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Profiling of Microbial-Derived Phenolic Metabolites in Human Feces after Moderate Red Wine Intake

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Supporting Information

ABSTRACT: A controlled and randomized trial study involving 41 healthy volunteers (33 intervention and 8 control subjects) was performed in order to establish changes in the microbial-derived phenolic metabolite profile of feces after moderate consumption of red wine (250 mL/day, 4 weeks). Out of the 35 phenolic metabolites identified, 10 compounds (mainly benzoic and 4-hydroxyvaleric acids) showed statistically significant increases ($P < 0.05$) after the wine intake. Also, the total phenolic metabolites content was significantly ($P < 0.05$) higher in the samples after the wine intake (625 ± 380 μg/g feces) in comparison to the samples before (358 ± 270 μg/g feces), and a tentative distribution of the volunteers into three groups could be established: <500, 500−1000, and >1000 μg/g feces. These results suggest that a different gut microbial capacity to metabolize wine polyphenols exists among the human population, as observed for polyphenols from other sources.

KEYWORDS: *red wine polyphenols, microbial metabolites, phenolic acids, feces, UPLC-ESI-MS/MS*

INTRODUCTION

The biological effects that phenolic compounds, including red wine polyphenols, exert at gut level, including anti-inflammatory activity, modulatory effects on the gut microbiota composition, and interaction with cells, among others, are a topic of current interest. It has become evident that these beneficial effects seem to be due more to phenolic metabolites formed in the gastrointestinal tract, mainly derived from the action of gut bacteria, rather than to the original forms found in food.[1,2] Therefore, there is a need to ascertain the phenolic profile and content in intestinal fluids, and, particularly, how they can be modified by diet. Given the difficulty in performing biopsies or any other intestinal fluid collection, a first approach can be to determine the phenolic profile in human feces.[3]

Polyphenols in wine comprise both flavonoid and nonflavonoid compounds, their molecular weight ranging from phenolic acids to highly polymerized proanthocyanidins. Flavonoids are more abundant in red wine and include flavan-3-ols and anthocyanins, as well as flavonols, flavanonols, and flavones, in lower proportions. The main nonflavonoids are phenolic acids (hydroxybenzoic and hydroxycinnamic acids), as well as other phenolic derivatives such as stilbenes. The intestinal metabolism of phenolic compounds starts in the epithelial cells with the hydrolysis, by intestinal β-glucosidases, of the glycosylated polyphenols such as anthocyanins, flavonols, and stilbenes and the subsequent absorption of the aglycon forms.[1,2] By contrast, monomeric flavanols and, in a lesser proportion, procyanidins dimers can be absorbed directly in the small intestine. Once absorbed, these polyphenols are first metabolized in the small intestine and then in the liver by phase II enzymes into methyl, glucuronide, or sulfate conjugates (phase II metabolites), which are preferentially excreted in the bile.[4] Other wine polyphenols, mainly oligomers with a degree of polymerization >3 and polymeric flavanols (proanthocyanidins or condensated tannins), hydroxycinnamic esters, and rhamnose-conjugated polyphenols, are not absorbed in their native forms. These compounds, together with phase II metabolites that reach the colon by enterohepatic recirculation, are catabolized by the colonic microbiota before their absorption.[5] Colonic catabolism involves the formation of simple phenols, phenolic and aromatic acids, and lactones with different degrees of hydroxylation and side-chain length, with often altered bioactivities compared to the parent compound,[6] that could be further absorbed and subsequently submitted to intestinal and hepatic metabolism by phase II enzymes.[1,7] Phenolic metabolites are excreted in urine and feces. So far, several human intervention studies have been carried out in order to study the colonic metabolism of wine polyphenols, reflected in plasma and urine.[8,9] In relation to feces, most of the knowledge acquired on the fecal profile of microbial metabolites comes from culture samples collected in in vitro fermentation studies.[10−13] To our knowledge, only a "pilot" study ($n = 8$ volunteers) has been conducted to ascertain the contents of phenolic metabolites in human feces after wine consumption.[14]

The study of biological responses due to a dietary intake of polyphenols cannot be managed without taking into consideration polyphenols−microbiota interactions. However, knowledge about the enzymatic activities/microbial population responsible for the degradation of polyphenols is still scarce.[2] But what is widely observed is the great interindividual variability in urine phenolic metaboloma after polyphenol-rich dietary interventions,[15−17] which has been attributed to

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dissimilarities in the populations of colonic bacteria. A first example of the different human capacity to metabolize polyphenols, as reflected in urine samples, was seen with isoflavones. In an intervention study with healthy premenopausal women (n = 60) who received soy-extract isoflavones, only some of the volunteers were able to metabolize daidzein to its active metabolite equol, which was attributed to differences in the composition of the intestinal microflora.[15] In addition, large interindividual variations were found in the equol-producing group, which was divided into two subgroups according to their ability to excrete more potent metabolites: high (approximately 25% of the volunteers) and low equol excretors.[15] Another example of the different human capacity to metabolize polyphenols refers to urolithins, microbial derived metabolites from ellagitannins. After an intervention with fresh strawberries and strawberry purée, urolithin A was found in the urine of all the volunteers (n = 20), but only three of them were found to produce and excrete urolithin B.[17] Moreover, volunteers were classified into three subgroups according to their levels of total urolithin A glucuronide excretion in urine.[17] A particular distribution of the volunteers according to their phenolic-metabolizing capacity was also suggested by Bolca et al.[18] in a dietary intervention (n = 150) with soymilk, soy germ, or hop. From their metabolite concentration in urine after the intervention, volunteers were phenotyped as poor, moderate, and strong producers of equol (derived from daidzein) or 8-prenylnaringenin (derived from isoxanthohumol).[18] Therefore, it is assumed that differences in human colonic microbiota lead to different polyphenol-metabolizing phenotypes, or "metabotypes", that in turn would govern the health effects of dietary polyphenols.[19] However, intervention/clinical studies with a large number of subjects are needed to establish these potential relationships between the consumption of foods rich in polyphenols and the production of phenolic metabolites.

To get a deeper understanding of the effects of wine consumption on human health, in the present work, a controlled and randomized trial study involving 41 healthy volunteers (33 intervention and 8 control subjects) was conducted in order to establish the changes in the phenolic metabolite profile of feces after the consumption of red wine polyphenols. Intervention was preceded by a 2-week baseline period with a restriction of wine and polyphenol consumption, and it was followed by a 4-week intervention period during which subjects consumed 250 mL of red wine per day. The changes in the fecal phenolic metabolites were determined using UPLC-ESI-MS/MS analysis and subjected to different statistical approaches. Our work has resulted in some improvements in the analysis of phenolic metabolites in feces by UPLC-ESI-MS/MS, invaluable data on the basal levels of phenolic metabolites in feces, in an assessment of significant changes in the contents and profile of phenolic metabolites in feces after wine consumption, and in a tentative attempt to distribute the population based on their capacity to metabolize wine polyphenols.

MATERIALS AND METHODS

Chemicals and Phenolic Standards. Acetic acid was purchased from Scharlau (Scharlau, Barcelona). 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).

Phenolic standards used in this study and corresponding to mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, anthocyanins, flavan-3-ols, flavonols, and stilbenes were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), Phytolab (Vestenbergsgreuth, Germany), or Extrasynthèse (Genay, France). The standards 5-(3′,4′,-dihydroxyphenyl)-γ-valerolactone and 5-(4′-hydroxyphenyl)-γ-valerolactone were previously synthesized.[20] The compound 4-hydroxybenzoic-2,3,5,6-d_4 acid, used as internal standard, was purchased from Sigma-Aldrich Chemical Co.

For the analysis of phenolic metabolites by UPLC-ESI-MS/MS, a stock solution of phenolic standars in acetonitrile/water (1:4, v/v) was prepared by weighting individual compounds to achieve different concentrations (25, 50, 100, 200, and 500 μg/mL), depending on their response in ESI-MS/MS.[3] Dilutions of the stock solution were prepared and used in the generation of the calibration curves: 15 different concentration levels from 2- to 20 000-fold dilutions of the initial pool solution were injected in triplicate (Table 1-OSM, Supporting Information).

Red Wine. The red wine used in this study was a young red wine (var. Pinot Noir, vintage 2010), kindly provided by Bodegas Miguel Torres S.A. (Catalonia, Spain). The wine was elaborated following the winery's own winemaking procedures and was selected because of its relatively high phenolic content: total polyphenols = 1758 mg of gallic acid equiv/L, total anthocyanins = 447 mg of malvidin-3-O-glucoside/L, and total catechins = 1612 mg of (+)-catechin/L. The antioxidant capacity of the wine measured as ORAC (oxygen radical absorbance capacity) was 35.5 mmol of Trolox equiv/L.

Human Intervention Study Design. A total of 41 healthy volunteers (22 women and 19 men; age range 20−65 years) were recruited. The participants were not suffering from any disease or intestinal disorder and were not receiving antibiotics or any other medical treatment for at least 6 months before the start of the study or during the study (including the washout period). All the participants were fully informed about the study and gave written informed consent.

A randomized and controlled 4-week intervention study involving 33 volunteers was performed in parallel to an observational study (no intervention) for 8 volunteers (control group).

For both intervention and observational studies, volunteers followed an initial washout period of 2 weeks (baseline) during which they did not consume any wine or any other alcoholic beverage and followed a low-polyphenols diet. After this period, the intervention group consumed 250 mL of red wine/day (equivalent to a dose of ∼450 mg of total polyphenols/day) divided into two doses, during 4 weeks. During this latter period, participants also maintained the restrictions for any other alcoholic beverages and followed a low-polyphenols diet. The control group followed the same pattern, with the exception that no wine was ingested during this 4-week period. Each participant provided samples of feces at two points: (a) after the washout period and (b) at the end of the study. Feces were immediately frozen and stored at −80 °C awaiting analysis.

Preparation of Fecal Solutions. For the preparation of fecal solutions, samples were thawed at room temperature and weighted (1.0 g) in 15 mL sterile conical tubes. Ten milliliters of sterile saline solution (NaCl 0.9%, Fresenius Kabi, Spain) spiked with the internal standard (IS) was added, vortexed, and centrifuged (10 min, 10 000 rpm, 4 °C) twice. The supernatant (fecal solution) was filtered (0.22 μm) and diluted with acetonitrile (1:4, v/v, acetonitrile/fecal solution). Saline solution contained 3.125 μg/mL of IS to achieve a final concentration of 2.5 μg/mL, and 2.0 μL of sample was injected onto the chromatographic system. Each analysis was realized in duplicate.

Analysis of phenolic metabolites. Phenolic metabolites were analyzed using an UPLC-ESI-MS/MS following a previously reported method.[3] The liquid chromatographic system was a Waters Acquity UPLC (Milford, MA) equipped with a binary pump, an autosampler thermostatted 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 (Milford, MA). 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 runtime of 18 min. The flow rate was set constant at 0.5 mL/min and injection volume was 2 μL.

The LC effluent was pumped to an Acquity TQD tandem quadrupole 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 ionization mode. For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific to each compound. The MS/MS parameters (cone voltage, collision energy and MRM transition) of the 60 phenolic compounds targeted in the present study (mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, 4-hydroxyvaleric acids, and valerolactones) were previously reported.[14] All metabolites were quantified using the calibration curves of their corresponding standards, except for 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric, 4-hydroxy-5-(3′-hydroxyphenyl)valeric, and 4-hydroxy-5-phenylvaleric acids, which were quantified using the calibration curves of 3-(3,4-dihydroxyphenyl)propionic, 3-(3-hydroxyphenyl)propionic, 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.

Analysis of Wine Phenolic Compounds. Analysis of both anthocyanin and nonanthocyanin phenolic compounds present in the wine used in this study was carried out by UPLC-ESI-MS/MS, using the equipment reported in the previous section. For the analysis of anthocyanins, the gradient consisted of A (water/formic acid; 90:10, v/v) and B (acetonitrile) applied as follows: 0−1 min, 5−15% B; 1−5.25 min, 15−24% B; 5.25−5.88 min, 24−100% B; 5.88−7.05 min, 100−5% B; 7.05−9.38 min, 5% B. The ESI parameters were the same as those reported above for the analysis of phenolic metabolites, but ESI was operated in positive ionization mode. The MRM transitions used for the detection of anthocyanins were cyanidin-3-*O*-glucoside (449 > 287), delphinidin-3-*O*-glucoside (465 > 303), petunidin-3-*O*-glucoside (479 > 317), peonidin-3-*O*-glucoside (463 > 301), malvidin-3-*O*-glucoside (493 > 331), cyanidin-3-*O*-(6″-*O*-acetyl)glucoside (491 > 287), delphinidin-3-*O*-(6″-*O*-acetyl)glucoside (507 > 303), petunidin-3-*O*-(6″-*O*-acetyl)glucoside (521 > 317), peonidin-3-*O*-(6″-*O*-acetyl)glucoside (505 > 301), malvidin-3-*O*-(6″-*O*-acetyl)-glucoside (535 > 331), cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside (595 > 287), delphinidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside (611 > 303), petunidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside (625 > 317), peonidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside (609 > 301), malvidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside (639 > 331). Cone voltage and collision energy were set at 35 and 20 V, respectively, for all compounds. All individual anthocyanins were quantified using the external calibration curve of malvidin-3-*O*-glucoside. Data acquisition and processing was carried out using MassLynx 4.1 software.

For the analysis of nonanthocyanins, the gradient and the ESI parameters were the same as those reported above for the analysis of phenolic metabolites. The ESI was also operated in negative ionization mode. The MRM transitions used for the detection of flavan-3-ols were catechin and epicatechin (289 > 245), epicatechin-3-*O*-gallate (441 > 289), procyanidin dimers (B1, B2, B3, B4, B5, and B7) (577 > 289) and procyanidins trimers (C1 and others) (865 > 577). 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), piceid (389 > 227), tryptophol (160 > 130), coutaric acid (295 > 163), and caftaric acid (311 > 179). In the absence of commercial standards, quantification of procyanidin B3, B4, B5, and B7 was carried out using the external calibration curve of procyanidin B1, and that of procyanidin trimers was based on procyanidin C1. Piceid was quantified using the external calibration curve of resveratrol. Coutaric acid and caftaric acid were quantified using the external calibration curves of *p*-coumaric acid and caffeic acid, respectively.

In addition, the wine was subjected to the analysis of phenolic metabolites as reported above to determine the content of benzoic acids, phenols, phenylacetic acids, phenylpropionic acids, and cinnamic acids. In all cases, analysis of wine (diluted 1:4, v/v, wine/water) was carried out in triplicate.

Statistical Analysis. The following statistical methods were used for the data analysis: *t* test for independent samples and its corresponding nonparametric Mann−Whitney test to evaluate differences in the means of the basal content of phenolic acid metabolites in feces between the control and intervention groups; *t* test for dependent samples and its corresponding nonparametric Wilcoxon matched-pairs test to evaluate differences in the means of the content of phenolic acid metabolites in feces before and after the 4-week study period, for both the control and intervention groups; the nonparametric Kruskal−Wallis test to compare the means of the content of phenolic metabolites between three groups of volunteers; and the Shapiro−Wilk test to verify the normal distribution of the data. 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

Improvement of the Analysis of Fecal Solutions by UPLC-ESI-MS/MS: Use of Internal Standard Calibration. The ultrahigh-pressure LC separation method used in this work was adopted from a previous method developed in our laboratory.[3] In this method, fecal solutions (1:10 w/v, feces/saline solution) were 2-fold diluted prior to injection onto the UPLC system. Under these conditions, no significant differences were observed in the quantification of microbial phenolic metabolites between external and internal standard calibration methods.[3] To increase detectability of phenolic metabolites, a solid-phase extraction (SPE) procedure was further applied as a preparative sample technique prior to UPLC analysis.[21] However, although detection of certain phenolic metabolites was improved, the procedure failed in the recovery of other phenolics of interest, which led us to discard SPE as a preparative technique in the analysis of fecal phenolic metabolites by UPLC-ESI-MS/MS.[21] In this work, detectability and quantification of phenolic metabolites in feces have been improved by reducing the dilution factor of the fecal solution and by using an IS. Thus, different dilution factors of fecal solutions were tested and ion suppression-enhancement for the IS (4-hydroxybenzoic-2,3,5,6-d_4 acid) was studied by comparing its MS response in fecal solutions with respect to its response in acetonitrile/water (1:4, v/v), at a concentration value of 2.5 μg/mL (results not shown). A compromised dilution factor of 1.25 (4:1, v/v, fecal solution/acetonitrile) was adopted in order to obtain the lowest matrix effect assuring the detectability of the compounds of interest. The instrumental limits of detection (LOD) and quantification (LOQ) followed the criterion of signal-to-noise ratio (S/N) ≥3 and ≥8, respectively, and the calibration curves were determined by injection of the pool standard dilutions (Table 1-OSM, Supporting Information). Also, the accuracy and precision of the method was calculated by using the 1:250 dilution of the initial pool solution. The accuracy for all compounds [expressed as 100 × (mean observed concentration)/(added concentration)] using the internal standard calibration method was realized by performing three different injections/day and ranged from 80 to 113% (Table 1-OMS, Supporting Information). The precision (interday assay, $n = 5$), expressed

Table 1. Mean Values and Standard Deviations (mg/L) of the Individual Phenolic Compounds of the Red Wine Used in this Study

compd	concn	compd	concn
benzoic acids		flavan-3-ols and others	
gallic acid	27.30 ± 0.20	(+)-catechin	51.60 ± 1.70
protocatechuic acid	3.88 ± 0.01	(−)-epicatechin	34.90 ± 2.90
3-*O*-methylgallic acid	1.06 ± 0.06	procyanidin B1	79.10 ± 0.90
4-hydroxybenzoic acid	0.57 ± 0.01	procyanidin B2	44.70 ± 0.60
vanillic acid	1.85 ± 0.03	procyanidin B3	16.00 ± 1.00
syringic acid	2.30 ± 0.13	procyanidin B4	12.90 ± 0.30
benzoic acid	1.14 ± 0.06	procyanidin B5	2.67 ± 0.01
salicylic acid	0.21 ± 0.01	procyanidin B7	5.75 ± 0.15
		procyanidin C1	14.00 ± 0.40
phenols		other trimers	7.96 ± 1.05
phloroglucinol	0.33 ± 0.03		
tyrosol	31.40 ± 1.40	flavonols	
dihidroxiphenylpropan-2-ol	0.30 ± 0.04	quercetin	1.92 ± 0.01
		myricetin	0.70 ± 0.028
phenylacetic and phenylpropionic acids			
4-hydroxy-3-methoxyphenylacetic acid	0.01 ± 0.01	anthocyanins	
3-(3,4-dihydroxyphenyl)-propionic acid	0.16 ± 0.02	delphinidin-3-*O*-glucoside	2.58 ± 0.11
		cyanidin-3-*O*-glucoside	0.76 ± 0.04
cinnamic acids		petunidin-3-*O*-glucoside	4.06 ± 0.13
caffeic acid	6.97 ± 0.26	peonidin-3-*O*-glucoside	18.90 ± 2.00
p-coumaric acid	1.39 ± 0.02	malvidin-3-*O*-glucoside	36.70 ± 3.40
ferulic acid	0.22 ± 0.02	delphinidin-3-*O*-(6″-acetyl)glucoside	0.14 ± 0.01
coutaric acid	8.64 ± 0.01	cyanidin-3-*O*-(6″-acetyl)glucoside	0.08 ± 0.01
caftaric acid	4.98 ± 0.33	petunidin-3-*O*-(6″-acetyl)glucoside	0.15 ± 0.01
		peonidin-3-*O*-(6″-acetyl)glucoside	0.87 ± 0.07
stilbenes		malvidin-3-*O*-(6″-acetyl)glucoside	1.17 ± 0.03
resveratrol	7.12 ± 0.29	delphinidin-3-*O*-(6″-*p*-coumaroyl)glucoside	0.05 ± 0.01
		cyanidin-3-*O*-(6″-*p*-coumaroyl)glucoside	0.07 ± 0.01
		petunidin-3-*O*-(6″-*p*-coumaroyl)glucoside	0.07 ± 0.01
		peonidin-3-*O*-(6″-*p*-coumaroyl)glucoside	1.09 ± 0.07
		malvidin-3-*O*-(6″-*p*-coumaroyl)glucoside	0.94 ± 0.04

as the relative standard deviations (% RSD), was lower than 15% in all cases (Table 1-OSM, Supporting Information).

Phenolic Metabolic Profile in Human Feces after Moderate Red Wine Intake. Phenolic compounds determined in the red wine consumed in this study included benzoic acids, phenols, phenylacetic and phenylpropionic acids, cinnamic acids, stilbenes, flavan-3-ols, flavonols, and anthocyanins, as determined by UPLC-ESI-MS/MS (Table 1). Of special note was the wine's richness in monomeric and oligomeric flavan-3-ols (270 mg/L).

Fecal samples from each volunteer before and after wine intake were analyzed by the described UPLC-ESI-MS/MS methods. Within the 60 targeted metabolic phenolics, a total of 35 compounds were quantified, although in a variable number of cases, for both control (after the 4-week observational period) and intervention groups (after the 4-week wine intervention period) (Table 2). Most of the metabolites were found in a higher number of volunteers after wine intake than before the intervention. This was especially noticeable for syringic acid, since it was quantified in only 4 samples before the wine intake and in 26 samples after the intervention. In a previous pilot study, only 22 phenolic metabolites were detected in feces after a wine intervention study (272 mL/day, 20 days),[14] which evidences either the improvements made on the method to enhance its sensitivity and/or the higher phenolic content of the wine used in the present study. No detectable amounts of the phenolic compounds presented in the wine (anthocyanins, flavan-3-ols, flavonols, or stilbenes) were found in the fecal samples. For a better understanding, mean values of phenolic metabolites concentration for the two groups (control and intervention groups) were calculated by excluding negligible cases (<LOQ) (Table 2). On the basis of these results, benzoic acid, phenylacetic acid, 3-(4′-hydroxyphenyl)propionic acid, 3-(3′-hydroxyphenyl)propionic acid, 3-phenylpropionic acid, and 4-hydroxy-5-phenylvaleric acid showed the highest concentrations for both groups before the 4-week study period. The mean values of phenolic compounds at baseline obtained in the present work were included within the concentration ranges previously reported in the literature,[22−27] in spite of the differences in methodology, number of volunteers, and sample preparation among studies. Table 2-OSM (Supporting Information) reports the basal concentrations in feces for most abundant phenolic metabolites reported in the literature, in comparison to the data reported in this work. Nevertheless, it is necessary to point out the high interindividual variability observed in the concentrations of fecal phenolic metabolites within each study, which evidence the difficulty in establishing reference values and the importance of carrying out these studies with a large group of volunteers, under controlled diets, in order to obtain reliable and consistent conclusions.

Table 2. Phenolic Metabolite Concentration in Human Feces for the Control and Wine Intervention Groups before and after Wine Intake

	metabolite concn[a] (μg/g feces)			
	control group (n = 8)		intervention group (n = 33)	
compd	before	after	before	after
mandelic acids				
3-hydroxymandelic acid	nd[b]	nd	1.77 (n = 1)[c]	1.77 ± 0.01 (n = 2)
benzoic acids				
gallic acid	2.05 ± 0.01 (n = 2)	2.02 ± 0.06 (n = 2)	2.36 ± 0.77 (n = 10)	2.19 ± 0.24 (n = 17)
3,5-dihydroxybenzoic acid	0.18 ± 0.21 (n = 8)	0.18 ± 0.13 (n = 7)	0.21 ± 0.16 (n = 26)	0.35 ± 0.21 (n = 32)
protocatechuic acid	0.99 ± 1.20 (n = 8)	1.14 ± 1.76 (n = 8)	0.79 ± 0.47 (n = 33)	1.25 ± 1.03 (n = 33)
3-*O*-methylgallic acid	0.627 (n = 1)	0.27 (n = 1)	0.50 ± 0.37 (n = 5)	0.37 ± 0.18 (n = 24)
4-hydroxybenzoic acid	1.15 ± 1.40 (n = 6)	0.90 ± 0.78 (n = 6)	0.74 ± 0.77 (n = 23)	0.69 ± 0.68 (n = 24)
4-*O*-methylgallic acid	nd	nd	1.15 ± 0.06 (n = 3)	1.24 ± 0.13 (n = 3)
3-hydroxybenzoic acid	0.95 ± 0.49 (n = 4)	0.59 ± 0.61 (n = 5)	0.63 ± 0.32 (n = 9)	1.04 ± 1.35 (n = 12)
vanillic acid	1.10 (n = 1)	0.98 ± 0.52 (n = 2)	1.08 ± 2.33 (n = 9)	1.12 ± 0.84 (n = 27)
syringic acid	0.42 (n = 1)	nd	0.77 ± 0.37 (n = 4)	1.84 ± 1.11 (n = 26)
benzoic acid	19.40 ± 15.00 (n = 8)	21.00 ± 22.50 (n = 8)	37.70 ± 25.50 (n = 33)	43.40 ± 35.90 (n = 33)
salicylic acid	0.18 ± 0.02 (n = 2)	0.38 ± 0.42 (n = 4)	0.92 ± 1.01 (n = 6)	0.69 ± 1.32 (n = 9)
phenols				
catechol/pyrocatechol	2.25 (n = 1)	nd	0.98 ± 0.25 (n = 3)	1.14 ± 0.55 (n = 6)
4-methylcatechol	nd	nd	0.97 ± 0.14 (n = 2)	1.78 (n = 1)
hippuric acids				
4-hydroxyhippuric acid	0.80 (n = 1)	0.33 (n = 1)	0.34 (n = 1)	0.35 (n = 1)
phenylacetic acids				
3,4-dihydroxyphenylacetic acid	4.77 ± 3.55 (n = 2)	2.28 (n = 1)	6.87 ± 10.04 (n = 5)	6.65 ± 8.16 (n = 7)
4-hydroxyphenylacetic acid	9.62 ± 13.50 (n = 6)	3.58 ± 3.32 (n = 7)	4.72 ± 7.62 (n = 31)	4.09 ± 4.99 (n = 31)
3-hydroxyphenylacetic acid	2.17 ± 1.22 (n = 7)	4.99 ± 5.80 (n = 8)	9.06 ± 8.19 (n = 29)	18.60 ± 20.10 (n = 33)
4-hydroxy-3-methoxyphenylacetic acid	0.40 (n = 1)	nd	4.60 ± 5.91 (n = 4)	6.29 ± 10.10 (n = 3)
phenylacetic acid	67.90 ± 39.20 (n = 8)	93.90 ± 59.70 (n = 8)	161.00 ± 141.00 (n = 33)	183.00 ± 167.00 (n = 32)
phenylpropionic acids				
3-(3′,4′-dihydroxyphenyl)-propionic acid	3.53 ± 2.46 (n = 5)	2.28 ± 0.61 (n = 4)	5.06 ± 7.62 (n = 13)	4.30 ± 5.09 (n = 10)
3-(4′-hydroxyphenyl)-propionic acid	30.70 ± 28.20 (n = 3)	12.70 (n = 1)	21.90 ± 15.60 (n = 5)	23.90 ± 17.70 (n = 7)
3-(3′-hydroxyphenyl)-propionic acid	17.60 ± 30.40 (n = 8)	12.30 ± 23.10 (n = 8)	48.30 ± 138.60 (n = 33)	48.00 ± 119.20 (n = 33)
3-phenylpropionic acid	30.10 ± 29.40 (n = 7)	30.00 ± 21.20 (n = 6)	62.40 ± 63.60 (n = 30)	81.60 ± 62.70 (n = 29)
valeric acids				
4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid	nd	1.46 ± 0.06 (n = 2)	1.42 ± 0.03 (n = 5)	1.65 ± 0.75 (n = 9)
4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid	0.12 ± 0.04 (n = 4)	0.16 ± 0.12 (n = 4)	0.12 ± 0.04 (n = 9)	0.12 ± 0.06 (n = 15)
4-hydroxy-5-phenylvaleric acid	12.90 ± 8.90 (n = 6)	18.70 ± 12.70 (n = 6)	30.20 ± 37.60 (n = 27)	241.00 ± 226.00 (n = 32)
valerolactones				
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	nd	2.64 (n = 1)	0.39 ± 0.66 (n = 5)	12.00 ± 11.80 (n = 5)
5-(3′-hydroxyphenyl)-γ-valerolactone	nd	nd	nd	24.30 ± 23.00 (n = 5)
5-(4′-hydroxyphenyl)-γ-valerolactone	nd	1.86 (n = 1)	11.80 ± 13.70 (n = 3)	5.12 ± 3.29 (n = 8)
cinnamic acids				
caffeic acid	2.03 ± 0.25 (n = 7)	2.08 ± 0.30 (n = 5)	2.13 ± 0.32 (n = 25)	2.07 ± 0.66 (n = 26)
p-coumaric acid	1.39 ± 0.20 (n = 8)	1.31 ± 0.04 (n = 8)	1.49 ± 0.35 (n = 30)	1.54 ± 0.34 (n = 32)
m-coumaric acid	nd	nd	0.33 ± 0.09 (n = 2)	0.34 ± 0.07 (n = 5)
ferulic acid	2.69 ± 2.15 (n = 6)	1.66 ± 0.19 (n = 7)	2.68 ± 2.30 (n = 30)	2.29 ± 1.35 (n = 28)
isoferulic acid	4.24 (n = 1)	nd	3.61 ± 0.21 (n = 4)	4.27 ± 1.03 (n = 3)

[a]Means and SD were calculated excluding negligible cases. [b]nd: not detected. [c]In parentheses is the number of cases considered for each compound.

Statistical analysis of data comprised both comparisons among the control and intervention groups before the 4-week study period (basal content) and comparisons before and after the 4-week study period within each group. For statistical comparison of means, all cases were considered, including negligible data (zero values). The t test for independent samples and its corresponding nonparametric Mann−Whitney test were realized to evaluate differences in the means of the basal content of phenolic acid metabolites in feces between the control and intervention groups. No significant differences (P > 0.05) were found in the content of phenolic metabolites between the two groups except for the 3-hydroxyphenylacetic acid (P < 0.05). However, when the sum of the concentration of all metabolites (total phenolic metabolite content) was considered, no statistically significant differences (P > 0.05) among groups were observed in this basal content (175 ± 98 and 358 ± 270 μg/g feces for the control and intervention groups, respectively). Hence, it can be concluded that the distribution of volunteers between the control and intervention groups was adequate.

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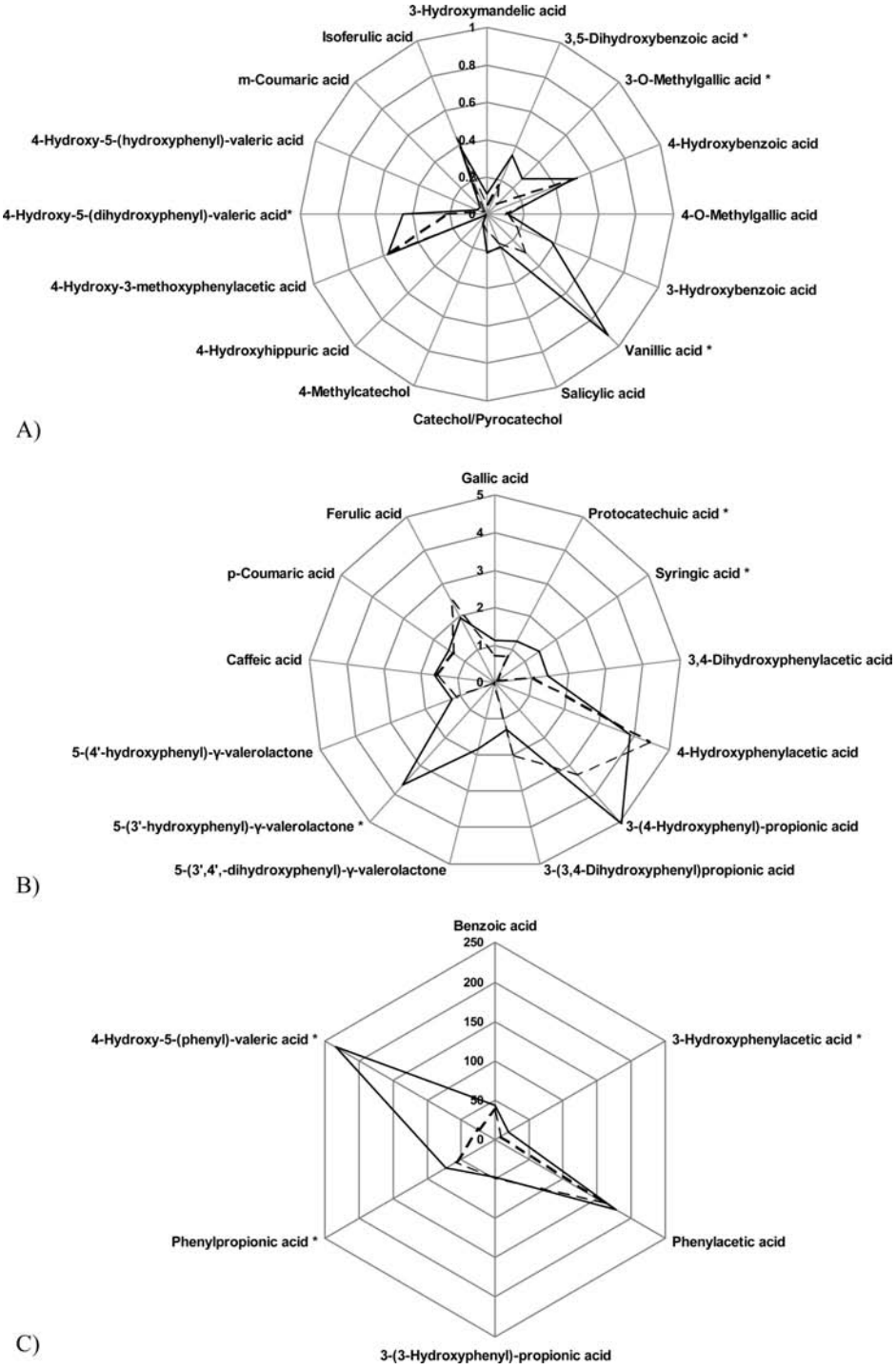

Figure 1. Phenolic metabolites in feces for the wine-intervention group (n = 33), before (dashed line) and after wine intake (solid line). (A) Metabolites with a content of (A) <1 μg/g, (B) 1−5 μg/g, and (C) 5−250 μg/g. *Mean values significantly different in concentration before and after wine intake ($P < 0.05$).

For the control group, the t test for dependent samples and the nonparametric Wilcoxon matched-pairs test showed no significant differences ($P > 0.05$) between samples before and after the 4-week study period, as expected. When the t test was applied to the intervention group, significant differences ($P < 0.05$) between samples before and after the wine intake were found for the content of 3,5-dihydroxybenzoic acid, protocatechuic acid, 3-*O*-methylgallic acid, vanillic acid, 3-hydroxyphenylacetic acid, syringic acid, 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid, and 4-hydroxy-5-phenylvaleric acid. Also, the total phenolic metabolites content was significantly higher in the samples after wine intake (625 ± 380 μg/g feces) compared to the samples before wine intake (358 ± 270 μg/g feces). Moreover, when the nonparametric Wilcoxon matched-pairs test was realized, aside from the previous compounds, significant differences were found for the content of 5-(3′-hydroxyphenyl)-γ-valerolactone and 3-phenylpropionic acid, whose data did not follow a normal distribution according to the Shapiro−Wilk test. To visualize changes in the fecal phenolic metabolite profile, radar diagrams including all

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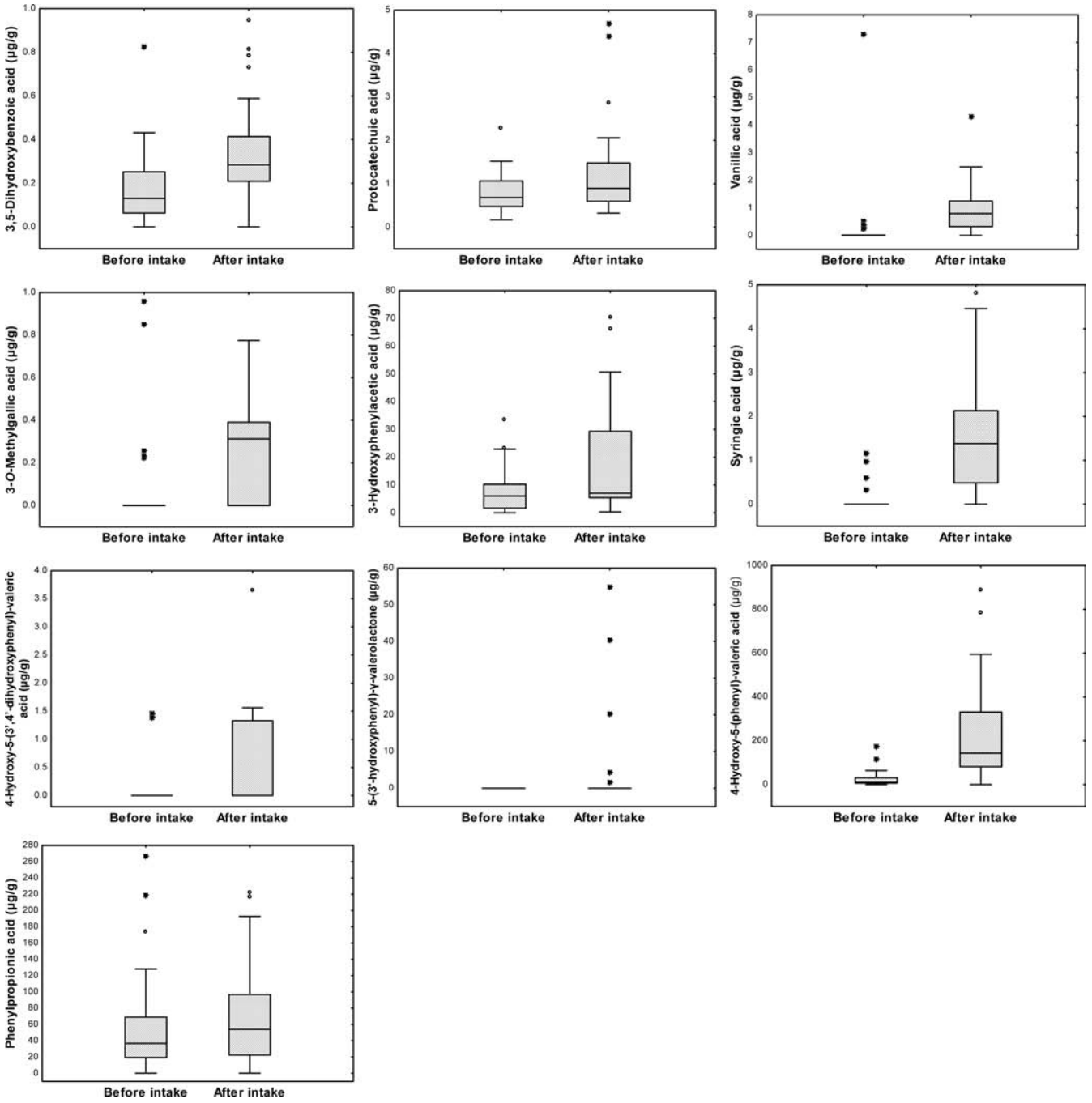

Figure 2. Box and whiskers plots (—, median; □, 25th−75th percentiles; |, nonoutlier range; ○, outliers; and *, extremes) of the phenolic metabolites found to be significantly different for the intervention group (n = 33) after wine intake.

metabolites detected in the fecal samples before (dashed line) and after wine intake (solid line) were constructed (Figure 1). Metabolites were classified into three groups according to their amount in fecal samples: (A) < 1 μg/g feces, (B) 1−5 μg/g feces, and (C) 5−250 μg/g feces. As can be seen, most of the compounds (n = 16) were found in low concentrations (<1 μg/g), with four of them showing a significantly different concentration level after wine intake (Figure 1). Three compounds among the metabolites with a concentration between 1 and 5 μg/g feces (n = 13) and three more metabolites among the metabolites with concentrations between 5 and 250 μg/g (n = 6) showed significant differences (P < 0.05) after intervention. Comparing the radar profiles between the fecal samples, before and after the wine intake, resulted in quite similar shapes for the three metabolite groups, with the exception of peaks corresponding to vanillic acid, 5-(3′-hydroxyphenyl)-γ-valerolactone, and 4-hydroxy-5-phenyl-valeric acid (quantified as propionic acid) in the fecal samples after the wine intake (Figure 1). Variability among volunteers is clearly shown in Figure 2, which displays the box and whiskers plots (median, 25th and 75th percentiles, nonoutlier range, outliers, and extremes) of the individual microbial metabolites mentioned above which showed significant differences (P < 0.05) before and after the red wine intervention. In general, the 25th−75th percentiles were greater for the samples after the wine intake than samples before the wine intake, indicating that

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wine polyphenol metabolism enhances interindividual variability further still.

Distribution of the Volunteers Based on Their Total Phenolic Metabolite Content in Their Feces. In an attempt to distribute the volunteers by looking at their capacity to metabolize wine polyphenols, frequency histograms for the total phenolic metabolite content in human feces for both the control and intervention groups were constructed (Figures 3 and 4, respectively). For the control group (n = 8), the distribution of the volunteers was similar before and after the 4-week intervention period. The frequency histogram for the intervention group (n = 33) became wider and moved toward higher values after the 4-week wine intake (Figure 4). On the basis of these histograms, the volunteers were tentatively distributed into three groups: (a) volunteers with a total phenolic metabolite content <500 μg/g feces, (b) volunteers with a total phenolic metabolite content between 500 and 1000 μg/g feces, and (c) volunteers with a total phenolic metabolite content >1000 μg/g feces. The group with total phenolic metabolite content <500 μg/g feces included 27 volunteers (82% of the total) before the wine intake, but only 13 (39% of the total) after the intake. In contrast, the number of volunteers in the group exhibiting a total phenolic content of 500−1000 μg/g feces increased from 5 to 13 volunteers after the wine intake. The same was observed for the group of highest phenolic metabolite content (>1000 μg/g feces), the number of volunteers increasing from 1 to 7 after the wine intake (Figure 4).

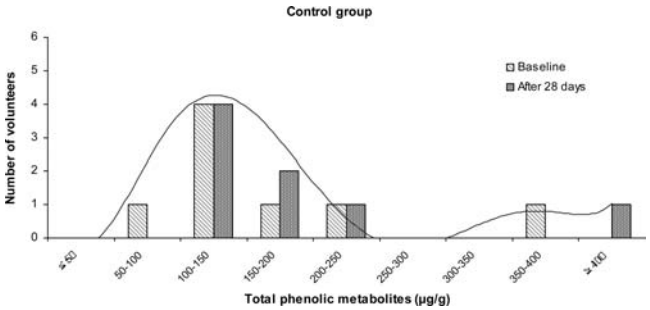

Figure 3. Distribution, by the total phenolic metabolites content, of the control group volunteers before and after the 28-day period.

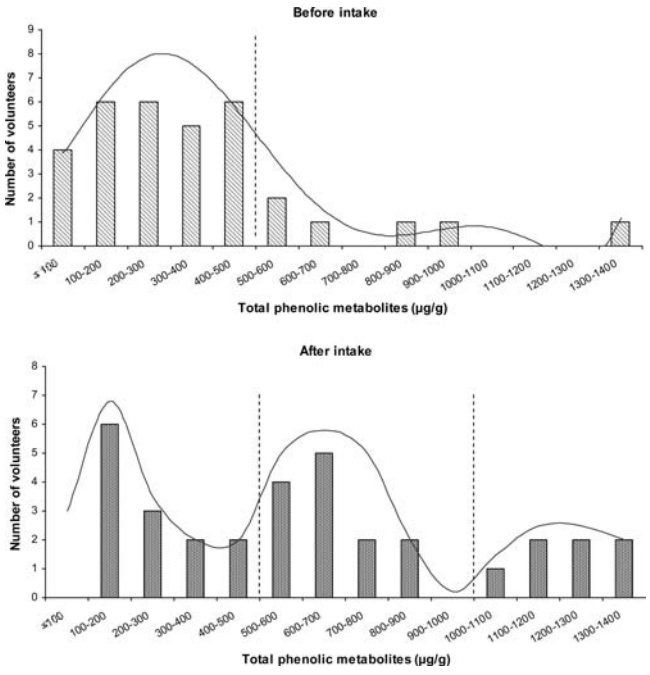

Figure 4. Distribution, by the total phenolic metabolites content, of the study group volunteers before and after the 28-day period of wine intake.

The means of the fecal contents of phenolic metabolites corresponding to the three groups of volunteers described above (<500, 500−1000, and >1000 μg/g feces) were compared after the 4-week wine intervention. The nonparametric Kruskal−Wallis test was applied once it was confirmed that the phenolic metabolite contents did not follow a normal distribution according to the Shapiro−Wilk test. Differences among the three groups (<500, 500−1000, and >1000 μg/g feces) were found for the contents of 3,4-dihydroxyphenylacetic acid, catechol/pyrocatechol, 3-(4-hydroxyphenyl)propionic acid, *m*-coumaric acid, 3-(3-hydroxyphenyl)propionic acid, phenylacetic acid, phenylpropionic acid, and 4-hydroxy-5-phenylvaleric acid, supporting the feasibility of the volunteer distribution proposed. As indicated in the Introduction, other authors have proposed classifying the human population into high, moderat,e and low metabolizers, taking into consideration the levels of a specific phenolic metabolite in urine after an intervention with a source rich in the corresponding precursors.[17,18] This paper shows that this different human capacity for metabolizing polyphenols is also reflected in the fecal metabolome, as seen with wine polyphenols. The volunteer classification made in this paper appears complex since it attempts to cover the global capacity of metabolizing wine polyphenols that encompass different phenolic groups. But even taking that into consideration, the volunteers could be tentatively classified into high, moderate, and low metabolizers based on the total phenolic content in feces after wine consumption.

On the other hand, to our knowledge, very few studies have reported changes in the content of fecal phenolic metabolites after a human intervention study.[25,28,29] Recently, Gill et al.[25] reported changes in the phenolic metabolite contents after an intervention study (n = 10) consisting of the intake of raspberry purée (200 g/day) during 4 days. From these published data in μg/mL, and considering an average fecal water content of 65%, we have expressed the means of fecal phenolic metabolites after this intervention as 145 μg/g for phenylacetic acid after raspberry purée, in comparison to the value of 183 μg/g reported in this paper after 4-weeks of wine intervention; 67.6 μg/g for 3-phenylpropionic acid, in comparison to the value of 81.6 μg/g reported in this paper; 20.8 μg/g for 3-(4′-hydroxyphenyl)propionic acid, in comparison to the value of 23.9 μg/g feces reported in this paper; 14.4 μg/g for 3-hydroxyphenylacetic acid, in comparison to the value of 18.6 μg/g reported in this paper; and 1.63 μg/g for 3,4-dihydroxyphenylacetic acid, in comparison to the value of 6.65 μg/g reported in this paper. Only the fecal contents of benzoic and isoferulic acids were significantly lower after the intervention with raspberry purée (200 g/day, 4 days) (2.83 and 0.411 μg/g, respectively) than after the red wine intervention (272 mL/day, 4 weeks) (43.4 and 4.27 μg/g, respectively), although finally concluding the consistency between both studies.

In conclusion, this paper reports valuable data on the basal levels of phenolic metabolites in feces, which are in accordance to those reported in the literature. Although showing a great interindividual variability, these data may be helpful for the design of in vitro and cell culture studies to address the biological effects that polyphenols exert at the gut level. Also, it has been proven that the moderate consumption of red wine significantly promotes changes in the metabolic prolife and content in feces, with may be related to positive biological effects. In parallel to this work, we are conducting studies of characterization and metabolic activity of the fecal microbiota from the same human samples reported here, with the final aim to go deeper in the understanding of the polyphenol–microbiota interactions.

■ ASSOCIATED CONTENT

Supporting Information

Table 1-OSM shows retention times and MS/MS parameters for the investigated phenolic metabolites and Table 2-OSM lists the content of phenolic compounds in feces at baseline reported in literature. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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