

Cite this: *Food Funct.*, 2020, **11**, 7012

Metabolism of different dietary phenolic compounds by the urolithin-producing human-gut bacteria *Gordonibacter urolithinifaciens* and *Ellagibacter isourolithinifaciens*†

 Rocío García-Villalba,  David Beltrán, María D. Frutos, María V. Selma, Juan C. Espín  and Francisco A. Tomás-Barberán *

Gordonibacter urolithinifaciens and *Ellagibacter isourolithinifaciens* are two human gut bacterial species that convert ellagic acid into urolithins. Urolithins are bioactive postbiotics produced by dehydroxylation reactions catalyzed by different catechol-dehydroxylases. The metabolic ability of these anaerobic bacteria on other dietary-phenolic compounds is unknown. In the present study, we evaluated the metabolism of flavonoids (quercetin, hesperetin, hesperidin, nobiletin, catechin, isoxanthohumol), isoflavonoids (daidzein), coumarins (esculetin, umbelliferone, scoparone), phenylpropanoids [caffeic acid; 3-(3',4'-dihydroxyphenyl)propanoic acid (dihydrocaffeic acid); rosmarinic acid, and chlorogenic acid], benzoic acid derivatives (gallic acid, ellagic acid), lignans (secoisolariciresinol diglucoside), stilbenes (resveratrol), and secoiridoids (oleuropein) by *G. urolithinifaciens* DSM 27213^T and *E. isourolithinifaciens* DSM 104140^T. Both strains metabolized ellagic acid leading to the characteristic urolithins. They also metabolized caffeic, dihydrocaffeic, rosmarinic, and chlorogenic acids. The rest of the phenolic compounds were not transformed. Catechol dehydroxylation and double bond reduction were prominent transformations observed during the incubations. The enzymatic activities seem to have a narrow substrate scope as many catechol- (quercetin, catechin, esculetin, gallic acid) and double bond-containing (resveratrol, esculetin, scoparone, umbelliferone) phenolics were not metabolized. The catechol-dehydroxylase activity was more efficient in *E. isourolithinifaciens*, while the reductase activity was more relevant in *G. urolithinifaciens*.

Received 24th June 2020,
Accepted 21st July 2020

DOI: 10.1039/d0fo01649g

rsc.li/food-function

Introduction

The bioavailability of dietary (poly)phenols (PPs) is very low.¹ Most of them reach the colon where they are catabolized by the gut microbes producing metabolites, recently named as postbiotics,^{2–4} that are much better absorbed than the original PPs, show relevant biological effects in the colon and systemically, and persist in the body for long periods (sometimes up to three or four days).⁵

In the case of ellagitannins and ellagic acid, the original PPs are converted to urolithins,⁶ and different metabolites are produced by the opening and decarboxylation of one of the two lactone rings in the ellagic acid molecule and the sequential removal of phenolic hydroxyls that lead to different urolithin metabolites.⁷ These biochemical conver-

sions involve dehydroxylation by different catechol-dehydroxylases.

Three consistent metabolotypes for the production of urolithins have been reported in humans,⁸ and bacterial strains producing this metabolic conversion have been discovered, characterized, and deposited in bacterial banks. *Gordonibacter urolithinifaciens* and *Gordonibacter pamelaee*, the first bacterial species that were reported to produce urolithins from ellagic acid, were able to provide urolithins M5 (3,4,8,9,10-pentahydroxy urolithin), M6 (3,8,9,10-tetrahydroxy urolithin) and C (3,8,9-trihydroxy urolithin),^{9,10} and the abundance of this genus within a complex human gut bacterial community positively correlated with the metabolotype A, while correlated negatively with metabolotype B.¹¹ However, the recently discovered *Ellagibacter isourolithinifaciens*^{12,13} was able to produce isourolithin A (3,9-dihydroxy urolithin) and positively correlated with metabolotype B.¹⁴

Although enzymes with catechol-dehydroxylase activity are well-known, some of these enzymes have only been recently isolated and characterized from human gut microbes.^{15,16} Thus, an enzyme for the regioselective *p*-dehy-

Laboratory of Food & Health, Research Group on Quality, Safety, and Bioactivity of Plant Foods, Department of Food Science and Technology, CEBAS-CSIC, P.O. Box 164, 30100 Campus de Espinardo, Murcia, Spain. E-mail: fatomas@cebas.csic.es
†Electronic supplementary information (ESI) available. See DOI: 10.1039/d0fo01649g



droxylation of dopamine to produce *m*-tyramine has been described from *Eggerthella lenta* strains.¹⁵ The removal of a *p*-hydroxyl group from hydrocaffeic acid [3-(3',4'-dihydroxyphenyl)-propanoic acid] and 3,4-dihydroxyphenylacetic acid (DOPAC) has also been recently reported.¹⁶ Another study has characterized the dehydroxylation of bidesmethyl-secoisolariciresinol to yield the mammalian lignan enterodiol by *G. pamelaee*.¹⁷ Besides, recent research has demonstrated considerable variability in the catechol-dehydroxylase activity across closely related gut microbiota strains, with a very narrow substrate scope, suggesting that different enzymes can dehydroxylate various catechol-containing phenolics.¹⁶

In the present study, we aimed to evaluate the metabolism of eighteen dietary phenolics, representative of different phenolic classes, by *G. urolithinifaciens* and *E. isourolithinifaciens*, and identify the metabolites produced.

Materials and methods

Chemicals

The PP standards selected (and their main dietary sources) were: gallic acid (3,4,5-trihydroxybenzoic acid, many foods and wine), hesperetin (5,7,3'-trihydroxy,4'-methoxyflavone, citrus), hesperidin (5,7,3'-trihydroxy-4'-methoxyflavone 7-O-rhamnosyl(1-6)glucoside, citrus fruits), quercetin (3,5,7,3',4'-pentahydroxyflavone, onion), daidzein (7,4'-dihydroxy-isoflavone, soybean), catechin (flavan-3-ol, many foods, tea), secoisolariciresinol diglucoside (lignan, flaxseed), isoxanthohumol (prenylated flavanone, hops and beer), resveratrol (stilbene, grapes, and wine); caffeic acid (hydroxycinnamic acid, coffee); rosmarinic acid (caffeic acid ester, rosemary), dihydrocaffeic acid [3-(3',4'-dihydroxyphenyl)propanoic acid] (gut microbiota metabolite derived from many dietary phenolics), oleuropein (dihydroxy-phenylethanol ester, olives and olive oil), esculetin (coumarin, barley), scoparone (coumarin methyl ether, citrus), umbelliferone (coumarin, carrot), nobiletin (5,6,7,8,3',4'-hexamethoxyflavone, citrus fruits), chlorogenic acid (caffeoyl-quinic derivative, coffee, and widespread in food), ellagic acid (gallic acid dimer, strawberry, pomegranate) (Fig. 1). Ferulic acid (3'-methoxy-4'-hydroxycinnamic acid), isoferulic acid (3'-hydroxy-4'-methoxy-cinnamic acid), *p*-coumaric acid (4'-hydroxy cinnamic acid), *m*-coumaric acid (3'-hydroxy cinnamic acid), 3-(4'-hydroxyphenyl)-propanoic acid, and 3-(3'-hydroxyphenyl)-propanoic acid were used for chromatographic comparisons. All standards were purchased from Sigma-Aldrich (St Louis, MO, USA). Methyl-caffeate was obtained by heating caffeic acid dissolved in methanol at 80 °C in a stoppered pressure tube under N₂ atmosphere. Stock solutions for each PP were prepared in methanol UPLC-MS quality (3 mM), and filtered through 0.22 µm polyvinylidene fluoride (PVDF) (Merck Millipore, Cork, Ireland) syringe filter for sterilization. The standards were injected independently in the analytical conditions (50 µM) to evaluate their response in DAD and MS detectors.

Bacterial strains

The two strains used in this study were *Gordonibacter urolithinifaciens* (DSM 27213^T = CCUG 64261^T)^{9,10} and *Ellagibacter isourolithinifaciens* (DSM 104140^T = CCUG 70284^T).^{12,13}

In vitro fermentation method

Preparation of bacterial strains and subsequent culturing experiments were made in an anaerobic chamber (Concept 400, Baker Ruskin Technologies, Ltd, Bridgend, South Wales, UK) with an atmosphere composed of N₂/H₂/CO₂ (85:5:10) at 37 °C. Both bacterial strains from an early stationary phase culture, were grown separately or in co-culture with an initial concentration of 7 log(cfu mL⁻¹) (determined by plate counting with an automatic counter), were inoculated into 5 mL of pre-reduced, anaerobe basal broth (Oxoid, Basingstoke, Hampshire, UK) containing 30 µM (a concentration reachable in the colon after dietary interventions) of the different PPs. Triplicate samples were done for each compound. As control samples, PPs were incubated without bacteria, and the bacterial strains were incubated without added PPs. All samples (5 mL) were incubated for seven days.

HPLC-DAD-MS analyses of the microbial metabolites

After incubations, 5 mL medium was extracted with 5 mL ethyl acetate LC-MS (Scharlau, Barcelona, Spain) acidified with 1.5% of formic acid (Panreac Química, Barcelona, Spain) using a refrigerated thermoblock shaker (VWR International, LLC, Radnor, PA, USA) at 20 °C for 10 min and 1500 rpm. Samples were centrifuged at 3500g and 4 °C for 10 min, and the supernatants evaporated in a speed vacuum concentrator (Savant SPD121P, ThermoScientific, Spain). The evaporated samples were re-dissolved in 250 µL methanol and filtered through a 0.22 µm PVDF filter before injecting into the HPLC-DAD-ESI-IT equipment.

Analyses were carried out on an Agilent 1100 HPLC system coupled in series to an ultraviolet-visible diode array detector (UV-Vis DAD) (Agilent Technologies, Waldbronn, Germany) and an ion trap (IT) mass spectrometer detector (model Esquire 1100 equipped with an electrospray interface (ESI)) (Brüker Daltoniks). The separation was achieved on a reverse-phase Poroshell 120 EC-18 column (100 × 3 mm, 2.7 µm particle size) (Agilent Technologies, Waldbronn, Germany), operating at room temperature and a flow rate of 0.5 mL min⁻¹. A volume of 5 µL of the sample was injected. The mobile phases used were water with formic acid (1%) (phase A), and acetonitrile (phase B) and the solvent gradient changed according to the following conditions: 0 min, 1% B, 0 to 15 min, 1–25% B; 15 to 28 min, 25–40% B; 28–30 min, 40–90% B; 30 to 31 min, 90% B; 31 to 32 min, 90 to 1% B and maintained at 1% 5 min. The UV-Vis spectra were acquired in the range of 200 to 550 nm, and the chromatograms were recorded at 360, 320, 305, and 280 nm. In the mass spectrometry detection, nitrogen was used as a drying and nebulizing gas with the following parameters: nebulizer pressure was set at 50 psi, dry



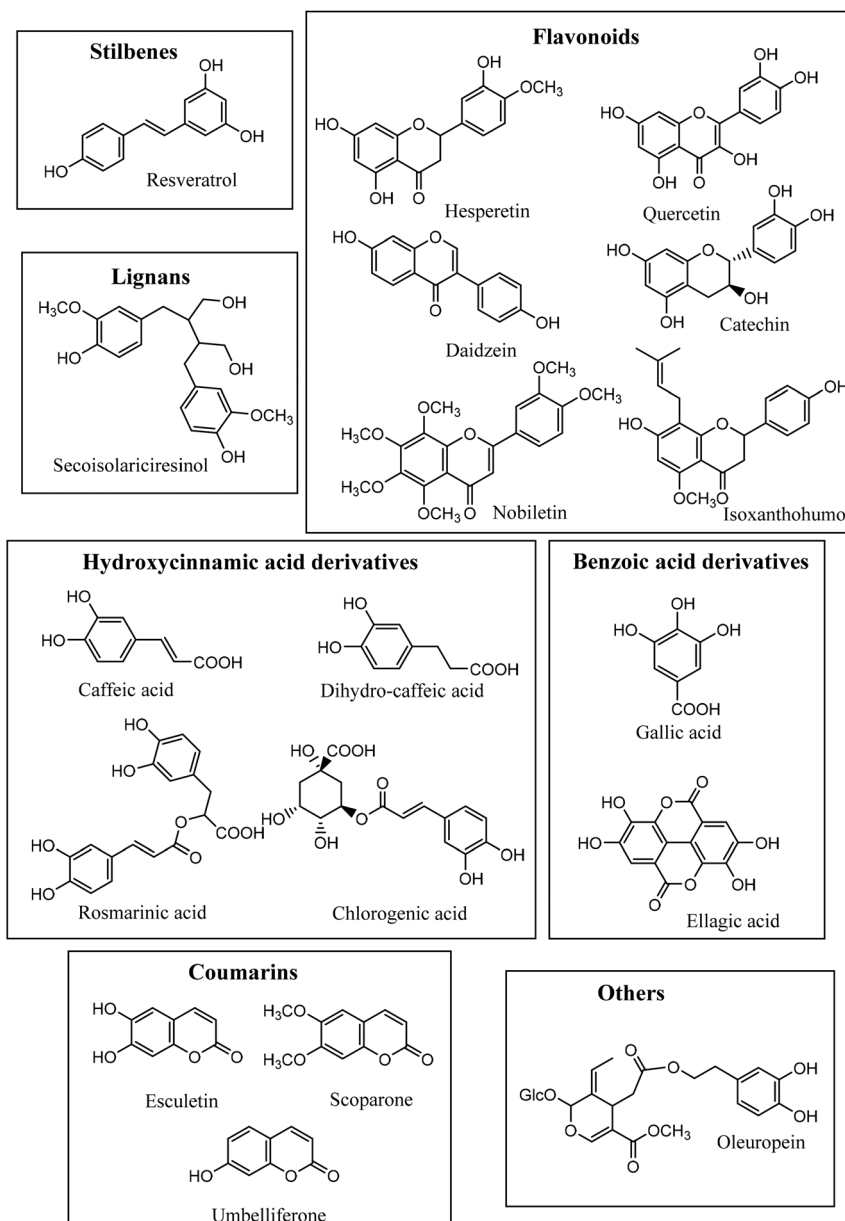


Fig. 1 Structures of the different (poly)phenols assayed.

gas flow 10 L min^{-1} , and dry gas temperature $350 \text{ }^\circ\text{C}$. Mass spectrometry data were acquired in the negative ionization mode, with a capillary voltage of 4 kV. Mass scan (MS) and MS-MS spectra were measured in the range of m/z 100–1100 with a target mass of 400 and compound stability of 75%. The maximum accumulation time of the ion trap was 50 ms. Auto MS-MS mode was applied with the following conditions: width of the isolation, 4.0; fragmentation amplitude, 1.00 V; and the number of parents, 3. The identification of the compounds was carried out through their elution order in the HPLC chromatograms, UV-Vis spectra, molecular weight, their MS-MS fragments, and, whenever possible, chromatographic comparison with authentic standards.

Results

Screening of (poly)phenols conversion

For the majority of the assayed PPs, the chromatographic integration of the PP peaks in the control incubations (PP in medium) and the bacterial incubations (PP with each bacterial strain and with a co-culture of both strains) were the same. The study showed that despite the prolonged exposure of the PP to the bacterial strains (7 days), most of them were not converted into other metabolites. When the chromatographic profiles of the control samples and those incubated with the bacterial isolates were compared at different UV wavelengths, no new peaks were observed (potential new metabolites pro-



duced) for most of the PPs. The MS analyses also showed no new metabolites after the incubation. As a positive control, both bacterial strains were active in the conversion of ellagic acid to produce the expected urolithin metabolites (Fig. 2), as was previously reported.^{6,9,10,12,13} *G. urolithinifaciens* yielded urolithin M5 (3,4,8,9,10 pentahydroxy urolithin), urolithin M6 (3,8,9,10-tetrahydroxy urolithin), and urolithin C (3,8,9-trihydroxy urolithin) while *E. isourolithinifaciens* transformed ellagic acid into urolithins M5, M6, C, and isourolithin A (3,9-dihydroxy urolithin). The incubations were repeated three times with identical results.

For the rest of the incubations with the selected PPs, new metabolites were only found after the incubation of caffeic (3',4'-dihydroxy-cinnamic acid), dihydrocaffeic [3-(3',4'-dihydroxyphenyl) propanoic], rosmarinic, and chlorogenic (5-caffeoyl-quinic) acids (Table 1). The study suggested that under the assayed conditions, both strains showed catechol-dehydroxylase, reductase, and lactonase/decarboxylase activities on some of the PPs. However, they did not show demethylase-, glucosidase-, rhamnosidase-, or single hydroxyl dehydroxylase-mediated conversions.

G. urolithinifaciens and *E. isourolithinifaciens* showed different conversion rates on the phenolics that were metabolized by these bacterial species. In Table 2, the percentage of conversion of the phenolic compounds by each bacterial species and the co-culture are included. Interestingly the percentage of conversion of ellagic acid into urolithins was much more relevant in *E. isourolithinifaciens* (89%) than in

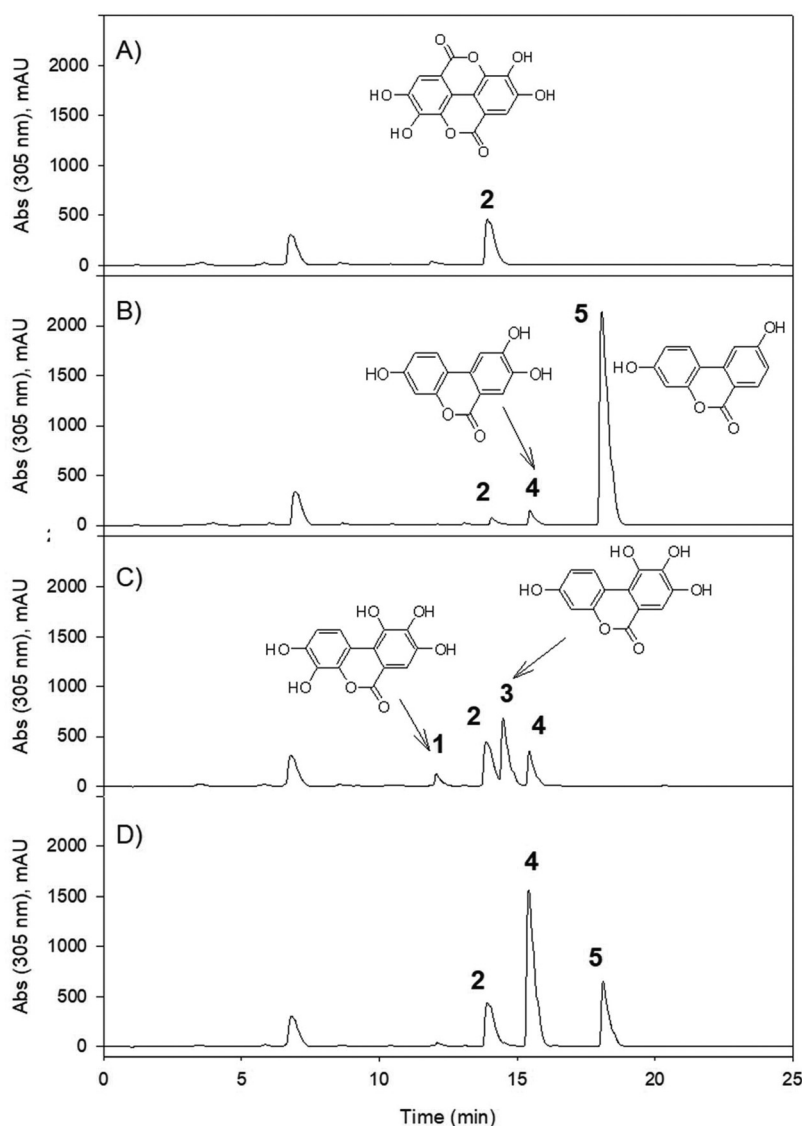


Fig. 2 HPLC-DAD chromatograms (305 nm) of the incubations of ellagic acid in the medium without bacterial strains (A), with *Ellagibacter isourolithinifaciens* (B), with *Gordonibacter urolithinifaciens* (C), and the co-culture of both strains (D). (1) Urolithin M5; (2) Ellagic acid; (3) Urolithin M6; (4) Urolithin C; (5) Isourolithin A.



Table 1 Main catabolic reactions and metabolites found after incubation of *E. isourolithinifaciens*, *G. urolithinifaciens* and co-culture with (poly) phenols from different classes

Phenolics	Catabolic reactions	<i>E. isourolithinifaciens</i>	<i>G. urolithinifaciens</i>	Co-culture
Ellagic acid	Decarboxylation Dehydroxylation	Urolithin M5 Urolithin C, isourolithin A	Urolithin M5 Urolithin M6, Urolithin C	Urolithin M5 Urolithin C isourolithin A
Caffeic acid	Dehydroxylation Reduction	<i>m</i> -Coumaric acid 3-HPPA	<i>m</i> -Coumaric acid 3-HPPA	<i>m</i> -Coumaric acid 3-HPPA
Dihydrocaffeic acid	Dehydroxylation	3-HPPA	3-HPPA	3-HPPA
Rosmarinic acid	Reduction	—	Dihydro rosmarinic acid	Dihydro rosmarinic acid
Chlorogenic acid	Quinic acid degradation Reduction Dehydroxylation	Methyl caffeate — 3-HPPA	— Dihydro chlorogenic acid 3-HPPA	— Dihydro chlorogenic acid 3-HPPA

3-HPPA: 3-(3-Hydroxyphenyl)propionic acid. (–) no metabolite produced. Other (poly)phenols tested that were not metabolized under the assayed conditions: coumarins (esculetin, umbelliferone, scoparone), flavonoids (quercetin, catechin, hesperidin, hesperetin, nobiletin, isoxanthohumol), isoflavones (daidzein), lignans (secoisolariciresinol diglucoside), stilbenes (resveratrol), other (oleuropein and hydroxytyrosol).

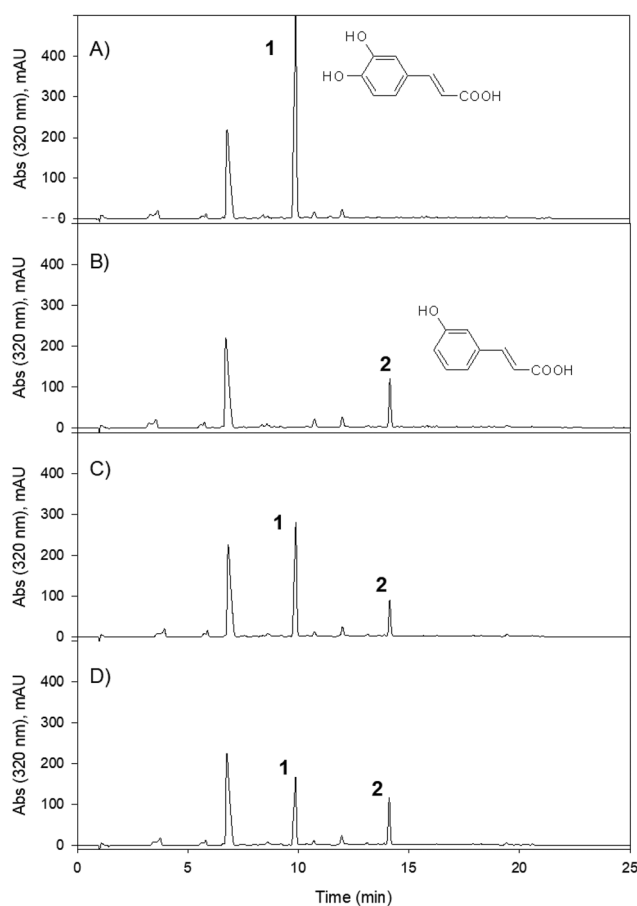
Table 2 Percentage of conversion of phenolic compounds by the gut bacterial species

PPs	<i>Elligibacter isourolithinifaciens</i>	<i>Gordonibacter urolithinifaciens</i>	Co-culture
Ellagic acid	89	7	2
Caffeic acid	100	48	69
Dihydrocaffeic acid	100	100	100
Rosmarinic acid	8	100	100
Chlorogenic acid	65	100	100

G. urolithinifaciens (7%) and in the co-culture (2%). These differences should be further studied to understand better this biochemical process.

Identification of the metabolites

From caffeic acid (Rt 9.84 min), a new metabolite was produced (Rt 13.79) by both bacterial species and the co-culture, and *E. isourolithinifaciens* was much more active producing the metabolite (100% conversion) than *G. urolithinifaciens* (48% conversion), as was evident by the remaining caffeic acid after the seven days of incubation (Fig. 3, Table 2). Caffeic acid decreased in the presence of *G. urolithinifaciens*, although it disappeared entirely in the presence of *E. isourolithinifaciens*, while a new metabolite appeared as a prominent peak in the chromatogram. The new metabolite had a $[M - H]^-$ at m/z 163, with a fragmentation profile 163/119 (–COO), while the fragmentation profile of caffeic acid was 179/135 (–COO). The new metabolite showed a difference of 16 mass units with caffeic acid, suggesting that the metabolite was a dehydroxylated derivative. The samples were also injected into a high-resolution UPLC-ESI-QTOF, and a peak of mass 163.0399 with a molecular formula $C_9H_8O_3$ consistent with a hydroxy-cinnamic acid was found. Following the two potential dehydroxylations of caffeic acid, the possible metabolites produced from caffeic acid by both strains were *p*-coumaric acid (4'-hydroxycinnamic acid) and *m*-coumaric acid (3'-hydroxycinnamic acid). The UV

**Fig. 3** HPLC-DAD chromatograms (320 nm) of the incubations of caffeic acid in the medium without bacterial strains (A), with *E. isourolithinifaciens* (B), with *G. urolithinifaciens* (C), and the co-culture of both strains (D). (1) Caffeic acid; (2) *m*-Coumaric acid.

spectrum of the metabolite (UV max nm 278, 316 sh) showed clearly that it was very different from that of *p*-coumaric acid (UV max nm 290 sh, 310), and the metabolite was confirmed to be *m*-coumaric acid by chromatographic comparison with an authentic standard. Besides, 3-(3'-hydroxy-phenyl) propa-



noic acid (3HPP) was also detected after the incubation with both species. However, dihydrocaffeic [3-(3',4'-dihydroxyphenyl) propanoic acid] was not detected, suggesting that the reductase activity was particularly active on *m*-coumaric acid as a substrate and not on caffeic acid.

Dihydrocaffeic acid [3-(3,4-dihydroxy-phenyl) propanoic acid] eluted at 9.2 min. Both bacterial species were very active to metabolize dihydrocaffeic (Table 2) yielding a metabolite eluting at 12.99 min, with a UV spectrum with maxima at 272, 276 sh nm, and a mass with $[M - H]^-$ at m/z 165 consistent with a loss of one hydroxyl from dihydrocaffeic $[M - H]^-$ (m/z 181). The chromatographic comparison with 3-hydroxy phenyl propanoic acid (UV 272, 276 sh nm) and 4-hydroxy phenyl propanoic acid (UV max 276 nm), and its UV spectrum clearly showed that the metabolite produced was the 3-hydroxy derivative (Fig. 4).

Rosmarinic acid, a dimeric molecule, including a caffeic acid residue and a dihydrocaffeic acid residue, was not metabolized by these bacterial species to produce dehydroxylated derivatives. The results show that although both species were able to dehydroxylate both caffeic and dihydrocaffeic acids, they were not able to dehydroxylate the dimer, suggesting that the rosmarinic acid molecule was probably too large to interact

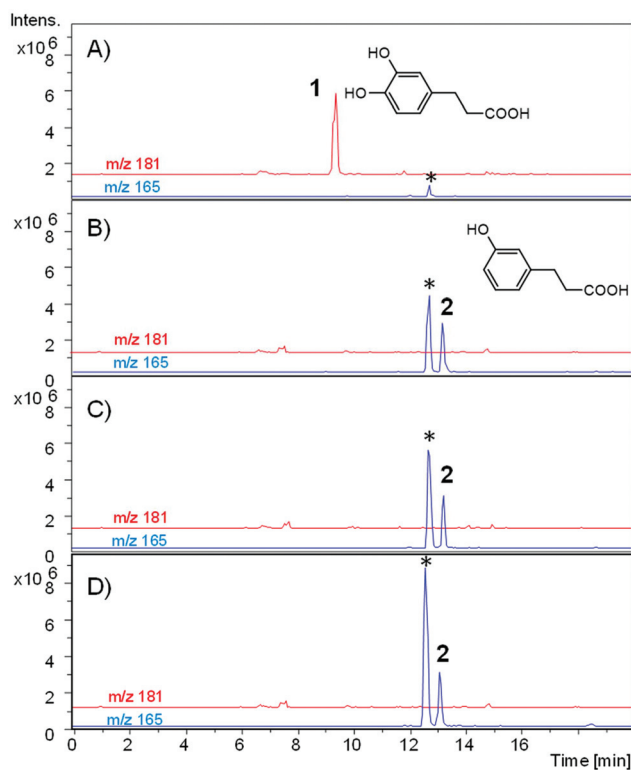


Fig. 4 HPLC-DAD-MS EIC chromatograms of the incubations of dihydrocaffeic acid in the medium without bacterial strains (A), with *E. isourolithinifaciens* (B), with *G. urolithinifaciens* (C), and the co-culture of both strains (D). Extracted ion chromatograms at m/z^- 165 [3-(3-hydroxyphenyl) propionic acid] and m/z^- 181 (dihydrocaffeic acid). * Unidentified metabolite present in all the samples.

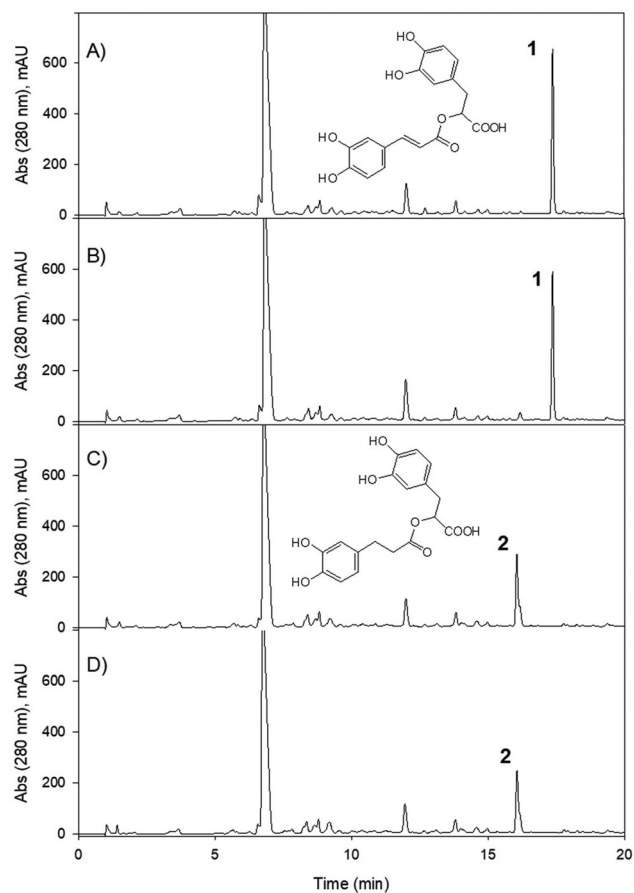


Fig. 5 HPLC-DAD chromatograms (280 nm) of the incubations of rosmarinic acid without cells (control) (A), with *Ellagibacter isourolithinifaciens* (B), with *Gordonibacter urolithinifaciens* (C) and with the co-culture of both strains (D). (1) rosmarinic acid; (2) dihydro-rosmarinic acid.

with the active center of the catechol-dehydroxylase (Fig. 5), or to be transported inside the cell for metabolism.

Rosmarinic acid (R_t 17.39 min) was only slightly metabolized by *E. isourolithinifaciens* (8% conversion, Table 2). However, it was completely converted (100%) by *G. urolithinifaciens* into a new metabolite at 16.06 min with a UV maximum at 280 nm and an $[M - H]^-$ at m/z 361 suggesting that the hydroxycinnamic residue was converted to a hydroxy-phenyl propanoic acid derivative by a reduction of the double bond (Fig. 5). This agrees with the changes observed in the UV spectrum of rosmarinic acid (328, 298sh nm) to yield a metabolite with UV spectrum with a maximum at 280 nm, and in the addition of two mass units (the two H added, m/z^- 361) to the mass of rosmarinic acid (m/z^- 359). The hydrogenation activity (reductase) is new for *Gordonibacter* and deserves further study. It seems that *G. urolithinifaciens* has a prominent reductase activity, although for *E. isourolithinifaciens* it does not seem to be a relevant metabolic transformation.

The metabolism of **chlorogenic acid** (5-caffeoyl-quinic acid) [R_t 9.5 min, $[M - H]^-$ at m/z 353, and MS-MS fragment at 191



(quinic acid)] was more complex. Its incubation as a control under the conditions used for conversion, but without the bacterial inoculum, showed that chlorogenic acid was partially isomerized into two different isomers. The isomers showed the same UV spectrum as chlorogenic acid, the same $[M - H]^-$ with m/z 353, and similar, although not identical, MS-MS fragments (ESI Fig. 1†). Thus, an isomer at 7.6 min showed MS/MS fragments at 191 (quinic acid), 179 (caffeic acid), and 135 (caffeic-COO). The isomer at 10.0 min showed fragments at 173 (quinic acid $-H_2O$) and 135, and other minor fragments at 191 and 179 (Fig. 6). A similar conversion was reported in a previous study with chlorogenic acid incubation with complex gut microbiota communities from different volunteers,¹⁹ and was also reported to occur after incubation at different pH values.²⁰ The Rts of the isomers suggested that they could be 3-caffeoyl quinic and 4-caffeoyl quinic isomers of 5-caffeoyl quinic acid (chlorogenic acid). These metabolites were identi-

fied by comparison with a green coffee bean extract,²¹ using this extract as a surrogate standard.²²

After incubation with *E. isourolithinifaciens*, chlorogenic acid and the isomers were substantially metabolized (65%, Table 2). A new metabolite appeared at 16.4 min, with the same UV spectrum of chlorogenic and caffeic acids and a $[M - H]^-$ ion at m/z 193. The UPLC-Q-TOF analysis showed a high-resolution mass of 193.0512 and a molecular formula of $C_{10}H_{10}O_4$. The mass, UV, and retention time suggest that the metabolite could be ferulic acid, isoferulic acid, or methyl caffeate. This conversion was consistently observed in three different incubation experiments. Chromatographic comparisons with authentic standards showed that this was methyl caffeate. This metabolite was only produced in the incubation with *E. isourolithinifaciens*, and it was not detected in its co-culture with *G. urolithinifaciens*, when chlorogenic acid was only incubated with *G. urolithinifaciens*, and in the control. An explanation for the formation of this metabolite is that *E. isourolithinifaciens* could have the ability to break the carbon-carbon bonds in the quinic acid residue, leaving finally a methyl fragment linked to the acid group of the caffeic acid. In addition, 3-(3-hydroxy-phenyl) propanoic acid (Rt 12.76 min) was also produced and confirmed that *E. isourolithinifaciens* can dehydroxylate catechol-containing groups and reduce the double bonds.

The incubation with *G. urolithinifaciens* showed the full conversion of chlorogenic acid and their isomers produced during the incubation (Table 2). It also showed the production of 3-(3-hydroxy-phenyl) propanoic acid (Rt 12.98 min). Three new peaks (Rts 7.4, 9.1, and 9.5 min, with $[M - H]^-$ at m/z 355) suggested the reduction of the double bond of the caffeic acid residue of the three caffeoyl-quinic acid isomers (Rts 7.6, 9.5 and 10.0 min) (Fig. 7). This was also consistent with the slight

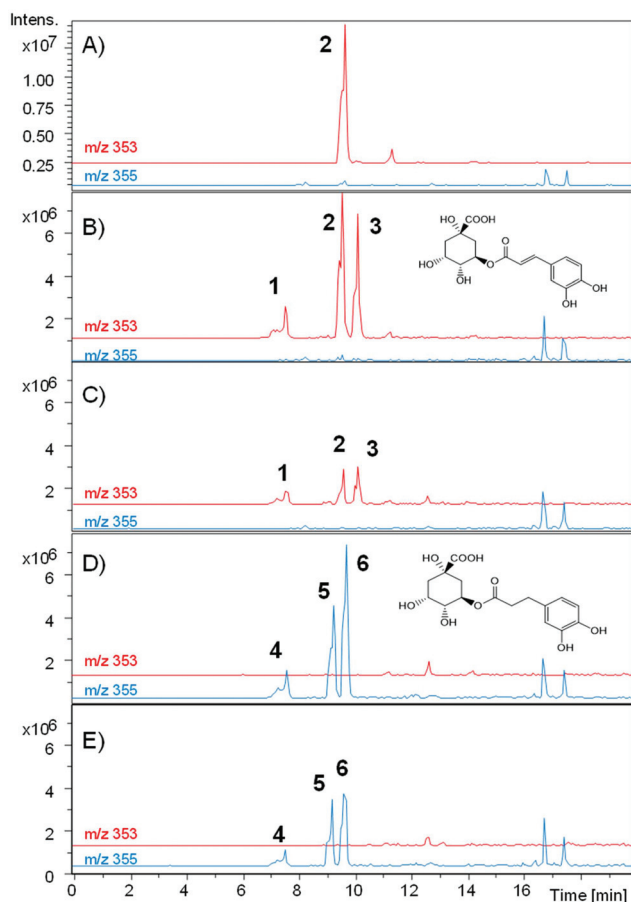


Fig. 6 HPLC-DAD-MS EIC chromatograms of the incubations of chlorogenic acid in the medium without bacterial strains at time 0 (A), in the medium without bacterial strains after 7 days (B), with *E. isourolithinifaciens* (C), with *G. urolithinifaciens* (D), and the co-culture of both strains (E). EICs at m/z 353 (caffeoyl quinic acids) and m/z 355 (dihydro-caffeoyl quinic acids). (1) 3-Caffeoyl-quinic acid; (2) 5-caffeoyl-quinic acid; (3) 4-caffeoyl-quinic acid; (4) 3-dihydrocaffeoyl-quinic acid; (5) 5-dihydrocaffeoyl-quinic acid; (6) 4-dihydrocaffeoyl-quinic acid.

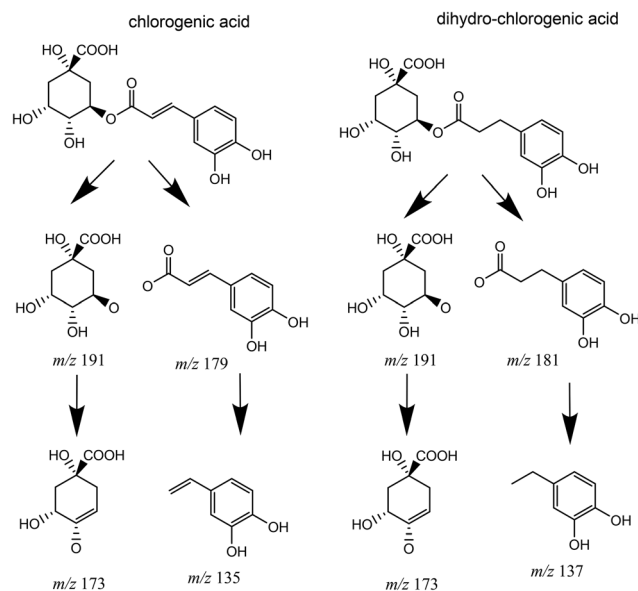


Fig. 7 MS-MS fragmentation of chlorogenic and dihydrochlorogenic acids.



decrease in *Rt* observed when comparing the chromatographic mobility of caffeic acid with that of dihydrocaffeic acid. The UV spectra of the new metabolites (UV max 280 nm) were also similar to that of dihydrocaffeic acid. The MS analysis showed the production of these metabolites only in the media incubated with *G. urolithinifaciens* (Fig. 6). The relative amount of the three metabolites also followed the same quantitative trend of the different isomers formed from chlorogenic acid in the incubation medium without bacterial inoculum (Fig. 7). The MS/MS fragments of the different dihydro chlorogenic acids [Rt 7.4, *m/z* 181 (dihydrocaffeic) and 137 (dihydrocaffeic-COO); Rt 9.1, *m/z* 181 (low), 173 (quinic acid-H₂O); Rt 9.5, 191 (quinic acid), 173 (quinic acid-H₂O)] were also consistent with those observed for their precursors (see above) (Fig. 6).

Discussion

The present study confirms the ability of *E. isourolithinifaciens* and *G. urolithinifaciens* to convert ellagic acid into isourolithin A (3,9-dihydroxy urolithin), and urolithin C (3,8,9-trihydroxy urolithin), respectively, through successive lactonase and decarboxylase activities, and sequential specific dehydroxylations that remove one hydroxyl group of different catechol moieties.

Remarkably, these human gut bacterial species can also catabolize other dietary-relevant phenolic compounds. Thus, *E. isourolithinifaciens* was able to dehydroxylate some catechol-containing phenolics, such as caffeic and dihydrocaffeic acids, preferentially by removing the hydroxyl group at the *para* position of the aromatic ring. The dehydroxylation, however, was not observed in related catechol-containing phenolics, such as rosmarinic acid and chlorogenic acid, showing that the size of the molecule can affect the interaction with the enzyme. This could be due to a limited uptake of the substrate by the bacterial strain and/or by a decreased catalytic efficiency of the enzyme towards the substrates. No dehydroxylation was observed either in other catechol-containing phenolics as quercetin, catechin, oleuropein, and esculetin or in pyrogallol-containing molecules like gallic acid (Fig. 8).

The regioselective removal of the hydroxyl in the *p*-position of the side chain of the catechol agrees with the metabolic conversions observed after the complex gut microbial communities' metabolism of catechol-phenylpropanoids produced after the breakdown of most flavonoids,¹⁸ and the catechol derivatives produced during the conversion of lignans,¹⁷ suggesting that both bacterial strains can be among those gut microbes responsible for this important conversion. A catechol-dehydroxylase for the dehydroxylation of dihydrocaffeic acid (*hcdh*) has recently been characterized from *Eggerthella lenta*.¹⁶

The results showed that *E. isourolithinifaciens* was also able to convert chlorogenic acid into methyl caffeate, which involved the ring-breakdown of the quinic acid cycle and the removal of carbon-carbon bonds. Something similar was already reported when chlorogenic acid was incubated with

human gut microbiota of different volunteers as caffeoyl-glycerol was detected after the fermentation, and suggested that the quinic acid part of the molecule can be partially degraded by the colonic bacterial communities.¹⁹ This activity deserves to be studied in future work.

G. urolithinifaciens, although had a catechol-dehydroxylase activity, also had a double-bond reductase activity as it was demonstrated for rosmarinic, chlorogenic, and caffeic acids. However, the double bond reduction was not observed on other phenolics that could potentially be reduced, like resveratrol and the coumarins esculetin, scoparone, and umbelliferone. It seems that this reduction is particularly active on larger molecules. The double-bond reductase activity had not been reported before in this bacterial genus.

Double bond reduction by some gut bacterial species had been reported for relevant biological transformations. Thus, *Eggerthella lenta*, also a member of the family Eggerthellaceae has been reported to be responsible for converting urocanate to imidazole propionate through the enzyme urocanate reductase, which reduced a double bond. This is a very relevant conversion as the production of imidazole propionate was associated with the development of type 2 diabetes.²³ *E. lenta* was also more abundant in subjects with type 2 diabetes. The same enzymatic activity was also observed in *Adlercreutzia equolifaciens*, another member of the Eggerthellaceae.²² *E. lenta* was also responsible for the reduction of a double bond in digoxin, leading to its inactivation.^{24,25} In a similar way, *Slackia equolifaciens*, and *A. equolifaciens* (with lower activity than *Slackia*), both members of the same family, were reported to be responsible for the conversion of the stilbene resveratrol to dihydro resveratrol.²⁶ Therefore, the double bond reductase activity observed in *G. urolithinifaciens* is relevant and could be a common feature of the Eggerthellaceae family. However, this activity could have a narrow substrate scope since no reduction of the double bond of resveratrol by *G. urolithinifaciens* or *E. isourolithinifaciens* was found in the present study.

The dehydroxylation of catechol-type PPs is a very unusual metabolic feature only observed in anaerobic bacteria occurring in the gastrointestinal tract.¹⁶ The enzyme responsible for the dehydroxylation that converts dopamine into *m*-tyramine, a molybdenum-dependent dehydroxylase (*Dadh*), has recently been described in *E. lenta*.¹⁵ This conversion, however, was not done by *G. pamelaeeae*, which as well as *G. urolithinifaciens* and *E. isourolithinifaciens*, is known to remove hydroxyl groups and to replace them with a hydrogen atom during the catabolism of ellagic acid into urolithins.⁹ However, *Gordonibacter* species can also dehydroxylate the lignan intermediate bi-desmethyl-secoisolariciresinol to enable the synthesis of the active metabolite enterodiol,¹⁷ and *E. lenta* is known to open the tetrahydrofuran rings in pinosresinol and lariciresinol leading to secoisolariciresinol.²⁷⁻²⁹

Our results showed that non-catechol phenolics (such as resveratrol, daidzein, isoxanthohumol, and umbelliferone) were not substrates for the catechol-dehydroxylase activity of both strains. However, the dehydroxylation of monohydroxy



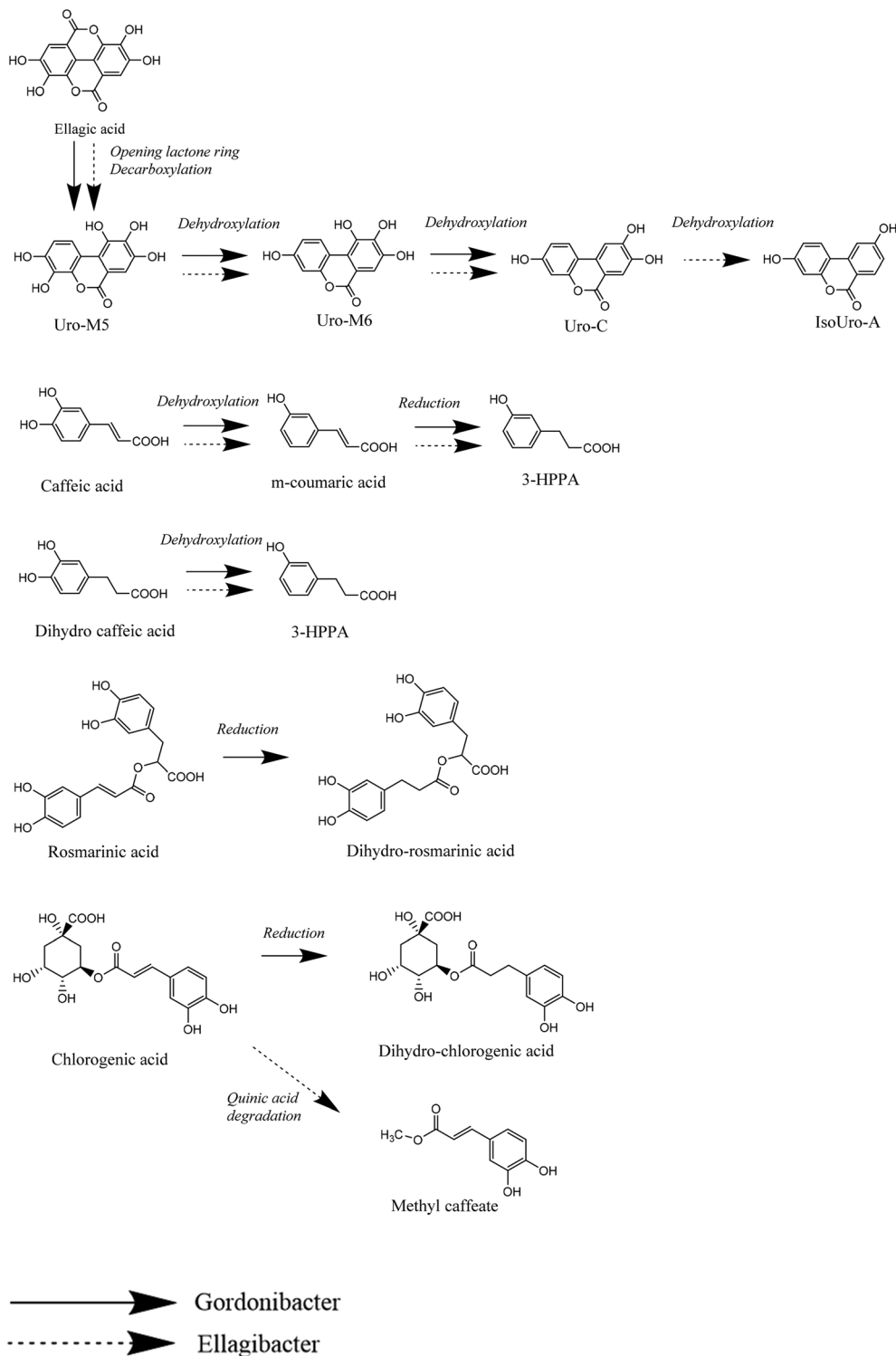


Fig. 8 Main catabolic reactions of *E. isourolithinifaciens* and *G. urolithinifaciens*.

phenolics is catalyzed by the xanthine-oxidase related *p*-hydroxy-benzoyl-CoA reductase, from anaerobic soil bacteria.³⁰ To the best of our knowledge, this activity has not been described in phenolic-metabolizing gut microbial strains so far.

Another relevant enzymatic activity of both bacterial species is the lactone-ring opening and decarboxylation activity that are the first steps in ellagic acid metabolism. This activity was not observed on other phenolics bearing a lactone ring as the coumarins that were not metabolized. Recent studies have



demonstrated that this decarboxylase activity to convert ellagic acid into urolithin M5 (3,4,8,9,10-pentahydroxy urolithin) can be completed by cell-free extracts, while the further dehydroxylations to produce urolithin C (3,8,9-trihydroxy urolithin) need the bacterial cells.^{16,31} The study of this catabolic activity also deserves further research.

Conclusions

In addition to ellagic acid, the urolithin-producing bacterial species *G. urolithinifaciens* and *E. isourolithinifaciens* can catabolize other dietary phenolics through the removal of hydroxyl groups from catechol moieties, and also by reducing double bonds. These activities, however, are not promiscuous and are specific in the metabolism of some phenolic compounds. Therefore, these bacterial species can be involved in some relevant steps of the metabolism of dietary phenolics when they are present in the gut bacterial ecology of individuals.

The abundance of several genera of the Eggerthellaceae family previously reported to be involved in polyphenol metabolism differ widely between individuals.¹⁸ Among them, *Gordonibacter* and *Ellagibacter* abundances in the human gut vary from 0 to 0.9% with average values of 0.03% and 0.08%, respectively.¹⁴

There are large inter-individual differences in the occurrence of both bacterial genera (*Gordonibacter* and *Ellagibacter*), and they are differentially distributed in the so-called 'urolithin metabolotypes'. Therefore, they could also contribute to the different responses of individuals to (poly)phenols consumption in randomized controlled trials.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This work has been supported by the Projects 19900/GERM/15 (Fundación Séneca, Spain), CSIC 201870E014, and PID2019-103914RB-I00 and AGL2015-73107-EXP (MINECO, Spain).

References

- 1 C. Manach, G. Williamson, C. Morand, A. Scalbert and C. Remesy, Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies, *Am. J. Clin. Nutr.*, 2005, **80**, 230S–242S.
- 2 A. Cortés-Martín, M. V. Selma, F. A. Tomás-Barberán, A. González-Sarrías and J. C. Espín, Where to look into the puzzle of polyphenols and health? The postbiotics and the gut microbiota associated with human metabolotypes, *Mol. Nutr. Food Res.*, 2020, e1900952.
- 3 K. Tslugiri and M. Rescigno, Postbiotics: What else?, *Benefic. Microbes*, 2013, **4**, 101–107.
- 4 J. N. Malagón-Rojas, A. Mantziari, S. Salminen and H. Szajewska, Postbiotics for preventing and treating common infectious diseases in children: A systematic review, *Nutrients*, 2020, **12**, 389.
- 5 A. González-Sarrías, J. C. Espín and F. A. Tomás-Barberán, Non-extractable polyphenols produce gut microbiota metabolites that persist in circulation and show anti-inflammatory and free radical-scavenging effects, *Trends Food Sci. Technol.*, 2017, **69**, 281–288.
- 6 F. A. Tomás-Barberán, A. González-Sarrías, R. García-Villalba, M. A. Núñez-Sánchez, M. V. Selma, M. T. García-Conesa and J. C. Espín, Urolithins, the rescue of 'old' metabolites to understand a 'new' concept; metabolotypes as a nexus between phenolic metabolism, microbiota dysbiosis and host health status, *Mol. Nutr. Food Res.*, 2017, **61**, 1500901.
- 7 R. García-Villalba, M. V. Selma, J. C. Espín and F. A. Tomás-Barberán, Identification of novel urolithin metabolites in human feces and urine after the intake of a pomegranate extract, *J. Agric. Food Chem.*, 2019, **67**, 11099–11107.
- 8 F. A. Tomás-Barberán, R. García-Villalba, A. González-Sarrías, M. V. Selma and J. C. Espín, Ellagic acid metabolism by human gut microbiota: Consistent observation of three urolithin phenotypes in intervention trials, independent of food source, age, and health status, *J. Agric. Food Chem.*, 2014, **62**, 6535–6538.
- 9 M. V. Selma, D. Beltrán, R. García-Villalba, J. C. Espín and F. A. Tomás-Barberán, Description of urolithin production capacity from ellagic acid of two human intestinal *Gordonibacter* species, *Food Funct.*, 2014, **5**, 1779–1784.
- 10 M. V. Selma, F. A. Tomás-Barberán, D. Beltrán, R. García-Villalba and J. C. Espín, *Gordonibacter urolithinifaciens* sp. nov., a urolithin-producing bacterium isolated from the human gut, *Int. J. Syst. Evol. Microbiol.*, 2014, **64**, 2346–2352.
- 11 M. V. Selma, M. Romo-Vaquero, R. García-Villalba, A. González-Sarrías, F. A. Tomás-Barberán and J. C. Espín, The human gut microbial ecology associated to overweight and obesity determines ellagic acid metabolism, *Food Funct.*, 2016, **7**, 1769–1774.
- 12 M. V. Selma, D. Beltrán, M. C. Luna, M. Romo-Vaquero, R. García-Villalba, A. Mira, J. C. Espín and F. A. Tomás-Barberán, Isolation of human intestinal bacteria capable of producing the bioactive metabolite isourolithin A from ellagic acid, *Front. Microbiol.*, 2017, **8**, 1521.
- 13 D. Beltrán, M. Romo-Vaquero, J. C. Espín, F. A. Tomás-Barberán and M. V. Selma, *Ellagibacter isourolithinifaciens* gen. Nov., sp. Nov. a new member of the family Eggerthellaceae, isolated from human gut, *Int. J. Syst. Evol. Microbiol.*, 2018, **68**, 1707–1712.
- 14 M. Romo-Vaquero, A. Cortés-Martín, V. Loria-Kohen, A. Ramirez-de-Molina, I. García-Mastrana, M. C. Collado, J. C. Espín and M. V. Selma, Deciphering the human gut microbiome of urolithin metabolotypes: Associations with



- enterotypes and potential cardiometabolic health implications, *Mol. Nutr. Food Res.*, 2019, **63**, 1800958.
- 15 V. M. Rekdal, E. N. Bess, J. E. Bisanz, P. J. Turnbaugh and E. P. Balskus, Discovery and inhibition of an interspecies gut bacterial pathway for levodopa metabolism, *Science*, 2019, **364**, 1055.
 - 16 V. M. Rekdal, P. N. Bernardino, M. U. Luescher, S. Kiamehr, C. Le, J. E. Bisanz, P. J. Turnbaugh, E. N. Bess and E. P. Balskus, A widely distributed metalloenzyme class enables gut microbial metabolism of host- and diet-derived catechols, *eLife*, 2020, **9**, e50845, DOI: 10.7554/eLife.50845.
 - 17 E. N. Bess, J. E. Bisanz, F. Yarza, A. Bustion, B. E. Rich, X. Li, S. Kitamura, E. Kaligurski, Q. Y. Ang, D. L. Alba, P. Spagiannopoulos, S. Nayfach, S. K. Koliwad, D. W. Wolan, A. A. Franke and P. J. Turnbaugh, Genetics basis for the cooperative bioactivation of plant lignans by *Eggethella lenta* and other human gut bacteria, *Nat. Microbiol.*, 2020, **5**, 56–66.
 - 18 M. V. Selma, J. C. Espín and F. A. Tomás-Barberán, Interaction between phenolics and gut microbiota: Role in human health, *J. Agric. Food Chem.*, 2009, **57**, 6485–6501.
 - 19 F. A. Tomás-Barberán, R. Garcia-Villalba, A. Quartieri, S. Raimondi, A. Amaretti, A. Leonardi and M. Rossi, *In vitro* transformations of chlorogenic acid by human gut microbiota, *Mol. Nutr. Food Res.*, 2014, **58**, 1122–1131.
 - 20 S. Deshpande, R. Jaiswal, M. F. Matei and N. Kuhnert, Investigation of acyl migration in mono- and di-caffeoyl quinic acids under aqueous basic, aqueous acid, and dry roasting conditions, *J. Agric. Food Chem.*, 2014, **62**, 9160–9170.
 - 21 F. A. Tomás-Barberán, J. Loaiza-Velarde, A. Bonfanti and M. E. Saltveit, Early wound- and ethylene-induced changes in phenylpropanoid metabolism in harvested lettuce, *J. Am. Soc. Hortic. Sci.*, 1997, **122**, 399–404.
 - 22 M. N. Clifford and N. E. Madala, Surrogate standards: A cost-effective strategy for identification of phytochemicals, *J. Agric. Food Chem.*, 2017, **65**, 3589–3590.
 - 23 A. Koh, A. Molinaro, A. Stalman, M. T. Khan, C. Schmidt, L. Manneras-Holm, H. Wu, A. Carreras, H. Jeong, L. E. Olofsson, P. O. Bergh, V. Gerdes, A. Hartstra, M. de Brauw, R. Perkins, M. Nieuwdorp, G. Bergstrom and F. Backhed, Microbially produced imidazole propionate impairs insulin signalling through mTORC1, *Cell*, 2018, **175**, 947–961.
 - 24 J. R. Saha, V. P. Butler, M. C. Neu and J. Linderbaum, Digoxin-inactivating bacteria: identification in human gut flora, *Science*, 1983, **220**, 325–327.
 - 25 P. Spagianopoulos, E. N. Bess, R. N. Carmody and P. J. Turnbaugh, The microbial pharmacist within us: a metagenomics view of xenobiotic metabolism, *Nat. Rev. Microbiol.*, 2016, **14**, 273–287.
 - 26 L. M. Bode, D. Bunzel, M. Huch, G. S. Cho, D. Ruhland, M. Bunzel, A. Bub, C. M. Franz and S. E. Kulling, *In vivo* and *in vitro* metabolism of *trans*-resveratrol by human gut microbiota, *Am. J. Clin. Nutr.*, 2013, **97**, 295–309.
 - 27 K. Struijs, J. P. Vincken and H. Gruppen, Bacterial conversion of secoisolariciresinol and anhydrosecoisolariciresinol, *J. Appl. Microbiol.*, 2009, **107**, 308–317.
 - 28 T. Clavel, G. Henderson, W. Engst, J. Doré and M. Blaut, Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside, *FEMS Microbiol. Ecol.*, 2006, **55**, 471–478.
 - 29 J. S. Jin, Y. F. Zhao, N. Nakamura, T. Aka, N. Kakiuchi, B. S. Min and M. Hattori, Enantioselective degradation of enterodiol and enterolactone precursors by human intestinal bacteria, *Biol. Pharm. Bull.*, 2007, **30**, 2113–2119.
 - 30 M. Unciuleac, E. Warkentin, C. C. Page, M. Ball and U. Ermler, Structure of a xanthine oxidase-mediated 4-hydroxybenzoyl-CoA reductase with an additional [4Fe-4S] cluster and an inverted electron flow, *Structure*, 2004, **12**, 2249–2256.
 - 31 H. Watanabe, S. Kishino, M. Kudoh, H. Yamamoto and J. Ogawa, Evaluation of electron-transferring cofactor mediating enzyme systems involved in urolithin dehydroxylation in *Gordonibacter urolithinfaciens* DSM 27213, *J. Biosci. Bioeng.*, 2020, **129**, 552–557.

