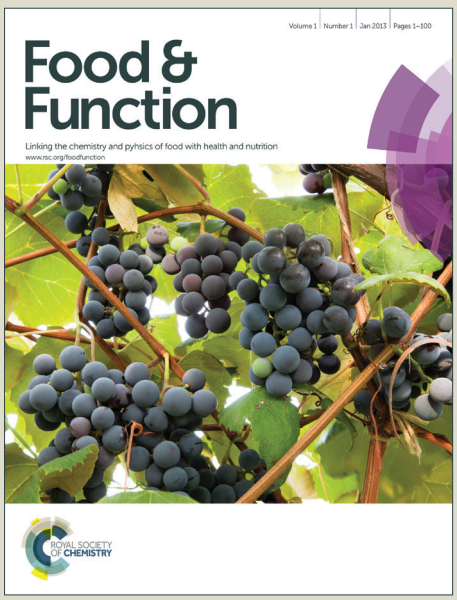
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Description of urolithin production capacity from ellagic acid of two human intestinal *Gordonibacter* species

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

Research Group on Quality, Safety and Bioactivity of Plant Foods, Department of Food Science and Technology, CEBAS-CSIC, 30100. Campus de Espinardo, Murcia, Spain.

Running title: Urolithin-producing bacteria.

*Corresponding author. E-mail address: mvselma@cebas.csic.es (M.V. Selma). Tel.: +34 968396102. Fax: +34 968396213.

Keywords: bioconversion, ellagitannin, *Gordonibacter*, gut bacteria, polyphenols.

Abstract

Ellagitannin and ellagic acid metabolism to urolithins in the gut shows a large human interindividual variability and this has been associated with differences in the colon microbiota. In the present study we describe the isolation of one urolithin-producing strain from human faeces of a healthy volunteer and the ellagic acid transformation to different urolithin metabolites by two species of intestinal bacteria. The isolate belongs to a new species described as *Gordonibacter urolithinfaciens*, sp. nov.. The type strain of *Gordonibacter* genus, *Gordonibacter pamelaeae* DSM 19378^T was also demonstrated to produce urolithins. Both human intestinal bacteria grew similarly in presence and absence of ellagic acid at 30 μM concentration. Ellagic acid catabolism and urolithin formation occurred during the stationary phase of the growth of the bacteria under anaerobic conditions. The HPLC-MS analyses showed the sequential production of pentahydroxy-urolithin (urolithin M-5), tetrahydroxy-urolithin (urolithin M-6) and trihydroxy-urolithin (urolithin C) while dihydroxy-urolithins (urolithin A and isourolithin A), and monohydroxy-urolithin (urolithin B), were not produced in pure cultures. Consequently, either other bacteria from the gut or the physiological conditions found *in vivo* are necessary for completing metabolism until the final urolithins (dihydroxy and monohydroxy urolithins). This is the first time that urolithin production capacity of pure strains has been demonstrated. The identification of the urolithin-producing bacteria is a relevant outcome as urolithin implication in health (cardiovascular protection, anti-inflammatory and anticarcinogenic properties) has been supported by different bioassays and urolithins can be used in the development of functional foods and nutraceuticals. The study represents an initial work that opens interesting possibilities of describing enzymatic activities involved in urolithin production that can help understanding both the human interindividual differences in

polyphenol metabolism, the microbial pathways involved, and the role of polyphenols in human health. The presence of urolithin producing bacteria can indirectly affect the health benefits of ellagitannin consumption.

1 Introduction

Dietary polyphenols have been suggested to be responsible for some health benefits associated with the consumption of fruit, vegetables and plant-derived foods.[1] Their bioavailability is however, rather limited and it is now well established that most of these compounds reach the colon where they are metabolized by the gut microbiota to produce bioactive molecules that are more readily absorbed than the original polyphenols.[2] The characterization of the metabolites produced from polyphenols by gut microbiota is a current trend in polyphenol research and in human nutrition due to the implication in health as a result of their antioxidant, anti-inflammatory, antiestrogenic, cardioprotective, cancer chemopreventive and neuroprotective properties.[3] Examples of these are hop (beer) isoxanthohumol which is transformed to 8-prenylnaringenin, soy isoflavones that are transformed to equol, cereal lignans that are transformed to mammalian-lignans (enterolactone, enterodiol), resveratrol which is transformed to dihydroresveratrol and ellagitannins that are transformed to urolithins.[2]

The identification of the bacteria responsible for the polyphenol and other organic compound transformation is also a relevant objective due to the potential development of functional foods and ingredients with health benefits on individuals with low production of some of these bioactive metabolites.[3] Several new bacterial species that are able to produce equol from isoflavones have been identified and these include *Adlercreutzia equolifaciens*,[4] *Eggerthella* strain Julong 732,[5] *Paraeggerthella hongkongensis,*[6] *Slackia equolifaciens,*[7] and *Slackia isoflavoniconvertens,*[8] The

transformation of the flavanone isoxanthohumol to prenylnaringenin by *Eubacterium limosum* as well as the deglycosylation of flavones and isoflavones by *Eubacterium cellulosolvens* has been described.[9, 10] Among dietary non-flavonoid polyphenols, the lignane secoisolariciresinol can be transformed to phytoestrogenic metabolites such as enterodiol and enterolactone by *Eggerthella lenta* and *Peptostreptococcus productus.*[11] More recently, the transformations of resveratrol into dihydroresveratrol by *S. equolifaciens* and *A. equolifaciens* as well as the hidrolisis of chlorogenic acid by *Bifidobacterium animalis* subsp. *lactis* have also been reported.[12, 13] Alternatively, human gut bacteria such as *Enterococcus casseliflavus* CP1 and *Escherichia coli* VL8 can metabolize glucosinolates to produce isothiocyanates as chemopreventive agents.[14] Apart from these examples, human gut bacteria involved in most dietary polyphenol transformations remain unknown. This is the "gap in knowledge" intended to be filled in part by the research.

Ellagitannins are present in berries of the genera *Rubus* (raspberry, blackberry, cloudberry, arctic bramble) and *Fragaria* (strawberry), and in pomegranates, tea, walnuts and some other nuts, oak-aged wines and muscadine grapes.[2, 15] Upon hydrolysis in the gut, ellagitannins undergo lactonization to produce ellagic acid (EA). It is generally accepted that the intestinal absorption of ellagitannins and EA is very low and that they reach the colon where they are catabolized into urolithins by the gut microbiota, these metabolites being much more absorbed.[16-18] Urolithins are bioavailable metabolites that can reach up to micromolar concentrations in the plasma of humans.[17] These metabolites exert anti-inflammatory[19-24] and cancer chemopreventive effects against colon cancer.[24-27] In addition, urolithins can also reach different target tissues as previously reported in both animal models (rats and pigs;[24, 28] and human prostate biopsies.[29] The main urolithin metabolite detected in human

prostate was urolithin A (range between 0.5 and 2 ng g^{-1} of tissue). In the case of animal models, up to 25 urolithin metabolites have been identified in the plasma, intestine, and bile from pigs.[28] In the rat, urolithins and their Phase-II conjugates can also reach organs including, the liver, prostate, brain, uterus, kidney, small intestine and colon.[24] The highest urolithin concentrations occur (in both pigs and rats) in the colon content (range from 6 to 168 μM of urolithin A, the most abundant urolithin) whereas the amount detected in the kidney and liver was much lower (0.12-0.20 μg g^{-1}, mainly urolithin A glucuronide). Urolithins undergo an active enterohepatic circulation and do not accumulate in organs.[28] In this context, chronic consumption of ellagitannin or EA containing foods can provide relevant concentrations of urolithins at both digestive (colon) and systemic levels. The poor bioavailability of ellagitannins or EA and the biological effects of urolithins in different *in vitro* assays suggest that urolithins can be the bioactive molecules *in vivo* and those responsible of the health effects observed after ellagitannins or EA intake.[30]

Urolithin production in the gut shows a large human interindividual variability and this has been associated with differences in the colon microbiota.[31] However, in scientific literature, nothing is found about the bacterial species responsible for urolithin production from ellagitannins, or other EA derived compounds. In the present study, we describe the metabolism of two species of human intestinal bacteria which can convert EA into different urolithins.

2 Materials and methods

2.1 Isolation of urolithin producing bacteria from human faeces

Faecal samples of a healthy woman (age 31) whose intestinal microbiota was capable of converting EA to urolithins were collected in stool cups in five moments of the year.

The study was conformed to ethical guidelines outlined in the Declaration of Helsinki and its amendments. The protocol (reference 03/2011) was approved by the Clinical Ethics Committee at Reina Sofia University Hospital (Murcia, Spain) and by the Spanish National Research Council's Bioethics Committee (Madrid, Spain). The volunteer provided written informed consent. Urolithins were analyzed and detected in urine, plasma and faeces of this volunteer after the intake of walnuts as described in one of our previous studies.[31] The faeces were processed for isolation of bacteria within 2 h. The samples were placed in filter bags, diluted 1/10 (w/v) in nutrient broth (NB, Oxoid, Basingstoke, Hampshire, UK) supplemented with 0.05% L-cysteine hydrochloride (Panreac química, Barcelona, Spain) and homogenized with a stomach homogenizer. The filtered suspension was diluted further in the same broth medium containing EA (Sigma-Aldrich, St. Louis, MO, USA) at 30 μM in order to determine the metabolic activity first. A portion of the culture, having metabolic activity, was seeded on differential reinforced clostridial medium (DRCM; Merck, Darmstadt, Germany) agar to improve the growth of anaerobic bacteria. Approximately 200 colonies were picked up, inoculated into 2 ml of a broth used to grow anaerobes (anaerobe basal broth, ABB; Oxoid) and after incubation; their conversion capacity of EA to urolithins was assayed. Urolithin-producing colonies were sub-cultured, until an urolithin-producing strains were isolated. The isolation procedure and incubation of plates was carried out under anaerobic 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 ºC.

2.2 Conversion testing of EA to urolithins

Colonies (200) isolated from human faeces described in the previous sectionwere used in *in vitro* experiments to investigate their capacity to grow and produce urolithins in

149 presence of EA. *Gordonibacter pamelaeae* DSM 19378^T obtained from DSMZ culture
150 collection, was reconstituted, maintained under the conditions specified in the DSMZ
151 online catalogue (http://www.dsmz.de) and its urolithin production capacity was also
152 tested because it is the only species belonging to the same genus that the urolithin-
153 producing isolates of the present study. Firstly, aliquots of the intestinal bacterial strains
154 were inoculated into 2 ml of ABB. After incubation, subcultures were inoculated into
155 fresh ABB containing EA (Sigma-Aldrich, St. Louis, MO, USA) at 30 μM. Duplicate
156 cultures were prepared in parallel from each bacterial strain suspension. In addition,
157 controls were used, some without bacteria and others without EA. The test culture was
158 incubated for 3 days at 37 ºC under anaerobic conditions at 37 ºC. Samples (5 ml) were
159 collected and prepared for LC analyses of urolithins as described below.
160

161 **2.3 Growth curve and time-course production of urolithins by *Gordonibacter***
162 ***urolithinfaciens* and *Gordonibacter pamelaeae***

163 The stocks of *G. pamelaeae* DSM 19378^T and the isolated strain able to produce
164 urolithins (*G. urolithinfaciens* strain CEBAS 1/15P), preserved frozen, were thawed and
165 incubated on ABB agar plate for 4 days. A single colony was then transferred to a 5 ml
166 ABB tube. One milliliter of diluted inoculum was transferred to ABB (50 ml) in 100 ml
167 flasks to give an initial concentration of about 100 cfu ml^{-1}. EA was added to the 50 ml
168 culture to achieve a final concentration of 9-10 μM. Flasks inoculated with *G.*
169 *pamelaeae* and *G. urolithinfaciens* were incubated for 8 days at 37 ºC in an anaerobic
170 conditions at 37 ºC. Samples (5 mL) were collected at appropriate time intervals during
171 8 day incubation at 37 ºC, and clean-up for LC analyses of urolithins as described
172 below. Similarly, 1 mL were collected every 2 h during 2 day incubation at 37 ºC.

Dilutions were made if necessary in NB and counts in ABB agar plates were performed. Triplicate growth curves were obtained.

2.4 LC-UV/Vis and LC-MS/MS analyses

Samples obtained in the fermentation experiments with colonies isolated from human faeces and with *G. pamelaeae* were extracted and analyzed by LC-UV/Vis and LC-MS as described previously.[31] Briefly, 5 ml of fermented medium was extracted with 5 ml of ethyl acetate (Labscan, Dublin, Ireland) acidified with 1.5% of formic acid (Panreac), vortexed for 2 minutes and centrifuged at 3500 *g* for 10 min. The organic phase was separated and evaporated and the dry samples were then re-dissolved in 250 µl of methanol (Romil, Barcelona, Spain). The analyses were performed using an HPLC system (1200 Series, Agilent Technologies, Madrid, Spain) equipped with a photodiode-array detector and a single quadrupole mass spectrometer detector in series (6120 Quadrupole, Agilent Technologies, Madrid, Spain) as described previously [28]. Calibration curves were obtained for EA (Sigma-Aldrich, St. Louis, MO, USA), urolithin A (chemically synthesized by Villapharma SL (Parque tecnológico de Fuente Álamo, Murcia, Spain) and urolithin C (Dalton Pharma Services (Toronto, Canada) with good linearity (R^2> 0.998). Urolithin A and C were quantified at 305 nm with their own standards; urolithins M-5 and M-6 with EA calibration curve at 360 nm and EA was quantified at 360 nm with its own standard.

2.5 Data modelling

Growth curves were fitted using the function of Baranyi et al.[32] 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.

3 Results

3.1 Identification of urolithin producing bacteria

One bacterial strain isolated from the faecal sample, named *G. urolithinfaciens* strain CEBAS 1/15P, obtained from a $1:10^6$ dilution plated on DRCM agar showed the capacity to convert EA to urolithins under anaerobic conditions. We have proposed the strain as a novel species belonging to the genus *Gordonibacter* based on several different physiological/biochemical, chemotaxonomic and phylogenetic characteristics from more closely related species.[33] The 16S rRNA gene sequence of *G. urolithinfaciens* has been deposited in the GenBank nucleotide sequence database under accession number HG000667. *G. urolithinfaciens* has been deposited in two public culture collections with the accession number (=DSM 27213^T =CCUG 64261^T). We also investigated the capacity of the type strain and only species of *Gordonibacter* genus (*G. pamelaeae* DSM 19378^T) to convert EA to urolithins under anaerobic conditions and also produced urolithins.

3.2 Analysis of urolithins produced by *Gordonibacter* species

The HPLC-MS analyses showed that pentahydroxy-urolithin (urolithin M-5), tetrahydroxy-urolithin (urolithin M-6) and urolithin C were produced from EA by both strains *G. urolithinfaciens* and *G. pamelaeae* cultured in separate (Fig. 1). 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 as reported previously.[31]

3.3 *In vitro* catabolism of EA by *Gordonibacter* species

G. urolithinfaciens and *G. pamelaeae* separately cultured grew similarly with and without EA at 30 μM, the lag phase being 4.87±0.81 h and 3.96±0.56 h while growth rate being 0.27±0.03 and 0.30±0.01 h^{-1}, in presence and absence of EA, respectively. EA catabolism and urolithin formation occurs during the stationary phase of the growth of the *G. urolithinfaciens* and *G. pamelaeae* species (Fig. 2A, 2B). A disappearance of EA from the medium was observed, at the same time that urolithins appeared. Urolithin M-5 was only observed in the sample obtained at day 3, suggesting that this is the first metabolite produced. Urolithin M-6 reached a maximum at day four, and then decreased steadily while urolithin C is produced reaching the complete transformation of EA into urolithin C at day seven (Fig. 2C, 2D). Longer incubation periods did not produce further hydroxyl removals from the urolithin nucleus.

4 Discussion

G. urolithinfaciens DSM 27213^T which is able to produce urolithins, was isolated from the human intestine of a healthy volunteer where it was found at high concentrations (≥ 10^7 cfu g^{-1} faeces). On the basis of 16S rRNA gene sequence identity, we tested the closest relatives of *G. urolithinfaciens* from the *Coriobacteriaceae* family including, *P. hongkongensis* HKU10^T (94.3%), *E. sinensis* HKU14^T (94.3 %) and *E. lenta* DSM 2243^T (93.0 %) and they were not able to produce urolithins [33]. In contrast, *G. pamelaeae* DSM 19378^T (97.0 % identity) was able to transform EA and the time-course production of urolithins is shown in the present study. *G. pamelaeae* DSM 19378^T was previously isolated by Würdemann et al.[34] from a patient with crohn's disease. However, strain ARC-1 whose 16S rRNA gene sequence (EF413639) has

100% identity with that of *G. pamelaeae* (AM886059) was isolated by Jin et al. from a healthy volunteer.[35] This suggests that *G. pamelaeae* could also be found in healthy human guts as occurs with *G. urolithinfaciens*.

Several studies have related species from the *Coriobacteriaceae* family with the transformation of different polyphenols to bioactive molecules such as equol, dihydroresveratrol, prenylnaringenin enterodiol and enterolactone,[4-12] but not with urolithin production. In the present study, the sequential production of urolithins M-5, M-6 and C by *G. urolithinfaciens* and *G. pamelaeae* was observed when the production of these metabolites was represented against time. Urolithin M-5 is transformed into urolithin M-6 by the removal of the hydroxyl at 9-position, which means that this dehydroxylase enzyme is more active than the one responsible for the opening of the lactone ring and sequential decarboxylation (Fig. 3). Urolithin C seems to be an end product for the catabolism of EA by *G. urolithinfaciens* and *G. pamelaeae* (Fig. 3). 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.[17, 31] Other metabolites such as urolithins M-5, M-6 and M-7 had been reported in rat faecal samples and other animal materials.[36-38] More recently, we reported the *in vitro* production of urolithins M-5, M-6 and M-7, E, C and A by human gut microbiota.[31] However, it is in the present study where two particular bacterial species from human gut microbiota are identified as producers of urolithins such as urolithins M-5, M-6 and C. Consequently, other bacteria from the gut are necessary for completing metabolism to urolithin A, urolithin B, and isourolithin A. Further studies should be carried out to find out if the absence of *Gordonibacter* species capable of transforming EA to urolithin C is the limiting factor in the production of urolithins *in vivo*.

No antimicrobial effect of EA at a concentration of 30 μM was observed during *in vitro* incubations of *G. urolithinfaciens and G. pamelaeae* as both bacteria grew similarly in presence and absence of this substrate. First urolithins were obtained at day three of incubation and seven days were necessary for the complete transformation of EA. This indicates urolithins are microbial secondary metabolites that do not play a role in growth because they are produced by *Gordonibacter* species during the stationary phase of growth. Other polyphenol transformations also occur in the stationary phase. This is the case of transformation of polyphenol lignans (dihydroxyenterodiol and dihydroxyenterolactone) to mammalian lignans (enterodiol and enterolactone) by strain ARC-1.[35] Equol production from daidzein by *Lactococcus garvieae* also occurs after the stationary phase of growth has been achieved.[39] The production of urolithins *in vivo* is also quite slow, and it is possible to detect urolithin metabolites in urine after the intake of ellagitannins for three or even more days.[17-19] This could be associated with the slow catabolism of ellagitannins and EA by the human gut microbiota, and by additional enterohepatic recirculation. Therefore, the results found in the present work suggest that the slow metabolism produced by the bacteria can be a relevant factor for the persistency of urolithins in urine after EA and ellagitannin intake.

Microbial secondary metabolites including antibiotics, antitumor agents, cholesterol-lowering drugs, and others are now increasingly being used against diseases previously treated only by synthetic drugs. Production of the metabolites directly by microbial fermentation is often more convenient than using synthetic chemistry. Present challenges in microbial pharmaceutical development are the discovery of novel secondary metabolites with significant biological activities.[40] The identification of bacteria responsible for the urolithin production is a relevant outcome due to the urolithin implication in health (cardiovascular protection, anti-inflammatory and

297 anticarcinogenic properties). Urolithin producing bacteria could potentially be applied
298 either as probiotics or in the industrial production of urolithins to developed food
299 ingredients, drinks, dietary complements, pharmaceuticals and/or functional foods
300 enriched in urolithins produced in a similar form as in the intestine (bacterial
301 metabolism from dietary polyphenols). However, further studies to demonstrate the
302 probiotical potential of these bacteria and also the health benefits to the host of the use
303 in functional foods should be carried out.

Acknowledgements

This work has been supported by the Projects CSD2007-00063 (Fun-C-Food; Consolider Ingenio 2010), Fundación Séneca de la Región de Murcia (grupo de excelencia GERM 06, 04486) and CICYT AGL2011-22447. R.G.V. holds a JAE-DOC grant from CSIC (Spain) co-financed by European Social Fund (ESF).

References

1 L. H. Yao, Y. M. Jiang, J. Shi, F. A. Tomás-Barberán, N. Datta, R. Singanusong and S. S. Chen, *Plant Foods Human Nutr.,* 2004, **59**,113-122.

2 M. V. Selma, J. C. Espín and F. A. Tomás-Barberán, *J. Agric. Food Chem*., 2009, **57**, 6485-6501.

3 F. A. Tomás-Barberán and C. Andrés-Lacueva, *J. Agric. Food Chem*., 2012, **60**, 8773-8775.

4 T. Maruo, M. Sakamoto, C. Ito, T. Toda and Y. Benno, *Int. J. Syst. Evol. Microbiol*., 2008, **58**, 1221–1227.

5 M. Kim, S. Kim, J. Han, X. Wang, D. Song and S. Kim, *Appl. Environ. Microbiol*., 2009, **75**, 3062–3068.

6 X. Wang, H. Hur, J. H. Lee, K. T. Kim and S. Kim, *Appl. Environ. Microbiol*., 2005, **71**, 214-219.

7 J. S. Jin, M. Kitahara, M. Sakamoto, M. Hattori and Y. Benno, *Int. J. Syst. Evol. Microbiol*., 2010, **60**, 1721-1724.

8 A. Matthies, M. Blaut and A. Braune, *Appl. Environ. Microbiol*., 2009, **75**, 1740–1744.

9 S. Possemiers, S. Rabot, J. C. Espín, A. Bruneau, C. Philippe, A. González-Sarrías, A. Heyerick, F. A. Tomás-Barberán, D. D. Keukeleire and W. Verstraete, *J. Nutr.,* 2008, **138**, 1310-1316.

10 A. Braune and M. Blaut, Intestinal bacterium *Appl. Environ. Microbiol*., 2012, **78**, 8151-8153.

11 T. Clavel, G. Henderson, C. A. Alpert, C. Philippe, L. Rigottier-Gois, J. Dore and M. Blaut, *Appl. Environ. Microbiol*., 2005, **71**, 6077–6085.

12 L. M. Bode, D. Bunzel, M. Huch, G. S. Cho, D. Ruhland, M. Bunzel, A. Bub, C. M. Franz and S. E. Kulling, *Am. J. Clin. Nutr.,* 2013, **97**, 295-309.

13 F. A. Tomás-Barberán, R. García-Villalba, A. Quartieri, S. Raimondi, A. Amaretti, A. Leonardi and M. Rossi, *Mol. Nutr. Food Res*., 2013, **00**, 1–10.

14 L. I. Vijitra, A. Narbad, C. Nueno-Palop, R. Mithen, M. Bennett and J. T. Rossiter, *Mol. Nutr. Food Res*., 2013, **00**, 1–9.

15 D. A. Marshall, S. J. Stringer and J. D. Spiers, *Pharmaceutical Crops*, 2012, *3*, 69-77.

16 B. Cerdá, R. Llorach, J. J. Cerón, J. C. Espín and F. A. Tomás-Barberán, *Eur. J. Nut*., 2003, **42**, 18-28.

17 B. Cerdá, J. C. Espín, S. Parra. P. Martínez and F. A. Tomás-Barberán, *Eur. J. Nut*., 2004, **43**, 205-220.

18 B. Cerdá, F. A. Tomás-Barberán, J. C. Espín, *J. Agric. Food Chem.,* 2005, **53**, 227-235.

19 M. Dell'Agli, G. V. Galli, M. Bulgari, M. Basilico, S. Romeo, D. Bhattacharya, D. Taramelli and E. Bosisio, *Malaria J*., 2010, **9**, 208.

20 J. A. Giménez-Bastida, A. González-Sarrías, M. Larrosa, F. A. Tomás-Barberán, J. C. Espín and M. T. García-Conesa, *Mol. Nut. Food Res.,* 2012, **56**, 784-796.

21 J. A. Giménez-Bastida, M. Larrosa, A. González-Sarrías, F. A. Tomás-Barberán, J. C. Espín and M.T. García-Conesa, *J. Agric. Food Chem*., 2012, **60**, 8866-8876.

22 A. González-Sarrías, M. Larrosa, F. A. Tomás-Barberán, P. Dolara and J. C. Espín, *Brit. J. Nutr.,* 2010, **104**, 503-512.

23 A. K. Kiss, S. Granica, M. Stolarczyk and M. F. Melzig, *Food Chem*., 2012, **131**, 1015-1020.

24 M. Larrosa, A. González-Sarrías, M. J. Yáñez-Gascón, M. V. Selma, M. Azorín-Ortuño, S. Toti, F. A. Tomás-Barberán, P. Dolara and J. C. Espín, *J. Nut. Biochem*., 2010, **21**, 717-725.

25 G. Cozza, A. Gianoncelli, P. Bonvini, E. Zorzi, R. Pasquale, A. Rosolen, L. A. Pinna, F. Meggio, G. Zagotto and S. Moro, *Chem. Med. Chem.* 2011, **6**, 2273–2286.

26 V. Furlanetto, G. Zagotto, R. Pasquale, S. Moro and B. Gatto, *J. Agric. Food Chem*., 2012, **60**, 9162−9170.

27 S. G. Kasimsetty, D. Bialonska, M. K. Reddy, C. Thornton, K. L. Willett and D. Ferreira, *J. Agric. Food Chem.* 2009, **57**, 10636–10644.

28 J. C. Espín, R. González-Barrio, B. Cerdá, C. Lopez-Bote, A. I. Rey and F. A. Tomás-Barberán, *J. Agric. Food Chem*., 2007, **55**, 10476-10485.

29 A. González-Sarrías, J. A. Giménez-Bastida, M. T. García-Conesa, M. B. Gomez-Sanchez, N. V. Garcia-Talavera, A. Gil-Izquierdo, C. Sanchez-Alvarez, L. Fontana-Compiano, J. P. Morga-Egea, F. A. Pastor-Quirante, F. Martinez-Diaz, F. A. Tomas-Barberan and J. C. Espín, *Mol. Nut. Food Res*., 2010, **54**, 311-322.

30 J. C. Espín, M. Larrosa, M. T. García-Conesa and F. A. Tomás-Barberán, *Evid. Based Complement. Alternat. Med*., 2013, **00**, 1-15.

31 R. García-Villalba, D. Beltrán, J. C. Espín, M. V. Selma and F. A. Tomás-Barberán, *J. Agric. Food Chem*., 2013, **61**, 8797–8806.

32 J. Baranyi, T. A. Roberts and P. McClure, 1993. *Food Microbiol*., **10**, 43–59.

33 M. V. Selma, F. A. Tomás-Barberán, D. Beltrán, R. García-Villalba, J. C. Espín. 2014. *Int. J. Syst. Evol. Microbiol.*, **64**, doi: 10.1099/ijs.0.055095-0.

34 D. Würdemann, B. J. Tindall, R. Pukall, H. Lünsdorf, C. Strömpl, T. Namuth, H. Nahrstedt, M. Wos-Oxley, S. Ott, S. Schreiber, K. N. Timmis and A. P. A. Oxley, 2009. *Int. J. Syst. Evol. Microbiol*., **59**, 1405–1415.

35 Jin J. S., Y. F. Zhao, N. Nakamura, T. Akao, N. Kakiuchi, B. S. Min and M. Hattori, 2007. *Biol. Pharm. Bull.*, **30**, 2113—2119.

36 R. González-Barrio, P. Truchado, H. Ito, J. C. Espín and Tomás-Barberán F. A. 2011. *J. Agric. Food Chem.,* **59**, 1152-1162.

37 R. González-Barrio, P. Truchado, R. García-Villalba, G. Hervás, P. Frutos, J. C. Espín, F. A. and Tomás-Barberán, 2012. *J. Agric. Food Chem.,* **60**, 3068-3077.

38 Ito, H., Iguchi, A., Hatano, T. 2008. *J. Agric. Food Chem.,* **56**, 393-400.

39 Uchiyama, S., Ueno, T. and Suzuki, T. 2007. *J. Intestinal Microbiol*., **21**, 217–220.

40 Wu, L. Z. and Hong, B., 2013. *Yao Xue Xue Bao*., **48**, 155-60.

392 **Fig. 1.** HPLC-DAD elution profile of *in vitro* metabolism of ellagic acid by
393 *Gordonibacter urolithinfaciens* and *Gordonibacter pamelaeae* under anaerobic
394 conditions. The insets show UV spectrum of ellagic acid and its metabolites. IS
395 (internal standard; 6,7-dihydroxycoumarin), 1: pentahydroxy-urolithin (urolithin M-5),
396 2: Ellagic acid. 3: tetrahydroxy-urolithin (urolithin M-6), 4: urolithin C. AU,
397 absorbance units.

398 **Fig. 2** Bacterial growth and time course ellagic acid (EA) conversion by
399 *Gordonibacter urolithinfaciens* (A, C) and *Gordonibacter pamelaeae* (B, D) to total
400 urolithins (UROs) and to urolithin C via pentahydroxy-urolithin (urolithin M-5) and
401 tetrahydroxy-urolithin (urolithin M-6).

402 **Fig. 3.** Proposed pathway for ellagitannins and ellagic acid metabolism by
403 *Gordonibacter urolithinfaciens* and *Gordonibacter pamelaeae* leading to urolithin
404 formation.
405

FIGURE 1

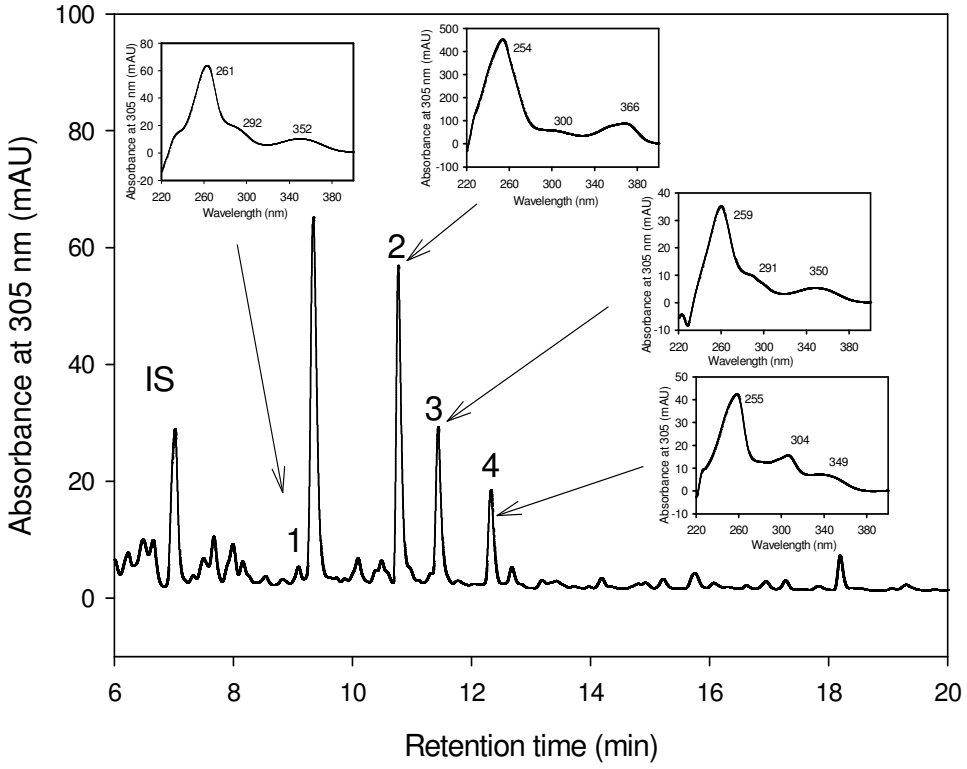


FIGURE 2

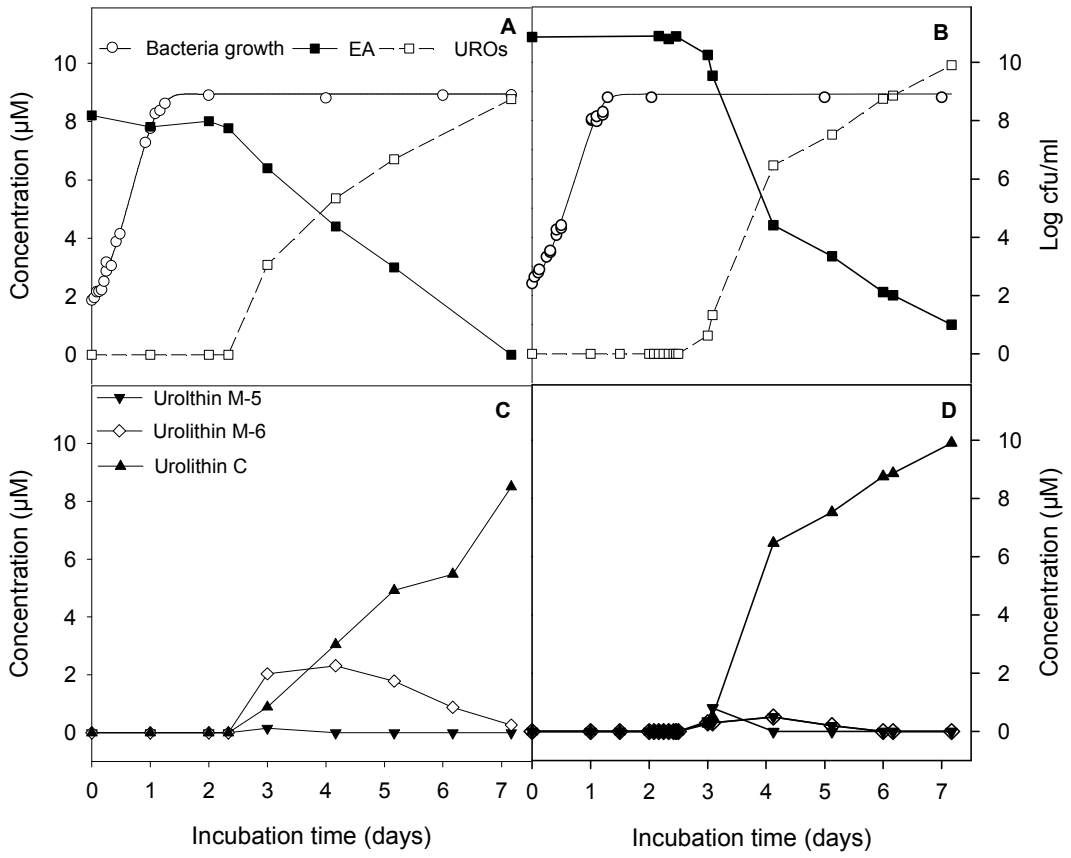


FIGURE 3