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In Vitro Fermentation of a Red Wine Extract by Human Gut Microbiota: Changes in Microbial Groups and Formation of Phenolic Metabolites

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ABSTRACT: An in vitro batch culture fermentation experiment was conducted with fecal inocula from three healthy volunteers in the presence and absence of a red wine extract. Changes in main bacterial groups were determined by FISH during a 48 h fermentation period. The catabolism of main flavonoids (i.e., flavan-3-ols and anthocyanins) and the formation of a wide a range of phenolic microbial metabolites were determined by a targeted UPLC-PAD-ESI-TQ MS method. Statistical analysis revealed that catechol/pyrocatechol, as well as 4-hydroxy-5-(phenyl)-valeric, 3- and 4-hydroxyphenylacetic, phenylacetic, phenylpropionic, and benzoic acids, showed the greatest increases in concentration during fermentation, whereas 5-(3′-hydroxyphenyl)-γ-valerolactone, its open form 4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid, and 3,4-dihydroxyphenylacetic acid represented the largest interindividual variations in the catabolism of red wine polyphenols. Despite these changes, microbial catabolism did not produce significant changes in the main bacterial groups detected, although a slight inhibition of the *Clostridium histolyticum* group was observed.

KEYWORDS: *red wine, flavan-3-ols, anthocyanins, fermentation, microbial catabolism, fecal bacteria*

INTRODUCTION

Since the postulation of the French paradox by Renaud and de Lorgeril,[1] moderate consumption of red wine has been associated with reduced risk of developing cardiovascular heart disease (CHD). Human intervention trials have suggested that red wine consumption provides antioxidant, antithrombotic, and anti-inflammatory effects, and may exert positive effects on vascular function and lipid metabolism, among other health benefits.[2−5] Such effects of wine in comparison to other alcoholic beverages have been mainly attributed to the presence of polyphenols.

Flavonoids are the most abundant, and among the most bioactive polyphenols present in red wine, mainly including flavan-3-ols and anthocyanins, as well as flavonols, flavanonols, and flavones, in lower proportion.[6] Flavan-3-ols occur as monomeric, oligomeric, or polymeric forms, and the latter two are also known as proanthocyanidins or condensed tannins. Main flavanol monomeric units include catechin, epicatechin, and epicatechin-3-*O*-gallate. Proanthocyanidins are constituted of flavanol units linked by C4−C6 or C4−C8 bonds, presenting a mean degree of polymerization (mDP) of between 3 and 7 units in the case of red wine.[7] The principal anthocyanins identified in red wine include the 3-*O*-monoglucosides and the 3-*O*-acylated monoglucosides of five main anthocyanidins: delphinidin, cyanidin, petunidin, peonidin, and malvidin. Such compounds can be acylated in the C-6 position of the glucose molecule through esterification with acetic, *p*-coumaric, and caffeic acids.

It is established that these flavonoids are metabolized by humans, and information has accumulated in the past few years to suggest that polyphenol metabolites, rather than the original forms present in wine, might be responsible for the health effects derived from wine consumption.[8] However, the different chemical structures of flavan-3-ols and anthocyanins directly influence bioavailability, for example, limiting the site of intestinal absorption either to the small intestine or colon, influencing their pharmacokinetics of absorption, extent of metabolism, and the amount finally excreted in urine[9,10]

Anthocyanin glycosides are first hydrolyzed by the intestinal β-glucosidase or cytosolic β-glucosidases (CBG) within the epithelial cells before absorption in the small intestine, although intact anthocyanins may also be found in plasma and urine.[9] However, monomeric flavan-3-ols and, to a lesser extent, dimeric procyanidins are directly absorbed in the small intestine with no prior chemical modification. Once absorbed, these polyphenols are first metabolized in the small intestine and then in the liver by phase II enzyme into methyl, glucuronide, or sulfate conjugates which are preferentially excreted in the

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bile.[9] In contrast to monomers, oligomers with DP > 3 and polymers of flavan-3-ols are not absorbed in their native forms. These compounds, together with phase-II metabolites reaching the colon by entero-hepatic recirculation, are catabolized by the colonic microbiota before absorption.[11,12] Colonic catabolism involves the formation of simple phenols, phenolic and aromatic acids, and lactones with different degrees of hydroxylation and side-chain length that could be further absorbed and subsequently submitted to intestinal and hepatic metabolism by phase II enzymes.[8,12−14] Consequently, colonic catabolism of red wine polyphenols may play a major role in the production of new phenolic compounds, which could be absorbed and have modified bioactivity compared to the parent compounds, providing systemic or local health effects. At the colonic level, changes in gut microbial population are expected to occur as a result of this polyphenol−bacteria interaction. Recent studies indicate that monomeric flavan-3-ols and flavan-3-ol-rich sources such as chocolate, green tea, and blackcurrant or grape seed extracts may modulate the intestinal microbiota in vivo, producing changes in beneficial bacteria such as *Lactobacillus* but inhibiting other groups such *Clostridium* spp.[15−19] However, evidence related to the effects of red wine polyphenols on gut microbiota is still scarce.[20]

Considering the importance that colonic catabolism of polyphenols may have in overall bioavailability and bioactivity of wine polyphenols, the aim of the present article was to look at the bacteria−polyphenol interactions involved in the colonic metabolism of red wine polyphenols using an in vitro batch culture fermentation model with human fecal microbiota. Changes in main microbial groups, as well as the changes in flavan-3-ols, anthocyanins, and phenolic microbial metabolites, were monitored during fermentation.

MATERIALS AND METHODS

Chemicals. Standards of phenolic compounds were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) (catechin, gallic acid, tyrosol, tryptophol, *trans*-resveratrol, γ-valerolactone, 3-hydroxyphenylacetic acid, phenylacetic acid, 3,4-dihydroxybenzoic acid (protocatechuic acid), syringic acid, 3-hydroxybenzoic acid, benzoic acid, catechol/pyrocatechol, and caffeic acid); from Koch-Light Laboratorie Ltd. (Colnbrook, Bucks, England) (ferulic acid); from Fluka (Buchs, Switherland) (epicatechin, myricetin, 3-(3,4-dihydroxyphenyl)-propionic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid (vanillic acid), and *p*-coumaric acid; from Phytolab (Vestenbergsgreuth, Germany) (Procyanidin C1); from Extrasynthèse (Genay, France) (epicatechin-3-*O*-gallate, quercetin, quercetin-3-*O*-glucoside (isoquercitrin), quercetin-3-*O*-galactoside (hyperoside), kaempferol, procyanidins B1 and B2, malvidin-3-*O*-glucoside, and phenylpropionic acid); from Janssen Chimica (Beerse, Belgium) (3-(4-hydroxyphenyl)-propionic acid and 4-hydroxyphenylacetic acids); and from Alfa Aesar (Karlruhe, Germany) (3-(3-hydroxyphenyl)-propionic acid). 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). Raftilose P95 fructo-oligosaccharide was purchased from Beneo (Tienen, Belgium). Isopore (0.22 μm) membrane filters were obtained from Millipore Corp. (Watford, Hertfordshire, UK). All oligonucleotide probes used for fluorescent in situ hybridization (FISH) were commercially synthesized and labeled 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.

Wine Phenolic Extract. A commercial wine phenolic extract, Provinols, was kindly provided by Safic-Alcan Especialidades, SAU (Barcelona, Spain). Its total phenolic content was 474 mg of gallic acid per gram, and its antioxidant capacity (ORAC value) was 14.5 mmol of Trolox per gram of product.

Fecal Batch-Culture Fermentation. Three independent fermentation experiments were carried out using feces from three different healthy volunteers, who had not ingested antibiotics for at least 6 months before the study and had no history of gastrointestinal disorder. 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 fecal slurries from each individual (i.e., fecal samples were not pooled) were used to inoculate batch-culture vessels. The protocol described by Tzounis et al.[15] was used for fecal fermentations. Briefly, three 300-mL glass fermenter vessels were filled with 135 mL of a prereduced sterile 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), $MgSO_4{\cdot}7H_2O$ (0.01 g/L), $CaCl_2{\cdot}6H_2O$ (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 medium was adjusted to pH 7.0 and continuously sparged with O_2-free N_2 overnight. pH was maintained at 6.8 and the temperature at 37 °C in order to mimic conditions that resemble the distal region of the human large intestine. Vessels were inoculated with 15 mL of fecal slurry (final concentration: 1% w/v). Then, the wine extract (0.6 mg/mL) and a known prebiotic compound (Raftilose P95 FOS, 1% w/v) used as positive control were added to separate stirring batch-culture vessels containing the fecal slurry. A further batch-culture vessel was prepared under the same conditions but without the addition of any compound (negative control). 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 analysis of phenolic metabolites. For this later analysis, samples were stored at −70 °C until required.

Bacterial Enumeration Using FISH. To assess differences in bacterial populations, FISH was used with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA. The probes were commercially synthesized and labeled with the fluorescent dye Cy3. The bacterial groups studied for enumeration were *Bifidobacterium* spp., *Lactobacillus/Enterococcus* spp., *Clostridium histolyticum* group, *Bacteroides* spp., and members of the domain *Bacteria* using the specific probes Bif164,[21] Lab158,[22] Chis150,[23] Bac303,[21] and EUBmix,[24] respectively. For total bacterial counts, 4,6-diamidino-2-phenylindole nucleic acid stain was utilized. Samples fixed in 4% (v/v) paraformaldehyde overnight at 4 °C were then centrifuged at 1500*g* for 5 min, washed twice with PBS (0.1 M; pH 7.0), resuspended in a mixture of PBS−99% ethanol (1:1, v/v), and stored at −20 °C for 1 h. For the hybridizations, 20 μL of each sample was pipetted onto Teflon- and poly-L-lysine-coated six-well slides (Tekdon Inc., Myakka City, FL). The samples were dried onto the slides at 46−50 °C for 15 min and afterward dehydrated in an alcohol series (50%, 80%, and 96%). The ethanol was allowed to evaporate from the slides before probes were applied to the samples. To permeabilize the cells for use with probes Bif164 and Lab158, samples were treated with 20 μL of lysozyme at room temperature for 15 min before being washed briefly (2−3 s) in water and afterward dehydrated in the ethanol series. A probe/hybridization buffer mixture (5 μL of a 50 ng/μL stock of probe plus 50 μL of hybridization buffer) was applied to the surface of each well. Hybridizations were performed for 4 h in an ISO20 oven (Grant Boekel). For the washing step, slides were placed in 50 mL of wash buffer containing 20 μL of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 50 ng/μL; Sigma) for 15 min. They were then briefly washed (2−3 s) in ice-cold water and dried under a stream of compressed air. Five microliters of ProLong Gold antifade reagent (Invitrogen) was added to each well and a coverslip applied. Slides were stored in the dark at 4 °C until cells were counted under a Nikon E400 Eclipse microscope. DAPI stained cells were examined under UV light, and a DM510 light filter was used to count specific bacteria hybridized with the probes. For each slide, 15 random different fields of view were counted. In order to determine changes in the bacterial populations between treatments, we used an "index of specific bacteria" (ISB). The ISB was calculated using the following equation: $ISB = ((Ns(T_1) - Ns(T_0)) - (Nc(T_1) - Nc(T_0))$, where *Ns* is the

number $\log_{10}$ of specific bacteria in a specific test sample, *Nc* is the number $\log_{10}$ of specific bacteria in the negative control (medium + fecal slurry), T_1 is a specific time point, and T_0 is the 0 h time point.

Targeted Analysis of Phenolic Metabolites. Two previously developed UPLC-ESI-TQ MS methods for the particular analysis of flavan-3-ols and their metabolites[25] and anthocyanins were applied to the analysis of the fermentation broths. A UPLC system coupled to an Acquity PDA eλ photodiode array detector and an Acquity TQD tandem quadrupole mass spectrometer (UPLC-PAD-ESI-TQ MS) (Waters, Milford, MA, USA) was used. Separation was performed on Waters BEH C18 columns (2.1 × 100 mm; 1.7 μm) at 40 °C and by applying a flow rate of 0.5 mL/min. For the analysis of flavan-3-ols and phenolic metabolites, a gradient composed of solvent A, water/acetic acid (98:2, v/v), and B, acetonitrile/acetic acid (98:2, v/v), was applied 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. For analysis of anthocyanins, the gradient consisted of A (water/formic acid; 90:10, v/v) and B (acetonitrile) applied as follows: 0−1 min, 5% B; 1−8.09 min, 5−30% B; 8.09−8.67 min, 30−100% B; 8.67−9.84 min, 100−5% B; 9.84−12.17 min, 5% B. In both methods, the ESI parameters were capillary voltage, 3 kV; source temperature, 130 °C; desolvation temperature, 400 °C; desolvation gas (N2) flow rate, 750 L/h; and cone gas (N2) flow rate, 60 L/h. The ESI was operated in negative mode for flavan-3-ol analysis and in positive mode for anthocyanin analysis. The MRM transitions used for detection of flavan-3-ols were 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).[26] The MRM transitions used for the detection of anthocyanins were cyanidin-3-*O*-glucoside (449 > 287), petunidin-3-*O*-glucoside (479 > 317), peonidin-3-*O*-glucoside (463 > 301), malvidin-3-*O*-glucoside (493 > 331), malvidin-3-*O*-(6″-*O*-acetyl)glucoside (535 > 331), and malvidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside (639 > 331). The MRM transitions used for the detection of other wine phenolic compounds were quercetin (301 > 151), kaempferol (285 > 93), myricetin (317 > 179), quercetin-3-*O*-glucoside (463 > 300), quercetin-3-*O*-galactoside (463 > 300), resveratrol (227 > 185), resveratrol-3-*O*-glucoside (389 > 227), tyrosol (137 > 106), tryptophol (160 > 130), cutaric acid (295 > 163), and caftaric acid (311 > 179). For detection of different structures of phenolic metabolites, the MRM transitions used were 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 phenylacetic acid (135 > 91); and for benzoic acids and others, gallic acid (169 > 125), benzoic acid (121 > 77), syringic acid (197 > 182), protocatechuic acid (153 > 109), vanillic acid (167 > 152), *p*-coumaric acid (163 > 119), caffeic acid (179 > 135), ferulic acid (193 > 134), and catechol/pyrocatechol (109 > 81).[25] In the absence of commercial standards, quantification of procyanidin B3 and B4 was carried out using the calibration curve of procyanidin B1, and that of procyanidin T2 was based on procyanidin C1. Procyanidin B2′-3-*O*-gallate was quantified using the epicatechin-3-*O*-gallate curve. Resveratrol-3-*O*-glucoside was quantified using the curve of resveratrol. Cutaric and caftaric acids were quantified using the curves of the free acids (*p*-coumaric and caffeic acids, respectively). Phenyl-γ-valerolactones derivatives were quantified as epicatechin and γ-valerolactone using its own 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. All individual anthocyanins were quantified using the calibration curve of malvidin-3-glucoside. Data acquisition and processing were carried out using MassLynx 4.1. software.

Before injection, samples were defrosted, centrifuged (15000*g*, 20 °C, 10 min), and filtered through 0.22 μm. The filtrate was diluted (1:1, v/v) with a mixture of water/acetonitrile (60:40, v/v), and 2 μL of the diluted sample was injected onto the column.

Nontargeted Analysis of Phenolic Compounds in the Red Wine Extract. In order to obtain an overview of the composition of the wine phenolic extract, some Direct-Injection Mass Spectrometry (DI-MS) experiments were carried out with an Agilent 1200 Series LC system (equipped with a binary pump, an autosampler, and an column oven) coupled to an 6520 quadrupole time-of-flight mass spectrometer using an electrospray interface (DI-ESI-QTOF MS instrument hereafter). All instruments were from Agilent Technologies (Santa Clara, CA). Five milligrams of the commercial wine phenolic extract was dissolved in 1 mL of methanol/water (50:50, v/v) and the solution filtered through 0.45 μm. Five microliters of the filtrate were injected using the autosampler into the LC system (without column) and carried through in acetonitrile/water (75:25, v/v) eluent at a flow rate of 100 μL/min. The ESI source parameters were previously optimized (Quintanilla-López et al, unpublished results) as follows: spray voltage, 4.5 kV; drying gas temperature, 300 °C; drying gas flow rate, 6 L/min; and nebulizer pressure, 30 psi. Nitrogen (99.5% purity) was used as the drying and nebulizer gas. Mass spectra were acquired in negative mode, recording from *m*/*z* 100 to 5000. Data acquisition and processing were done using Agilent Mass Hunter Workstation Acquisition v. B.02.00 software.

Statistical Analysis. A paired Student's *t* test was used to test for significant differences in the bacterial group populations between the negative control and the wine extract fermentations. Also, principal component analysis (PCA), from standardized variables, was used to summarize changes in concentrations of microbial phenolic metabolites resulting from batch culture fermentation of the wine phenolic extract. All statistical analyses were carried out using the STATISTICA program for Windows, version 7.1 (StatSoft. Inc. 1984−2006, www.statsoft.com).

RESULTS AND DISCUSSION

Composition of the Red Wine Extract. Main phenolic compounds present in the red wine extract were flavan-3-ols and anthocyanins (Table 1), as determined by UPLC-ESI-TQ MS. Among flavan-3-ols, monomers and dimers (including galloylated forms) were present in very similar proportion (44 and 47% of total flavan-3-ol quantified, respectively), whereas trimers accounted for 6%. Procyanidin B1 was the most abundant compound followed by catechin and epicatechin. Galloylated monomers and trimers were present in low amounts. Considering anthocyanins, malvidin-3-*O*-glucoside (55% of total anthocyanins quantified) was the main anthocyanin in the red wine extract, as expected from *Vitis vinifera* spp.,[6] followed by peonidin and petunidin-3-*O*-glucosides, which were present in concentrations similar to those of the acylated forms of malvidin-3-*O*-glucoside.

The QTOF spectra obtained by direct injection analysis of the sample, gave a more complete overview of the composition of the wine phenolic extract (Figure 1). Main *m*/*z* signals corresponded to $[M - H]^-$ ions from phenolic compounds, which indicated the richness of the extract in these compounds. Besides monomers (*m*/*z* 289.08), dimers (*m*/*z* 577.15), dimer gallates (*m*/*z* 729.17), and trimers (*m*/*z* 865.22) also detected by UPLC-ESI-TQ MS, the QTOF spectra revealed the presence of flavan-3-ols constituted up to 7 units by both (epi)catechins and (epi)gallocatechins (i.e., prodelphinidins): trimers (2 (epi)catechins + 1 (epi)gallocatechin: *m*/*z* 881.21), tetramers (4 (epi)catechins: *m*/*z* 1153.28; 3 (epi)catechins + 1 (epi)gallocatechin: *m*/*z* 1169.28), pentamers (5 (epi)catechins:

Table 1. Phenolic Composition (mg/g) of the Red Wine Extract Used in This Study[a]

flavan-3-ols and others		anthocyanins	
gallic acid	1.06 ± 0.05	delphinidin-3-*O*-glucoside	0.568 ± 0.012
catechin	9.90 ± 0.32	cyanidin-3-*O*-glucoside	0.265 ± 0.010
epicatechin	6.87 ± 0.15	petunidin-3-*O*-glucoside	1.47 ± 0.03
epicatechin-3-*O*-gallate	0.226 ± 0.018	peonidin-3-*O*-glucoside	1.78 ± 0.01
B1	11.1 ± 0.1	malvidin-3-*O*-glucoside	9.01 ± 0.50
B2	4.69 ± 0.10	malvidin-3-*O*- (6″-acetyl) glucoside	1.92 ± 0.02
B3	1.23 ± 0.02	malvidin-3-*O*-(6″-*p*-coumaroyl) glucoside	1.24 ± 0.01
B4	0.827 ± 0.018		
B2-3-*O*-gallate	0.0271 ± 0.0106	flavonols	
B2-3′-*O*-gallate	0.0258 ± 0.0028	quercetin	22.4 ± 0.6
C1	1.07 ± 0.04	kaempherol	0.0366 ± 0.0055
T2	1.24 ± 0.09	myricetin	2.55 ± 0.07
		quercetin-3-*O*-glucoside	0.137 ± 0.023
alcohols		quercetin-3-*O*-galactoside	0.107 ± 0.006
tyrosol	18.9 ± 1.3		
		stilbenes	
esters		resveratrol	0.427 ± 0.020
cutaric acid	2.00 ± 0.12	resveratrol-3-*O*-glucoside	9.17 ± 0.17
caftaric acid	0.192 ± 0.071		

[a]Mean value (n = 2) ± SD.

m/z 1441.35; 4 (epi)catechins + 1 (epi)gallocatechin: m/z 1457.35, hexamers (6 (epi)catechins: m/z 1729.41; 5 (epi)-catechins + 1 (epi)gallocatechin: m/z 1745.41), and heptamers (7 (epi)catechins: m/z 2017.48; 6 (epi)catechins + 1 (epi)gallocatechin: m/z 2033.49). In the case of procyanidins, these findings seem to be in accordance to those reported by Fulcrand et al.[7] However, to our knowledge prodelphinidins with DP up to 7 have not been detected before in red wine.

Effect of the Red Wine Extract on Human Bacteria. For the three volunteers, populations of the different bacterial groups were measured by FISH during the experimental time course (5, 10, 24, 30, and 48 h) of the in vitro fermentation of the red wine extract, as well as for the negative (medium + fecal slurry) and positive (medium + fecal slurry + prebiotic) controls (Figure 2). All data of bacterial populations were expressed relative to the negative control.

Bifidobacterium spp. (Bif164 probe), *Lactobacillus/Enterococcus* spp. (Lab158 probe), *Bacteroides* spp. (Bac303), and members of the domain *Bacteria* (EUBmix probe) remained constant during the time course of fermentations in the presence of the wine phenolic extract. A slight inhibition in the *C. histolyticum* group (Chis150 probe) was observed at 30 and 48 h of fermentation in comparison to remaining times, but these changes were not significant compared to the negative control. This trend seems to be in line with that reported for other in vitro studies performed with monomeric flavan-3-ols and cocoa flavan-3-ols using the same batch culture model,[15,19] as well as with in vivo studies conducted with flavan-3-ol-rich sources.[16−18] However, in contrast to these studies, we did not find a positive effect on the growth of other bacteria groups such as *Lactobacillus/Enterococcus* spp. Differences between studies could be attributed to variability in the flavan-3-ol concentration used. For example, a concentration of 600 mg of red wine extract used here provides ca. 5.94, 4.12, and 20.94 mg/L of catechin, epicatechin and flavan-3-ols DP1−2 (monomers + dimers), respectively, in comparison to 150−1000 mg/L of catechin and epicatechin standards used by Tzounis et al.[15] or 219 mg/L of flavan-3-ols (DP1−2) from cocoa used by Tzounis et al.[19] Therefore, despite the fact that the red wine extract has high total polyphenol content (474 mg/g), the proportion of flavan-3-ols may be not enough to produce significant changes in microbiota during the course of a 48 h fermentation period. On the other hand, this fact also

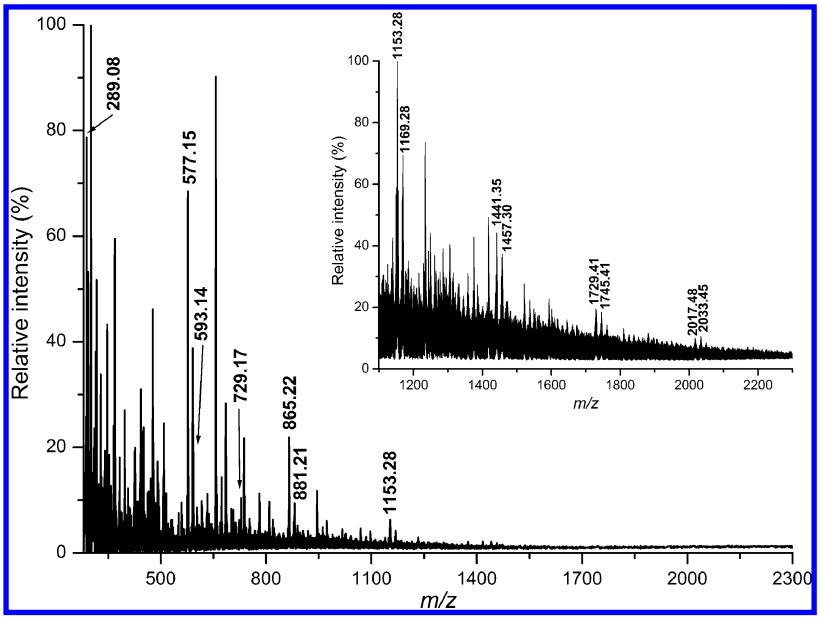

Figure 1. Direct injection QTOF spectrum of the red wine extract used in this study. The inset shows an enlarged section of 1100−2300 m/z range.

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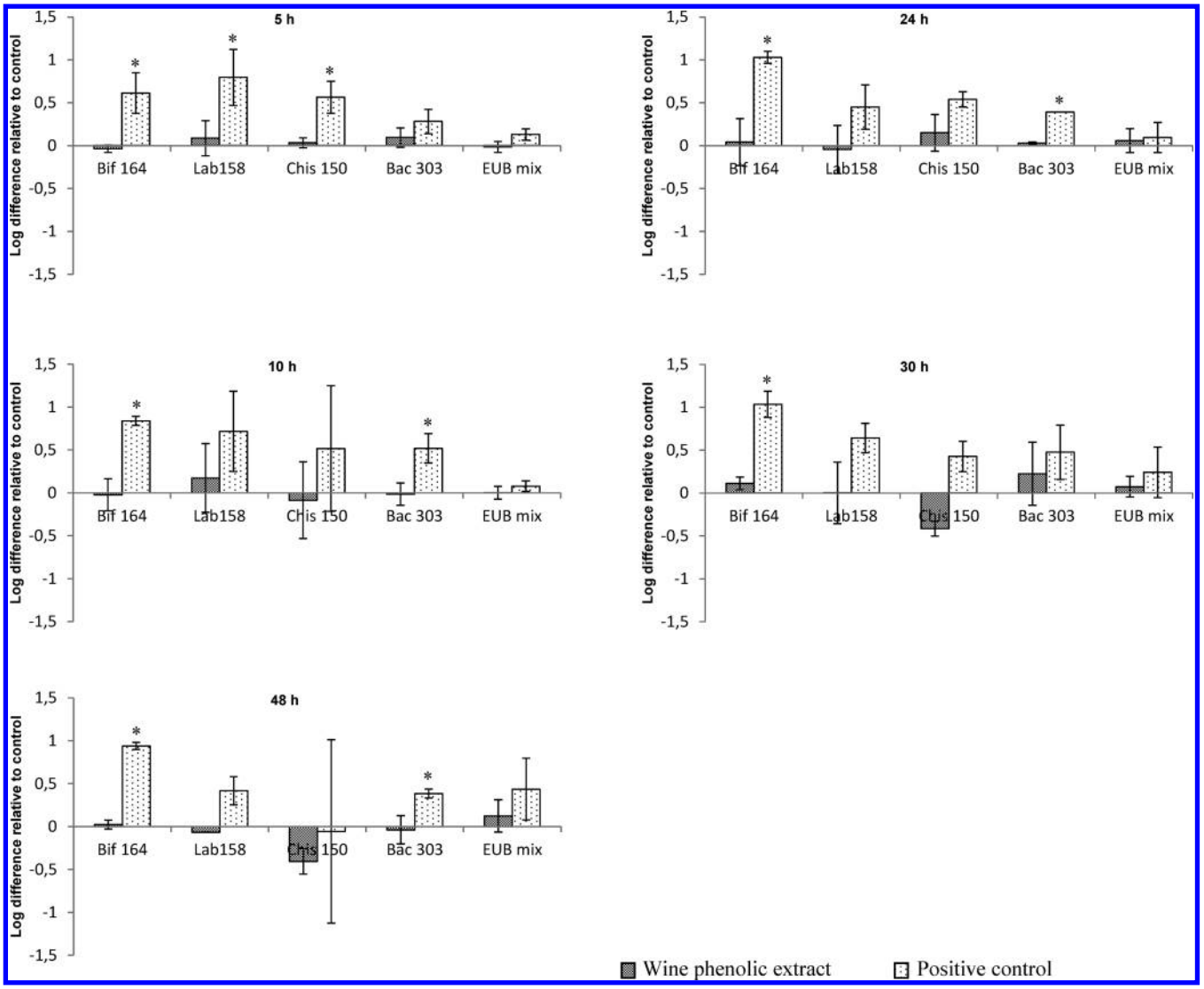

Figure 2. Changes in bacterial group populations during the fermentation of the red wine extract (600 mg/L) in a pH-controlled fecal batch culture. Histograms indicate the mean log difference (n = 3) in the bacterial population relative to the negative control and the bars the standard deviation.

suggests that anthocyanins themselves may not be able to produce significant changes in bacterial population in comparison to flavan-3-ols.

Another factor that may influence microbiota changes is the time of exposure to polyphenols and origin of microbiota used. Dolara et al.[20] found that rats fed with red wine polyphenols (50 mg/kg, equivalent to ca. 1.2 mg of catechin + epicatechin/day) during 15 weeks had significantly lower levels of *Clostridium* spp. and higher levels of *Lactobacillus* spp. Therefore, exposure to lower concentration of flavan-3-ols for a prolonged period of time may promote positive changes in bacterial populations.

Microbial Transformations of Red Wine Polyphenols. In order to follow up the fermentation of red wine extract with human fecal microbiota, main flavonoid compounds (flavan-3-ols and anthocyanins) were monitored by UPLC-DAD-ESI-TQ MS during the time course of the experiments. To better illustrate changes, representative evolutionary trends of the different compounds are shown for Volunteer 1 (V1) (Figures 3 and 4). Concerning flavan-3-ols, monomers, catechin and epicatechin, and procyanidin B2 were completely degraded by the microbiota after 5 h of fermentation, while for procyanidin B1, this was attained at 10 h (Figure 3A). This trend was also observed for procyanidin B3, whereas B4 showed a lower degradation rate, attaining complete degradation after 30 h of fermentation (Figure 3B). Trimeric procyanidins C1 and T2 showed a rapid decrease in concentration during the first 10 h, afterward showing slight transitions in concentration up to 48 h (Figure 3B).

Gallic acid was also degraded by 30 h of fermentation, and no accumulation of this compound was observed during fermentation, in contrast to previous studies performed with other sources of galloylated flavan-3-ols (i.e., green tea and grape seed extract),[26,27] despite the decline observed in (−)epicatechin-3-*O*-gallate and B2-3′-*O*-gallate, at 10 h and 5 h of fermentation, respectively (Figure 3C).

As observed for most flavan-3-ols, maldivin-3-glucoside and its acylated forms showed a rapid decline in concentration during the first 5 h of fermentation, although complete degradation of the main anthocyanin was not observed until 30 h of fermentation (Figure 4A). This was also the trend observed for peonidin- and cyanidin -3-*O*-glucosides which were rapidly degraded during the first 5 h, although no complete degradation was observed up to 30 h of fermentation.

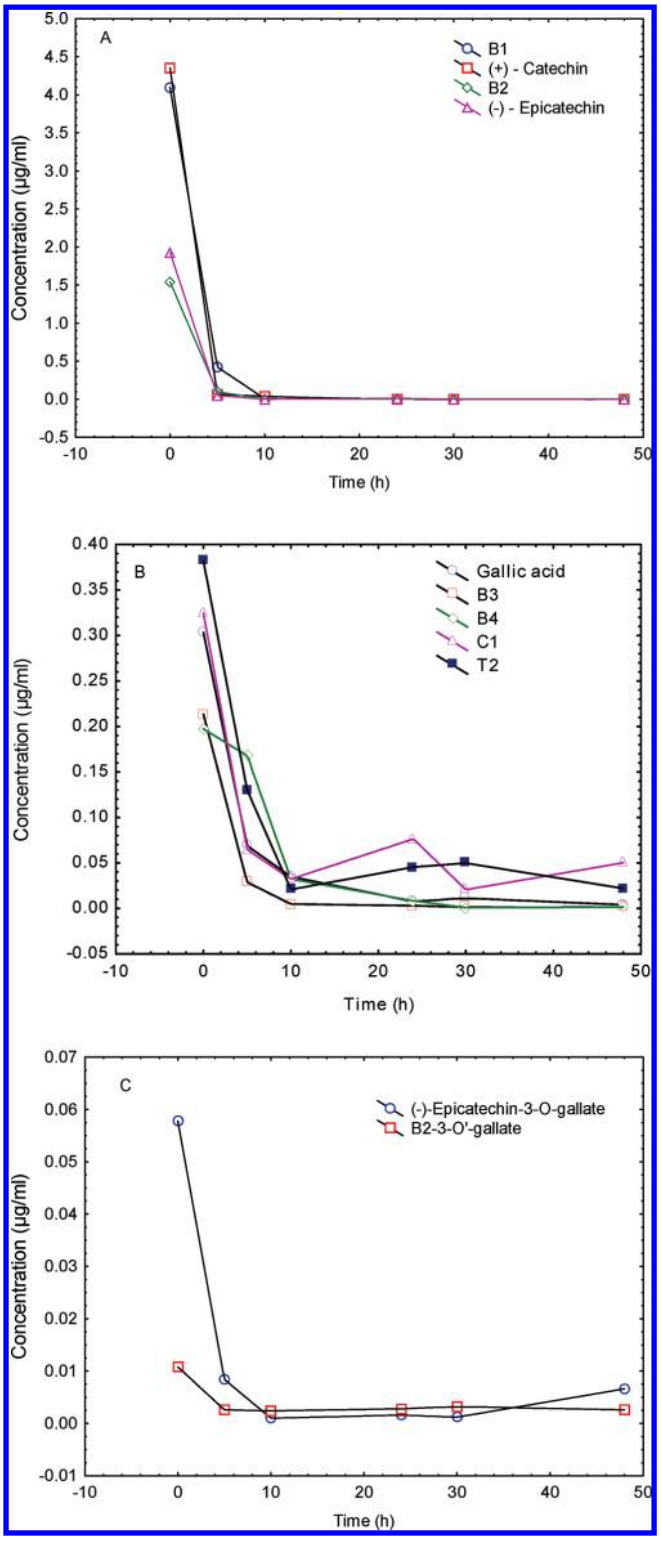

Figure 3. Changes in gallic acid and flavan-3-ols during fecal fermentation (volunteer V1) of the red wine extract: (A) catechin, epicatechin, and procyanidins B1 and B2; (B) gallic acid and procyanidins B3, B4, C1, and T2; (C) epicatechin-3-*O*-gallate and procyanidin B2-3′-*O*-gallate.

However, petunidin-3-*O*-glucoside showed a faster degradation rate attaining complete degradation after 10 h (Figure 4B). This fast catabolism of anthocyanins during the early hours of fermentation has been mainly attributed to the β-glucosidase activity of the gut microbiota.[28]

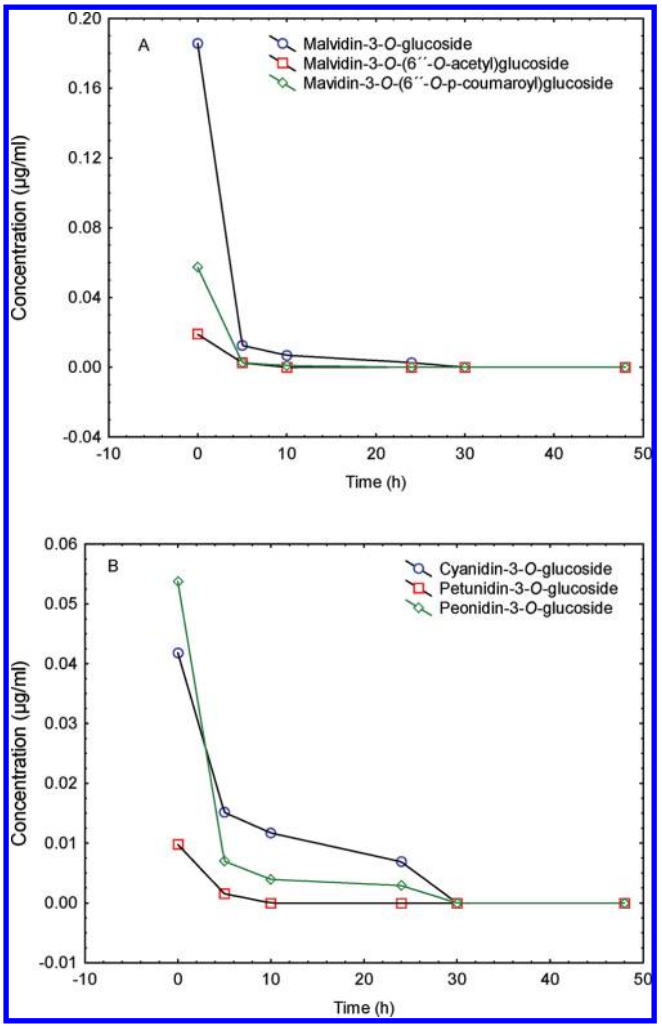

Figure 4. Changes in anthocyanins during fecal fermentation (volunteer V1) of the red wine extract: (A) malvidin-3-*O*-glucoside, malvidin-3-*O*-(6″-*O*-acetyl)glucoside, and malvidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside; (B) cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, and peonidin-3-*O*-glucoside.

According to Keppler and Humpf,[29] peonidin-3-glucoside gave a slower degradation rate than cyanidin- and malvidin-3-glucosides; however, no clear effect of the hydroxylation pattern of the different anthocyanidins on bacterial degradation rate was observed in the present study. However, considering the acylation pattern, it appeared that the acylated forms of maldivin-3-glucoside presented a faster decline in comparison to the remaining ones. Incubation of the red wine extract in the absence of bacteria (chemical control) did not reveal significant changes in the concentration of these anthocyanins despite the fact that they are known to be more susceptible to chemical hydrolysis than the no-acylated ones. In any case, no transitional increase in the concentration of maldivin-3-glucoside was observed as a consequence of the possible hydrolysis of the acetyl or *p*-coumaroyl moieties of the acylated forms, probably due to dynamism of the bacterial catabolism and rapid turnover into other degradation products.

Formation of Microbial Phenolic Metabolites. A wide range of potential phenolic metabolites derived from the catabolism of flavan-3-ols, including intermediate metabolites (i.e., phenyl-γ-valerolactones and phenylvaleric acid derivatives) and end products (simple phenols, hydroxyphenylpropionic,

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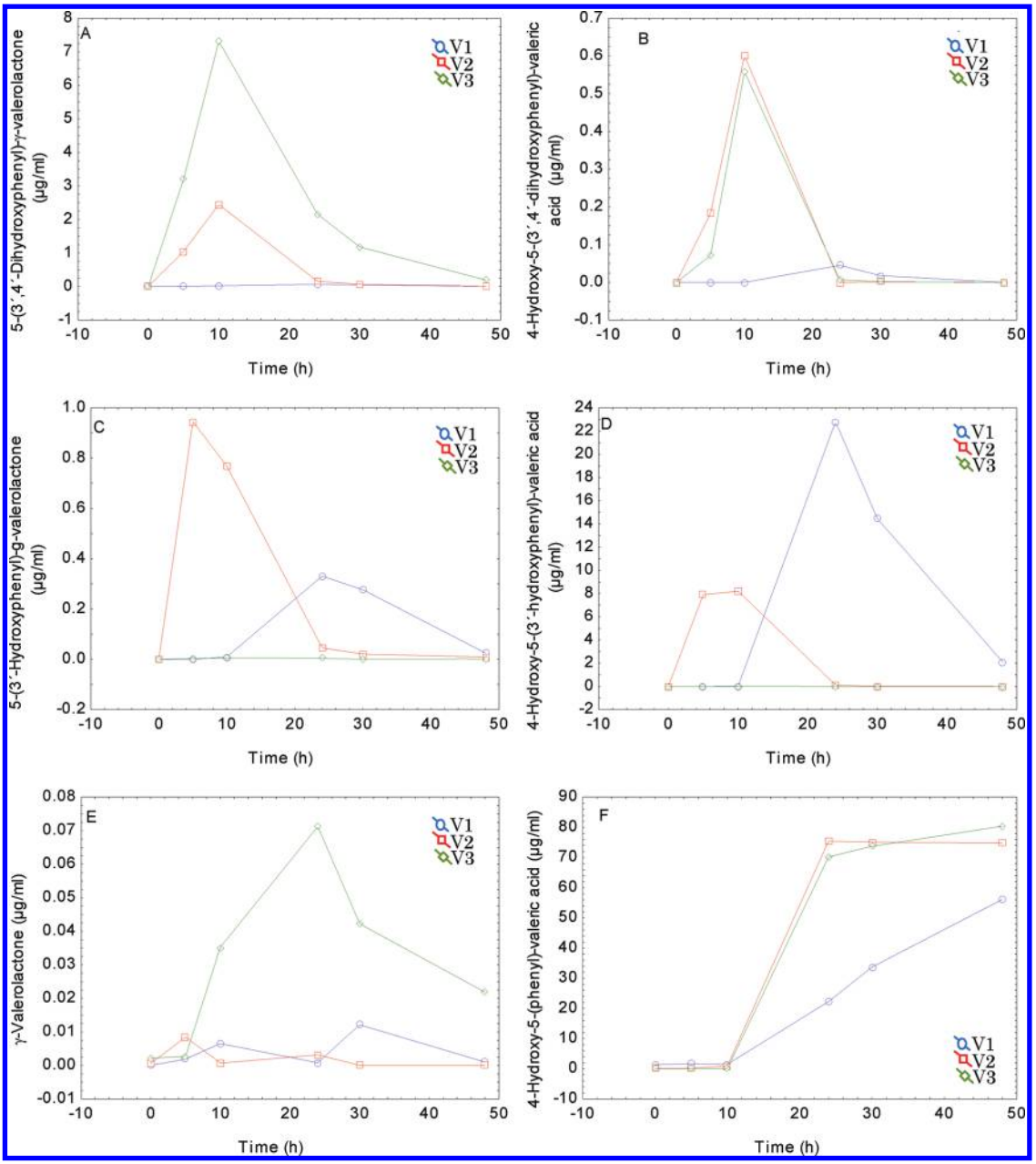

Figure 5. Changes in phenyl-γ-valerolactone and 4-hydroxy-5-(phenyl)-valeric acid derivatives during fecal fermentation (volunteers V1, V2, and V3) of the red wine extract. (A) 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone; (B) 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid; (C) 5-(3′-hydroxyphenyl)-γ-valerolactone; (D) 4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid; (E) γ-valerolactone; (F) 4-hydroxy-5-(phenyl)-valeric acid.

hydroxyphenylacetic, hydroxycinnamic, and hydroxybenzoic acids), some of these latter compounds being also common to the catabolism of anthocyanins, were targeted by UPLC-DAD-ESI-TQ MS during the time course of batch culture fermentations.[25,26]

Phenyl-γ-valerolactone and 4-Hydroxy-5-(phenyl)-valeric Acid Derivatives. The formation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, a characteristic metabolite arising from first steps in the microbial degradation of flavan-3-ols, was seen between 0 and 5 h of fermentation reaching a maximum concentration after 10 h (Figure 5A), a time period which coincided with the largest decline of precursor flavan-3-ols (Figure 3). Although the time at which the maximum formation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone occurs was variable, depending on the origin of the microbiota (i.e., human or animal) and structural features of the precursor compound used in purified form (i.e., degree of polymerization and galloylation),[27,30−32] this finding seems to be in line with previous studies carried out with grape seed extract in the same in vitro fermentation model.[26] The open form, 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid (Figure 5B), also gave peak concentration levels after 10 h, which is in accordance with its possible equilibrium with the lactone form.[30] Large interindividual differences were observed in the formation of both metabolites, showing high (for V3), intermediate (for V2), and low (for V1) maximum concentration levels. For this latter V1, the levels of the metabolites were low, but the time at which the peak concentration was reached was shifted up to 24 h of fermentation, probably due to a low microbial enzymatic activity.

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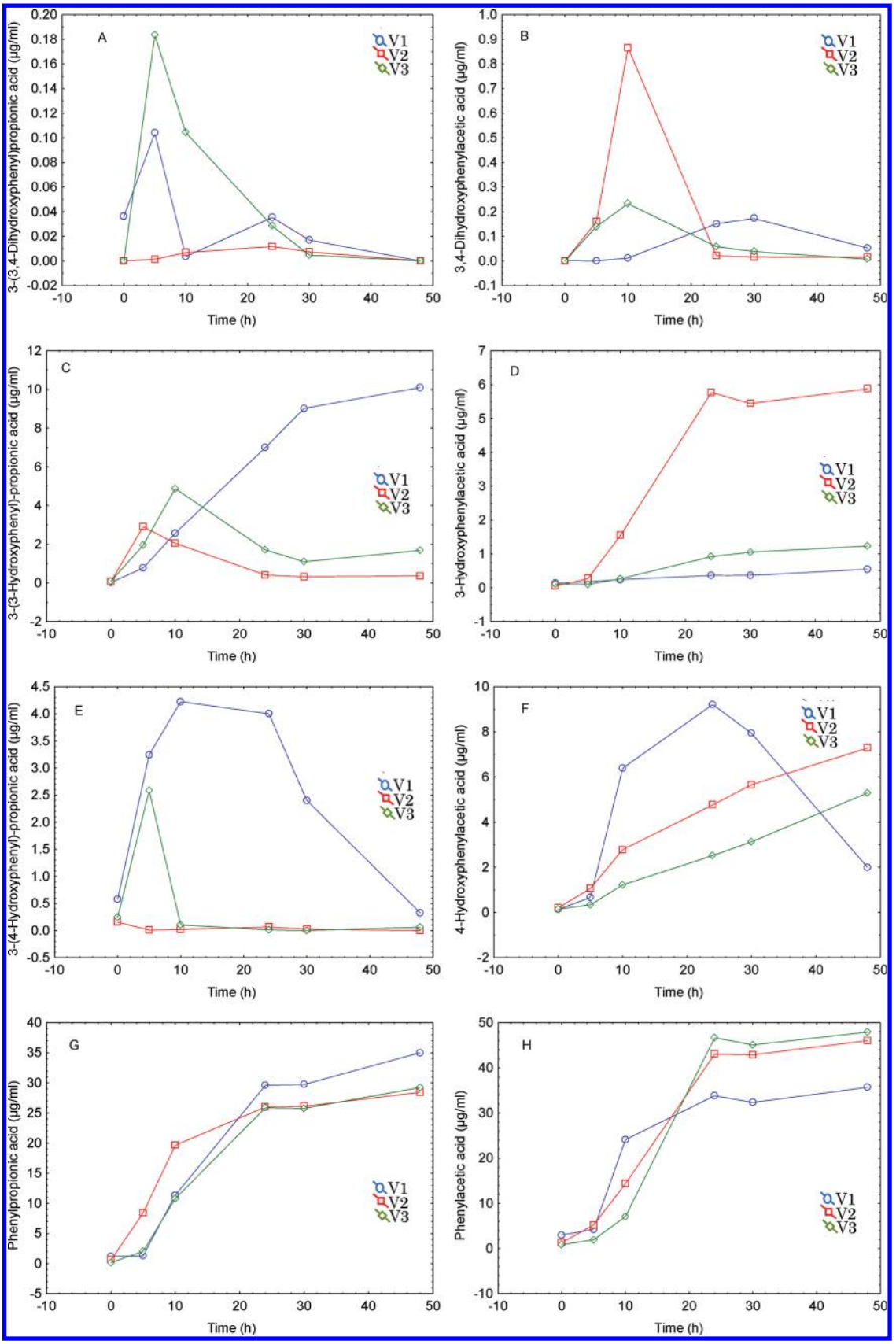

Figure 6. Changes in phenylpropionic and phenylacetic acid derivatives during fecal fermentation (volunteers V1, V2, and V3) of the red wine extract. (A) 3-(3,4-Dihydroxyphenyl)-propionic acid; (B) 3,4-dihydroxyphenylacetic acid; (C) 3-(3-hydroxyphenyl)-propionic acid; (D) 3-hydroxyphenylacetic acid; (E) 3-(4-hydroxyphenyl)-propionic acid; (F) 4-hydroxyphenylacetic acid; (G) phenylpropionic acid; (H) phenylacetic acid.

However, a different scenario was observed for the monohydroxylated form since V3, which produced the highest levels of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, was a low producer of 5-(3′-hydroxyphenyl)-γ-valerolactone (Figure 5C). This was also observed for 4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid (Figure 5D). Also of note was reversion in activity of V1 from a low to a high producer of the latter metabolite. Again, differences were observed in the time of peak concentration between volunteers in keeping with the formation of the precursor metabolites.

The formation of the nonhydroxylated form, 4-hydroxy-5-(phenyl)-valeric acid, was not detected until 24 h of fermentation and was progressively accumulated up to 48 h in the case of V1 and V3 (Figure 5F), indicating its formation from 4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid. However, the corresponding nonhydroxylated form of phenyl-γ-valerolactone was not detected, but instead low levels of the nonphenyl form, γ-valerolactone, were observed in particular for V3 (Figure 5E).[26]

Phenylpropionic and Phenylacetic Acid Derivatives. The formation of 3-(3,4-dihydroxyphenyl)-propionic and 3,4-dihydroxyphenylacetic acids was registered concurrently with the formation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid, although the time at which maximum concentration was reached varied among volunteers in comparison to the precursor metabolites (Figure 6A and B). Phenylpropionic acid derivatives are considered to arise from β-oxidation of phenylvaleric acid derivatives, whereas phenylacetic acid derivatives may arise from α-oxidation of the former derivatives,[30,31] although alternative different routes have been proposed depending on the degree of polymerization of flavan-3-ol and origin of the microbiota used.[33] It is important to highlight that red wine extracts contain other flavanoid compounds such as flavonols,[34] which although in minor proportion could also be metabolized by the intestinal microbiota into phenylacetic acids.[35]

The evolution pattern of the monohydroxylation forms differed for phenylpropionic and phenylacetic acids (Figures 6C, D, E, and F). Dehydroxylation reactions of 3-(3,4,-dihydroxyphenyl)-propionic acid favored the formation of 3-(3-hydroxyphenyl)-propionic acid in comparison to that of 3-(4-hydroxyphenyl)-propionic acid (Figures 6C and E), as described in previous studies.[36] In the case of V2 and V3, 3-(3-hydroxyphenyl)-propionic acid accumulated during the first 5 h of fermentation (Figure 6C) and then declined as observed for its precursor, 3-(3,4,-dihydroxyphenyl)-propionic acid. In contrast, V1, which exhibited the lowest levels of precursor metabolites, showed a progressive accumulation up to 48 h of fermentation as well as the highest peak concentration levels. This trend was also observed for the corresponding phenylacetic acid form (i.e., 3-hydroxyphenylacetic), in particular for V2, which generated an earlier and higher accumulation than V1 and V3, indicating a posterior formation from 3-(3,4-dihydroxyphenyl)-acetic acid (Figure 6D).

Formation of the 4-monohydroxylated forms resulting from dehydroxylation at C-3 (i.e., 3-(4-hydroxyphenyl)-propionic acid and 4-hydroxyphenylacetic acid) was also apparent for V1, rapidly accumulating up to 10−24 h and declining afterward (Figure 6E). However, differences between both metabolites were observed for V2 and V3. It is important to highlight that these compounds also originated from fermentation of the basal medium, as reported in previous in vitro experiments.[26,37] The case was similar for phenylpropionic and phenylacetic acids (Figure 6G and H), the nonhydroxylated forms, which showed a progressive increase during the time-course of fermentation for all volunteers, despite the interindividual differences described above for the other metabolites.[26] These metabolites have been reported to be intermediate products of the metabolic pathway of certain aminoacids.[38]

Simple Phenols and Benzoic and Hydroxycinnamic Acids. As a result of bacterial metabolism, the heterocyclic C-ring of anthocyanins is broken and degraded into phloroglucinol derivatives (from A-ring) and benzoic acids (from the B-ring). Both phloroglucinol aldehyde and its oxidation product, phloroglucinol acid, have been detected following the degradation of anthocyanins.[29] As a consequence of the fermentation of the red wine extract, phloroglucinol was not detected, but instead, the dihydroxylated benzene, catechol/pyrocatechol, was progressively formed from 10 to 30 h of fermentation (Figure 7A). This simple phenol may also originate from the catabolism of galloylated flavan-3-ols after release of the gallic acid moiety and further dehydroxylation;[26] however, in the case of the red wine extract it is most likely that this metabolite originated from the catabolism of anthocyanins, due to their relatively higher abundance in the extract (Table 1).

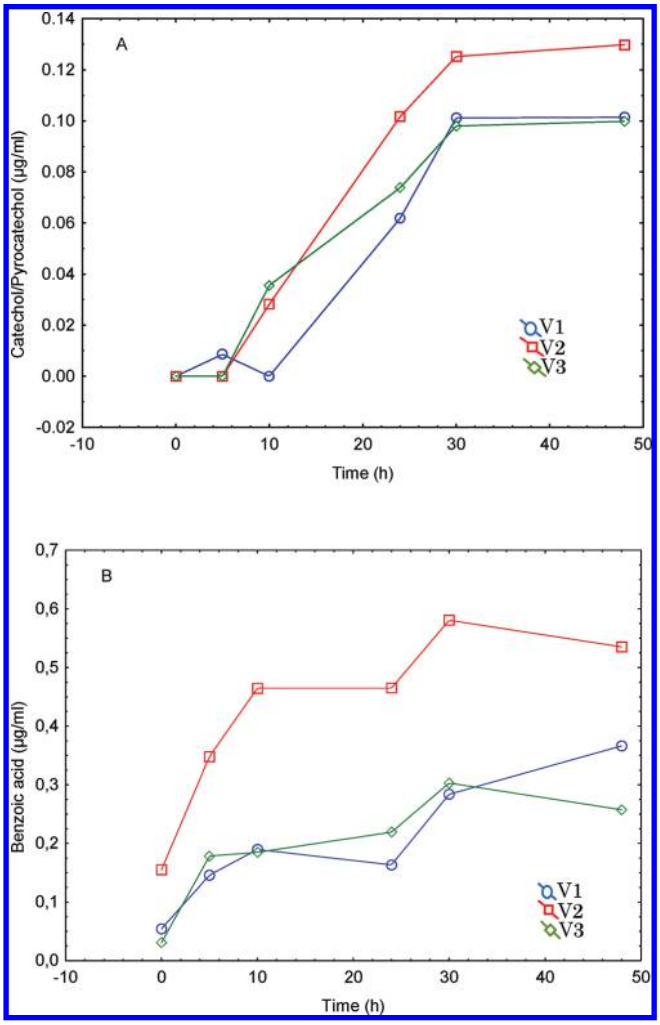

Figure 7. Changes in simple phenols and benzoic acid derivatives during fecal fermentation (volunteers V1, V2, and V3) of the red wine extract. (A) Catechol/pyrocatechol; (B) benzoic acid.

The B-ring of malvidin would be degraded into 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid), whereas as that of cyanidin and peonidin would be degraded into 3,4-dihydroxybenzoic acid (protocatechuic acid) and 4-hydroxy-3-methoxybenzoic acid (vanillic acid), respectively.[8,28,29,39] Such metabolites have been reported to be formed during the first 2 h of fermentation, decreasing afterward due to further catabolism by the microbiota.[29] In the present study, a transitory increase in syringic acid was observed after 5 h of fermentation for V2 and V3; however, no detectable increases in concentration were observed for protocatechuic and vanillic acids (data not shown). *O*-Demethylation of syringic and vanillic acids into gallic and protocatechuic acids, respectively, have also been reported during fecal fermentation of anthocyanins. However, we did not observe a later increase in these acids. In fact, according to Keppler and Humpf,[29] O-demethylated metabolites were only detected from 2 to 4 h of fermentation in very small amounts. Instead, a progressive increase in benzoic acid during fecal fermentation of the red wine extract was observed (Figure 7B), indicating that *O*-demethylated metabolites may be extensively dehydroxylated giving rise to benzoic acid as the final degradation product. The formation of benzoic acid by fecal microbiota is also in line with the results from previous studies reporting that 3- and 4-hippuric acids were strong urinary biomarkers of the intake of either a red wine/red grape juice mix or red grape juice.[40]

Other phenolic acids originating from the catabolism of flavan-3-ols, including the hydroxycinnamic acids *p*-coumaric, caffeic, and ferulic acids, were also monitored during fecal fermentation of the red wine extract. Small amounts of these acids were originally present in the extract as they usually occur in red grapes.[6] No increase in the concentration of these acids was observed, but instead, they progressively decreased during fecal fermentation (data not shown).

Overview of Changes in Microbial Metabolites during Fecal Fermentation of the Red Wine Extract. In order to summarize changes in phenolic microbial metabolites resulting from fecal fermentation of the red wine extract, a Principal Component Analysis (PCA) was applied. Two principal components (PC1 and PC2), which explained 53.1% of the total variance of the data, were obtained. To show 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 8).

The first principal component (PC1), explaining 33.9% of total variance, was positively correlated (loadings ≥0.7) with metabolites: catechol/pyrocatechol (r = 0.9510), and 4-hydroxy-5-(phenyl)-valeric (0.9342), 4-hydroxyphenylacetic (0.7006), 3-hydroxyphenylacetic (0.7298), phenylacetic (0.9528), phenylpropionic acid (0.8914), and benzoic acids (0.7179). Increases observed in PC1 values indicated that the concentration of the latter metabolites progressively increased during fermentation and that the largest changes were observed between 10 and 24 h, in particular for V2 and V3. In other words, PC1 reflected major changes in the formation of microbial metabolites occurring during the time-course of the catabolism of the red wine extract, arising from both flavan-3-ol and anthocyanin precursors. These findings seem to agree with the global profile of phenylacetate and phenylpropionate formation observed after in vitro fermentation of a red wine/grape juice extract,[37] as well as from other flavan-3-ol-rich sources such as chocolate, green tea, or almond skins.[36,41,42]

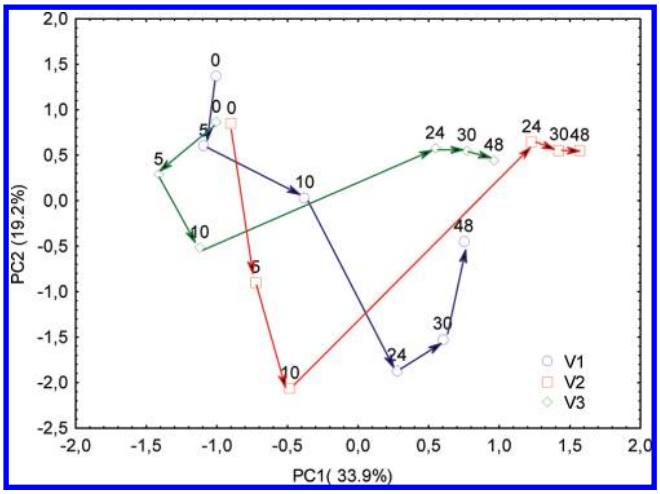

Figure 8. Representation of the samples in the plane defined by the first two principal components (PC1 and PC2) resulting from a PCA of microbial-derived phenolic metabolites (n = 16) for the three volunteers (V1, V2, and V3) at different incubation times (0, 5, 10, 24, 30, and 48 h).

The second principal component (PC2), explaining 11.4% of the total variance, was negatively correlated with 5-(3′-hydroxyphenyl)-γ-valerolactone (r = −0.7205), 4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid (−0.8258), and 3,4-dihydroxyphenylacetic acid (−0.7215). Negative values in PC2 increased during the first 10 h (for V2 and V3) or 24 h (for V1) of fermentation, indicating that these latter metabolites were formed during the first few hours of fermentation and sharply decreased afterward. It is important to highlight that variation in this component reflected interindividual differences in the microbial metabolism of the red wine extract among the 3 volunteers, suggesting that formation of intermediate metabolites such as 5-(3′-hydroxyphenyl)-γ-valerolactone and its open form 4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid and its subsequent transformation into 3,4-dihydroxyphenylacetic acid could be critical steps delimiting the rate and extent of the catabolism of red wine polyphenols and therefore the absorption and further bioactivity of these compounds. In fact, not until very recently bacteria with ability to form 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone from the catabolism of flavan-3-ols have been identified,[43] supporting the idea that formation of phenyl-γ-valerolactone derivatives by gut microbiota could be largely variable among individuals. Previous studies also demonstrated that the microbial catabolism of red wine/grape juice extract was highly variable among individuals, although phenyl-γ-valerolactone derivatives were not considered as a possible biomarker of red wine/grape microbial catabolism.[37] Our findings support the results from previous in vitro fermentation experiments with grape seed extract[26] and indicate that flavan-3-ols could largely contribute to the profile of microbial metabolites derived from the catabolism of red wine polyphenols and therefore to their overall bioavailability and in vivo bioactivity.

In conclusion, microbial metabolism of the red wine extract produced a wide range of microbial metabolites, some of them characteristic of the catabolism of flavan-3-ols, whereas other were also common to the catabolism of anthocyanins and other flavonoids present in the red wine extract. The metabolites formed from the catabolism of anthocyanins appeared to be rapidly degraded into simpler compounds. In contrast, flavan-3-

ols metabolites such as phenyl-γ-valerolactone derivatives may deserve further consideration as a possible biomarker of red wine consumption linked to in vivo health effects. Nevertheless, the large interindividual differences in catabolism observed among the volunteers is an important factor to consider in this matter. Despite the bacteria−polyphenol interaction, as evidenced by the decline in flavonoids and formation of microbial metabolites, only subtle changes in the different bacterial groups were observed. Factors such as the initial flavan-3-ol concentration, the flavan-3-ol/anthocyanin ratio, as well as the original microbiota composition used may have affected the modulation effect of polyphenols in comparison to other studies conducted with the same in vitro model. At the moment, further in vitro and in vivo trials are being conducted to determine the effect of moderate consumption of red wine on gut microbiota and their possible benefits on microbiota-related diseases.

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Notes

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REFERENCES

(1) Renaud, S.; De Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523−1526.

(2) Cuevas, A. M.; Guasch, V.; Castillo, O.; Irribarra, V.; Mizon, C.; San Martin, A.; Strobel, P.; Perez, D.; Germain, A. M.; Leighton, F. A high-fat diet induces and red wine counteracts endothelial dysfunction in human volunteers. *Lipids* **2000**, *35*, 143−148.

(3) Pignatelli, P.; Ghiselli, A.; Buchetti, B.; Carnevale, R.; Natella, F.; Germanò, G.; Fimognari, F.; Di Santo, S.; Lenti, L.; Violi, F. Polyphenols synergistically inhibit oxidative stress in subjects given red and white wine. *Atherosclerosis* **2006**, *188*, 77−83.

(4) Pignatelli, P.; Lenti, L.; Pulcinelli, F. M.; Catasca, R.; Saccani, G.; Germanò, G.; Marcoccia, A.; Silvestri, M. A.; Ghiselli, A.; Violi, F. Red and white wine differently affect collagen-induced platelet aggregation. *Pathophysiol. Haemostasis Thromb.* **2002**, *32*, 356−358.

(5) Estruch, R.; Sacanella, E.; Badia, E.; Antúnez, E.; Nicolás, J. M.; Fernández-Solá, J.; Rotilio, D.; De Gaetano, G.; Rubin, E.; Urbano-Márquez, A. Different effects of red wine and gin consumption on inflammatory biomarkers of atherosclerosis: A prospective randomized crossover trial: Effects of wine on inflammatory markers. *Atherosclerosis* **2004**, *175*, 117−123.

(6) Monagas, M.; Bartolomé, B.; Gómez-Cordovés, C. Updated knowledge about the presence of phenolic compounds in wine. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 85−118.

(7) Fulcrand, H.; Remy, S.; Souquet, J. M.; Cheynier, V.; Moutounet, M. Study of wine tannin oligomers by on-line liquid chromatography electrospray ionization mass spectrometry. *J. Agric. Food. Chem.* **1999**, *47*, 1023−1028.

(8) Williamson, G.; Clifford, M. N. Colonic metabolites of berry polyphenols: the missing link to biological activity ? *Br. J. Nutr.* **2010**, S48−s66.

(9) Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727−747.

(10) Donovan, J. L.; Manach, C.; Faulks, R. M.; Kroon, P. A. Absorption and Metabolism of Dietary Plant Secondary Metabolites. In *Plant Secondary Metabolites. Occurrence, Structure and Role in the Human Diet*; Crozier, A., Clifford, M. N., Ashihara, H., Eds.; Blackwell Publishing Ltd: Oxford, UK, 2006; pp 303−341.

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

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

(13) Aura, A. M.; Mattila, I.; Seppänen-Laakso, T.; Miettinen, J.; Oksman-Caldentey, K. M.; Oresic, M. Microbial metabolism of catechin stereoisomers by human faecal microbiota: Comparison of targeted analysis and a non-targeted metabolomics method. *Phytochem. Lett.* **2008**, *1*, 18−22.

(14) Selma, M. V.; Espín, J. C.; Tomás-Barberán, F. A. Interaction between phenolics and gut microbiota: Role in human health. *J. Agric. Food. Chem.* **2009**, *57*, 6485−6501.

(15) Tzounis, X.; Vulevic, J.; Kuhnle, G. G. C.; George, T.; Leonczak, J.; Gibson, G. R.; Kwik-Uribe, C.; Spencer, J. P. E Flavanol monomer-induced changes to the human faecal microflora. *Br. J. Nutr.* **2008**, *99*, 782−792.

(16) Hara, Y. Influence of tea catechins on the digestive tract. *J. Cell. Biochem.* **1997**, *67*, 52−58.

(17) Molan, A. L.; Liu, Z.; Kruger, M. The ability of blackcurrant extracts to positively modulate key markers of gastrointestinal function in rats. *World J. Microbiol. Biotechnol.* **2011**, *26*, 1735−1743.

(18) Viveros, A.; Chamorro, S.; Pizarro, M.; Arija, I.; Centeno, C.; Brenes, A. Effects of dietary polyphenol-rich grape products on intestinal microflora and gut morphology in broiler chicks. *Poult. Sci.* **2011**, *90*, 566−578.

(19) 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. *Am. J. Clin. Nutr.* **2011**, *93*, 62−72.

(20) Dolara, P.; Luceri, C.; De Filippo, C.; Femia, A. P.; Giovannelli, L.; Caderni, G.; Cecchini, C.; Silvi, S.; Orpianesi, C.; Cresci, A. Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. *Mut. Res., Fundam. Mol. Mech. Mutagen.* **2005**, *591*, 237−246.

(21) Manz, W.; Amann, R.; Ludwig, W.; Vancanneyt, M.; Schleifer, K. H. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology* **1996**, *142*, 1097−1106.

(22) Harmsen, H. J. M.; Elfferich, P.; Schut, F.; Welling, G. W. A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in faecal samples by fluorescent in situ hybridization. *Microb. Ecol. Health Dis.* **1999**, *11*, 3−12.

(23) Franks, A. H.; Harmsen, H. J. M.; Raangs, G. C.; Jansen, G. J.; Schut, F.; Welling, G. W. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* **1998**, *64*, 3336−3345.

(24) Daims, H.; Brühl, A.; Amann, R.; Schleifer, K. H.; Wagner, M. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: Development and evaluation of a more comprehensive probe set. *Syst. Appl. Microbiol.* **1999**, *22*, 434−444.

(25) Sánchez-Patán, F.; Monagas, M.; Moreno-Arribas, M. V.; Bartolomé, B. Determination of microbial phenolic acids in human faeces by UPLC-ESI-TQ MS. *J. Agric. Food. Chem.* **2011**, *59*, 2241−2247.
(26) Sánchez-Patán, F.; Cueva, C.; Monagas, M.; Walton, G. E.; Gibson, G. R.; Martín-Álvarez, P. J.; Moreno-Arribas, M.-V.; Bartolomé, B. Gut microbial catabolism of grape seed flavan-3-ols by human faecal microbiota. Targeted analysis of precursor compounds, intermediate metabolites and end products. *Food Chem.* **2012**, *131*, 337−347.
(27) Roowi, S.; Stalmach, A.; Mullen, W.; Lean, M. E. J.; Edwards, C. A.; Crozier, A. Green tea flavan-3-ols: Colonic degradation and urinary excretion of catabolites by humans. *J. Agric. Food. Chem.* **2010**, *58*, 1296−1304.
(28) Aura, A. M.; Martin-Lopez, P.; O'Leary, K. A.; Williamson, G.; Oksman-Caldentey, K. M.; Poutanen, K.; Santos-Buelga, C. In vitro metabolism of anthocyanins by human gut microflora. *Eur. J. Nutr.* **2005**, *44*, 133−142.
(29) Keppler, K.; Humpf, H. U. Metabolism of anthocyanins and their phenolic degradation products by the intestinal microflora. *Bioorg. Med. Chem.* **2005**, *13*, 5195−205.
(30) Stoupi, S.; Williamson, G.; Drynan, J. W.; Barron, D.; Clifford, M. N. Procyanidin B2 catabolism by human fecal microflora: Partial characterization of 'dimeric' intermediates. *Arch. Biochem. Biophys.* **2010**, *501*, 73−78.
(31) Meselhy, M. R.; Nakamura, N.; Hattori, M. Biotransformation of (-)-epicatechin 3-O-gallate by human intestinal bacteria. *Chem. Pharm. Bull.* **1997**, *45*, 888−893.
(32) Takagaki, A.; Nanjo, F. Metabolism of (-)-epigallocatechin gallate by rat intestinal flora. *J. Agric. Food. Chem.* **2010**, *58*, 1313−1321.
(33) Appeldoorn, M. M.; Vincken, J. P.; Aura, A. M.; Hollman, P. C. H.; Gruppen, H. Procyanidin dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-γ-valerolactone as the major metabolites. *J. Agric. Food. Chem.* **2009**, *57*, 1084−1092.
(34) Diebolt, M.; Laflamme, K.; Labbe, R.; Auger, F. A.; Germain, L.; Andriantsitohaina, R. Polyphenols modulate calcium-independent mechanisms in human arterial tissue-engineered vascular media. *J. Vasc. Surg.* **2007**, *46*, 764−72.
(35) Aura, A. M. Microbial metabolism of dietary phenolic compounds in the colon. *Phytochem. Rev.* **2008**, *7*, 407−429.
(36) Gonthier, M. P.; Cheynier, V.; Donovan, J. L.; Manach, C.; Morand, C.; Mila, I.; Lapierre, C.; Rémésy, C.; Scalbert, A. 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. *J. Nutr.* **2003**, *133*, 461−467.
(37) Gross, G.; Jacobs, D. M.; Peters, S.; Possemiers, S.; Van Duynhoven, J.; Vaughan, E. E.; Van De Wiele, T. In vitro bioconversion of polyphenols from black tea and red wine/grape juice by human intestinal microbiota displays strong interindividual variability. *J. Agric. Food. Chem.* **2010**, *58*, 10236−10246.
(38) Curtius, H. C.; Mettler, M.; Ettlinger, L. Study of the intestinal tyrosine metabolism using stable isotopes and gas chromatography-mass spectrometry. *J. Chromatogr., A* **1976**, *126*, 569−580.
(39) Fleschhut, J.; Kratzer, F.; Rechkemmer, G.; Kulling, S. E. Stability and biotransformation of various dietary anthocyanins in vitro. *Eur. J. Nutr.* **2006**, *45*, 7−18.
(40) van Dorsten, F. A.; Grün, C. H.; van Velzen, E. J. J.; Jacobs, D. M.; Draijer, R.; van Duynhoven, J. P. M The metabolic fate of red wine and grape juice polyphenols in humans assessed by metabolomics. *Mol. Nutr. Food Res.* **2009**, *54*, 897−908.
(41) Rios, L. Y.; Gonthier, M. P.; Rémésy, C.; Mila, I.; Lapierre, C.; Lazarus, S. A.; Williamson, G.; Scalbert, A. Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects. *Am. J. Clin. Nutr.* **2003**, *77*, 912−918.
(42) Ward, N. C.; Croft, K. D.; Puddey, I. B.; Hodgson, J. M. Supplementation with grape seed polyphenols results in increased urinary excretion of 3-hydroxyphenylpropionic acid, an important metabolite of proanthocyanidins in humans. *J. Agric. Food. Chem.* **2004**, *52*, 5545−5549.
(43) Kutschera, M.; Engst, W.; Blaut, M.; Braune, A. Isolation of catechin-converting human intestinal bacteria. *J. Appl. Microbiol.* **2011**, *111*, 165−75.