Comparative Study of Microbial-Derived Phenolic Metabolites in Human Feces after Intake of Gin, Red Wine, and Dealcoholized Red Wine

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Ⓢ *Supporting Information*

ABSTRACT: The analysis of microbial phenolic metabolites in fecal samples from in vivo studies is crucial to understanding the potential modulatory effects derived from polyphenol consumption and its overall health effects, particularly at the gut level. In this study, the composition of microbial phenolic metabolites in human feces collected after regular consumption of either red wine, dealcoholized red wine, or gin was analyzed by UPLC-ESI-MS/MS. Red wine interventions produce a change in the content of eight phenolic acids, which are probably derived from the catabolism of flavan-3-ols and anthocyanins, the main flavonoids in red wine. Moreover, alcohol seemed not to influence the formation of phenolic metabolites by the gut microbiota. A principal component analysis revealed large interindividual differences in the formation of microbial metabolites after each red wine polyphenol intervention, but not after the gin intervention, indicating differences in the gut microbial composition among subjects.

KEYWORDS: *red wine polyphenols, gut microbiota, microbial metabolites, feces, UPLC-ESI-MS/MS*

■ INTRODUCTION

Recent studies seem to indicate that the biological activity and health effects derived from the consumption of polyphenol-rich foods, such as wine, are mainly due to the phenolic metabolites formed in the gastrointestinal tract rather than the original forms present in foods.[1−5] The contribution of either phase II metabolism or colonic catabolism to the overall bioavailability of polyphenols is directly influenced by their chemical structure. Wine polyphenols comprise a wide range of nonflavonoid and flavonoid compounds. Flavan-3-ols and anthocyanins are among the most abundant and bioactive flavonoids in red wine. Flavan-3-ols occur as monomeric, oligomeric, or polymeric forms; the latter two are also known as proanthocyanidins or condensed tannins. Main flavanol monomeric units include (+)-catechin, (−)-epicatechin, and (−)-epicatechin-3-O-gallate. 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 glucose molecule through esterification with acetic, p-coumaric, and caffeic acids.

Among the different structures, monomeric flavan-3-ols and anthocyanin glucosides are mainly absorbed in the small intestine and then reach the colon through heterohepatic recirculation in conjugated form, whereas proanthocyanidins with a degree of polymerization ≥3 directly reach the colon in their native form.[4]

Although the colonic microbial catabolic pathways of these compounds are still under consideration and hence, microbial phenolic metabolites derived from wine consumption are not fully elucidated, in vitro fermentations carried with human feces in the presence of grape seed, wine extracts or individual compounds, have allowed the identification of a wide range of metabolites including phenyl-valerolactones and phenolic acids of different side chain length and hydroxylation pattern.[3]

Recent in vitro studies suggest that the polyphenol food source influences the amount and profile of microbial metabolite that may be produced by the colonic microbiota.[6−9] In fact, in vivo studies have revealed the differential impact of black tea or red wine/grape juice polyphenolic extracts on the urinary human metabolome.[10,11] These changes in the metabolome may have profound health effect implications at the gut level and systemically, because for some polyphenols microbial catabolism constitutes a way of their conversion into more bioactive forms.[3] Although the known bioactive properties of microbial metabolites are still limited, some metabolites have revealed antioxidant, antithrombotic, anti-inflammatory, and antiproliferative activ-

ities, as well as inhibition of pathogenic bacteria and modulation of lipid metabolism.[3]

Moreover, considering that the colon is the site where the bacteria−polyphenol interaction occurs, modulation of the microbiota growth and metabolism is excepted to occur at the colon level.[12] Recent studies indicate that flavan-3-ol-rich sources such as chocolate, green tea, black currant or grape seed extracts may modulate the intestinal microbiota in vivo, producing changes in beneficial bacteria such as *Lactobacillus*, but inhibiting other groups such as *Clostridium* spp.[13−17] In the case of red wine polyphenols, evidence is still scarce.[18] Despite these modulatory effects found in vivo, very few studies have attempted to study the metabolome of the corresponding fecal samples,[19,20] limiting the analysis to urine samples. On the other hand, most of the knowledge accumulated on the profile of microbial metabolites in feces comes from culture samples collected in in vitro fermentation studies.[3] The integration of both microbiome and metabolome analyses in fecal samples from in vivo polyphenol feeding trials studies is crucial not only to link bacteria to the production of certain metabolites but also to understand how the modulatory effects of these compounds result in benefits to the bacteria−host mutualism, in favor of disease prevention or health improvement.

In a recent study carried out by our group and comprising three different interventions (red wine, dealcholized red wine, and gin), it was found that the daily consumption of red wine polyphenol for 4 weeks by human subjects significantly increased the number of *Enterococcus, Prevotella, Bacteroides, Bifidobacterium, Bacteroides uniformis, Eggerthella lenta*, and *Blautia coccoides−Eubacterium rectale* groups in fecal samples compared to baseline.[21] In addition, it was found that positive changes in cholesterol and C-reactive protein concentration were linked to changes in the *Bifidobacterium* number.

Because of the impact of regular wine consumption on the modulation of the microbiota composition and its correlation with some biomarkers of health effect, in the present work fecal samples were chosen to study the profile of microbial phenolic metabolites derived from red wine consumption and compared to that from samples collected after dealcoholized red wine and gin consumption. For this purpose, a powerful UPLC-ESI-MS/MS method was validated and used for the screening of more than 60 metabolites in fecal samples.

■ MATERIALS AND METHODS

Standards and Reagents. Phenolic compounds targeted in this study ($n = 60$) are listed in Table 1-OSM in the Supporting Information. Standards of mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, and cinnamic acids were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), Phytolab (Vestenbergsgreuth, Germany), or Extrasynthèse (Genay, France). Valerolactone standards (5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(4′-hydroxyphenyl)-γ-valerolactone) were previously synthesized.[22] Signals of 4-hydroxyvaleric acids (4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric, 4-hydroxy-5-(3′-hydroxyphenyl)valeric acid, 4-hydroxy-5-(phenyl)valeric acid and 4-hydroxy-5-(3′,4′,5′-trihydroxyphenyl)-valeric acid), 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone and other metabolites (dihydroresveratrol, 1-(3′,4′,5′-trihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol, 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol) were also considered. Acetic acid was purchased from Scharlau (Barcelona, Spain). Acetonitrile (HPLC grade) was purchased from Labscan (POCH S.A, Gliwice, Poland). Ultrapure water was obtained using a Milli-Q system (Waters Millipore, Milford, MA, USA).

Stock solutions of phenolic standards (250 μg mL^{-1}) were prepared by exact weighing of the analytes and dissolution with acetonitrile/water (1:4, v/v). Diluted solutions of 5 and 50 μg mL^{-1} were prepared to optimize the MS/MS parameters. According to their response, analytes were classified in five different groups[7] (from highest to lowest response), and a stock standard pool solution was prepared by weighing individual compounds to achieve the following starting concentrations: 25, 50, 100, 200, and 500 μg mL^{-1}. Other solutions were prepared via serial dilutions and used in the generation of the calibration curves (11 different calibration levels from 10- to 10000-fold dilutions of the initial pool solution).

Design of the Human Intervention Study. A randomized, crossover, controlled intervention study was performed to study the influence of moderate red wine intake on the gut microbiota.[21] The study involved eight healthy adults (ages ranging from 45 to 50 years). The participants were not receiving treatment for diabetes, hypertension, or dyslipidemia, nor did they have any acute or chronic inflammatory diseases, infectious diseases, viral infections, cancer, or a previous cardiovascular event at study entry. They had not received any antibiotic therapy, prebiotics, probiotics, symbiotics, or vitamin supplements or any other medical treatment influencing intestinal microbiota during the 3 months before the start of the study or during the study (including the washout period). The study was divided into four consecutive periods: an initial washout period of 2 weeks (baseline) during which the participants did not consume any alcohol or red wine, followed by three consecutive periods of 20 days during which the participants drank only dealcoholized red wine (272 mL/day), red wine (272 mL/day), or gin (100 mL/day).

Red wine and dealcoholized red wine used in this study were produced with the Merlot grape variety, from the Penedès appellation (Spain). The dealcoholized red wine had the same composition and polyphenolic compounds as the red wine, except for the ethanol (only 0.42%).[21]

Each participant provided four different fecal samples: a first baseline sample after the washout period and a sample at the end of each 20 day period. The participants were asked not to change their dietary pattern and lifestyle habits during the study. The subjects were asked to avoid alcoholic beverages during the study. All of the participants gave written informed consent.

Sample Collection and Preparation of Fecal Solutions. Fecal samples were stored at −80 °C awaiting analysis. For preparation of fecal solutions, samples were thawed at room temperature and weighed (1.0 g) in 15 mL sterile conical tubes. Ten milliliters of sterile saline solution (NaCl 0.9%, Fresenius Kabi, Spain) was added and vortexed and centrifuged (10 min, 10000 rpm, 4 °C) two times. The supernatant (fecal water) was filtered (0.22 μm) and diluted with acetonitrile (1:4, v/v, acetonitrile/fecal water). Two microliters of sample was injected into the chromatographic system.

Analysis of Phenolic Metabolites in Fecal Solutions. Phenolic metabolites were analyzed by UPLC-ESI-MS/MS.[20] The liquid chromatographic system was a Waters Aquity UPLC (Milford, MA, USA) equipped with a binary pump, an autosampler thermostated at 10 °C, and a heated column compartment (40 °C). The column employed was a BEH-C18, 2.1 × 100 mm, and 1.7 μm particle size from Waters. The mobile phases were 2% acetic acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient program was as follows: 0 min, 0.1% B; 1.5 min, 0.1% B; 11.17 min, 16.3% B; 11.5 min, 18.4% B; 14 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B. Equilibrium time was 2.4 min, resulting in a total run time of 18 min. The flow rate was set constant at 0.5 mL min^{-1}, and the injection volume was 2 μL.

The LC effluent was pumped to an Acquity TQD tandem quadruple mass spectrometer equipped with a Z-spray electrospray ionization (ESI) source operated in negative polarity mode. The ESI parameters were set as follows: 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. The MS/MS parameters (cone voltage and collision energy) of each analyte were initially optimized by direct infusion experiments using 10 μg mL^{-1} solutions at a flow rate of 5 μL min^{-1}, and the most sensitive transition (precursor and product ions) was selected for quantification purposes using the multiple reaction monitoring (MRM) mode. Apart from the phenolic standards, signals of valeric acids (4-
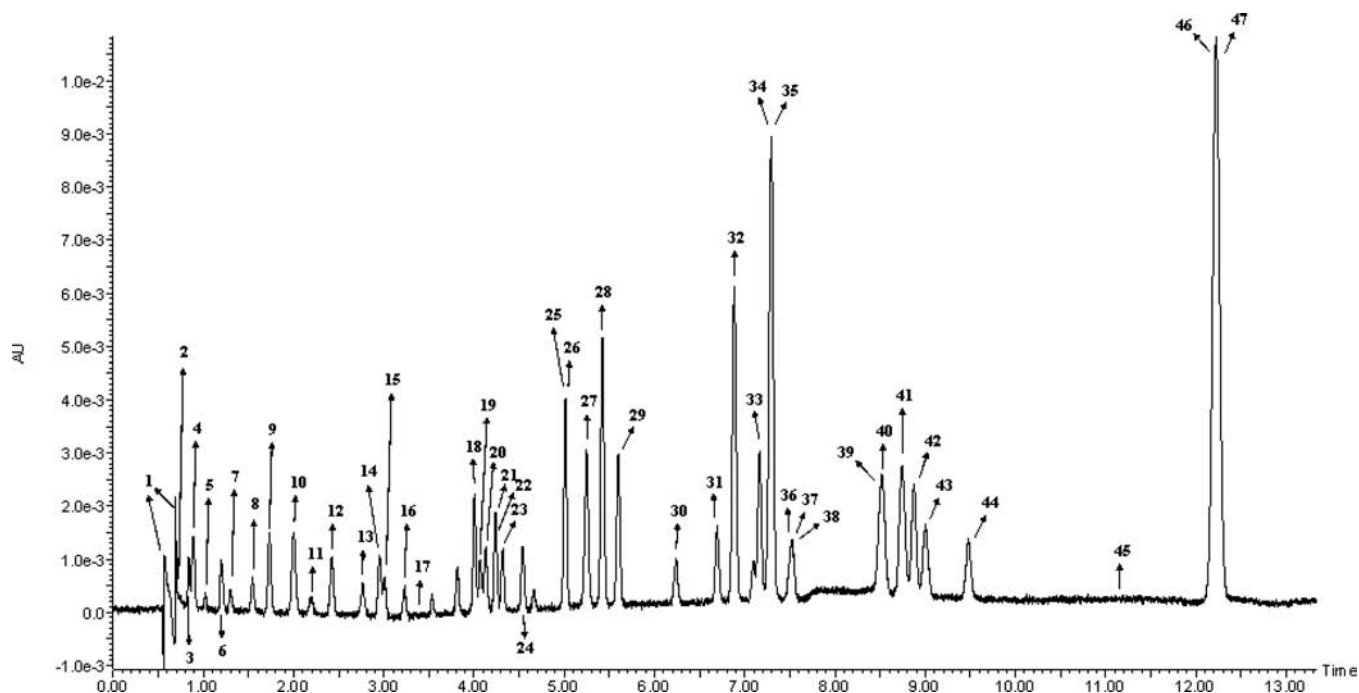
Figure 1. UPLC-DAD chromatogram of a solution of phenolic standards (n = 47). Peaks: (1) 3,4-dihydroxymandelic acid; (2) phloroglucinol; (3) 4-hydroxymandelic acid; (4) gallic acid; (5) pyrogallol; (6) 4-hydroxy-3-methoxymandelic acid; (7) 3-hydroxymandelic acid; (8) 3,5-dihydroxybenzoic acid; (9) protocatechuic acid; (10) 3-hydroxy-4-methoxymandelic acid; (11) 4-hydroxyhippuric acid; (12) catechol/pyrocatechol; (13) 3,4-dihydroxyphenylacetic acid; (14) 3-*O*-methylgallic acid; (15) 4-hydroxybenzoic acid; (16) 4-*O*-methylgallic acid; (17) mandelic acid; (18) 4-hydroxyphenylacetic acid; (19) 3-(3,4-dihydroxyphenyl)propionic acid; (20) 3-hydroxybenzoic acid; (21) hippuric acid; (22) caffeic acid; (23) vanillic acid; (24) 3-hydroxyphenylacetic acid; (25) syringic acid; (26) 4-hydroxy-3-methoxyphenylacetic acid; (27) 4-methylcatechol; (28) 3-(4-hydroxyphenyl)propionic acid; (29) *p*-coumaric acid; (30) 3-(3-hydroxyphenyl)propionic acid; (31) ferulic acid; (32) *m*-coumaric acid; (33) 3,4-dimethoxybenzoic acid; (34) benzoic acid; (35) isoferulic acid; (36) phenylacetic acid; (37) salicylic acid; (38) 3,4-dimethoxyphenylacetic acid; (39) 4-methoxyphenylacetic acid; (40) 4-methoxybenzoic acid; (41) 4-ethylcatechol; (42) 3,4,5-trimethoxybenzoic acid; (43) 3-methoxybenzoic acid; (44) 3-(3,4-dimethoxyphenyl)propionic; (45) phenylpropionic acid; (46) 3,4,5-trimethoxycinnamic acid; (47) *trans*-cinnamic acid.

hydroxy-5-(3′,4′-dihydroxyphenyl)valeric, 4-hydroxy-5-(3′-hydroxyphenyl)valeric, and 4-hydroxy-5-(phenyl)valeric acids) were optimized using samples from in vitro fermentations of flavan-3-ols.[7] These latter metabolites were quantified using the calibration curves of 3-(3,4-dihydroxyphenyl)propionic, 3-(3-hydroxyphenyl)propionic acids, and propionic acids, respectively. 5-(3′-Hydroxyphenyl)-γ-valerolactone was quantified using the calibration curve of 5-(4′-hydroxyphenyl)-γ-valerolactone. Data acquisition and processing were realized with MassLynx 4.1 software.

Statistical Analysis. The statistical methods used for the data analysis were as follows: *t* test for dependent samples and its corresponding nonparametric Wilcoxon matched pairs test to evaluate differences in means of the content of phenolic acid metabolites in feces between two levels of the four interventions (baseline, dealcoholized red wine, red wine, and gin); and principal component analysis (PCA), from correlation matrix, to examine the relationships between analyzed variables and samples. A value of $P = 0.05$ was fixed for the level of significance of the tests. The Statistica program for Windows, version 7.1 (StatSoft Inc., 1984–2006, www.statsoft.com), was used for data processing.

■ RESULTS AND DISCUSSION

Validation of the UPLC-ESI-MS/MS Method for Analysis of Phenolic Metabolites in Fecal Solutions. Ultrahigh-pressure LC improves chromatographic resolution, speed, and sensitivity and, when coupled to MS, facilitates rapid and high-throughput analysis. Therefore, a UPLC column was used, and the choice for the mobile phase was adopted from a previous method developed in our laboratory.[20] The chromatographic optimization allowed the separation, within 15.5 min, of the phenolic standards selected for this work, including phenols and

mandelic, benzoic, hippuric, phenylacetic, phenylpropionic, valeric, and cinnamic acids (Figure 1).

Flow injection analysis of the studied compounds was performed by directly injecting individual standard solutions into the source operated in negative mode. The deprotonated molecular ion $[M - H]^-$ was selected as the precursor ion in all cases. Further identification of the most abundant product ions and selection of the optimum collision energies for each analyte were carried out in the product ion scan mode. Table 1-OMS in the Supporting Information reports the MS/MS parameters (MRM transitions, cone voltages, and collision energies) for the 60 phenolic metabolites targeted. These compounds were selected on the basis of previous studies reported in the literature, availability, analogous chemical structures, etc.

The method validation was carried out in terms of linearity, precision and accuracy, and limits of detection (LOD) and quantification (LOQ). Calibration curves were prepared according to the method of external standard at 11 different concentration levels in triplicate. According to their response, five different starting concentrations were employed (25, 50, 100, 200, and 500 μg mL^{-1}), and dilutions from 1:10 to 1:10000 were made. Weighted least-squares regression analysis was applied. As expected from the differences observed in MS responses, the linear concentration range largely varies between the different analytes, even between the ones included in the same calibration range. LOD and LOQ were determined by injection of the pool standard dilutions and following the criterion of signal-to-noise ratio (S/N) $\geq$3 and $\geq$8, respectively. The accuracy and precision study was made by using the 1:100 dilution of the initial stock

Table 1. Mean ± Standard Deviation Values ($n = 8$) of the Microbial Phenolic Concentration (Micrograms per Gram) in Human Feces of the Studied Subjects at Baseline and after the Three Treatments

compound	baseline (washout period)	dealcoholized red wine	red wine	gin
benzoic acids				
gallic acid	ND[a]	0.364 ± 0.825	0.266 ± 0.445	0.121 ± 0.224
3,5-dihydroxybenzoic acid	0.010 ± 0.029	0.046*[b] ± 0.056	0.150* ± 0.189	0.026 ± 0.028
protocatechuic acid	0.277 ± 0.270	0.554 ± 0.408	1.080* ± 1.020	0.565 ± 0.300
3-O-methylgallic acid	ND	0.134* ± 0.145	0.293* ± 0.273	0.082 ± 0.165
4-hydroxybenzoic acid	0.227 ± 0.218	0.267 ± 0.197	0.447 ± 0.361	0.270 ± 0.219
3-hydroxybenzoic acid	0.061 ± 0.068	0.219 ± 0.293	0.249 ± 0.429	0.082 ± 0.121
vanillic acid	0.018 ± 0.034	0.151* ± 0.170	0.343* ± 0.363	0.063 ± 0.093
syringic acid	ND	0.495* ± 0.592	1.270* ± 1.390	0.461 ± 0.654
benzoic acid	47.344 ± 47.317	69.735 ± 79.125	93.000 ± 114.132	33.761 ± 31.376
salicylic acid	0.206 ± 0.450	0.069 ± 0.094	0.093 ± 0.165	0.026 ± 0.055
phenylacetic acids				
4-hydroxyphenylacetic acid	1.900 ± 2.510	1.480 ± 1.270	2.040 ± 1.290	1.320 ± 0.560
3-hydroxyphenylacetic acid	1.340 ± 2.910	4.920 ± 6.590	6.150 ± 6.580	2.290 ± 3.670
phenylacetic acid	35.395 ± 23.871	37.508 ± 26.739	37.600 ± 9.772	31.872 ± 20.803
phenylpropionic acids				
3-(3,4-dihydroxyphenyl)propionic acid	0.016 ± 0.046	0.149 ± 0.234	0.107 ± 0.258	0.089 ± 0.173
3-(3-hydroxyphenyl)propionic acid	4.410 ± 10.600	1.140 ± 1.580	0.899 ± 0.886	0.828 ± 0.556
phenylpropionic acid	5.950 ± 5.260	16.700* ± 13.200	11.900* ± 4.200	9.450 ± 7.920
valeric acids				
4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid	ND	0.017 ± 0.033	0.069 ± 0.198	0.003 ± 0.008
4-hydroxy-5-(3′-hydroxyphenyl)valeric acid	0.041 ± 0.116	ND	1.170 ± 3.310	0.347 ± 0.728
4-hydroxy-5-(phenyl)valeric acid	6.270 ± 9.270	29.700* ± 30.600	31.400* ± 21.100	33.200 ± 49.400
cinnamic acids				
caffeic acid	0.265 ± 0.376	0.302 ± 0.178	0.605 ± 0.713	0.346 ± 0.293
p-coumaric acid	0.049 ± 0.032	0.415* ± 0.389	0.728* ± 0.573	0.347 ± 0.393
ferulic acid	0.557 ± 0.527	1.860 ± 2.380	2.200 ± 2.490	2.490 ± 4.350

[a]ND, not detected. [b]The asterisk indicates the mean value is significantly different from the baseline concentration ($p < 0.05$).
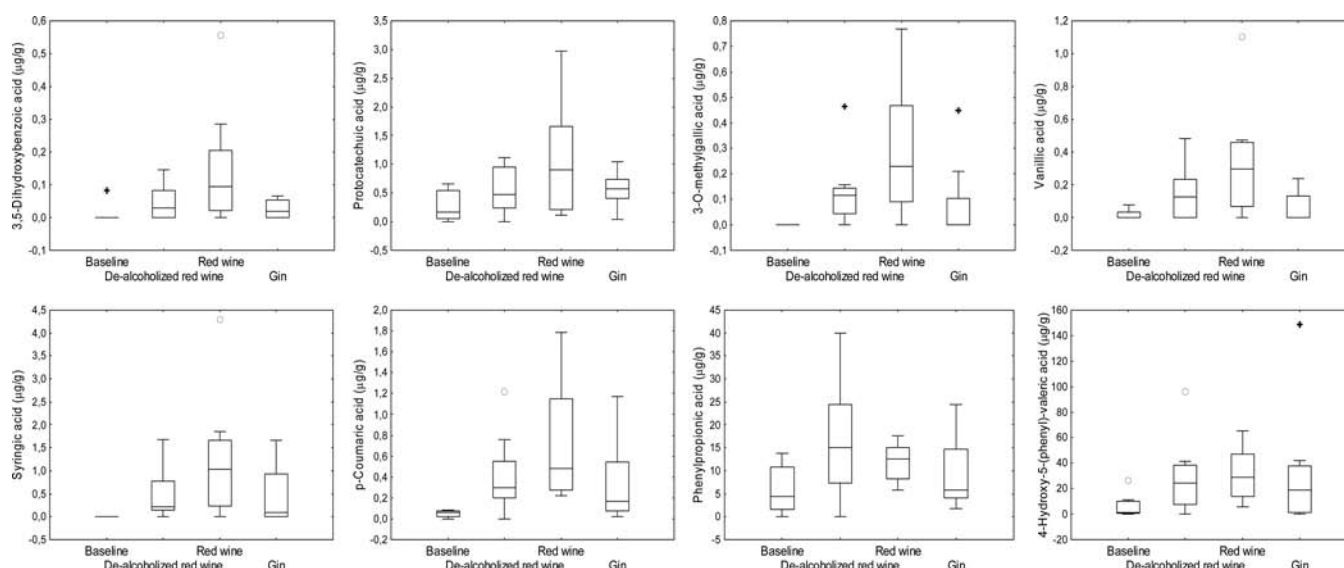

Figure 2. Box and whiskers plots (median, 25th and 75th percentiles, nonoutlier range) of the microbial metabolite content in feces (μg/g) at baseline and after dealcoholized red wine, regular red wine, and gin interventions. The outliers (o) and extremes (∗) are also included.

standard pool solution (0.25, 0.50, 1.0, 2.0, and 5.0 μg mL^{-1}), performing three different injections/day on three different days. The accuracy of the method (expressed as 100 times the mean observed concentration/added concentration) ranged from 80 to 126%. The precision (interday assay), expressed as the relative standard deviation (% RSD), was <15% in all cases. Results of the method validation (concentration range, linearity, LOD, LOQ,

accuracy and precision) agreed with those from our previous publication.[20]

Analysis of Phenolic Acid Metabolites in Fecal Solutions. Fecal samples from the volunteers ($n = 8$) at baseline and after the three consecutive periods of beverage consumption (dealcoholized red wine, red wine, and gin) were analyzed with the described method. Results expressed as the
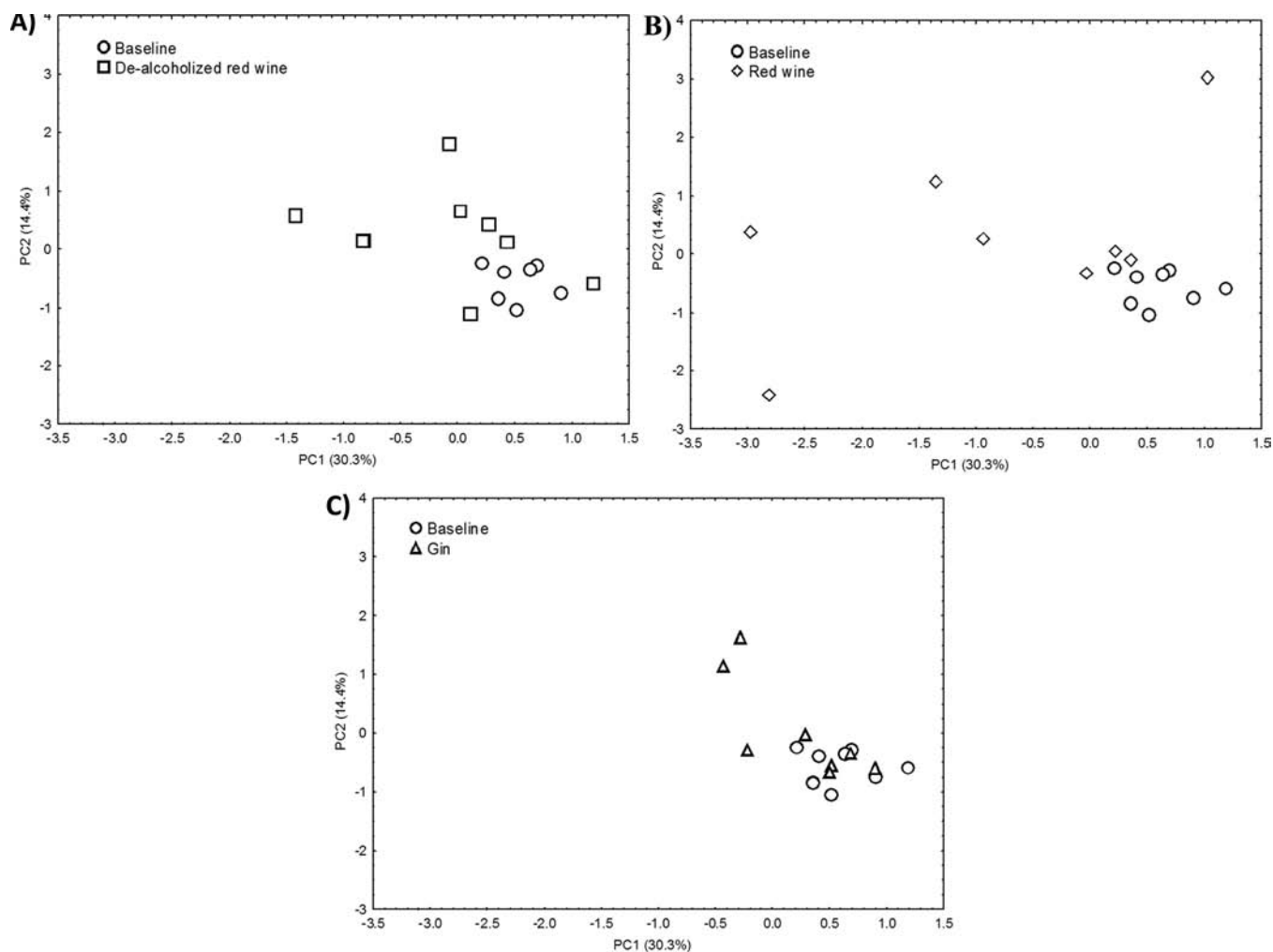
Figure 3. Representation of the samples in the plane defined by the first two principal components (PC1 and PC2) resulting from a PCA of both baseline and after dealcoholized red wine (A), red wine (B), and gin (C) interventions.

mean ± standard deviation (SD) are shown in Table 1. Among the microbial metabolites, benzoic acid, phenylacetic acid, phenylpropionic acid, 4-hydroxy-5-phenylvaleric acid, and ferulic acid were the most abundant metabolites of each group at baseline. In a study carried out by Jener et al.,[19] a concentration range of 15.6−149.5 mg/L of phenylacetic acid was found in fecal water (that is, the upper water layer after homogenization of feces in a stomacher), which is equivalent to a content of 10.1−97.2 μg/g of fresh feces considering an average fecal water content of 65%. These data are in line with our findings because the content of phenylacetic acid at baseline varied from 17.02 to 73.16 μg/g. In the same paper,[19] a concentration range of 0.85−42.8 μg/g was found for phenylpropionic acid, which is in accordance with the range of 3.16−13.75 μg/g obtained in the present work. The content of benzoic acid reported[19] is included within the range of 1.56−10.6 μg/g, considerably lower than the mean value obtained by us (47.3 μg/g). This is due to the high content of benzoic acid found for one of the volunteers of the study (142.8 μg/g), which substantially increased the mean value of this compound.

Both the t test for dependent samples and the Wilcoxon matched pairs test showed significant differences in phenolic content between the baseline and the red wine intervention for eight metabolites: 3,5-dihydroxybenzoic acid, 3-O-methylgallic acid, p-coumaric acid, phenylpropionic acid, protocatechuic acid,

vanillic acid, syringic acid, and 4-hydroxy-5-(phenyl)valeric acid (Table 1). When comparing data at baseline and after dealcoholized red wine intervention, the t test and the Wilcoxon matched pairs showed significant differences for the content of 3,5-dihydroxybenzoic acid, 3-O-methylgallic acid, p-coumaric acid, phenylpropionic acid, vanillic acid, syringic acid, and 4-hydroxy-5-phenylvaleric acid (Table 1). Therefore, except for protocatechuic acid, the same metabolites were changed after the consumption of polyphenols in the form of either red wine or dealcoholized wine. Moreover, no significant differences were found for any of these metabolites when samples from the red wine and dealcoholized red wine interventions were compared (data not shown), indicating that the alcoholic matrix of wine does not seem to affect the profile of microbial metabolites and, thus, the bioavailabity and biotransformation of red wine polyphenols, as previously reported for resveratrol metabolites.[23] Finally, as expected, no significant differences were found in the content of any metabolite between samples at baseline and after gin intervention.

Figure 2 illustrates the box and whiskers plots (median, 25th and 75th percentiles, nonoutlier range) of the microbial metabolites mentioned above, which showed significant differences ($P < 0.05$) between the baseline and the red wine interventions. The outliers (o) and extremes ($*$) values are also shown. Although, as mentioned above, differences between the

red wine and dealcoholized red wine interventions were not significant for these compounds, in all cases a slightly higher content was found after the red wine intervention compared to the dealcoholized red wine intervention, with the exceptions of phenylpropionic and 4-hydroxy-5-(phenyl)valeric acids. One of the main limitations of our study is that no washout period was carried out between interventions, so these findings could be due to a possible cumulative effect derived from the previous intervention.

On the basis of the proposed catabolic pathway of flavan-3-ols, 4-hydroxy-5-(phenyl)valeric acids are considered to arise from the first steps of the microbial degradation of flavan-3-ols as a result of the fission of the heterocyclic C-ring of the flavonoid molecule. Shortening of the side-chain length by subsequent β-oxidation reactions results in phenylpropionic, phenylacetic, and benzoic acid derivatives. Phenylpropionic acid usually results from the dehydroxylation of 3-(3,4-dihydroxyphenyl)propionic acid, which has been reported as one of the most abundant final microbial metabolites derived from flavan-3-ols, both in vivo and in vitro.[6,7,24] O-Methylated benzoic acids including syringic and vanillic acids could arise from the catabolism of red wine anthocyanins.[25−28] The B-ring of malvidin, the major anthocyanin in red wine, could be degraded into 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid), whereas peonidin could be degraded into 4-hydroxy-3-methoxybenzoic acid (vanillic acid). Cyanidin, a nonmethylated form, would result in 3,4-dihydroxybenzoic acid (protocatechuic acid). 4-O-Methylgallic acid is a potential urinary biomarker of red wine consumption, resulting from hepatic or renal metabolism of gallic acid,[29,30] which is first released from hydrolysis of grape seed galloylated flavan-3-ols.[7] Likewise, both 4-O- and 3-O-methylgallic acids have been reported as important urinary biomarkers of black tea consumption.[31] However, the presence of 3-O-methylgallic acid in fecal samples is most likely to arise from the catabolism of petunidin. Finally, p-coumaric acid could be formed from the hydrolysis of p-coumaroyl-acylated anthocyanins, which are also very abundant in red wine.[32] Therefore, metabolites significantly changing after the red wine interventions come from the catabolism of both flavan-3-ols and anthocyanins, the major flavonoids in red wine.

To summarize the changes in the phenolic metabolites as a consequence of moderate intake of red wine polyphenols, a PCA was applied. Figure 3 represents the plane defined by the first two principal components (PC1 and PC2) that resulted from the PCA using the data from samples at baseline and after dealcoholized red wine (A), red wine (B), and gin (C) interventions, respectively. The first principal component (PC1), explaining 30.3% of the total variance, was negatively correlated (loadings ≤ −0.7) with 3,5-dihydroxybenzoic acid, protocatechuic acid, 3-O-methylgallic acid, vanillic acid, syringic acid, and p-coumaric acid. The second principal component explained 14.4% of the total variance. Panels A and B of Figure 3 show that samples collected after the intake of either red wine or dealcoholized red wine could be easily differentiated from those at baseline. On the other hand, Figure 3C shows no differentiation between samples at baseline and after the gin intervention. Therefore, interindividual variability between subjects considerably increased as a result of the different wine interventions in comparison to baseline conditions, but not after the gin intervention, reflecting variations in the microbiota composition between subjects. These findings indicate that red wine polyphenols have a profound impact on the fecal metabolite profile. Although some connections have been made between

certain bacterial clusters including *Actinobacteria* and *Clostridium* with metabolites arising from the first stages of microbial degradation of flavan-3-ols, such as phenylvalerolactones,[33] the association between changes in metabolic profile and microbial groups is still difficult to establish and would require in-depth studies at the metagenomic or transcriptomic levels.

In conclusion, the validated method allowed the analysis of 60 microbial phenolic metabolites in fecal solutions. Results obtained indicate that the microbial metabolic profile of feces is significantly modified after moderate intake of red wine polyphenols. Also important was the fact that the presence of alcohol in red wine did not seem to affect the performance of the microbial catabolism of polyphenols and, therefore, the potential bioavailability of these compounds. Metabolites derived from the catabolism of both flavan-3-ols and anthocyanins seem to contribute to explain changes in the fecal metabolic profile. Some of these metabolites may help to explain some of the numerous health benefits derived from red wine polyphenols, including the modulation of the microbial population reported in our previous study.[21] Although our results need to be further corroborated with a larger number of volunteers, our study showed that the regular consumption of red wine could affect the metabolic profile of feces and produce positive changes in the microbiota composition.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information
Table 1-OSM. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
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■ ACKNOWLEDGMENTS

■ REFERENCES

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