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Identification of Urolithin A as a Metabolite Produced by Human Colon Microflora from Ellagic Acid and Related Compounds

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Dietary ellagic acid and related polyphenols are metabolized in humans to dibenzopyran-6-one derivatives, and the microbial origin of these metabolites has been suggested. However, this has not been demonstrated so far. Fecal samples donated by six volunteers were incubated under anaerobic conditions, and aliquots were used to evaluate the fecal metabolism of ellagic acid, the ellagitannin punicalagin, and an ellagitannin rich extract from walnuts. The isoflavone daidzein was also incubated with the same fecal samples to follow the production of the microbial metabolites previously reported (dihydrogenistein, *O*-demethylangolensin, and equol) as a positive control of the system and to evaluate similarities between isoflavone and ellagic acid fecal flora metabolism. After fermentation the metabolite "urolithin A" (3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one) was produced from ellagic acid, punicalagin, and the ellagitannin extract in all the fecal cultures from different volunteers, but with very different production rates and concentrations. This large variability in the concentration of metabolite and kinetics of metabolite production is consistent with the large variability found in the excretion of these metabolites in urine in vivo after human consumption of ellagitannins, and with differences in the composition of the fecal microflora. No correlation between isoflavone and ellagic acid metabolism by fecal microflora was observed. The present study confirms the microbial origin of the recently reported in vivo generated hydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one derivatives in humans and is a further step in the study of the bioavailability and metabolism of ellagic acid and ellagitannins.

KEYWORDS: Ellagitannin; ellagic acid; polyphenol; metabolism; colonic microflora; dihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one; urolithin A; walnut; pomegranate; punicalagin

INTRODUCTION

Dietary habits rich in plant food products have been related to a decrease in mortality by cardiovascular diseases and cancer (*1*, *2*), and polyphenols have been the focus of the constituents that could be responsible for these properties (*3*−*5*). The bioavailability of food phenolic compounds has been reported to be rather small for most natural compounds (*6*−*9*). In fact it has been recently reported that many of these natural compounds are metabolized by the fecal microflora in the colon, where the metabolites produced are then absorbed or excreted (*6*−*9*). It has also been suggested that the biological activity attributed to food phenolics is more likely related to their colonic metabolites, rather than to the original compounds that are poorly absorbed (*6*−*9*).

In recent papers it has been reported that phenolic metabolites of the ellagic acid group from different food products (pomegranate, strawberry, raspberry, walnut, and red wine) show a high antioxidant activity in vitro (*10*−*13*) but they are not absorbed in humans (*14*). Their consumption is associated with the urinary excretion of dibenzopyran-6-one metabolites, mainly urolithin A (3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]-pyran-6-one), which are also observed in plasma as conjugates after consumption of ellagic acid derivatives (*15*). The large interindividual variability observed in the production and excretion of these metabolites suggested their microbial origin and their production in the colon of those individuals with the appropriate microflora to achieve the ellagitannin degradation and transformation in dibenzopyran-6-one metabolites (*14*−*16*).

The aim of the present work was to evaluate whether human fecal microflora are able to metabolize ellagic acid and related products (the ellagitannin punicalagin and ellagitannin-rich sources such as walnut extract) to produce dibenzopyran-6-one derivatives and particularly urolithin A. This was tested by adding ellagic acid and ellagitannin samples to human colon microflora cultures produced with feces donated by several healthy volunteers, and the production of urolithin A was followed by HPLC−MS−MS. The occurrence of urolithin A in

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feces donated by a volunteer after having walnuts was also analyzed.

In order to test that the fecal fermentation assay was working properly, the metabolism of the isoflavone daidzein by the fecal fermentation preparations was also tested and the results obtained were compared with those obtained after fermentation of ellagic acid and related compounds.

MATERIALS AND METHODS

Chemicals. Ellagic acid, daidzein, and L-cysteine were obtained from Sigma (St. Louis). Methanol, diethyl ether, dimethyl sulfoxide and sodium hydroxide were obtained from Merck (Darmstadt, Germany). Brain heart infusion (BHI) medium was obtained from Pronadisa (Torrejón de Ardoz, Madrid, Spain), and resazurin was obtained from Aldrich (Steinheim, Germany). Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this experiment.

Walnut Extract Preparation. Three grams of walnuts (edible part) were homogenized in an Ultraturrax T-25 equipment (Janke and Kunkel, Ika-Labortechnick, Staufen, Germany) at 24 000 rpm for 1 min with 20 mL of MeOH:H_2O (80:20; v:v). The extracts were centrifuged at 5000*g* for 10 min in a Centromix centrifuge (Selecta, Barcelona, Spain). Methanol was evaporated under reduced pressure (45 °C), and the ellagitannins were identified and quantified by HPLC−DAD−MS−MS as previously reported (*14*).

Punicalagin Preparation. Pure punicalagin standard was obtained from pomegranate peel according to the protocol previously reported (*16*).

Fecal Samples for Microflora Cultures. Human fecal samples were donated by six healthy volunteers (three men and three women) aged 25 to 30 years. They had no history of gastrointestinal disease and had not used antibiotics in the 3 months before sample collection. Subjects were nonsmokers, not vegetarians, with no history of gastrointestinal disease or any chronic disease and not involved in a weight-reducing dietary regimen. Female volunteers were neither pregnant nor lactating. All the donors filled out a questionnaire about their common dietary habits prior to participation in the study, i.e., weekly intake of milk, cheese, vegetables, yogurt, fruits, coffee, wine, olive oil, cereals, etc. No correlation was found between the dietary habits and the ability to produce the urolithin derivatives by the fecal flora (results not shown). The study complied with the Helsinki Declaration, and the protocol was fully explained to the donors who gave their written consent prior to participation. The intake of ellagitannin containing sources such as berries (strawberry, raspberry, blackberry, etc. and derived foodstuffs such as jams), pomegranates, chocolate, nuts, wine, and spirits was strictly forbidden for 1 week before the experiment. Samples were processed within 30 min of defecation.

Polyphenol Fermentation Assay. The growth medium (sterilized at 121 °C for 20 min) consisted of brain heart infusion (BHI) medium (37 g/L), L-cysteine (0.5 g/L), and resazurin (1 mg/L), in distilled water and adjusted to pH 7.4 with NaOH. Fecal suspensions were prepared by mixing human fecal samples (1 g) (freshly collected) in the basal medium (100 mL).

Solutions in dimethyl sulfoxide (DMSO) of ellagic acid, punicalagin, walnut extract, and daidzein were sterilized by filtration on Millex-GS sterile units (25 mm, 0.22 μm) (Millipore Corp.) and added to the fecal suspensions. The final concentration in the medium of ellagic acid was 10 μg/mL, of punicalagin was 100 μg/mL, of ca. 110 μg/mL ellagic acid equivalents from walnut extracts, and of daidzein was 1 μg/mL. Five hundred microliters of the different solutions were added to 100 mL of medium to reach the final concentrations indicated. In the control samples, 0.5 mL of DMSO was added to the medium. This amount of DMSO did not affect bacteria growth.

The fecal suspensions with the polyphenols were incubated in sealed jars (Oxoid Ltd, Basingstoke, Hampshire, England) under anaerobic conditions using AnaeroGen sachets (Oxoid Ltd, Basingstoke, Hampshire, England) at 37 °C. Samples (from different jars) were taken at 5, 24, 48, and 72 h.

Three controls were performed, i.e., polyphenols incubated in the basal medium without flora; flora in the basal medium without polyphenols; flora heat-inactivated (121 °C, 20 min) and in the basal medium with polyphenols. All experiments were conducted in triplicate.

Metabolite Recovery from Fecal Suspensions. Twenty milliliters of the fecal suspensions were extracted with diethyl ether (12 mL × three times). A fourth extraction did not extract any detectable metabolite. The organic phases were pooled and evaporated under reduced pressure until dryness and redissolved in 1 mL of MeOH. This extract contained urolithin A and other aglycon metabolites. With the objective of detecting water soluble conjugates (glucuronic acid and sulfate conjugations), the aqueous phase remaining after diethyl ether extraction was concentrated using a Sep-Pak reverse phase C-18 extraction cartridge (Waters Millipore). The cartridges were previously activated with 10 mL of MeOH and 10 mL of water. After eluting the sample volume, the cartridge was washed with 10 mL of water. The metabolites remaining in the cartridge were eluted with 2 mL of MeOH. Samples of 100 μL of each methanolic fraction, from both organic and aqueous phases, were analyzed by LC−MS−MS after filtration through a 0.45 μm filter.

Metabolite Extraction from Feces. One of the volunteers ingested 35 g of walnuts. Samples of feces were donated 29 and 55 h after walnut intake. One gram of feces was homogenized with 10 mL of MeOH:H_2O:HCOOH (80:19.9:0.1, v:v). The mixture was homogenized with an Ultraturrax T25 (Henkel, Germany) and filtered through sterilized cheesecloth. The filtrate was centrifuged for 10 min at 4 °C and 14000*g*. The supernatant was filtered through a 0.45 μm filter, and the filtrate was analyzed by HPLC−DAD−MS−MS.

HPLC−MS−MS analysis. Chromatographic separations of culture samples were carried out on a 250 × 4 mm, particle size 5 μm reverse phase C_{18} LiChroCART column (Merck, Darmstadt, Germany) using water/formic acid (99:1, v:v) (A) and MeOH (B) as the mobile phases at a flow rate of 1 mL/min. The gradient started with 20% B in A to reach 70% B in A at 25 min and 95% B in A at 30 min. UV chromatograms were recorded at 255, 305, and 360 nm. The same chromatographic conditions were used for UV and MS−MS detection. The metabolite urolithin A was identified by its retention time, UV spectrum, and MS−MS analyses, and its occurrence was confirmed by chromatographic comparison using the corresponding purified metabolite previously isolated from human urine (*15*).

The HPLC system equipped with both a photodiode-array detector (DAD) and mass detector in series consisted of a HPLC binary pump, autosampler, and degasser controlled by software from Agilent Technologies (Waldbronn, Germany). The mass detector was an ion-trap mass spectrometer (Agilent) equipped with an electrospray ionization (ESI) system (capillary voltage 4 kV, dry temperature 350 °C). Mass scan (MS) and MS−MS daughter spectra were measured from *m*/*z* 150−500. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the alternating positive/negative ionization mode.

RESULTS AND DISCUSSION

Test of the Fecal Microbial Transformation Assay. In order to check that the in vitro human fecal fermentation system used for polyphenols was working properly, the isoflavone daidzein was metabolized by fecal samples donated by six volunteers and the metabolites were evaluated by HPLC−MS−MS analyses after 5, 24, 48, and 72 h. The metabolites dihydrodaidzein (UV 312, 274 nm; MS^+ *m*/*z* 257, 163) and *O*-demethylangolensin (UV 314, 278 nm; MS^+ *m*/*z* 259, 241, 213, 165) were detected after 5 h incubation. The metabolites were tentatively identified by their UV spectra and MS spectra recorded with the HPLC−DAD−MS−MS analyses. No equol was detected under these conditions. No metabolites were observed in the three controls carried out (i.e. isoflavones in basal medium without flora; flora in the basal medium without polyphenols; flora heat-inactivated and basal medium with polyphenols) showing that the metabolites obtained were produced by the living fecal flora under the fermentation conditions of the in

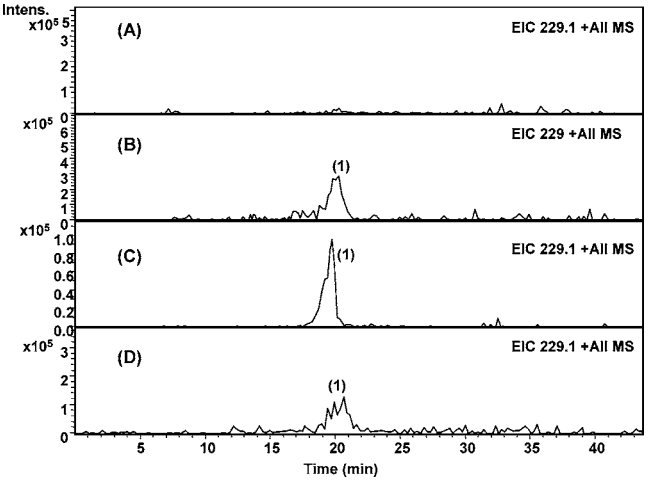

Figure 1. Extracted ion chromatograms (EIC) of extracts after 24 h fermentation of different phenolics by fecal microflora: (**A**) control; (**B**) ellagic acid (10 μg/mL); (**C**) walnut extract (ca. 110 μg/mL); (**D**) punicalagin (100 μg/mL). (**1**) Urolithin A.

vitro system. This study showed that the experimental method used for the fecal incubation of the extracts was working correctly and the metabolites produced coincided with those reported by other authors (*17*, *18*).

Identification of Urolithin A. Ellagic acid, punicalagin, and a walnut extract containing a number of ellagitannins as the main phenolic compounds [pedunculagin, casuarictin, valoneic acid dilactone, glansrin A and C, strictinin, isostrictinin, tellimagrandin I and II, rugosin C, and casuarinin; identified by HPLC−MS−MS and consistent with those reported previously (*13*)] were added to the incubation fecal medium, and the production of metabolites was followed during 5, 24, 48, and 72 h. The occurrence of 3,8-dihydroxy-dibenzopyran-6-one (urolithin A) was analyzed in the extracts obtained from the incubation media. None of the original phenolic compounds added to the fecal medium were detected after 5 h incubation. This could be due (apart from the colonic metabolism) to a precipitation or transformation of these polyphenols in the medium conditions, as this medium is rich in proteins and has a pH of 7.4.

The HPLC extracted ion chromatograms of the extracts obtained from the control medium and those of the incubation of the different isolated compounds (ellagic acid and punicalagin) and the walnut extract with the fecal microflora clearly showed the generation of urolithin A after 24 h of incubation in the three samples in which ellagic acid or ellagitannins had been added (**Figure 1**).

No urolithin A derived glucuronides or sulfates, the principal metabolites detected in urine and plasma in volunteers after ellagitannin rich food ingestion (*14*, *15*), were produced by the fecal microflora at any time, suggesting that the conjugates are produced after absorption of the aglycon metabolite in the intestinal cells and/or in the liver as has been demonstrated for other phenolic compounds (*19*).

When walnut polyphenol fecal fermentation metabolites were analyzed by HPLC−MS−MS (**Figure 2**), the metabolite urolithin A was also present. The ms^2 fragmentation of the metabolite at 19.7 min ($M + H^+$ *m*/*z* 229) produced the characteristic fragments of urolithin A (*14*, *15*). This suggested that urolithin

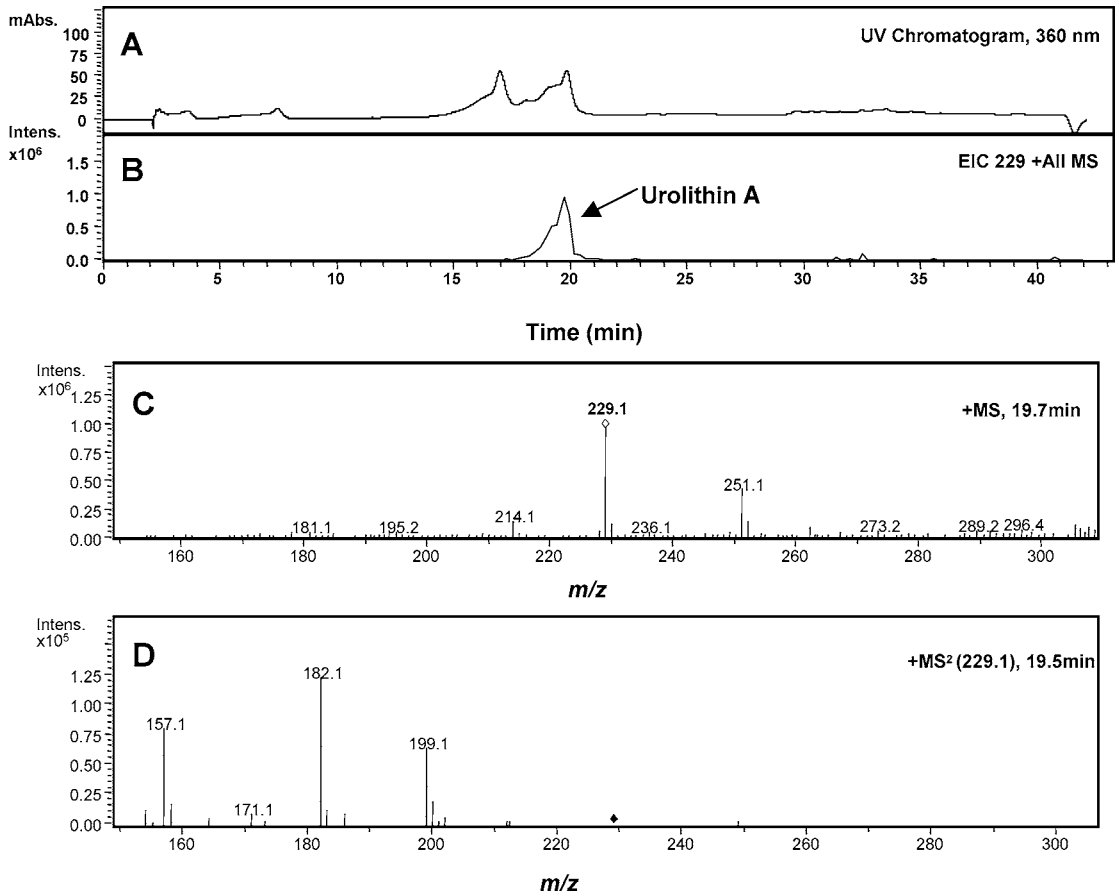

Figure 2. LC–MS–MS analysis of walnut extract fecal microbial degradation metabolites: (**A**) UV chromatogram at 360 nm; (**B**) extracted ion chromatogram (EIC) at m/z^+ 229 showing the metabolite urolithin A; (**C**) MS scan at the retention time of the above EIC; (**D**) MS–MS daughter spectra from urolithin A.

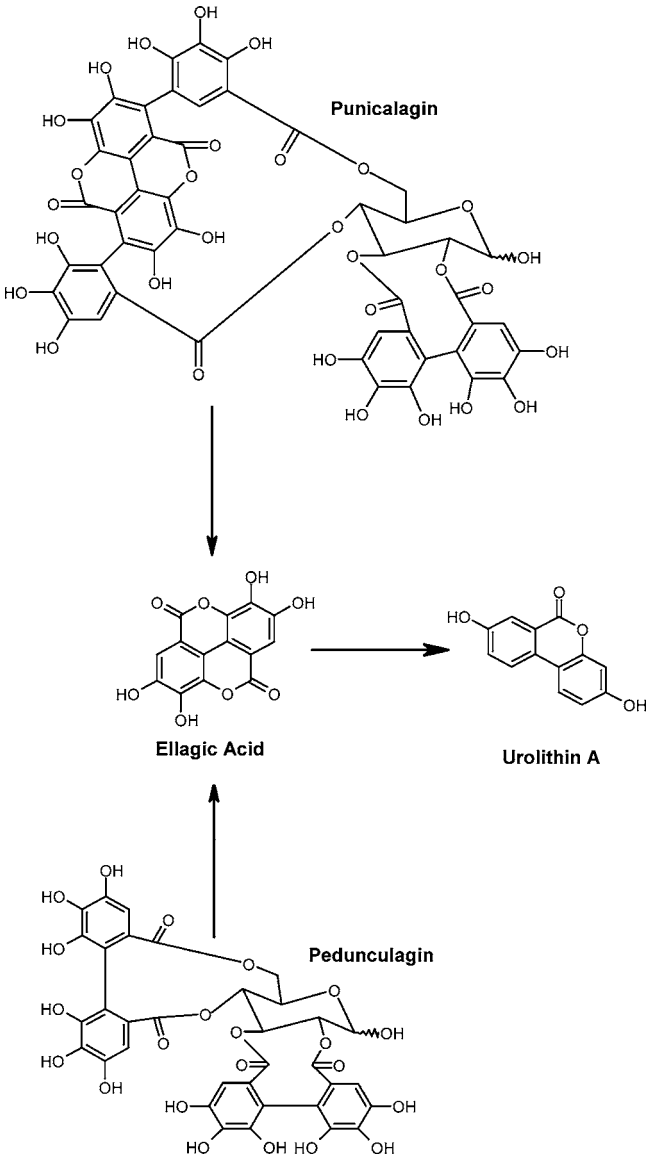

Figure 3. Proposed fecal microflora metabolism of ellagitannins.

A was produced by fecal microflora from complex ellagitannins, through ellagic acid as an intermediate (**Figure 3**).

Urolithin A was also detected in the extracts obtained from feces donated by a volunteer 29 h after ingestion of 35 g of walnuts. This was the main metabolite present in the extracts, and no glucuronide nor sulfate conjugates of urolithin A were detected, which could suggest that no bile excretion of the conjugated metabolites is produced in vivo (**Figure 4**). However, the β-glucuronidase activity of the intestine could hydrolyze the glucuronide derivatives produced in the liver and excreted with bile to yield the corresponding urolithin A aglycon. Therefore, the excretion of urolithin β-glucuronides in bile requires further investigation, and the analysis of the bile content in animal models, such as pig, should be undertaken to verify this pathway.

Table 1. Transformation of Ellagic Acid into Urolithin A by the Fecal Flora of Volunteers[a]

volunteer	0 h	5 h	24 h	48 h	72 h
V1	nd	ne	ne	7.02	106.27
V2	nd	27.92	103.75	294.81	101.56
V3	nd	47.70	538.19	620.65	322.89
V4	nd	t	t	t	t
V5	nd	nd	nd	nd	2.86
V6	nd	nd	nd	nd	t

[a] Values are expressed as μg/100 mL fecal culture; ne, not evaluated; nd (below the detection limit 0.03 μg/mL); t, traces (the metabolite was detected but not in enough amount to allow its quantification).

Interindividual Production of Urolithin A. Ellagic acid (at a concentration of 10 μg/mL) and walnut extract (providing a concentration of ca. 110 μg/mL ellagic acid equivalents) were fermented individually by fecal microflora from six healthy donors, and samples were taken after 5, 24, 48, and 72 h. The metabolite urolithin A was produced by the fecal microflora from all volunteers, although with very different rates and concentrations in the medium. The fecal microflora from two volunteers (V2 and V3) was able to produce urolithin A from ellagic acid after 5 h fermentation, and the concentration of this metabolite continued to increase during the next 48 h, decreasing in the 72 h sample (**Table 1**). This decrease could be explained either by a further microbial degradation of urolithin A to simpler metabolites (i.e. short chain aliphatic acids) or by precipitation (low solubility in aqueous media) or combination of the metabolites to form polymers that are no longer detected in the chromatograms. The fecal microflora from some volunteers (V2, V3) started to produce urolithin A in detectable quantities from ellagic acid after 5 h while in that of V5 the metabolite was only detected after 72 h. The flora of other donors (V4, V6) only produced traces of urolithin A. The large

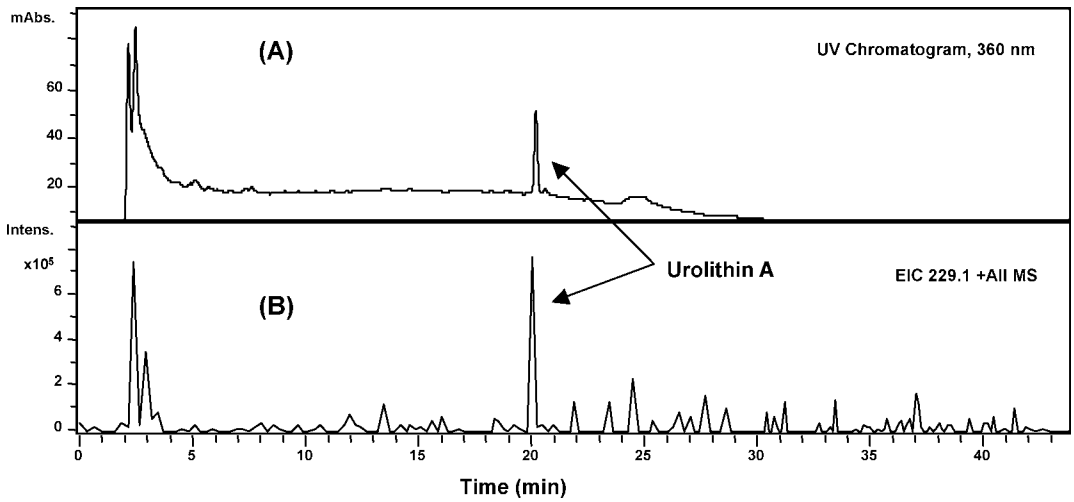

Figure 4. LC–MS–MS analysis of fecal extracts from a donor 29 h after walnut ingestion: (**A**) UV chromatogram at 360 nm; (**B**) extracted ion chromatogram (EIC) at m/z^+ 229 showing the metabolite urolithin A.

Table 2. Transformation of Walnut Extract Ellagitannins into Urolithin A by the Fecal Flora of Volunteers[a]

volunteer	0 h	5 h	24 h	48 h	72 h
V1	nd	ne	369.58	341.18	313.72
V2	nd	t	248.85	386.37	258.29
V3	nd	21.78	288.85	321.55	294.57
V4	nd	t	76.76	110.28	95.01
V5	nd	nd	nd	75.04	224.06
V6	nd	nd	nd	nd	3.92

[a] Values are expressed as μg/100 mL fecal culture; ne, not evaluated; nd (below the detection limit 0.03 μg/mL); t, traces (the metabolite was detected but not in enough amount to allow its quantification).

Table 3. Transformation of the Isoflavone Daidzein into Dihydrodaidzein and *O*-Demethylangolensin by the Fecal Flora of Volunteers[a]

volunteer	0 h	5 h	24 h	48 h	72 h
			Dihydrodaidzein		
V1	nd	ne	60.70	1723.33	1214.37
V2	nd	48.59	279.05	413.36	232.07
V3	nd	106.22	1190.36	314.84	189.29
V4	nd	t	37.67	71.65	nd
V5	nd	93.58	436.37	181.43	ne
V6	nd	nd	199.42	314.01	ne
			O-Demethylangolensin		
V1	nd	ne	43.55	35.17	63.64
V2	nd	nd	nd	nd	nd
V3	nd	nd	nd	60.32	27.01
V4	nd	nd	nd	nd	nd
V5	nd	nd	78.81	t	ne
V6	nd	nd	nd	t	ne

[a] Values are as μg/100 mL fecal culture; ne, not evaluated; nd (below the detection limit 0.1 μg/mL); t, traces (the metabolite was detected but not in enough amount to allow its quantification).

variability in metabolite production is consistent with differences in the composition of the fecal microflora from the different donors as has been previously demonstrated in the case of isoflavones (*17*, *18*, and agrees with published data that shows a large interindividual variability in the urinary excretion and plasma concentration of the metabolites after ingestion of food rich in ellagitannins (*14*, *15*). No correlation between the dietary habits of the different donors and the ability to transform ellagic acid into urolithin A has been found (data not shown).

Similar results were obtained when a mixture of walnut complex phenolics (ellagitannins) was fermented by the fecal microflora from the six donors (**Table 2**). In this case, urolithin A was produced in quantifiable amounts by the microflora from the six volunteers after 72 h fermentation. A large variability was also observed both in the concentration of the metabolite produced and in the kinetics of metabolite production. The metabolites were already produced after 5 h fermentation in 3 out of the 6 fecal cultures. This large variability confirms the importance of the fecal microflora in the ellagitannin metabolism in vivo (*14*, *15*).

Comparison of Isoflavone and Ellagitannin Fecal Microflora Fermentation. The fermentation rates of the isoflavone daidzein were also evaluated using the same fecal microflora cultures that were used for ellagitannin fermentation in order to test similarities and differences in the fermentation behavior of different polyphenols. All the microflora cultures were able to metabolize daidzein into dihydrodaidzein, and this transformation was observed for all the samples after 5 h, with the exception of the sample V6 (**Table 3**). The transformation of dihydrodaidzein into *O*-demethylangolensin was much more variable and confirmed previous findings that reported that both steps were produced by different bacteria (*20*, *21*). No equol was produced in detectable amounts, and therefore, these volunteers could be classified as "non equol producers" (*22*). There was no correlation between the ability to produce the isoflavone metabolites and the fermentation of ellagitannins to produce urolithin A suggesting that different degradation metabolic pathways should be involved. This is evident in the samples from donors V4, V5, and V6 that produce very small amounts of urolithin A, although the microflora is able to produce isoflavone metabolites soon after starting the fermentation (**Tables 1**, **2**, and **3**).

In summary, the present study confirms the microbial origin of the hydroxy-6*H*-dibenzopyran-6-one derivatives detected in plasma and urine of humans after food ellagitannin intake (*14*, *15*) and is a further step in the study of the bioavailability and metabolism of ellagic acid and related molecules in humans (*14*, *15*).

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