is the first time that γ-valerolactone is being reported as microbial metabolites derived from the catabolism of flavan-3-ols.

Phenylpropionic and phenylacetic acid derivatives

Parallel to the previous interconversion and dehydroxylation reactions, β-oxidation of phenylvaleric acid derivatives are considered to occur resulting in phenylpropionic acid derivatives. During the *in vitro* fermentation of the grape seed extract, 3-(3,4-dihydroxyphenyl)-propionic acid, which has been reported as one of the most abundant final microbial metabolites derived from the catabolism of flavan-3-ols, both *in vivo* and *in vitro* (Gonthier et al., 2003a; Rios et al., 2003; Ward et al., 2004), was detected (Figure 4A). This metabolite showed a rapidly increase from 0 to 5 h of fermentation, reaching a peak level at 10 h and markedly declining at 24 h (in the case of V2 and V3), whereas V1 again showed a more discrete evolution trend. In contrast to our results, Stoupi et al. (Stoupi et al., 2010) reported that the formation of 3-(3,4-dihydroxyphenyl)-propionic acid started to be registered much later (12 h and 24 h of the fermentation in the case (-)-epicatechin and procyanidin B2, respectively). It is of note that the profile observed was similar to that of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, indicating the possible coexistence of different catabolic pathways leading to formation of this metabolite, as proposed by Appeldoorn et al. (Appeldoorn

et al., 2009). Nevertheless, some limitations of the batch culture models should also be considered when evaluating metabolite profiles, in particularly the fact that metabolites accumulated during fermentation, whereas in the *in vivo* case they are concurrently being produced and absorbed from the colonocytes. On the other hand, it is also of note that metabolomic studies carried out with urine collected after the intake of flavan-3-ols rich extracts by humans, revealed that the contribution of 3-(3',4'-dihydroxyphenyl)-propionic acid to urinary metabolome could occur very early (0-5 h after the intake) for its glucuronide conjugates, but later (10-24 h) for its sulfate conjugates (Llorach et al., 2010).

Dehydroxylation of 3-(3,4-dihydroxyphenyl)-propionic acid into 3-(3-hydroxyphenyl)-phenylpropionic acid further occurred during the time course of fermentation, leading to a marked increase in concentration of this metabolite from 10-24 h (Figure 4C). It is of note that the evolution trend of 3-(3-hydroxyphenyl)-propionic acid observed for each volunteer was very similar to that of their corresponding phenyl-γ-valerolactone derivative, suggesting once again a high dehydroxylation activity for the microbiota of V1 and V2, in comparison to that of V3. Some other metabolites resulted from dehydroxylation reactions, in particularly 3-(4-hydroxyphenyl)-propionic acid and phenylpropionic acid (non-hydroxylated form) (Figure 4E and 4G), were also found to be formed during the fermentation of the basal medium (Online Supporting Information, Figure S1A, for phenylpropionic acid), as has been reported in previous *in vitro* experiments (Gross et al., 2010). These metabolites have been reported to be intermediate products of metabolic pathway of phenylalanine (Curtius, Mettler & Ettlinger, 1976).

Another important series of microbial metabolites are phenylacetic acid derivatives. These metabolites are considered to arise from the α-oxidation of phenylpropionic acid derivatives (Meselhy et al., 1997; Stoupi et al., 2010), although an alternative pathway supports that they could exclusively arise from the microbial cleavage of the top unit of dimeric procyanidins (Appeldoorn et al., 2009). The evolutionary trend of 3,4-dihydroxyphenylacetic acid was near to that of 3-(3,4-dihydroxyphenyl)-propionic acid, suggesting once again concurrent formation rather than posterior (Figure 4B). Previous *in vitro* studies have shown that the evolution trend of 3,4-dihydroxyphenylacetic acid largely varies depending on the original substrate to be fermented, green or black tea (Gao et al., 2006). Dehydroxylation at C-4 leads to the production of 3-hydroxyphenylacetic acid, which showed an increase from 10 to 24 h of fermentation, particularly in V2 (Figure 4D). On the other hand, the product resulting from the dehydroxylation at C-3 (4-hydroxyphenylacetic acid) (Figure 4F) was shown to be largely formed from the fermentation of basal medium (Figure S1B). Similar to this was the case of phenylacetic acid (Figure 4H and Figure S1C) (Gross et al., 2010). Both metabolites have been reported to be formed from the metabolism of certain aminoacids such as tyrosine and phenylalanine, respectively (Curtius et al., 1976).

Benzoic acid derivatives and other metabolites

Finally, benzoic acid derivatives are among the end products of the microbial catabolism of flavan-3-ols. They usually arise from the β-oxidation of phenylpropionic acid derivatives. However, in the presence of galloylated flavan-3-ols, as is the case of grape seed extract, microbial esterase activity results in the formation of gallic acid during the initial phases of fermentation, as could be observed from its rapid increase in concentration from 0-5 h (Figure 5A), which is consistent with a sharp decline observed

in monomeric and dimeric galloylated flavan-3-ols during the same period of time (Figures 1 and 2). This profile is in agreement with that observed after the *in vitro* fermentation of black tea and green tea catechins (Gross et al., 2010; Roowi et al., 2010). However, the posterior decarboxylation into pyrogallol was not detected in our study. Instead, catechol/pyrocatechol was detected from 10 h of fermentation on, progressively increasing up to 30 h in the case of V1 and V3, and up to 48 h for V2 (Figure 5B).

Other metabolites, including 4-hydroxybenzoic and benzoic acids were also detected during time-course of fermentation (Figures 5D and 5E, respectively). The origin of 4-hydroxybenzoic acid seems to be more in line with its corresponding phenylpropionic acid derivative (Figure 4E). On the other hand, benzoic acid showed a non-uniform tendency during fermentation, but its formation partly aroused from the fermentation of the basal medium (data not shown). 4-Hydroxybenzoic and benzoic acids are important precursors of the hepatic metabolites 4-hydroxyhippuric and hippuric acids, respectively, which are among the most abundant end products of the metabolism of flavan-3-ols.

Another metabolite which deserves consideration is 4-hydroxymandelic acid. Although this metabolite has been reported to arise from non-phenolic sources such as tyramine and similar biogenic amines (Scheline, 1991), we did not detected any formation of 4-hydroxymandelic acid with the incubations carried out with the medium lacking the grape seed extract (data not shown), indicating that it arose from the microbial degradation of the grape seed flavan-3-ols. Recently, this metabolite has also been

detected in human urine after the intake of red wine/red juice polyphenols (van Dorsten et al., 2009) , although its origin was not clearly attributed to polyphenols.

Integrated summary of changes detected in the phenolic profile during faecal fermentation of the grape seed extract

A Principal Component Analysis (PCA) was performed in order to summarize changes in the concentrations of both precursor flavan-3-ols and microbial phenolic metabolites resulting from the batch culture fermentation of the grape seed extract. Two principal components (PC1 and PC2), which explained 66.2 % of the total variance of the data, were obtained. To show the changes over time, scores of the samples in the different time periods (0, 5, 10, 24, 30 and 48 h) for the 3 volunteers were plotted in the plane defined by the first two principal components (Figure 6).

The first principal component (PC1), explaining 54.8% of the total variance, was negatively correlated (loadings ≤ -0.7) with precursor flavan-3-ols: (+)-catechin, (-)-epicatechin, procyanidins B1, B2, B3 and B4, (-)-epicatechin-3-*O*-gallate, B2-3-*O*-gallate, B2-3'-*O*-gallate, procyanidins C1 and T2. On the other hand, it was positively correlated (loadings ≥ 0.7) with the following microbial phenolic metabolites: catechol/pyrocatechol, 5-(3'-hydroxyphenyl)-γ-valerolactone, 4-hydroxy-5-(phenyl)-valeric acid, 3-(3-hydroxyphenyl)-propionic acid, phenylpropionic acid, 4-hydroxyphenylacetic acid and phenylacetic acid. Finally, the second principal component (PC2), explaining 11.4% of the total variance, was negatively correlated with 5-(3', 4'-dihydroxyphenyl)-γ-valerolactone.

Changes observed in PC1 values during first 10 h of fermentation were explained by precursor flavan-3-ols (negatively correlated with PC1), whereas as those occurring from 10-48 h were explained by microbial phenolic metabolites (positive correlated with PC1). In other words, PC1 reflected overall changes (decrease in precursor compounds and increase in microbial phenolic metabolites) occurring during the time-course of the microbial catabolism of the grape seed extract. In the case of PC2, negative values increased during the first 10 h of fermentation, indicating an increase in the concentration of 5-(3', 4'-dihydroxyphenyl)-γ-valerolactone, which was the only compound negative correlated with this component. As fermentation time progressed from 10 h to 48 h, values in PC2 became positive and were indicative of the decreased observed in this metabolite during the latter phases of fermentation. It is of note that this component reflected a marked inter-individual variation in microbial catabolism of the grape seed extract, suggesting that formation of 5-(3', 4'-dihydroxyphenyl)-γ-valerolactone, as an intermediate metabolite, could be a critical step in the rate and extent of flavan-3-ol catabolism and therefore, in delimiting the absorption and further bioactivity of these compounds. Our findings reinforce the idea that 5-(3',4'-dihydroxyphenyl)-γ-valerolactone could be a potential biomarker of flavan-3-ols intake, as suggested from previous metabolomic studies (Llorach et al., 2010). However, as far as we are aware, there is still no reports of bacteria with the ability to form 5-(3', 4'-dihydroxyphenyl)-γ-valerolactone from the catabolism of flavan-3-ols. The human bacterium *Eubacterium* sp. SDG-2 was able to open the ring of the *3R* [(-)-catechin and (-)-epicatechin] and the *3S* [(+)-catechin and (+)-epicatechin] forms of monomeric flavan-3-ols into 1,3-diphenylpropan-2-ols, but was incapable of producing the same in their galloylated esters (Wang et al., 2001). However, this bacteria was unable to continue the catabolism up to the formation of 5-(3',4'-dihydroxyphenyl)-γ-

valerolactone (Wang et al., 2001). Apparently, structural features, including stereochemistry, as well as the own antimicrobial properties of flavan-3-ols, could have been a limitation in the progress of the identification of flavan-3-ol-metabolizing bacteria, which could be crucial in the activation of the microbial catabolism of flavan-3-ols (Monagas et al., 2010).

CONCLUDING REMARKS

The approach of targeting both changes in precursor flavan-3-ols and microbial phenolic metabolites during faecal fermentation of the grape seed extract followed by the application multivariate statistical analysis, provided an overall picture of distinct phases of the microbial degradation of flavan-3-ols and helped to explain inter-individual variations in microbial catabolism among volunteers. The first phase (0-10 h) was mainly characterized by the formation of the dihydroxylated forms of 5-(phenyl)-γ-valerolactone and 4-hydroxy-5-(phenyl)-valeric acid derivatives and coincided with practically the total consumption of flavan-3-ol precursors by the faecal microbiota. Accumulation of the dihydroxylated forms of phenylpropionic and phenylacetic acids also occurred during this period. Final phases (10-48 h) involved dehydroxylation reactions of metabolites formed in early phases into their mono-hydroxylated, non-hydroxylated forms, and even non-phenolic forms. Our results suggest that although the end products of the microbial catabolism of flavan-3-ols are common to that of other flavonoids, the formation of intermediate characteristic metabolites such as 5-(3', 4'-dihydroxyphenyl)-γ-valerolactone, should be considered a key step in delimiting the rate and extent of flavan-3-ol bioavailability and potential bioactivity.

ACKOWLEDGEMENTS

This work has been funded by the Spanish Ministry for Science and Innovation (AGL2009-13361-C02-01, AGL2010-17499 and CSD2007-00063 Consolider Ingenio 2010 FUN-C-FOOD Projects), Comunidad de Madrid (ALIBIRD P2009/AGR-1469 Project), and Spanish Research Council (CSIC) (Programa Intramural Especial 200470E641). FSP is the recipient of a contract from the JAE-Doc Program (CSIC) and CC is the recipient of fellowships from the FPI-MEC program.

REFERENCES

Appeldoorn, M. M., Vincken, J. P., Aura, A. M., Hollman, P. C. H., & Gruppen, H. (2009). Procyanidin dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-?- valerolactone as the major metabolites. *Journal of Agricultural and Food Chemistry, 57*(3), 1084-1092.

Arts, I. C. W., Jacobs Jr, D. R., Harnack, L. J., Gross, M., & Folsom, A. R. (2001). Dietary catechins in relation to coronary heart disease death among postmenopausal women. *Epidemiology, 12*(6), 668-675.

Aura, A. M. (2008). Microbial metabolism of dietary phenolic compounds in the colon. *Phytochemistry Reviews, 7*(3), 407-429.

Curtius, H. C., Mettler, M., & Ettlinger, L. (1976). Study of the intestinal tyrosine metabolism using stable isotopes and gas chromatography-mass spectrometry. *Journal of Chromatography A, 126*(C), 569-580.

Déprez, S., Brezillon, C., Rabot, S., Philippe, C., Mila, I., Lapierre, C., & Scalbert, A. (2000). Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular-weight phenolic acids. *Journal of Nutrition, 130*(11), 2733-2738.

Gao, K., Xu, A., Krul, C., Venema, K., Liu, Y., Niu, Y., Lu, J., Bensoussan, L., Seeram, N. P., Heber, D., & Henning, S. M. (2006). Of the major phenolic acids formed during human microbial fermentation of tea, citrus, and soy flavonoid supplements, only 3,4-dihydroxyphenylacetic acid has antiproliferative activity. *Journal of Nutrition, 136*(1), 52-57.

Garrido, I., Urpi-Sarda, M., Monagas, M., Gómez-Cordovés, C., Martín-Álvarez, P. J., Llorach, R., Bartolomé, B., & Andrés-Lacueva, C. (2010). Targeted analysis of conjugated and microbial-derived phenolic metabolites in human urine after consumption of an almond skin phenolic extract. *Journal of Nutrition, 140*(10), 1799-1807.

Gonthier, M. P., Cheynier, V., Donovan, J. L., Manach, C., Morand, C., Mila, I., Lapierre, C., Rémésy, C., & Scalbert, A. (2003a). Microbial aromatic acid metabolites formed in the gut account for a major fraction of the polyphenols excreted in urine of rats fed red wine polyphenols. *Journal of Nutrition, 133*(2), 461-467.

Gonthier, M. P., Donovan, J. L., Texier, O., Felgines, C., Remesy, C., & Scalbert, A. (2003b). Metabolism of dietary procyanidins in rats. *Free Radical and Biological Medicine, 35*(8), 837-844.

Grimm, T., Schäfer, A., & Hoögger, P. (2004). Antioxidant activity and inhibition of matrix metalloproteinases by metabolites of maritime pine bark extract (Pycnogenol). *Free Radical Biology and Medicine, 36*(6), 811-822.

Groenewoud, G., & Hundt, H. K. L. (1986). The microbial metabolism of condensed (+)-catechins by rat-caecal microflora. *Xenobiotica, 16*(2), 99-107.

Gross, G., Jacobs, D. M., Peters, S., Possemiers, S., Van Duynhoven, J., Vaughan, E. E., & Van De Wiele, T. (2010). In vitro bioconversion of polyphenols from black tea and red wine/grape juice by human intestinal microbiota displays strong interindividual variability. *Journal of Agricultural and Food Chemistry, 58*(18), 10236-10246.

Grün, C. H., van Dorsten, F. A., Jacobs, D. M., Le Belleguic, M., van Velzen, E. J. J., Bingham, M. O., Janssen, H. G., & van Duynhoven, J. P. M. (2008). GC-MS methods for metabolic profiling of microbial fermentation products of dietary polyphenols in human and in vitro intervention studies. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 871*(2), 212-219.

Hertog, M. G. L., Feskens, E. J. M., & Kromhout, D. (1997). Antioxidant flavonols and coronary heart disease risk. *Lancet*, *349*(9053), 699.

Knekt, P., Isotupa, S., Rissanen, H., Heliövaara, M., Järvinen, R., Häkkinen, S., Aromaa, A., & Reunanen, A. (2000). Quercetin intake and the incidence of cerebrovascular disease. *European Journal of Clinical Nutrition*, *54*(5), 415-417.

Kohri, T., Suzuki, M., & Nanjo, F. (2003). Identification of metabolites of (-)-epicatechin gallate and their metabolic fate in the rat. *Journal of Agricultural and Food Chemistry*, *51*(18), 5561-5566.

Lambert, J. D., Rice, J. E., Hong, J., Hou, Z., & Yang, C. S. (2005). Synthesis and biological activity of the tea catechin metabolites, M4 and M6 and their methoxy-derivatives. *Bioorganic and Medicinal Chemistry Letters*, *15*(4), 873-876.

Li, C., Lee, M. J., Sheng, S., Meng, X., Prabhu, S., Winnik, B., Huang, B., Chung, J. Y., Yan, S., Ho, C. T., & Yang, C. S. (2000). Structural identification of two metabolites of catechins and their kinetics in human urine and blood after tea ingestion. *Chemical Research in Toxicology*, *13*(3), 177-184.

Llorach, R., Garrido, I., Monagas, M., Urpi-Sarda, M., Tulipani, S., Bartolome, B., & Andres-Lacueva, C. (2010). Metabolomics study of human urinary metabolome modifications after intake of almond (Prunus dulcis (Mill.) D.A. Webb) skin polyphenols. *Journal of Proteome Research*, *9*(11), 5859-5867.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: Food sources and bioavailability. *American Journal of Clinical Nutrition*, *79*(5), 727-747.

Meng, X., Sang, S., Zhu, N., Lu, H., Sheng, S., Lee, M. J., Ho, C. T., & Yang, C. S. (2002). Identification and characterization of methylated and ring-fission metabolites of tea catechins formed in humans, mice, and rats. *Chemical Research in Toxicology*, *15*(8), 1042-1050.

Meselhy, M. R., Nakamura, N., & Hattori, M. (1997). Biotransformation of (-)-epicatechin 3-O-gallate by human intestinal bacteria. *Chemical and Pharmaceutical Bulletin*, *45*(5), 888-893.

Monagas, M., Urpi-Sarda, M., Sanchez-Patan, F., Llorach, R., Garrido, I., Gomez-Cordoves, C., Andres-Lacueva, C., & Bartolome, B. (2010). Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. *Food & Function*, *1*(3), 233-253.

Rios, L. Y., Gonthier, M. P., Rémésy, C., Mila, I., Lapierre, C., Lazarus, S. A., Williamson, G., & Scalbert, A. (2003). Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects. *The American journal of clinical nutrition*, *77*(4), 912-918.

Roowi, S., Stalmach, A., Mullen, W., Lean, M. E. J., Edwards And, C. A., & Crozier, A. (2010). Green tea flavan-3-ols: Colonic degradation and urinary excretion of catabolites by humans. *Journal of Agricultural and Food Chemistry*, *58*(2), 1296-1304.

Sánchez-Patán, F., Monagas, M., Moreno-Arribas, M. V., & Bartolomé, B. (2011). Determination of microbial phenolic acids in human faeces by UPLC-ESI-TQ MS *Journal of Agricultural and Food Chemistry*, 59(6), 2241–2247.

Scheline, R. R. (1991). *Handbook of mammalian metabolism of plant compounds*. Boca raton, FL: CRC Press.

Selma, M. V., Espín, J. C., & Tomás-Barberán, F. A. (2009). Interaction between phenolics and gut microbiota: Role in human health. *Journal of Agricultural and Food Chemistry*, *57*(15), 6485-6501.

Stalmach, A., Mullen, W., Steiling, H., Williamson, G., Lean, M. E. J., & Crozier, A. (2010). Absorption, metabolism, and excretion of green tea flavan-3-ols in humans with an ileostomy. *Molecular Nutrition and Food Research*, *54*(3), 323-334.

Stoupi, S., Williamson, G., Drynan, J. W., Barron, D., & Clifford, M. N. (2010). Procyanidin B2 catabolism by human fecal microflora: Partial characterization of 'dimeric' intermediates. *Archives of Biochemistry and Biophysics*.

Takagaki, A., & Nanjo, F. (2010). Metabolism of (-)-epigallocatechin gallate by rat intestinal flora. *Journal of Agricultural and Food Chemistry*, *58*(2), 1313-1321.

Tzounis, X., Rodriguez-Mateos, A., Vulevic, J., Gibson, G. R., Kwik-Uribe, C., & Spencer, J. P. E. Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. *American Journal of Clinical Nutrition*, *93*(1), 62-72.

van't Slot, G., & Humpf, H. U. (2009). Degradation and metabolism of catechin, epigallocatechin-3-gallate (EGCG), and related compounds by the intestinal microbiota in the pig cecum model. *Journal of Agricultural and Food Chemistry*, *57*(17), 8041-8048.

van Dorsten, F. A., Grün, C. H., van Velzen, E. J. J., Jacobs, D. M., Draijer, R., & van Duynhoven, J. P. M. (2009). The metabolic fate of red wine and grape juice polyphenols in humans assessed by metabolomics. *Molecular Nutrition and Food Research*, *54*(7), 897-908.

Wang, L. Q., Meselhy, M. R., Li, Y., Nakamura, N., Min, B. S., Qin, G. W., & Hattori, M. (2001). The heterocyclic ring fission and dehydroxylation of catechins and related compounds by Eubacterium sp. strain SDG-2, a human intestinal bacterium. *Chemical and Pharmaceutical Bulletin*, *49*(12), 1640-1643.

Ward, N. C., Croft, K. D., Puddey, I. B., & Hodgson, J. M. (2004). Supplementation with grape seed polyphenols results in increased urinary excretion of 3-hydroxyphenylpropionic acid, an important metabolite of proanthocyanidins in humans. *Journal of Agricultural and Food Chemistry*, *52*(17), 5545-5549.

Williamson, G., & Clifford, M. N. (2010). Colonic metabolites of berry polyphenols: the missing link to biological activity ? *British Journal of Nutrition*(104), S48-s66.

FIGURE LEGENDS

Figure 1. Changes in monomeric flavan-3-ols during faecal fermentation of the grape seed extract. **A)** (+)-Catechin; **B)** (-)-Epicatechin, and **C)** (-)-Epicatechin-3-*O*-gallate.

Figure 2. Changes in dimeric and trimeric procyanidins during faecal fermentation of the grape seed extract. **A)** B1; **B)** B2, **C)** B3; **D)** B4; **E)** B2-3-*O*-gallate; **F)** B2-3'-*O*-gallate; **G)** C1, and **H)** T2.

Figure 3. Changes in phenyl-γ-valerolactone and 4-hydroxy-5-(phenyl)-valeric acid derivatives during faecal fermentation of the grape seed extract. **A)** 5-(3',4'-Dihydroxyphenyl)-γ-valerolactone; **B)** 4-Hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid; **C)** 5-(3'-Hydroxyphenyl)-γ-valerolactone; **D)** 4-Hydroxy-5-(3'-hydroxyphenyl)-valeric acid; **E)** γ-Valerolactone; **F)** 4-Hydroxy-5-(phenyl)-valeric acid.

Figure 4. Changes in phenylpropionic and phenylacetic acid derivatives during faecal fermentation of the grape seed extract. **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.

Figure 5. Changes in benzoic acid derivatives and other metabolites during faecal fermentation of the grape seed extract. **A)** Gallic acid; **B)** Catechol/pyrocatechol; **C)** 4-Hydroxymandelic acid 4-; **D)** 4-Hydroxybenzoic acid; **E)** Benzoic acid.

Figure 6. Representation of the samples in the plane defined by the first two principal components (PC1 and PC2) resulted from a PCA of both precursor flavan-3-ols and microbial-derived phenolic metabolites for three volunteers (V1, V2, V3) at different incubation times (0, 5, 10, 24, 30 and 48 h).

Table 1. Phenolic composition of the Vitaflavan® grape seed extract.

	mg/g
Gallic acid	9.11 ± 0.01
(+)-Catechin	74.57 ± 0.09
(-)-Epicatechin	67.68 ± 0.75
(-)-Epicatechin-3-*O*-gallate	26.21 ± 0.41
B1	60.99 ± 1.42
B2	45.13 ± 0.95
B3	20.39 ± 0.33
B4	15.04 ± 0.13
B2-3-*O*-gallate	1.80 ± 0.06
B2-3'-*O*-gallate	1.61 ± 0.01
C1	7.07 ± 0.08
T2	6.81 ± 0.06

Mean value (n=3) ± SD

Table 2. MS/MS parameters, injection range, limit of detection (LOD) and quantification (LOQ) of the monomeric and dimeric flavan-3-ols.

Name	MW	Rt (min)	Transition (*m/z*)	Cone (V)	Collision (V)	Injection range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
(+) -Catechin	290	3.84	289>245	40	16	0.005-50	0.005	0.016
(-) -Epicatechin	290	5.27	289>245	38	14	0.005-50	0.010	0.025
(-)-Epicatechin-3-*O*-gallate	442	7.11	441>289	38	14	0.0025-25	0.009	0.023
B1	578	3.56	577>289	45	25	0.0025-25	0.004	0.009
B2	578	4.68	577>289	45	25	0.0025-25	0.012	0.027
C1	866	5.69	865>577	50	25	0.01-100	0.090	0.200