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Time Course Production of Urolithins from Ellagic Acid by Human Gut Microbiota

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ABSTRACT: Ellagic acid (EA) is converted to urolithins by gut microbiota. Urolithins have beneficial biological effects in humans, but differences in urolithin production capacity among individuals have been shown. Therefore, the identification of the urolithin production pathways and the microorganisms implicated is of high interest. EA was incubated with gut microbiota from two volunteers able to produce urolithins but with different in vivo urolithin profiles (urolithin A and isourolithin A producers). The metabolic capabilities observed in vivo were retained in vitro. Both individuals showed a much higher abundance of *Clostridium leptum* group of *Firmicutes* phylum than *Bacteroides/Prevotella*. EA was either dissolved in DMSO or suspended in water. DMSO increased EA solubility but decreased urolithin production rate due to a delay in growth of some microbial groups, principally, *Clostridium coccoides*. This allowed the detection of catabolic intermediates [urolithins M-5, M-6, M-7, C, and 2,3,8,10-tetrahydroxy urolithin (urolithin E)]. Bacteria from *C. coccoides* group (or genera co-occurring in vivo with this group) seem to be involved in production of different urolithins.

KEYWORDS: *ellagitannins, colon microbiota, metabolism, dibenzopyranones, polyphenols, human health*

INTRODUCTION

Ellagitannins and ellagic acid (EA) are plant secondary metabolites that have relevant antioxidant activities in vitro, potential cardiovascular protection, and anticarcinogenic and anti-inflammatory effects.[1−3] These phytochemicals are relevant constituents in different foods including pomegranates, berries (strawberry, raspberry, blackberry, camu−camu, etc.), nuts (walnuts, acorns, chestnuts, etc.), muscadine grapes, oak-aged wines, and medicinal plants and tisanes (geranium, oak leaves, etc.). They are not absorbed in the gut and are metabolized in vivo by the gut microbiota to produce a series of metabolites known as urolithins.[4,5] Therefore, urolithins have been suggested as biomarkers of intake for strawberries, raspberries, pomegranates, walnuts, and oak-aged red wines.[6] We have previously suggested that urolithins, that are much better absorbed than the original ellagitannins, could be responsible for the systemic biological effects of ellagitannins.[2,5] Additional evidence supports this suggestion as urolithins and their glucuronide and sulfate metabolites have shown relevant effects at the concentrations found in vivo.[7−9] Urolithins share a nucleus of a dibenzo-pyran-one, with different hydroxyl substitutions. The first reported urolithins were urolithin A and urolithin B that were found in kidney stones of sheep.[10] In a recent study, it has been demonstrated that other mammals also produce urolithins from ellagitannins, although they have different structures and different hydroxylation patterns on the urolithin nucleus.[11] Thus, isourolithin A has been found as the main in vivo metabolite in beef cattle after oak leaf intake,[12] and a series of tetrahydroxy and trihydroxy urolithin metabolites were produced by rats after *Geranium* extracts intake.[13]

Recently, it has been reported that some humans are able to produce isourolithin A as the main in vivo metabolite instead of urolithin A after the intake of strawberries[14] and raspberries.[15] Furthermore, marked person-to-person differences have been observed in the level of in vivo urolithin production following consumption of ellagitannin-containing food. In vitro anaerobic metabolism of EA to urolithin A in human fecal suspensions has also been reported.[16] More recently, in vitro anaerobic incubation of EA with human fecal suspensions demonstrated conversion to some other urolithins, urolithins B, C, and isourolithin A.[17] However, neither comparison between the in vitro and the in vivo urolithin patterns of these volunteers nor their endogenous microbiota has been analyzed in any of these studies.

In the present study, we evaluated the in vitro time course production of urolithins from EA by human fecal microbiota from two volunteers who have urolithin A and isourolithin A urinary excretion patterns. Detailed in vitro and in vivo experiments were performed to identify the intermediary and final catabolites of EA conversion by the human gut microbiota to elucidate interindividual urolithin production routes and to determine if EA metabolism could be linked to the microbiota composition of these individuals.

MATERIALS AND METHODS

Chemicals. EA and 6,7-dihydroxycoumarin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Urolithins A and B were chemically synthesized by Villapharma SL (Parque Tecnológico de Fuente Álamo, Murcia, Spain). Urolithins C (3,7,8-tetrahydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one, Uro-C; > 95% purity) and D (2,3,7,8-tetrahydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one, Uro-D; > 95% purity) were purchased from Dalton Pharma Services (Toronto, Canada). Methanol and acetonitrile were purchased from Romil (Barcelona, Spain) and ethyl acetate and dimethyl sulfoxide from Labscan (Dublin,

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Ireland). Formic acid and hydrochloric acid was obtained from Panreac (Barcelona, Spain). Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this experiment. Nutrient broth (NB) was from Oxoid (Basingstoke, Hampshire, UK). L-Cysteine hydrocloride was from (Panreac Química, Barcelona, Spain). All chemicals and reagents used in the preparation of buffers, macromineral, micromineral, and reducing solutions were obtained from Sigma-Aldrich, Panreac, and Scharlab.

Collection of Human Fecal Samples. These were donated by a healthy female (age 31) and a healthy male (age 36) in the Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC, Murcia, Spain). These two volunteers were identified as urolithin A and isourolithin A producers, respectively, in a previous study in which urine samples were analyzed after the intake of strawberries and strawberry jam,[18] although these metabolic groups were not reported. To conduct fermentation experiments in duplicate, each volunteer donated two fecal samples. They were stored at 4 °C and were further processed within 1 h of donation, as no differences in microbial concentrations were observed in fresh samples before and after processing samples for fermentation experiments. Anoxic conditions were maintained by using the AnaerobeGen compact system (Oxoid).

Fermentation Medium. This was prepared as described by Jaganath et al.[19] with some modifications. Briefly, 2 g of tryptone, 2 g of glucose, 1 g of maltose and 2 g of yeast extract were mixed in 400 mL of distilled water and 100 μL of micromineral solutions (consisting of 13.2 g of $CaCl_2{\cdot}2H_2O$, 10 g of $MnCl_2{\cdot}4H_2O$, 1 g of $CoCl_2{\cdot}6H_2O$, 8 g of $FeCl_3{\cdot}6H_2O$, and distilled water up to 100 mL), 200 mL of buffer solution (2 g of NH_3CO_3, 17.5 g Na_2CO_3, and distilled water up 500 mL), 200 mL of macromineral solution (2.85 g of NA_2HPO_4, 3.1 g KH_2PO_4, 0.3 g $MgSO_4{\cdot}7H_2O$, and distilled water up to 500 mL), 1 mL of 1% (w/v) of resazurin solution (a redox indicator) and 0.5 mg/L of Vit K_1, 5 mg/L hemin and 625 mg/L L-cysteine hydrochloride. The medium was adjusted to pH 7 using HCl, dispensed into the fermentation vessels, and autoclaved at 121 °C for 15 min and allowed to cool under anaerobic conditions.

Conversion Experiments of EA into Urolithins with Human Fecal Cultures. Preparation of fecal suspensions and subsequent culturing experiments were conducted under anoxic conditions in an anaerobic chamber (Don Whitley Scientific Limited, Shipley, UK) with an atmosphere consisting of $N_2/H_2/CO_2$ (80:10:10) at 37 °C. Aliquots of fecal samples (10 g) were diluted 1/10 (w/v) in NB supplemented with 0.06% L-cysteine hydrochloride and homogenized by stomacher in filter bags. Aliquots of filtered fecal suspensions (2 mL) were inoculated into 200 mL of prereduced fermentation medium containing EA at 30 μM with and without 1% DMSO. DMSO was used in order to increase EA solubility, and EA dissolved in water instead of DMSO was used as control. Duplicate cultures were prepared in parallel from each fecal suspension. In addition, controls were used; ones without fecal suspension and others without EA. Samples (5 mL) were collected at appropriate time intervals during 8 day incubation at 37 °C, extracted with 5 mL of ethyl acetate acidified with 1.5% formic acid and stored at 4 °C until further HPLC analysis. Similarly, 1 mL were collected every 2 h during 2 day incubation at 37 °C and stored at −20 °C until further qPCR analysis.

Sample Cleanup for LC Analyses. Feces and urine of the two volunteers were extracted and analyzed. Urine samples were defrosted, vortexed, and centrifuged at 14000*g* for 10 min at 4 °C. The supernatant was filtered through a 0.45 μm PVDF filter and analyzed by HPLC-DAD-IT MS. When the intensity was not enough to distinguish the metabolites, urine samples were 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. Then 25 mL of urine acidified with 250 μL of formic acid (1%) were passed through the cartridge that was then dried with air. The metabolites remaining in the cartridge were eluted with 3 mL of $MeOH/H_2O$ (50:50, v/v). Samples were analyzed after filtration through 0.45 μm PVDF filter. Feces samples (1 g) were defrosted and homogenized with 10 mL of $MeOH/DMSO/H_2O$ (40:40:20) with 0.1% HCl using an Ultra-Turrax for 1 min at 24000 rpm. The mixture was centrifuged at 5000*g* for 10 min at room temperature and the supernatant filtered through a 0.45 μm PVDF filter before analysis.

Samples (5 mL) obtained in fermentation experiments with human faecal suspensions were extracted with 5 mL of ethyl acetate acidified with 1.5% formic acid. The mixture was vortexed for 2 min and centrifuged at 3500*g* for 10 min. The organic phase was separated and evaporated under reduced pressure until dryness. The dry samples were then redissolved in 250 μL of methanol and filtered through a 0.45 μm PVDF filter. Then 5 μL of 100 μg/mL of internal standard (6,7-dihydroxycoumarin) was added to 50 μL of sample prior to the injection onto a column for LC-UV/vis and LC-MS analysis under the conditions described below.

LC-UV/Vis and LC-MS/MS Analyses. The analyses were performed using an Agilent 1100 HPLC system equipped with a photodiode array detector and an ion-trap mass spectrometer detector in series (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was carried out on a reverse phase LiChroCART C-18 column (Merck, Darmstadt, Germany) (250 mm × 4 mm, 4.5 μm particle size) using water with 1% formic acid (A) and acetonitrile (B) as the mobile phases. The gradient profile was: 0−20 min, 5−30% B, 20−30 min, 30−55% B, 30−38 min, and 55−90% B, and this percentage was maintained for 2 min and then came back to the initial conditions. A volume of 10 μL of sample was injected onto the column operating at room temperature and a flow rate of 1 mL/min. UV chromatograms were recorded at 280, 360, and 305 nm.

The HPLC system was coupled in series to an ion trap mass spectrometer (IT) equipped with an electrospray interface (ESI). Nitrogen was used as drying gas with flow of 11 L/min and temperature of 350 °C and nebulizing gas at pressure of 65 psi. The capillary voltage was set at 4 kV. Mass scan (MS) and daughter (MS-MS) spectra were recorded in negative mode in the range of m/z 100−700 with target mass of 300. Maximum accumulation time of ion trap and the number of MS repetitions to obtain the MS average spectra were set at 200 ms and 3, respectively. Compound stability was set at 75%.

Identification of all metabolites was carried out by direct comparison (UV spectra and MS) with pure standards and confirmed by their spectral properties and molecular mass. Calibration curves were obtained for EA, urolithin A, urolithin B, and urolithin C with good linearity (R^2 > 0.998). EA was quantified at 360 nm and urolithins at 305 nm. The limits of detection (LODs) were determined based on a signal-to-noise ratio (S/N) of 3 and of 10 for the limit of quantification (LOQ). EA, urolithin A, and urolithin B showed LODs of 0.5 μM and LOQs of 1.67 μM and urolithin C an LOD of 0.2 μM and an LOQ of 0.67 μM. Repeatability was evaluated by injecting 20 μM of a mixture of standards four times in the same day (intraday repeatability) and in four different days (interday repeatability). The results expressed as the relative standard deviation (RSD) of peak area were ≤5% for intraday repeatability and ≤8% for interday repeatability. The recovery of the compounds was calculated, spiking the medium in the presence of inactivated bacteria with a standard solution of EA, urolithin A, and urolithin B in DMSO at a final concentration of 20 μM. Recoveries of 75%, 90%, and 83% were obtained for EA, urolithin A, and urolithin B, respectively. Isourolithin A was quantified at 305 nm with the urolithin A calibration curve and urolithins M-5, M-6, and M-7 at 360 nm with the EA calibration curve.

Fecal Dry Weight Determination and DNA Extraction. To determine fecal moisture content, approximately 0.5 g (wet weight) of each fecal specimen was placed in a vacuum dryer for 3 d and reweighed. Percent fecal dry weight was calculated. Total DNA was extracted from human fecal samples using a commercial DNA extraction kit (QIAampR DNA Stool Mini Kit, Qiagen Inc., Valencia, CA). Although this kit did not contain beads, an additional step of vigorous shaking using the FastPrep Instrument was carried out. Briefly, 10 mg of fresh fecal samples (25.3 ± 8.9% dry matter) were added to 2 mL tubes containing specialized beads (MP Biomedicals, LLC, Ohio, USA) and homogenized in the lysing matrix using the FastPrep Instrument for 30 s at a speed setting of 5.5. The following steps were carried out attending protocol supplied with the kit: briefly, incubation temperatures (95 °C, 5 min and 70 °C, 10 min), cell lysis

Table 1. Primer and Probe Sequences Used for Detection of Main Bacterial Groups in Human Feces by Quantitative Real-Time Polymerase Chain Reaction

target organism groups and species[24]	primers and probes	sequence 5′−3′	refs
Clostridium leptum group	F_Clept 09	CCT TCC GTG CCG SAG TTA	24
Clostridium leptum	R_Clept 08	GAA TTA AAC CAC ATA CTC CAC TGC TT	
Faecalibacterium prausnitzii	P_Clep 01[a]	6FAM-CAC AATAAG TAA TCC ACC	
Ruminococcus albus			
Bifidobacterium genus	F_Bifid 09c	CGG GTG AGT AAT GCG TGA CC	24
Bifidobacterium adolescentis	R_Bifid 06	TGA TAG GAC GCG ACC CCA	
Bifidobacterium breve	P_Bifid[a]	6FAM-CTC CTG GAA ACG GGT G	
Bifidobacterium infantis			
Clostridium coccoides group	F_Ccoc 07	GAC GCC GCG TGA AGG A	24
Clostridium coccoides	R_Ccoc 14	AGC CCC AGC CTT TCA CAT C	
Ruminococcus gnavus	P_Erec482[a]	VIC-CGG TAC CTG ACT AAG AAG	25
Ruminococcus hansenii			
Eubacterium rectale			
Bacteroides/Prevotella group	F_Bacter 11	CCT WCG ATG GAT AGG GGT T	24
Bacteroides fragilis	R_Bacter 08	CAC GCT ACT TGG CTG GTT CAG	
Bacteroides ovatus	P_Bac303[a]	VIC-AAG GTC CCC CAC ATT G	26
Bacteroides thetaiotaomicron			
Bacteroides uniformis			
Bacteroides vulgatus			
Bacteroides caccae			
Bacteroides eggerthii			
Prevotella oralis			
Prevotella buccae			
Prevotella albensis			
Escherichia coli species	E.coli F	CAT GCC GCG TGT ATG AAG AA	27
	E.coli R	CGG GTA ACG TCA ATG AGC AAA	
Lactobacillus/Leuconostoc/Pediococcus group	F_Lacto 05	AGC AGT AGG GAA TCT TCC A	24
Lactobacillus acidophilus	R_Lacto 04	CGC CAC TGG TGT TCY TCC ATA TA	
Lactobacillus casei			
Lactobacillus paracasei			
Lactobacillus delbrueckii			
Lactobacillus fermentum			
Lactobacillus johnsonii			
Lactobacillus plantarum			
Lactobacillus rhamnosus			
Lactobacillus helvetic			
Pediococcus inopinatus			
Pediococcus parvulus			
Pediococcus cellicola			
Pediococcus acidilactici			
Leuconostoc mesenteroides			
Leuconostoc pseudomesenteroides			

[a]Probe sequences.

and homogenization (centrifugation at 14,000 rpm, 1 min), adsorption of inhibitors (InhibitEx tablet), and approximate time to completion (60−80 min).

Real-Time qPCR. DNA from dominant groups of fecal bacteria was quantified with real-time qPCR and primers as summarized in Table 1. Real-time qPCR was performed using an ABI 7500 sequence detection system. Amplification and detection were carried out in 96-well plates with TaqMan Universal PCR 2× master mix (Applied-Biosystems) or with SYBR-Green PCR 2× master mix (Applied Biosystems). Each reaction was run in duplicate in a final volume of 25 μL with 0.2 mM final concentration of each primer, 0.25 mM final concentration of each probe, and 10 mL of appropriate dilutions of DNA samples. Probes were designed with molecular-groove binding nonfluorescence quencher (MGBNFQ). Amplifications were carried out using the following ramping profile: 1 cycle at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. For SYBR-Green amplifications, the following dissociation protocol (a hold at 95 °C for 15 s, a hold at 60 °C for 20 s, and a slow ramp for 20 min from 60 to 95 °C) was followed after the last cycle in order to check the expected amplification products.

When PCR was performed on unknown fecal samples, the cycle threshold of each sample was compared with a standard curve

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(performed in triplicate) made by diluting genomic DNA (6-fold serial dilutions of *Bifidobacterium longum* DSM 20088T for *Bifidobacterium* genus, *Escherichia coli* CECT 515T for *E. coli* species, *Clostridium leptum* DSM 753T and *Clostridium coccoides* DSM 935T for *C. leptum* and *C. coccoides* groups, *Bacteroides ovatus* DSM 1896T for *Bacteroides/Prevotella* group and *Lactobacillus plantarum* CECT 748T for *Lactobacillus/Leuconostoc/Pediococcus* group). Selected bacteria strains were cultured anaerobically on selective broth as recommended by DSMZ. For each culture, the total number of bacteria, in terms of CFU, was determined by plating. Aliquots of 1 mL of culture were used for DNA extraction by DNeasy blood and tissue kit (Quiagen) following the manufacturer's instructions.

Statistical Analyses and Data Modeling. Fecal samples were analyzed in triplicate, and the quantitative estimates represent mean values ± standard error. Data were subjected to statistical analysis of variance (ANOVA). Normality of variances was tested by the Shapiro–Wilk test before determining the ANOVA. Statistical analyses were performed using SPSS V.14 for Windows.

Microbial growth curves were fitted using the function of Baranyi et al.[20] to estimate the main growth parameters (maximum specific growth rate, lag time of microorganisms before the onset of growth, and estimated correlation coefficient that indicates the goodness of fit of the parameters derived from experimental data). Only growth curves with at least 10 data points were used for modeling, as suggested by the authors.

RESULTS

In Vivo Metabolism of EA and Ellagitanins by Human Gut Microbiota. Two volunteers who had different routes of EA metabolism in vivo were selected for the present study. Volunteer 1 produced urolithin A, and volunteer 2 produced isourolithin A. We had previously identified these two urolithin metabolic profile groups in urine samples from different volunteers after the intake of walnuts, strawberries, and raspberries (unpublished data). The analyses of fecal samples from both volunteers after the intake of walnuts (0.6 g/kg body weight per day for 5 days) showed that volunteer 1 had a chromatographic profile characterized by urolithin A that was identified by its MS spectrum, its characteristic UV spectrum,[11] and by chromatographic comparisons with an authentic standard, while volunteer 2 produced isourolithin A as a main metabolite (Figure 1). The UV spectra of urolithin A and isourolithin A allow a clear and fast identification of both metabolites.[11] In addition, other minor metabolites were also detected in fecal samples of both volunteers [urolithin M-6 (**7**) and urolithin C (**8**)] (Table 2). When the urine of the same volunteers was analyzed, their metabolic profiles were consistent with those found in feces, and volunteer 1 excreted mainly urolithin A glucuronide (**1**), while volunteer 2 excreted two isomers of isourolithin A glucuronide (**2**, **3**) instead (Figure 2). Trace amounts of urolithin B-glucuronide (**10**) were also detected in the urine of volunteer 2 (Figure 2B).

Bacterial Populations of Human Fecal Samples Used in This Study. Fecal bacteria densities of the two volunteers, who donated feces for the investigation of the metabolic fate of EA in vivo, were also analyzed. Interindividual differences in fecal microbiota were analyzed by qRT-PCR using primers and probes (Table 1) that target the main bacterial groups: *Bifidobacterium* genus; *C. leptum* group (*C. leptum*, *Faecalibacterium prausnitzii*, and some species of *Ruminococcus* genus); *C. coccoides* group (*C. coccoides*, *Eubacterium rectale* spp., and some species of *Ruminococcus* genus); *E. coli* species; *Bacteroides/Prevotella* group; *Lactobacillus/Leuconostoc/Pediococcus* group (Table 1). All bacteria results were presented as the mean of the log CFU per g feces. Fecal dry weight (%) was 31.7 and

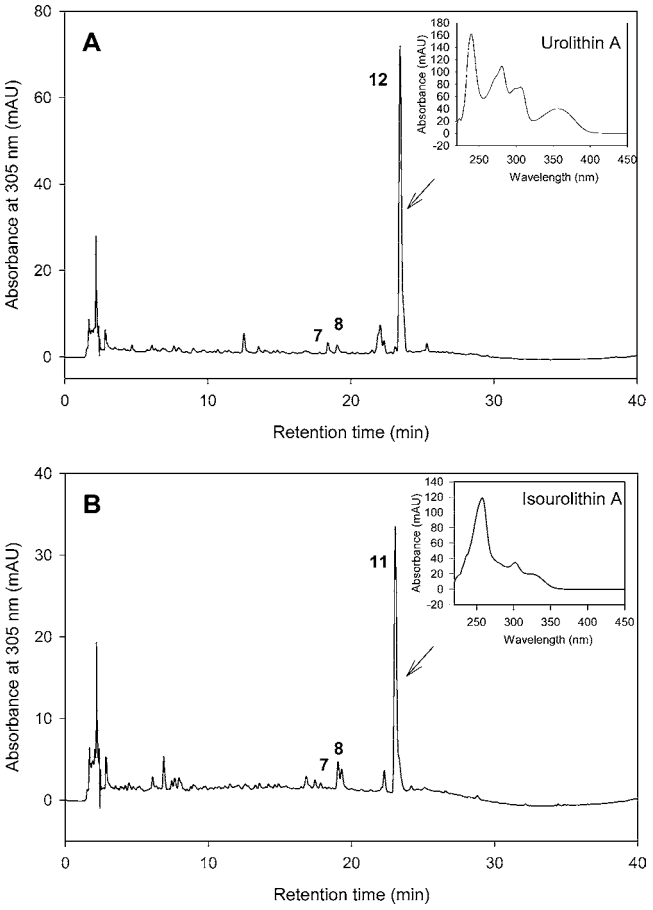

Figure 1. HPLC-UV analysis at 305 nm of fecal samples of volunteer 1 (A) and volunteer 2 (B) after the intake of walnuts (0.6 g/kg body weight per day for 5 days). (**7**) urolithin M-6; (**8**) urolithin C; (**11**) isourolithin A; (**12**) urolithin A.

19.0% in volunteers 1 and 2. To overcome the fact that fecal samples contained different water, a correction factor (19/31.7 = 0.6) was applied to microbial data of volunteer 1.

Fecal sample analyses from both urolithin A (volunteer 1) and isourolithin A (volunteer 2) producers were analyzed (Figure 3). This study showed that one of the most highly represented bacterial groups, in human stools of both volunteers, was the *C. leptum* group of the *Firmicutes* phylum. No significant differences in levels of *C. leptum* group ($7-34 \times 10^7$ CFU/g) and *C. coccoides* group ($(5-8) \times 10^6$ CFU/g) of the *Firmicutes* phylum as well as the *Bacteroides/Prevotella* group ($5-12 \times 10^5$ CFU/g) of the *Bacteroidetes* phylum were observed between fecal samples from volunteers 1 and 2. However, concentrations of other bacterial groups in the volunteer 1 fecal sample were noticeably different from those of the volunteer 2 fecal sample ($P \leq 0.01$) (Figure 3). Thus, the microbiota from volunteer 1 showed a higher concentration of *Bifidobacterium* genus (6×10^8 CFU/g) of the *Actinobacteria* phylum when compared with that of volunteer 2 (1×10^6 log CFU/g) (Figure 3A). Furthermore, in volunteer 1, the fecal sample contained a higher concentration of *E. coli* species (1×10^6 CFU/g) of the *Proteobacteria* phylum than fecal sample of volunteer 2 (8×10^4 CFU/g). In contrast, a lower abundance of *Lactobacillus/Leuconostoc/Pediococcus* group of the *Firmicutes* phylum was found in volunteer 1 (2×10^4 CFU/g) which were 2.7 log units lower than counts for fecal sample of volunteer 2 (1×10^7 CFU/g) (Figure 3). These studies showed that the

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Table 2. Metabolites identified in urine (U), Feces (F), Fermentation Medium Incubated with Human Fecal Suspensions in the Presence of Ellagic Acid (FM) or in the Presence or Ellagic Acid Dissolved in 1% DMSO (FM-DMSO) (sh, Shoulder)[a]

no.	compds	volunteers	origins	R_t (min)	$[M-H]^-$	λ_{max}
1	urolithin A glucuronide	1	U	15.75	403	246, 278, 303, 350
2	isourolithin A glucuronide	2	U	15.52	403	255, 299sh, 325
3	isourolithin A glucuronide	2	U	16.14	403	255, 299sh, 325
4	urolithin M-5	1	FM and FM-DMSO	14.65	275	261, 292sh,352
		2	FM-DMSO			
5	urolithin E	1	FM-DMSO	16.55	259	252, 276sh,365
6	ellagic acid	1 and 2	FM and FM-DMSO	17.62	301	254, 300sh, 366
7	urolithin M-6	1 and 2	F and FM and FM-DMSO	17.94	259	259, 291sh, 350
8	urolithin C	1	F and FM	18.83	243	255, 304, 349
		2	F and FM and FM-DMSO			
9	urolithin M-7	1	FM and FM-DMSO	20.49	243	250, 280sh, 367
		2	FM-DMSO			
10	urolithin B glucuronide	2	U	20.56	387	249, 273, 297, 326
11	isourolithin A	2	F and FM and FM-DMSO	23.06	227	256, 302, 329
12	urolithin A	1	F and FM and FM-DMSO	24.11	227	246, 280, 305, 356
		2	FM and FM-DMSO			
13	urolithin B	2	FM	28.21	211	249, 276, 301, 333

[a]Internal standard (6,7-dihydroxycoumarin), $[M-H]^-$ 177; retention time, 11.52 min.

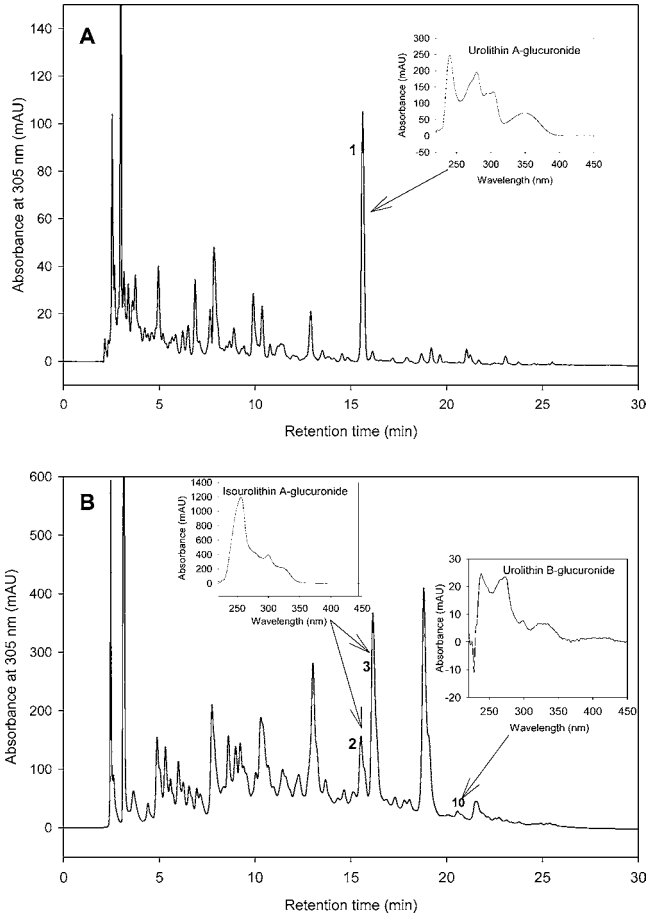

Figure 2. HPLC-UV analysis at 305 nm of urine samples of volunteer 1 (A) and volunteer 2 (B) after the intake of walnuts (0.6 g/kg body weight per day for 5 days). (**1**) urolithin A glucuronide; (**2**, **3**) isourolithin A glucuronide; (**10**) urolithin B glucuronide.

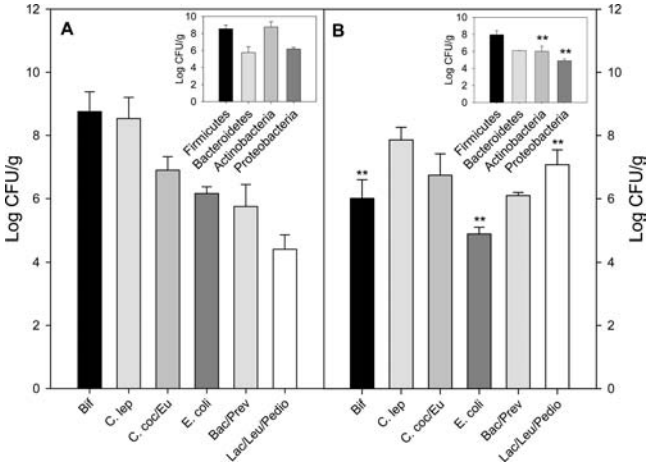

Figure 3. Quantities of dominant groups of bacteria in fecal samples of volunteer 1 (A) and volunteer 2 (B) as determined by real-time polymerase chain reaction. The mean (±SD) counts from triplicate determinations (three DNA samples isolated from the same feces) were used to quantify the bacterial groups. (Bif) *Bifidobacterium* genus; (C. lep) *Clostridium leptum* group; (C. coc/Eu) *Clostridium coccoides* group; (*E. coli*) *E. coli* species; (Bac/Prev) *Bacteroides/Prevotella* group; (Lac/Leu/Pedio) *Lactobacillus/Leuconostoc/Pediococcus* group. **Significant differences ($P \leq 0.01$) in bacteria levels between volunteer 1 (A) and volunteer 2 (B). An estimation of the total amount of *Firmicutes* was obtained by adding bacterial values obtained from *C. coccoides*, *C. leptum*, and *Lactobacillus/Leuconostoc/Pediococcus*.

most highly represented bacterial group in volunteer 2 was the *C. leptum* of the *Firmicutes* phylum, while *C. leptum* and *Bifidobacterium* genus of the *Firmicutes* and *Actinobacteria* phyla were the major representative in volunteer 1.

In Vitro Catabolism of EA by Human Gut Microbiota. The production of urolithins by the gut microbiota from the two volunteers was followed in vitro in order to study the time course of the production of the two microbial metabolite patterns found in vivo and identify potential intermediate catabolites in the route from EA to urolithin A or isourolithin A (Figure 4). The first metabolites observed when EA was incubated with the volunteer 1 (urolithin A producer) fecal microbiota were urolithin M-5 (pentahydroxy-urolithin) and urolithin M-6 (tetrahydroxy-urolithin), which peaked at 12 h

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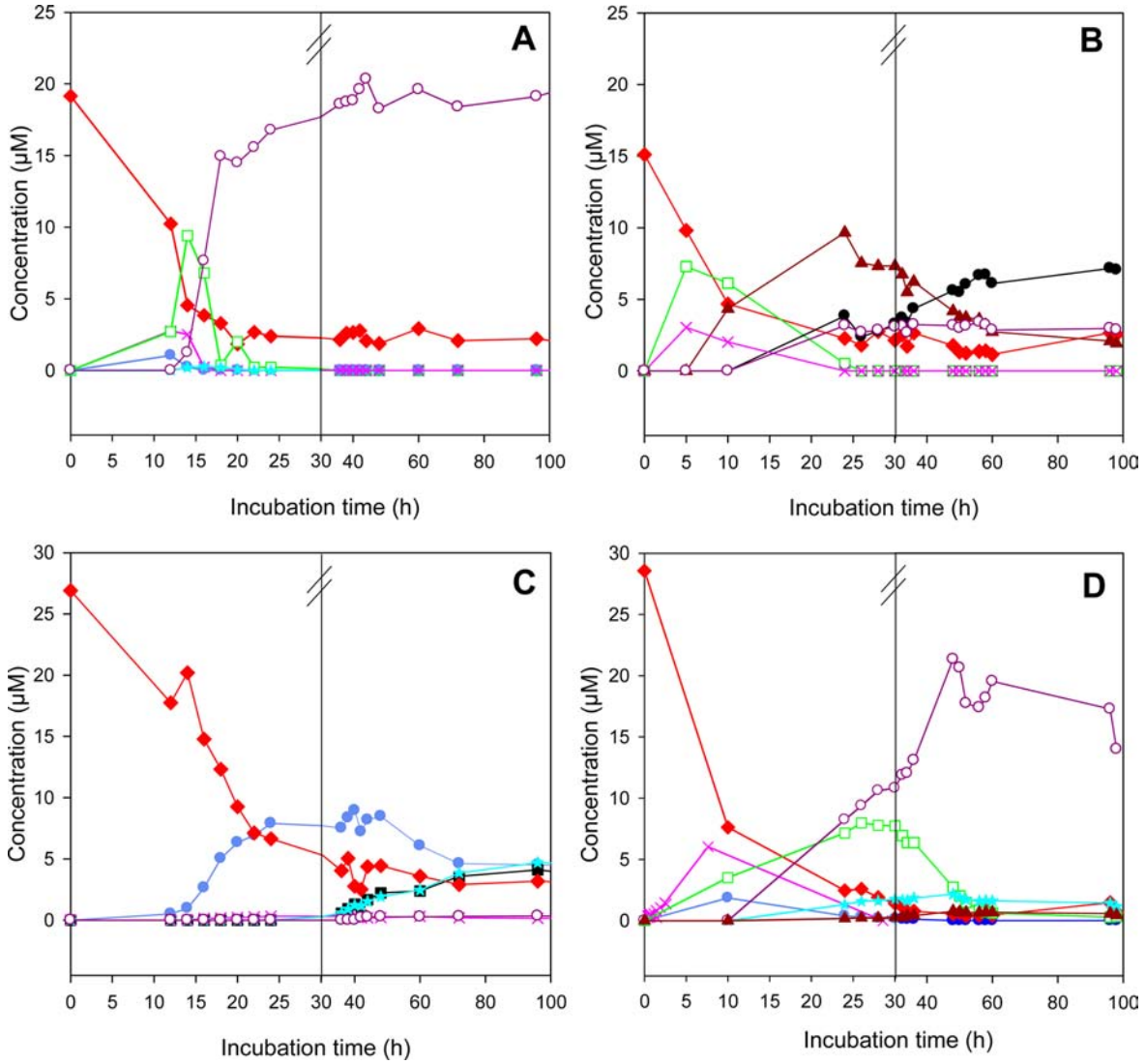

Figure 4. In vitro conversion of EA during batch fermentation with human feces from two volunteers. Human fecal cultures consisted of (A) volunteer 1 fecal suspension in fermentation medium with 30 μM EA; (B) volunteer 2 fecal suspension in fermentation medium with 30 μM EA; (C) volunteer 1 fecal suspension in fermentation medium with 30 μM EA dissolved with 1% DMSO; (D) volunteer 2 fecal suspension in fermentation medium with 30 μM EA dissolved with 1% DMSO. Ellagic acid (red line with red solid diamonds); urolithin M-5 (blue line with blue solid circles); urolithin E (black lines with black solid squares); urolithin M-6 (magenta lines with magenta Xs); urolithin M-7 (cyan lines with cyan solid stars); urolithin C (green lines with green open squares); urolithin A (purple lines with purple open circles); isourolithin A (brown lines with brown solid triangles); urolithin B (black lines with black solid circles).

with a maximum concentration of 1 and 2.5 μM, respectively (Figure 4A). Then, urolithin C (trihydroxy-urolithin) started to be accumulated reaching 9.4 μM at 14 h. At that moment, a small amount of another trihydroxy-urolithin (urolithin M-7) started to be produced with a maximum of 0.29 μM at 16 h. Urolithin A was detected after 14 h incubation, and a maximum of 19.6 μM was reached at 42 h, then a plateau was maintained (Figure 4A). A large proportion of the EA supplied (30 μM) was finally converted into urolithin A (21 μM), although some EA remained unmetabolized in the medium (1.3 μM).

In the case of volunteer 2 (isourolithin A producer), no urolithin M-5 was detected and both urolithin C and urolithin M-6 appeared with a maximum after 5 h (7.5 and 3.5 μM, respectively) (Figure 4B). Then isourolithin A started to be produced, reaching a maximum of 10 μM after 24 h. Isourolithin A then decreased, and urolithin B increased to reach 6.5 μM after 60 h and then reached a plateau. At the same time as urolithin B was produced, some urolithin A was also observed, although it reached concentrations much lower than those found for volunteer 1 (3 μM). The maximum EA conversion to urolithins was lower in the case of volunteer 2 fecal fermentation (15 μM) (Figure 4B) than in the case of volunteer 1 fecal fermentation (21 μM) (Figure 4A) and some EA remained unmetabolized in the medium (2 μM).

The urolithin production from EA when this was dissolved in DMSO and added to the fermentation medium (1%) to increase its solubility, and potentially its bacterial accessibility, was also evaluated. The EA catabolism was very different from that observed when EA was added to the fermentation medium without adding any organic solvent, and significant changes were observed in growth parameters of some bacterial groups analyzed as described in the section below. In the case of volunteer 1, the main in vitro metabolite produced in the presence of DMSO was urolithin M-5 (pentahydroxy-urolithin)

instead of urolithin A, which started to be detected after 12 h, with a maximum concentration of 9 μM at 40 h incubation. Urolithin M-6, urolithin M-7, and urolithin A were also observed but not before 16, 36, and 42 h incubation, respectively, and in much smaller maximal concentrations (0.3, 1.3, and 0.5 μM, respectively) (Figure 4C) than in the absence of DMSO (2.5, 0.3, and 19.6 μM, respectively) (Figure 4A). Urolithin C was not produced in the presence of DMSO (Figure 4C). Despite the smaller and slower urolithin production in presence of the DMSO, a large proportion of EA supplied was metabolized in the medium (24 μM) (Figure 4C). A relevant unidentified tetrahydroxy urolithin was started to be produced after 36 h of incubation, and this was named as urolithin E (Figure 4C). This had the same mass spectrum as urolithin M-6. To identify the structure of urolithin E, this was compared with the other two available tetrahydroxy-urolithins, urolithin D and urolithin M-6. All of them had the same MS spectrum but differed in the UV spectrum (Figure 5). Urolithin M-6 had a characteristic UV spectrum, and urolithin D had a similar spectrum to urolithin A. Urolithin E had a different UV spectrum, with a significant band I at 360 nm, a characteristic of those urolithins without a hydroxyl at 9-position (Figure 5).[11] Therefore, urolithin E was tentatively identified as 3,4,8,10-tetrahydroxy-urolithin, a new urolithin metabolite. NMR analysis of this urolithin was not possible due to the small amount produced.

In the case of volunteer 2, when EA added was dissolved in DMSO, this started to be converted to urolithin M-5, urolithin M-6, and urolithin C with a maximum concentration of 2, 6, and 7.5 μM after 7.5, 10, and 25 h, respectively. Then urolithin A started to be produced, reaching a maximum (22 μM) after 40 h (Figure 4D). In this case, only a small concentration of isourolithin A was detected (1 μM). Urolithin M-7 was also observed and reached maximal concentrations of 3 μM after 50 h. Most EA was metabolized, and only low concentrations (1 μM) remained unmetabolized in the medium (Figure 4D).

Changes in Human Gut Microbiota in Vitro. Changes in the fecal microbiota of volunteers 1 and 2 were followed during the in vitro metabolism of EA in order to elucidate which microbial groups could be implicated in the production of urolithins. The growth characteristics of the main bacterial groups, determined by q-PCR, during the conversion of EA in vitro batch fermentation are shown in Table 3. In the absence of DMSO, the main differences between fecal microbiota development of volunteers 1 and 2 were detected in the *Bifidobacterium* genus. In fact, the bifidobacteria of volunteer 2 were not able to grow in the in vitro conditions despite urolithins being produced. *Lactobacillus/Leuconostoc/Pediococcus* group also grew slower in volunteer 2 than in volunteer 1, but a higher level of bacteria was obtained at the end of the growth (N_{max}) in volunteer 2 (5.0 log CFU/mL) than in volunteer 1 culture (4.4 log CFU/mL). In contrast, *C. coccoides* group reached a higher level in volunteer 1 (N_{max}: 6.1 log CFU/mL) than in volunteer 2 culture (N_{max}: 5.0 log CFU/mL) (Table 3). In the same way, urolithin concentrations were higher in volunteer 1 than in volunteer 2 culture.

In the presence of DMSO, a delay in the growth of *C. coccoides* group was observed in both volunteer fecal fermentations. Thus, the lag period was extended in 9.7 and 22 h in the case of volunteers 1 and 2, respectively (Table 3). For human fecal suspensions of volunteer 1, the extension of the lag phase in 9.7 h of *C. coccoides* group in the presence of DMSO was accompanied by a delay of 10 h in the beginning of urolithin production. A similar tendency was observed in fecal suspension of volunteer 2, where the presence of DMSO lengthens both lag period (24.6 h) and time needed to achieve the maximum urolithin concentration. In contrast, the *Bacteroides/Prevotella* group achieved higher levels in the absence of DMSO (N_{max}: 7.2 log CFU/mL) than when DMSO was present (N_{max}: 6.8 log CFU/mL) in the case of volunteer 2 (Table 3).

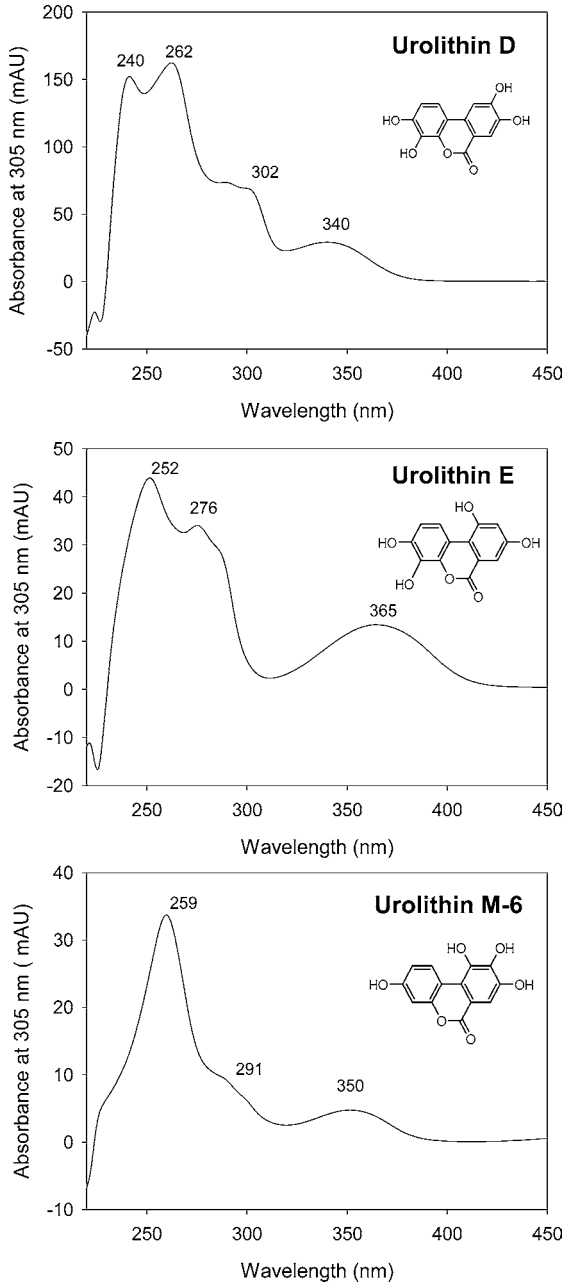

Figure 5. UV spectra of the three tetrahydroxy urolithins ($[M - H]^-$ m/z 259): urolithin D, urolithin E, and urolithin M-6.

DISCUSSION

The studies carried out so far with human biological fluids (urine and plasma) have allowed the identification of urolithin A, urolithin B, urolithin C, and isourolithin A.[5] Other metabolites such as urolithins M-5, M-6, and M-7 had been reported in rat fecal samples and other animal materials.[11−13]

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Table 3. Growth Characteristics of Main Bacterial Groups Determined by q-PCR during Conversion of Ellagic Acid in Vitro Batch Fermentation with Human Fecal Suspensions[a]

microbial groups	volunteer	fermentation medium + EA	lag time (h)	Gr_{max} (log CFU/h)	N_{max} (log CFU/mL)	correlation coefficient (R^2)
Clostridium coccoides group	1	without DMSO	0	0.12	6.08	0.99
		with DMSO	9.66**[a]	0.05**[a]	5.87	0.99
	2	without DMSO	2.59	0.10	5.04	0.99
		with DMSO	24.60**[a]	0.14	5.08	0.99
Bifidobacterium genus	1	without DMSO	0	0.15	7.91	0.92
		with DMSO	0	0.10	7.93	0.83
	2	without DMSO	no growth	no growth	no growth	
		with DMSO	no growth	no growth	no growth	
Clostridium leptum group	1	without DMSO	0	0.07	6.89	0.95
		with DMSO	0	0.04**[a]	6.60*[a]	0.87
	2	without DMSO	0	0.11	7.08	0.99
		with DMSO	0	0.09	7.12	0.97
Lactobacillus/Leuconostoc/Pediococcus	1	without DMSO	0	0.16	4.40	0.99
		with DMSO	0	0.18	4.34	0.99
	2	without DMSO	0	0.02	5.00	0.99
		with DMSO	0	0.05**[a]	4.96	0.99
Bacteroides/Prevotella group	1	without DMSO	0	0.12	6.40	0.96
		with DMSO	0	0.24**[a]	6.41	0.98
	2	without DMSO	0	0.21	7.17	0.99
		with DMSO	0	0.21	6.81*[a]	0.99
E. coli species	1	without DMSO	0	0.45	8.22	0.98
		with DMSO	0	0.40	8.30	0.99
	2	without DMSO	0	0.31	7.37	0.99
		with DMSO	0	0.27	7.83*[a]	0.99

[a]Fecal cultures consisted of volunteer 1 and 2 fecal suspensions in fermentation medium with 30 μM ellagic acid (EA) dissolved or not with 1% DMSO. Significant differences in growth without and with DMSO addition. * ($P \leq 0.05$), ** ($P \leq 0.05$).

However, this is the first time that urolithin M-5, urolithin M-6, urolithin M-7, urolithin C, and the new identified metabolite urolithin E are reported to be produced by human gut microbiota. Urolithin M-6 (tetrahydroxy-urolithin) was detected with a maximum concentration between those of urolithin M-5 and two trihydroxy-urolithins (urolithin C and urolithin M-7) as could be expected for the catabolic sequence (Figure 6). This shows that these metabolites occur in vivo in human gut, although they can be quickly metabolized into urolithin A, which is the main metabolite found in plasma and urine. Analysis of human feces after the intake of ellagitannins and EA should be carried out in order to evaluate the occurrence of these urolithin metabolites in the gut conditions.

Degradation of EA by human gut microbiota leads to similar urolithin metabolites in vitro (fermentation medium containing EA at 30 μM) than in vivo (after consumption of 0.6 g walnuts/kg body weight per day for 5 days). Thus, both urolithin A and isourolithin A producers in vivo (volunteers 1 and 2) led to urolithin A and isourolithin A in vitro, respectively (Figure 4A,B). Despite the fact that urolithin metabolic profiles found in vivo were consistent with those found in vitro, further studies should be done in order to study the time course of the production of the two microbial metabolite patterns when EA containing foods are used in place of EA. This would elucidate the influence of other food constituents such as fiber on urolithin production rate.

In the human fecal culture of volunteer 1 (in vivo urolithin A producer), EA was 62% converted to urolithins at 36 h, while in volunteer 2 (in vivo isourolithin A producer) the conversion efficiency to urolithins was much lower (46% at 36 h). Taking into account the higher EA conversion efficiency of volunteer 1 and the fact that of the *C. coccoides* group achieved 1 log higher concentration in fecal fermentation sample of volunteer 1 than in volunteer 2, this suggests that this microbial group or other in vivo co-occurring bacteria could be involved in EA catabolism. In contrast, the lack of growth of *Bifidobacterium* in volunteer 2 could indicate that this microbial group is not involved in urolithin production.

Because of solubility problems of EA, this was alternatively dissolved in DMSO and incorporated into the medium at 1% v:v. In human fecal cultures where DMSO was added, different EA conversion was observed (28% and 74% in the case of volunteer 1 and 2, respectively). In the case of volunteer 1, the EA catabolism did not reach the whole transformation to urolithin A, and different intermediates were obtained. Urolithin M-5 was observed as the first step in urolithin production from EA to continue with urolithin M-6 and then urolithin M-7 to end with urolithin A but in much lower concentration than in the absence of DMSO. Furthermore, the addition of DMSO prevented the conversion of urolithin M-6 into urolithin C being one trihydroxy-urolithin (urolithin M-7), the only precursor found for the final catabolite (urolithin A)

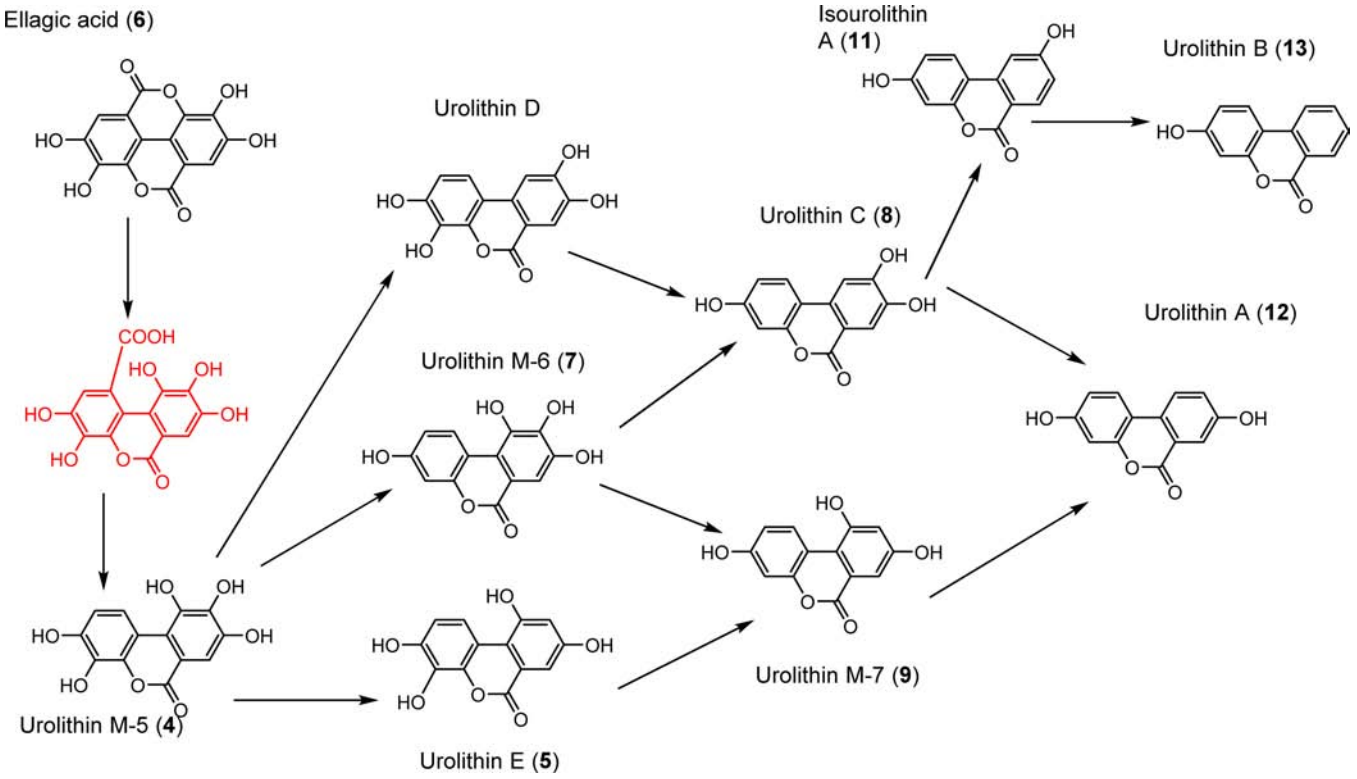

Figure 6. Structures of the main urolithin metabolites detected and suggested catabolic routes of formation by gut microbiota.

(Figure 6). Therefore, the use of DMSO in fecal cultures offered the possibility of studying other metabolic intermediates between EA and urolithin A, such as urolithin E, another tetrahydroxy-urolithin precursor of urolithin M-7 (Figure 6). Despite the smaller urolithin production in presence of the DMSO, most of the EA was metabolized in the medium (24 μM). This indicates that DMSO delayed urolithin producing bacteria development, allowing the action of other bacteria able to metabolize EA to simple organic acids such as phenyl acetic acid and phenylpropionic acid. However, further studies should be done in order to elucidate if higher abundance of *Bacteroidetes* and less abundance of *Firmicutes* such as *C. coccoides* are correlated with a lower urolithin production in vivo.

In the case of human fecal culture of volunteer 2, it seems that the catabolic route of EA to urolithins was modified by the effect of DMSO. In fact, much lower concentration of isourolithin A and much higher concentration of urolithin A were detected in the presence of DMSO (Figure 4D). This suggests that the microbial strains responsible for isourolithin A production in volunteer 2 grew with difficulty under these in vitro conditions. Furthermore, urolithin B was not detected, which suggests isourolithin A and not urolithin A is its only precursor and confirms this theory that was first proposed in cattle after the administration of oak leaves.[12] In addition, this is also in agreement with a previous in vitro study where urolithin B was only detected in those human fermentation cultures where isourolithin A were detected.[17] A negative effect of DMSO on the growth of some bacterial groups was also observed, particularly on *C. coccoides* bacteria. A similar delay on growth of this bacterial group and the time needed to achieve the maximum urolithin concentration was observed, suggesting that this bacterial group could be involved in the EA metabolism of both volunteers.

In the literature, nothing is found about bacterial species or groups responsible for urolithin production from ellagitanins. In the present study, *C. coccoides* group (*C. coccoides* spp., *E. rectale* spp., and some species of *Ruminococcus* genus) from the *Firmicutes* phylum, or genera co-occurring in vivo with this group, are proposed as the microorganisms involved in urolithins production. *Clostridium* and *Eubacterium* genera are a common element of the metabolism of several phenolic compounds such as flavonoids.[21] Given the phylogenetic association between these two genera, the association of them in the phenolic transformations is not surprising. The fecal samples corresponding to in vivo urolithin A and isourolithin A producers showed a higher abundance of either *C. coccoides* or *C. leptum* than that of the *Bacteroides/Prevotella* group. Recently, three robust clusters, referred to as "enterotypes", which are not nation or continent specific have been identified. Assignment of an individual microbiome into a given enterotype is based upon the relative enrichment of that microbiome in one of three genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), or *Ruminococcus* (enterotype 3).[22] Further studies should be done in order to elucidate existent relations between human urolithins production capacity and relative abundance of bacteria either in the different phyla or in the different enterotypes.

Over the past decade, there has been enormous public and scientific interest in urolithins because of their proposed health-promoting effects, which, nonetheless, are far from being fully understood.[23] Future studies should take the newly identified urolithin metabolites into account when investigating EA absorption and metabolism as well as their physiologic effects in humans. Isolation and identification of bacteria strains implicated in urolithins production also require further investigation.

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Notes

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