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Metabolism of Oak Leaf Ellagitannins and Urolithin Production in Beef Cattle

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Supporting Information

ABSTRACT: Oak leaves have a high concentration of ellagitannins. These phytochemicals can be beneficial or poisonous to animals. Beef cattle are often intoxicated by oak leaf consumption, particularly after suffering feed restriction. The severity of the poisoning has recently been associated with the ruminal microbiota, as different bacterial populations were found in animals that tolerated oak leaves and in those that showed clinical and pathological signs of toxicity. Intoxication has previously been linked to the production of phenolic metabolites, particularly catechol, phloroglucinol, and resorcinol. This suggested that the microbial metabolism of ellagitannins could also be associated with its tolerance or intoxication in different animals. Therefore, it is essential to understand the metabolism of ellagitannins in cattle. Here we show that ellagitannins are metabolized in the cattle rumen to urolithins. Different urolithins were detected in ruminal fluid, feces, urine, and plasma. Oak leaf ellagitannins declined as they were converted to urolithins, mainly isourolithin A and urolithin B, by the ruminal and fecal microbiota. Urolithin aglycons were observed in rumen and feces, and glucuronide and sulfate derivatives were detected in plasma and urine. Sulfate derivatives were the main metabolites detected in plasma, while glucuronide derivatives were the main ones in urine. The main urolithins produced in cattle were isourolithin A and urolithin B. This is a relevant difference from the monogastric mammals studied previously in which urolithin A was the main metabolite produced. Low molecular weight phenolics of the benzoic, phenylacetic, and phenylpropionic groups and metabolites such as catechol, resorcinol, and related compounds were also detected. There was a large variability in the kinetics of production of these metabolites in individual animals, although they produced similar metabolites in all cases. This large variability could be associated with the large variability in the rumen and intestine microbiota that has previously been observed. Further studies are needed to demonstrate if the efficiency in the metabolism of ellagitannins by the microbiota could explain the differences observed in susceptibility to intoxication by the different animals.

KEYWORDS: *urolithins, ellagitannins, metabolism, polyphenols, hydrolyzable tannins, oak leaves, cattle intoxication*

INTRODUCTION

Oak leaves contain substantial amounts of hydrolyzable tannins including both gallotannins and ellagitannins.[1] High concentrations of tannins in fodder plants inhibit gastrointestinal bacteria and reduce ruminant performance, mainly by reducing intake and nutrient digestibility.[2] However, tannins are considered to be able to exert both adverse and beneficial effects in ruminants, depending on the type and the amount consumed.[3,4] Ellagitannins and ellagic acid have received attention during the past few years due to their high in vitro antioxidant activity[5,6] and the biological properties associated with the consumption of ellagitannin-rich diets. Beneficial health effects, such as improved plasma lipid profile, reduction of oxidative stress, and induction of apoptosis in cancer cells, which are associated with a decrease in the risk of various chronic diseases including cardiovascular diseases and cancer, have been reported.[7−9] These effects have been suggested to be due to urolithins that are metabolites of ellagitannins produced by the gut microbiota that are absorbed and circulate in plasma conjugated as glucuronides and sulfates.[10−12]

Leaves from oak trees, shrubs, and saplings are often consumed by grazing ruminants when there is a shortage of other feed resources. In the hill areas of northern Spain, cases of intoxication of beef cattle occur recurrently in the spring when the animals consume large amounts of immature oak (*Quercus pyrenaica*) leaves. These leaves are rich in hydrolyzable tannins,[13] which may cause poisoning in animals if sufficiently large amounts are consumed.[14,15] However, most reports on tannin intoxications in the literature fail to state the type of tannin responsible, and only a few studies have investigated the toxicity of specific tannins. For example, administration of castalagin and vescalagin, ellagitannins from chestnut, to lambs did not provoke toxic effects.[16] Punicalagin, the main ellagitannin in pomegranate, has been reported to cause liver necrosis and nephrotoxicity in cattle when consuming *Terminalia oblongata*.[14] However, Cerdá et al.[17] did not observe any toxic effects in

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rats upon consumption of large amounts of pomegranate ellagitannins for 5 weeks.

The metabolism of ellagic acid and ellagitannins has been investigated both in animal models (rat and pig)[17,18] and humans.[19] These studies have reported extensive transformation of ellagic acid and ellagitannins to urolithins,[20] and these metabolites were the products of intestinal microbiota metabolism[11,21] that can reach systemic organs such as the human prostate.[12] In a previous study, cattle were fed oak leaves to evaluate the role of the rumen microbial community in the intoxication and to monitor the variations in some bacterial groups that were potentially able to resist or metabolize tannins. Rumen bacterial populations were studied throughout the trial (before, during, and after the oak leaf administration) and related to the different clinical and pathological responses of the animals to the oak leaf consumption.[22] Differences in the individual composition of the microbiota colonizing the rumen of intoxicated and tolerant animals were found,[23] particularly in *Selenomonas*, *Streptococcus*, and *Prevotella* species.

The toxicity observed in cattle after oak leaves consumption[14,15] has previously been associated with the microbial metabolism of ellagitannins in the rumen, which may result in the formation of absorbable low molecular weight metabolites such as gallic acid, catechol, resorcinol, pyrogallol, and phloroglucinol, which are considered to be potentially toxic to ruminants.[24,25] The present study was, therefore, undertaken to assess the metabolism of oak leaf ellagitannins in bulls using HPLC−MS and GC−MS analyses. This was used to understand ellagitannin metabolism in ruminants for the first time.

MATERIAL AND METHODS

Chemicals and Reagents. Methanol (MeOH), acetonitrile, formic acid, and hydrochloric acid were from Merck (Darmstadt, Germany). 3-Hydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, hydroferulic acid, and ferulic acid were from Fluka (Milan, Italy). Ellagic acid, resorcinol, pyrogallol, phloroglucinol, 3-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, hippuric acid, caffeic acid, phenylacetic acid, phenylpropionic acid, 3-hydroxyphenylpropionic acid, 4-hydroxyphenylpropionic acid, gallic acid, and β-glucuronidase (type H-2 crude solution from *Helix pomatia*) were from Sigma-Aldrich (St. Louis, MO). Urolithins A and B were chemically synthesized (Kylolab, Murcia, Spain). Milli-Q system (Millipore Corp.) ultrapure water was used throughout this experiment. For the extraction procedure, sodium acetate and ethyl acetate (Panreac, Barcelona, Spain) and hydrochloric acid (Scharlau, Barcelona, Spain) were used. *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS) (Supelco, Bellefonte PA) was used as derivatization reagent. These reagents were used from freshly opened 1 mL bottles.

Oak Leaves. Very young oak (*Q. pyrenaica*) leaves were manually collected from saplings during the spring on a country estate in the northwest of Spain (Sahechores, León; latitude 42°37′49″, longitude 5°09′25″), at a mean altitude of about 900 m, and frozen within 1 h at −30 °C until used. The tannin contents were 230 g of tannic acid equivalents/kg dry matter (DM) for total tannins and 18.8 and 11.0 g/kg DM for gallic and ellagic acid, respectively.[13] The characterization of the different ellagitannins, gallotannins, and flavonoids present in the oak leaves used for this study were analyzed by HPLC−DAD−ESI-MS−MS.

Animals and Diets. The experiment was performed in accordance with Spanish Royal Decree 1201/2005 for the protection of animals used for experimental and other scientific purposes. Three young Brown Swiss bulls (animals 1−3) (about 1.8 years old, 498 ± 22.2 kg live weight at the beginning of the experiment), each equipped with a ruminal cannula of 10-cm internal diameter (Ankom Technology Corp., Macedon, NY), were selected for this study. They were individually penned and fed approximately 5 kg of grass hay (dry matter; DM = 875 g/kg) per animal and day for a 14-day adaptation period.

It had been observed, under practical conditions, that a feed restriction previous to the oak leaf consumption (which occurs due to a shortage of feed resources) favors the intoxications.[22] Therefore, a very limited amount of the grass hay was then offered for a feed restriction period of 8 days (on average 4 kg for two days, 3 kg for one day, 2 kg for the next four days, and one day of fasting; days −8 to −1). Afterward, they were offered daily 14 g DM of grass hay plus 14 g DM of oak leaves/kg of metabolic weight (on average 1.7 kg of hay and 5 kg of oak leaves; oak leaf DM = 298 g/kg) for 6 days (days 1−6), until the animals developed clinical signs of intoxication. The oak leaves, defrosted and slightly chopped, were administered twice per day (at 8.30 and 20.00 h) through the rumen cannula to ensure that all animals received the established amount. Finally, all bulls received again the same amount of grass hay offered during the adaptation period (on average 5 kg/animal) for 12 more days (days 7−18) until they were euthanized. Clean water and vitamin−mineral blocks were always available.

Collection of Biological Samples. Samples of ruminal fluid were taken at approximately 8:30 a.m. (immediately before the feed supply), via the rumen cannula, throughout the experiment: before starting the severe feed restriction (day −10), after the restriction period (day 1), after 2 and 5 days of oak leaf administration (days 3 and 6), and 4 and 12 days after the last oak leaf administration (days 10 and 18).

Blood samples were collected from the caudal vein into heparinized evacuated tubes (Venoject, Terumo Europe N.V., Leuven, Belgium) for plasma analysis. They were taken on days −10, 1, 3, 5, 7, 9, 11, 13, 15, and 18 of the experiment.

Urine samples were collected early in the morning using a plastic bag attached to a harness on days −9, −1, 1, 3, 5, 7, 9, 11, 13, 15, and 17.

Spot samples of feces were collected by rectal grab sampling the last day of the severe feed restriction (day −1) and on days 1, 3, 5, 7, 9, 11, 13, 15, and 17.

Extraction and Analysis of Oak Leaf Phenolic Compounds. Oak leaves were frozen at −70 °C and then freeze-dried. The freeze-dried samples (0.5 g) were homogenized with 20 mL of MeOH:HCl:-water (79.9/0.1/20, v/v/v) using an Ultra-Turrax T-25 basic homogenizer (IKA Werke KG, Staufen, Germany) for 1 min at 24 000 rpm and further extracted by sonication for 10 min using an ultrasonic bath Branson 5510 (Bransonic). The mixture was centrifuged for 5 min at 5000*g* in a Centromix centrifuge (Selecta, Barcelona, Spain) at room temperature. The supernatant was filtered through a 0.45 μm membrane filter Millex-HV13 (Millipore Corp., Bedford, MA) and analyzed by HPLC−DAD-MS−MS.

Extraction of Phenolic Compounds and Their Metabolites from Biological Samples. *Rumen.* Samples (1.5 mL) were defrosted, vortexed, and centrifuged in a Sigma 1−13 microcentrifuge (Braun Biotech International, Melsungen, Germany) at 14 000*g* for 10 min at 4 °C. The supernatants were collected and mixed with 10 mL of distilled water plus 10 μL of 6 N HCl and loaded on to a 360 mg Sep-Pak C_{18} cartridge (Waters, Milford, MA) which was washed with 10 mL of distilled water before elution with 2 mL methanol. The pellets were re-extracted using 1 mL of MeOH, sonicated, and centrifuged at 14 000*g* for 10 min at 4 °C. The methanolic eluates and the supernatant obtained from the re-extracted pellet were combined and reduced to dryness under nitrogen flow at room temperature. The dry samples were then resuspended in 100 μL of MeOH plus 100 μL of 1% aqueous formic and filtered before analysis by HPLC−DAD−MS−MS.

Feces. Samples were extracted as reported previously.[18] Briefly, samples (1 g) were defrosted and homogenized with 10 mL of MeOH:HCl:water (79.9/0.1/20, v/v/v) using an Ultra-Turrax homogenizer for 1 min at 24 000 rpm. The mixture was centrifuged at 5000*g* for 5 min at room temperature and the supernatant filtered through a 0.45 μm membrane filter Millex-HV13 before analysis by HPLC−DAD−MS−MS. For GC−MS analysis, fecal extracts were prepared by adding 4 mL of Milli-Q water to 150 mg of thawed feces. The mixture was homogenized by vortex mixing for 5 min and centrifuged

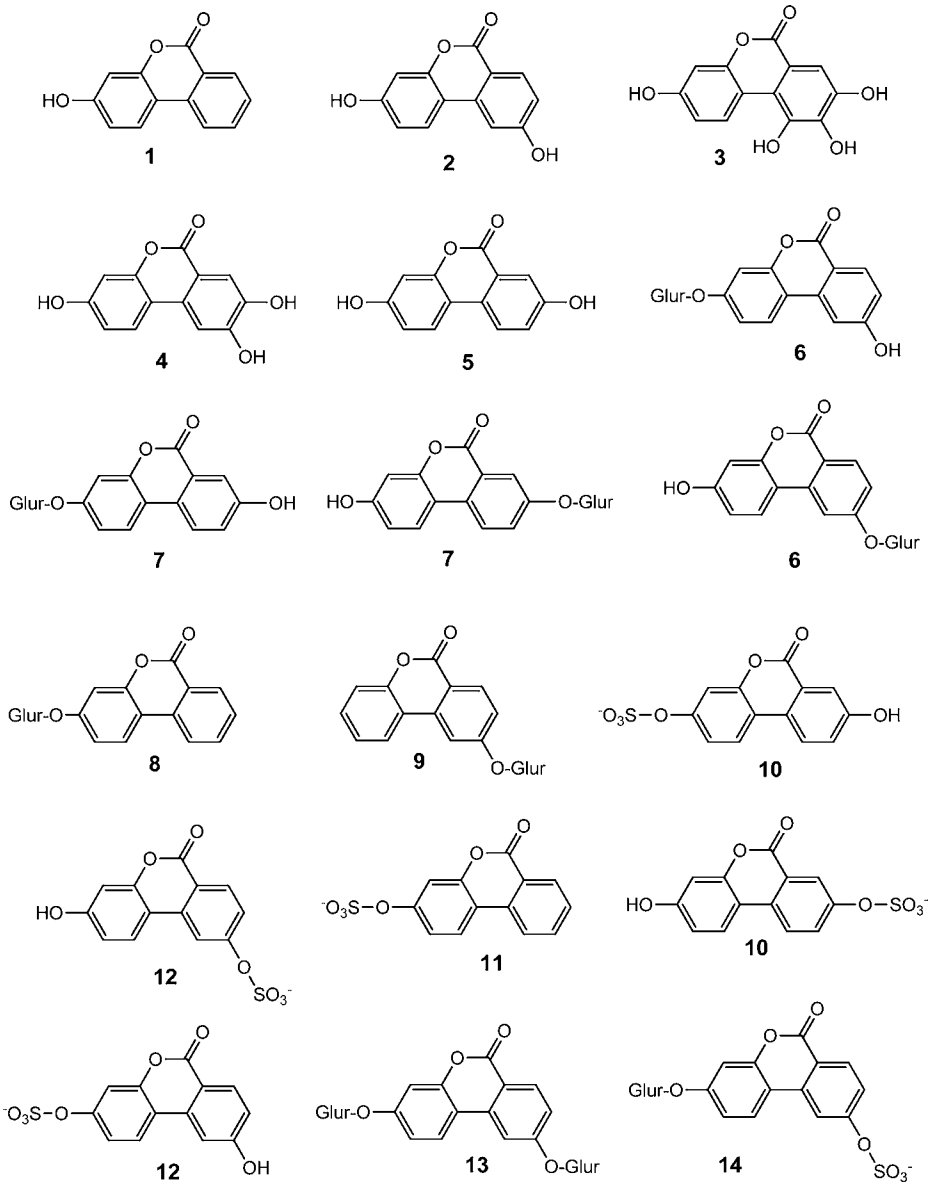

Figure 1. Urolithin metabolites detected after oak leaf ellagitannin administration: urolithin B (**1**), isourolithin A (**2**), urolithin M-6 (**3**), urolithin C (**4**), urolithin A (**5**), isourolithin A glucuronide (**6**), urolithin A glucuronide (**7**), urolithin B glucuronide (**8**), isourolithin B glucuronide (**9**), urolithin A sulfate (**10**), urolithin B sulfate (**11**), isourolithin A sulfate (**12**), isourolithin A diglucuronide (**13**), and isourolithin A glucuronide sulfate (**14**).

at 5000*g* for 10 min. The supernatant was extracted by adding 200 μL of 0.5 N HCl and 3 mL of ethyl acetate. After 10 min of stirring in an ultrasonic bath and 10 min centrifugation at 5000*g*, the supernatant was evaporated to dryness using a speed vacuum centrifuge. The dried samples were reconstituted in 100 μL of methanol.

Plasma. Samples (600 μL) were defrosted and extracted with 1.5 mL of acetonitrile:formic acid (99:1, v/v) by continuous shaking for 10 min and further extracted by sonication using an ultrasonic bath for 10 min. The mixture was centrifuged at 14 000*g* for 10 min at 4 °C, the supernatant decanted, and the pellet re-extracted with 1 mL of MeOH, sonicated, and centrifuged for 10 min at 14 000*g* at 4 °C. The two supernatants were combined and reduced to dryness under nitrogen flow at room temperature. The dry samples were then resuspended in 100 μL of MeOH plus 100 μL of 1% aqueous formic and filtered before analysis by HPLC–DAD–MS–MS. For GC–MS, after centrifugation, 200 μL of plasma was mixed with 80 μL of 1 M sodium acetate buffer and 10 μL of β-glucuronidase from *Helix pomatia* and incubated for 4 h at 37 °C. After cooling at room temperature, phenolic compounds were extracted as described for urine but adding 80 μL of 0.5 N HCl and 1 mL of ethyl acetate. Finally, dried samples were reconstituted in 100 μL of methanol.

Urine. Samples were defrosted, vortexed, centrifuged at 14 000*g* for 10 min at 4 °C, filtered, and analyzed by HPLC–DAD–MS–MS. For GC–MS analysis, 1 mL of urine was adjusted to pH 5 by the addition of 400 μL of 1 M sodium acetate buffer. An aliquot of 25 μL of β-glucuronidase from *H. pomatia* was added to the sample and incubated for 4 h at 37 °C. After cooling the samples to room temperature, they were acidified with 150 μL of 0.5 M HCl, and a liquid–liquid extraction with 3 mL ethyl acetate was carried out. After 10 min of stirring in an ultrasonic bath and 10 min centrifugation at 5000*g*, the supernatant was evaporated to dryness using a speed vacuum centrifuge. The dried sample was reconstituted in 100 μL of methanol.

HPLC–DAD–MS–MS. A HPLC system equipped with a photodiode array detector (1100 series, Agilent Technologies, Waldbronn, Germany) was place in series with an ion-trap mass spectrometer detector (Bruker Daltonics, Bremen, Germany). Chromatographic separations of samples were carried out on a 250 mm × 4 mm i.d., 5 μm, LiChroCART RP-18 (Merck, Darmstadt, Germany) using

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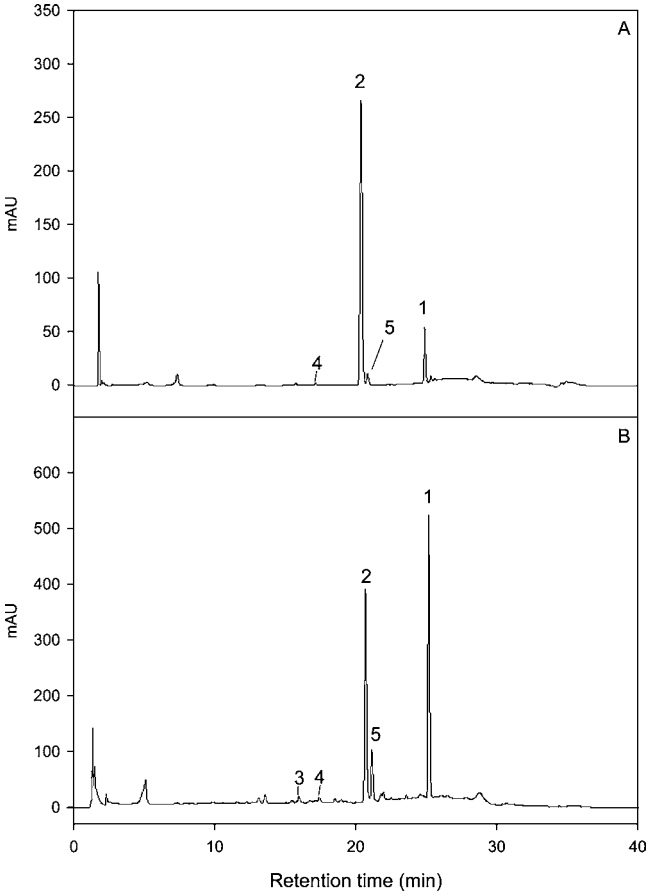

Figure 2. Representative HPLC analysis (305 nm) of urolithin metabolites: (A) ruminal content of animal 2 on day 6 after the administration of oak leaves and (B) feces of animal 1 at day 7 after oak leaves intake; urolithin B (**1**), isourolithin A (**2**), urolithin M-6 (**3**), urolithin C (**4**), and urolithin A (**5**).

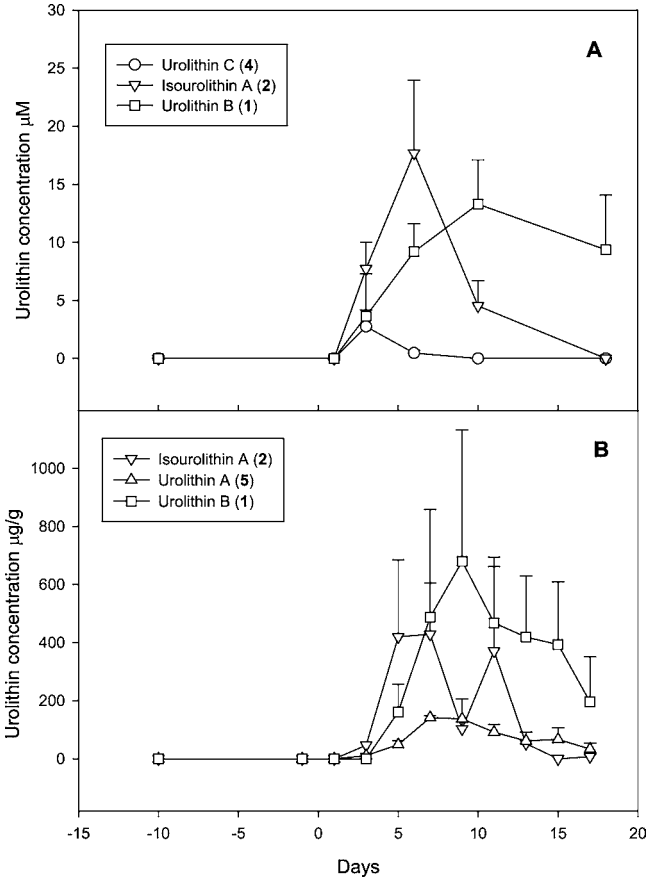

Figure 3. Concentration of urolithins (μM) in ruminal fluids (A) and feces (μg/g) (B). Values are the mean of the animals studied (n = 3). Error bars are standard errors.

water:formic acid (99:1, v:v) (A) and acetonitrile (B) as the mobile phases at a flow rate of 1 mL/min. The gradient started with 5% B in A to reach 30% B at 20 min, 55% B at 30, and 90% at 38 min and returned to the initial conditions (5% B in A) in 4 min. UV chromatograms of samples were recorded at 280, 305, and 360 nm. The ion-trap mass spectrometer was equipped with an electrospray interface (ESI, capillary voltage, 4 kV). Nitrogen was used as nebulizer gas at a pressure of 65 psi and a dry gas at a flow rate and temperature of 9 L/min and 350 °C respectively. Analyses were carried out using full scan mass and data dependent MS^2 scanning from m/z 100 to 700 using the Ultra scan mode (26 000 m/z s^{-1}). Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with a collision energy of 1 V. Mass spectrometry data were acquired in negative ionization mode. A combination with authentic standards, where available, of absorbance spectra and mass spectra, using MS^2, was used to confirm the identity of compounds previously reported in the literature.[11,18−21,26] Quantitative estimates of urolithins in the biological samples were based on the absorbance response at 305 nm and were quantitated by reference curve as urolithin A and B.

Analysis of Low Molecular Weight Phenolic Metabolites by GC−MS. *Derivatization Reaction.* A 50 μL portion of the methanolic extract was dried under a stream of nitrogen and the derivatization reaction was carried out by adding 50 μL of BSTFA plus 1% TMCS. After adding the derivatization reagent the solution was vortexed for 2 min and the trimethylsilylation reaction was performed at room temperature for 1 h. The stability of BSTFA-derivatized samples, kept at ambient temperature (20−25 °C), was determined periodically by injecting samples for up to 96 h. Data proved to be rather consistent from 0 to 48 h.

GC−MS. The GC−MS system consisted of an Agilent 7890A (Agilent Technologies, Palo Alto, CA), equipped with a multipurpose sampler MPS2 (Gerstel, Mülheim, Germany) and coupled to an Agilent 5975C mass selective detector. Acquisition was done using Chemstation software (Hewlett−Packard, Palo Alto, CA). The GC was fitted with a fused silica capillary column HP-5-MS (30 m, 0.25 mm i.d., 0.25 μm film thickness) and helium was used as the carrier gas at 1 mL/min. Injection was made in the splitless mode with an injection volume of 1 μL and an injector temperature of 250 °C. The column temperature was initially kept at 120 °C for 5 min and then increased from 120 to 280 °C at 5 °C/min and kept at this temperature for 5 min. The detector was operated in electron impact ionization mode (electron energy 70 eV), scanning the range m/z 50−600. The temperature of the ion source was 230 °C, the quadrupole 150 °C, and the interface 280 °C. To identify the phenolic compounds the retention time and the assistance of the libraries NIST08 and Wiley7n were used (match quality higher than 95%).

RESULTS AND DISCUSSION

Characterization of Urolithin Metabolites. The different urolithin (dibenzo[*b*,*d*]pyran-6-one) derivatives present in the samples were detected and characterized by their UV spectra and HR-MS as reported previously.[20] Aglycons (**1**−**5**), glucuronides (**6**−**9**), sulfates (**10**−**12**), diglucuronides (**13**), and glucuronide sulfate (**14**) derivatives were detected (Figure 1). Two possible isomers of urolithin A sulfate (**10**), isourolithin A sulfate (**12**), and isourolithin A glucuronide sulfate (**14**) were feasible, and the same number was given to both isomers, as it

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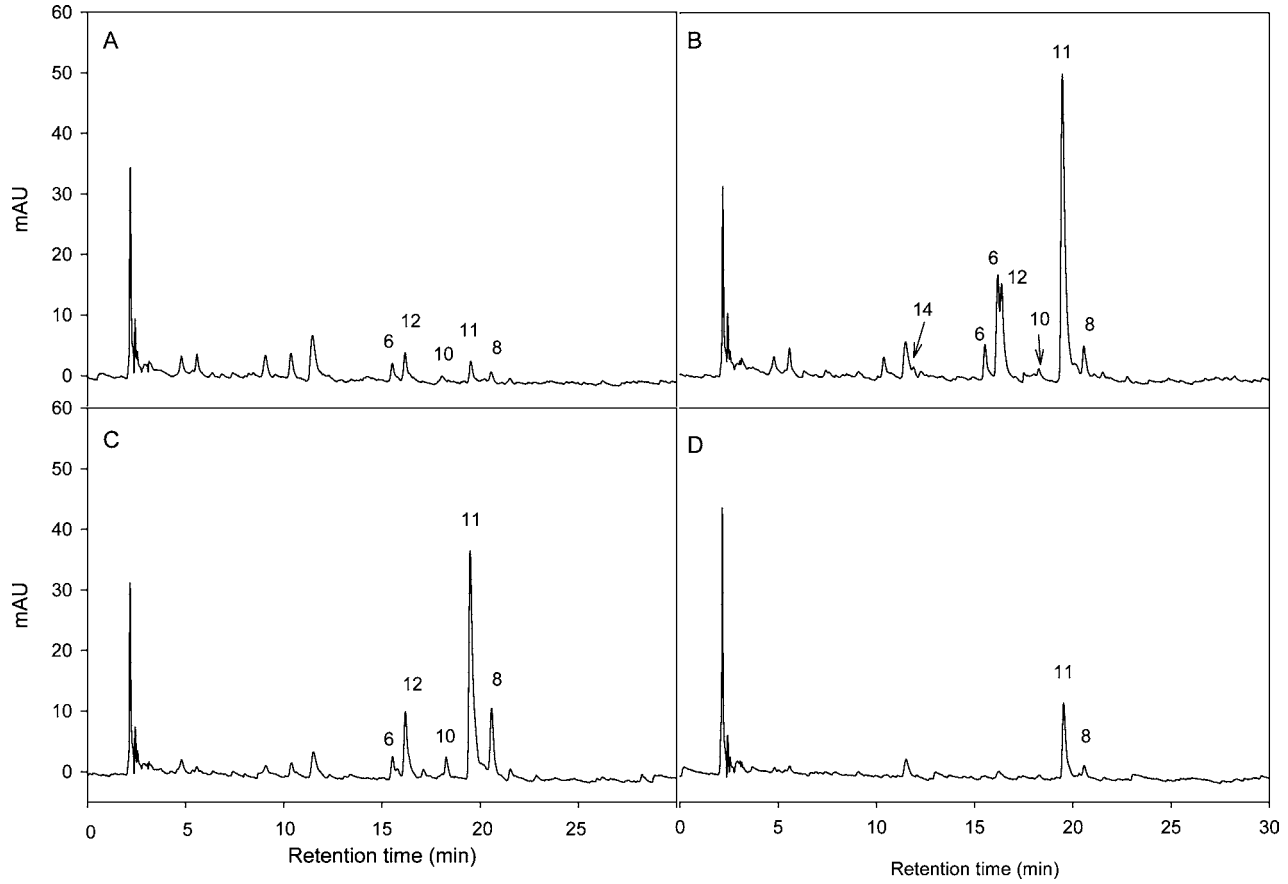

Figure 4. Representative HPLC analyses (305 nm) of animal plasma samples (A) 3 days after oak leaf administration, (B) 5 days after oak leaf administration, (C) 7 days after oak leaf administration, and (D) 9 days after oak leaves administration: isourolithin A glucuronide (**6**), urolithin B glucuronide (**8**), urolithin A sulfate (**10**), urolithin B sulfate (**11**), isourolithin A sulfate (**12**), isourolithin glucuronide sulfate (**14**).

was not possible to differentiate among them. The chemical characterization of the different metabolites was previously reported.[20] Urolithin A and isourolithin A derivatives were easily differentiated by their characteristic UV spectra.[20]

The calibration graphs were constructed by plotting peak area versus concentration of urolithin A in methanol solution. Good linearity was achieved in the range from LOQ to 200 μM with significant correlation ($r^2 \geq 0.9996$) (urolithin A, $y = 55\,462x - 18\,661$; urolithin B: $y = 28\,341x - 45\,16.7$). The limits of detection (LOD) and quantitation (LOQ) were obtained by injecting successively more diluted standard solutions and were calculated according to the IUPAC method based on a signal-to-noise ratio (S/N) of 3 for the LOD and of 10 for the LOQ. The results showed LODs for urolithin A and B of 0.5 μM and LOQs for urolithin A and B of 1.7 μM.

The recovery of the compounds during sample preparation was calculated using control samples of urine, feces, and plasma spiked with the standard solution of urolithin A and B at three final concentrations (5, 20, and 50 μM). After vortexing, the samples were extracted as described above. The recovery percentages expressed as an average of the different concentrations were 83.2% in urine, 89.5% in feces, and 83.5% in plasma. For urolithin B they were 84.5% in urine, 85.2% in feces, and 77.5% in plasma.

Regarding the repeatability, it was evaluated by injecting 20 μM of urolithin A and B in the urine matrix three times in the same day (intraday repeatability) and in three different days (interday repeatability). The results expressed as the relative standard deviation (RSD) of peak areas were 0.27% for intraday repeatability and 1.59% for interday repeatability. For urolithin B the results were 0.49% for intraday repeatability and 0.82% for interday repeatability.

Metabolism of Oak Leaf Ellagitannins in the Rumen. The analysis of rumen samples by HPLC−MS showed the presence of free ellagic acid and small amounts of the flavonol aglycons (quercetin, kaempferol, and isorhamnetin) as a consequence of the hydrolysis of ellagitannins and flavonol glycosides that are abundant in oak leaves. Some ellagitannins were also detected (vescalagin/castalagin; pedunculagin; trigalloyl-hexahydrodiphenoyl-glucose, and digalloyl-hexahydroxydiphenoyl-glucose). Urolithin metabolites were not detected in the rumen samples after 1 day of the oak leaf ellagitannin administration. The metabolites detected in rumen were urolithin C (trihydroxydibenzo[*b*,*d*]pyran-6-one; *m*/*z* 243) (**4**), isourolithin A (dihydroxydibenzo[*b*,*d*]pyran-6-one; *m*/*z* 227) (**2**); urolithin A (dihydroxydibenzo[*b*,*d*]pyran 6-one; *m*/*z* 227) (**5**) and urolithin B (monohydroxydibenzo[*b*,*d*]pyran-6-one; *m*/*z* 211) (**1**) (Figure 2A). These metabolites started to be detected in the rumen samples taken after 3 days and reached a maximum in the sample taken at day 6 (Figure 3A). Interestingly, urolithin A, the main gut microbiota ellagitannin metabolite reported in humans and monogastric animals (rats, pigs, etc.), was only observed as a minor metabolite in the rumen samples. The kinetics of urolithin production indicates that urolithin C is produced first (concentration peak at day 3) and isourolithin A (peak at 6 days) and urolithin B (peak at 10 days) were

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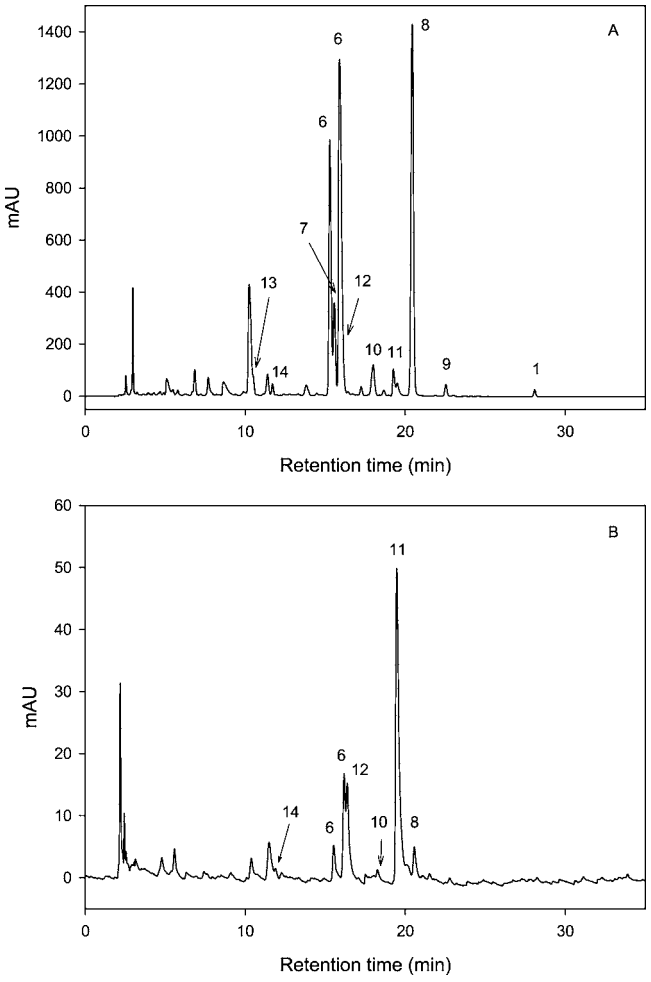

Figure 5. HPLC chromatogram (305 nm) of (A) urine 7 days after oak leaves administration and (B) plasma 5 days after oak leaves administration: isourolithin A glucuronide (**6**), urolithin A glucuronide (**7**), urolithin B glucuronide (**8**), isourolithin B glucuronide (**9**), urolithin A sulfate (**10**), urolithin B sulfate (**11**), isourolithin A sulfate (**12**), isourolithin A diglucuronide (**13**), isourolithin A glucuronide sulfate (**14**), and urolithin B (**1**).

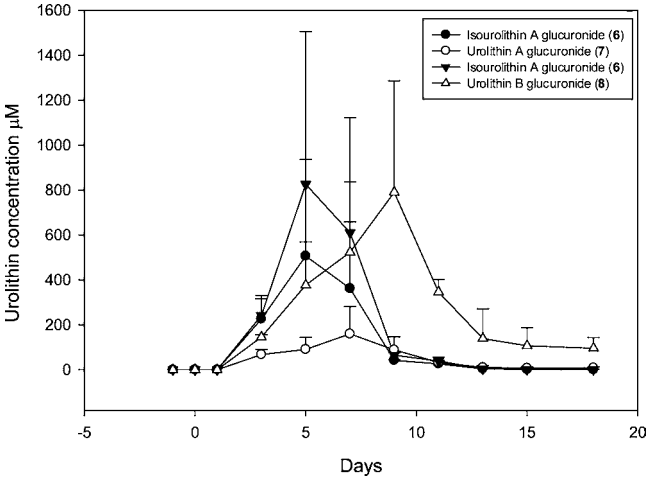

Figure 6. Concentrations of urolithins (μM) in urine. Values are the mean of the animals studied (n = 3). Error bars are standard errors.

produced sequentially by successive removal of the corresponding hydroxyls by the dehydroxylases of the rumen microbiota

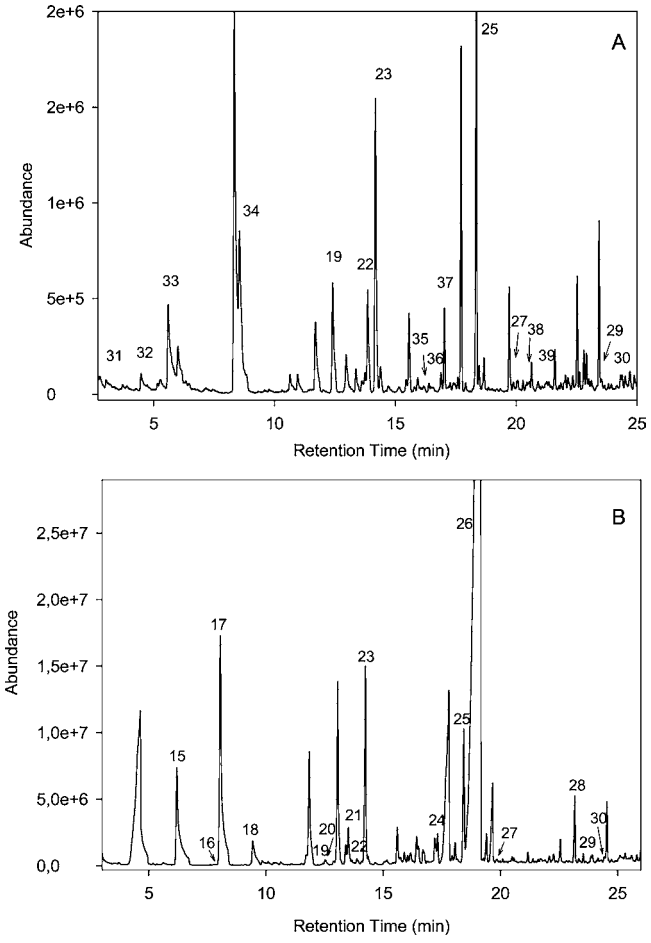

Figure 7. GC−MS analysis of phenolic metabolites in (A) the feces of animal 1 at day 7 after oak leaf administration and (B) the urine of animal 3 at day 7 after the intake of oak leaf administration: catechol (**15**), resorcinol (**16**), methylcatechol (**17**), 2-methylhydroquinone (**18**), 3-hydroxybenzoic acid (**19**), 2-hydroxybenzoic acid (**20**), 3-hydroxyphenylacetic acid (**21**), 4-hydroxybenzoic acid (**22**), 4-hydroxyphenylacetic acid (**23**), 2,5-dihydroxybenzoic acid (**24**), 3,5-dihydroxybenzoic acid (**25**), hippuric acid (**26**), hydroferulic acid (**27**), 2-hydroxyhippuric acid (**28**), ferulic acid (**29**), caffeic acid (**30**), 1-methyl-3-hydroxybenzene (**31**), benzoic acid (**32**), phenylacetic acid (**33**), phenylpropionic acid (**34**), 3-hydroxyphenylpropionic acid (**35**), 4-hydroxyphenylpropionic acid (**36**), 4-hydroxy-3-methoxybenzoic acid (**37**), hydroxycinnamic acid (**38**), and gallic acid (**39**).

(Figure 3A). The main ellagitannin metabolites detected in all rumen samples were isourolithin A and urolithin B. The maximal concentrations of these metabolites in ruminal content were 18 and 13 μM, respectively.

Fecal Metabolism of Oak Ellagitannins. The analysis of fecal samples from all animals showed the presence of tetra-, tri-, di-, and monohydroxydibenzo[*b*,*d*]pyran-6-ones (urolithin M-6, urolithin C, urolithin A and isourolithin A, and urolithin B) (Figure 2B). Urolithin A, a minor metabolite in the ruminal fluid samples, was present in more relevant amounts in most fecal samples. The maximum concentration of urolithins in feces occurred between 7 and 9 days after feeding with oak leaves. However, significant amounts of urolithins, and particularly urolithin B, were observed in these samples until the end of the experiment (Figure 3B). The analysis of urolithins in feces was also consistent with the sequential production of urolithins from ellagic acid, by the gut microbiota removing specific hydroxyls leading to metabolites with less hydroxyl substituents.

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Table 1. Concentrations (μM) of Low Molecular Weight Microbial Phenolic Metabolites Excreted in Urine After 9 Days of Oak Leaf Administration (n = 3)[a]

no.	compd	Rt (min)	$[M]^+$	metabolites in urine
15	catechol[b]	6.2	254	84.67 ± 34.16
16	resorcinol	7.8	254	1.22 ± 0.30
17	4-methylcatechol[b]	8.1	268	76.33 ± 67.48
18	2-methylhydroquinone[b]	9.4	268	23.69 ± 12.63
19	3-hydroxybenzoic acid	12.4	282	0.28 ± 0.28
20	2-hydroxyphenylacetic acid[c]	12.5	296	2.36 ± 1.06
21	3-hydroxyphenylacetic acid	13.5	296	6.85 ± 6.37
22	4-hydroxybenzoic acid	13.9	282	2.95 ± 0.20
23	4-hydroxyphenylacetic acid	14.2	296	23.26 ± 19.73
24	2,5-dihydroxybenzoic acid[d]	17.2	370	11.28 ± 10.46
25	3,5-dihydroxybenzoic acid[d]	18.4	370	39.02 ± 18.93
26	hippuric acid	19.1	251	409.44 ± 202.73
27	hydroferulic acid	19.9	340	0.92 ± 0.21
28	2-hydroxyhippuric acid[e]	23.2	339	76.87 ± 56.60
29	ferulic acid	23.5	338	1.25 ± 0.39
30	caffeic acid	24.4	396	5.86 ± 5.07

[a]Compounds were identified as trimethylsilyl derivatives. [b]Quantified as resorcinol equivalent. [c]Quantified as 3-hydroxyphenyl acetic acid equivalent. [d]Quantified as 3-hydroxybenzoic acid equivalent. [e]Quantified as hippuric acid equivalent.

Table 2. Concentrations (μg/g) of Low Molecular Weight Microbial Phenolic Metabolites Excreted in Feces 5 Days After Oak Leaf Administration (n=3)[a]

no.	compds	Rt (min)	$[M]^+$	metabolites in feces
31	1-methyl-3-hydroxybenzene[b]	3.1	180	2.92 ± 0.97
32	benzoic acid[b]	4.5	194	1.96 ± 0.46
33	phenylacetic acid	5.6	208	23.73 ± 4.75
34	phenylpropionic acid	8.6	222	15.59 ± 5.40
19	3-hydroxybenzoic acid	12.5	282	5.92 ± 1.30
22	4-hydroxybenzoic acid	13.9	282	3.40 ± 0.31
23	4-hydroxyphenylacetic acid	14.2	296	5.53 ± 3.52
35	3-hydroxyphenylpropionic acid	16.2	310	0.76 ± 0.12
36	4-hydroxyphenylpropionic acid	16.9	310	0.68 ± 0.06
37	4-hydroxy-3-methoxybenzoic acid	17.0	312	4.46 ± 0.29
25	3,5-dihydroxybenzoic acid[b]	18.4	370	22.93 ± 15.87
27	hydroferulic acid	19.8	340	1.15 ± 0.29
38	hydroxycinnamic acid[c]	20.6	308	1.79 ± 0.06
39	gallic acid	21.3	458	0.74 ± 0.39
29	ferulic acid	23.5	338	1.19 ± 0.02
30	caffeic acid	24.4	396	0.76 ± 0.07

[a]Compounds identified as trimethylsilyl derivatives. [b]Quantified as 3-hydroxybenzoic acid equivalent. [c]Quantified as cinnamic acid equivalent.

Metabolites Detected in Systemic Circulation. The analyses of plasma samples showed the presence in circulation of isourolithin A glucuronide (two isomers **6**), urolithin B glucuronide (**8**), and also isourolithin A sulfate (**12**), urolithin A sulfate (**10**), urolithin B sulfate (**11**), and a small amount of isourolithin A glucuronide sulfate (**14**) (Figure 4). After 3 days of oak leaf administration, small amounts of glucuronides and sulfates of urolithin A and B and isourolithin A were detected. These continued to increase after 5 and 7 days of the oak leaf supply with a decrease in plasma metabolites at day 9, when the oak leaf administration had finished. The plasma concentrations increased with time of oak leaf treatment. Interestingly, the concentrations of sulfates in plasma (isourolithin A sulfate, 0.17−6.71 μM; urolithin A sulfate, 0.27−1.75 μM; urolithin B-sulfate, 1.10−26.19 μM) were generally higher than those of the glucuronides (isourolithin A glucuronide, 1.04−4.76 μM; urolithin B glucuronide, 0.51−5.23 μM).

Table 3. Concentrations (μM) of Low Molecular Weight Microbial Phenolic Metabolites Detected in Plasma 9 Days After Oak Leaf Administration (n = 3)[a]

no.	compd	Rt (min)	$[M]^+$	metabolites in plasma
22	4-hydroxybenzoic acid	13.9	282	3.0 ± 0.08
23	4-hydroxyphenylacetic acid	14.2	296	1.4 ± 0.13
26	hippuric acid	18.4	251	4.2 ± 0.69
27	hydroferulic acid	19.8	340	1.3 ± 0.01
29	ferulic acid	23.5	338	1.3 ± 0.66
30	caffeic acid	24.4	396	1.2 ± 0.01

[a]Compounds identified as trimethylsilyl derivatives.

Metabolites Excreted in Urine. The HPLC analyses of urine samples showed the presence of glucuronide metabolites of urolithin A (**7**), two isourolithin A isomers (**6**), urolithin B (**8**), and isourolithin B (**9**). Urolithin A sulfate (**10**), isourolithin A sulfate (**12**), urolithin B sulfate (**11**), isourolithin A diglucuronide (**13**), isourolithin A glucuronide sulfate (**14**), and urolithin B (**1**) were also detected although in smaller concentrations (Figure 5A). The chromatographic (HPLC−MS−MS) comparison of plasma and urine samples showed that urine had higher levels of urolithin B glucuronide (**8**), while plasma had larger amounts of the sulfate (**11**) instead (Figure 5). The kinetics of the excretion of the different metabolites shows that the isourolithin A glucuronides were the main metabolites excreted during the first 5 days of the experiment, in accordance with the production of isourolithin A in the rumen, then the excretion of urolithin B glucuronide increased to reach a maximum at day 9, this being consistent with the results observed in rumen and feces (Figure 6). Urolithin A glucuronide (**7**) was a minor metabolite, with a maximal excretion at day 7.

Analysis of Phloroglucinol, Pyrogallol, Resorcinol, and Other Simple Phenolics by GC−MS. Hydrolyzable tannins were reported to be degraded by the microbiota to low molecular weight metabolites, such as gallic acid, pyrogallol, and other products[24,25] that are potentially toxic to ruminants.

In addition, gallic acid, resorcinol, and pyrogallol have been proposed as products of hydrolyzable tannins degradation by ruminal microbes.[27] Despite extensive investigation with HPLC−DAD−MS−MS in a number of modes to enhance sensitivity for low molecular weight phenolics (ESI or APCI in both positive and negative ionization mode), the analyses revealed that these compounds were poorly ionized and they were difficult to detect in all the samples collected in the current study. An alternative analytical strategy that is more sensitive was therefore employed, based on the method of Borges et al.,[28] which involved forming trimethylsilyl derivatives and analyzing samples by GC−MS.

The analysis of fecal samples of the three animals collected after 5 days of the oak leaf consumption (Figure 7A) showed that no catechol, resorcinol, or phloroglucinol was detected in

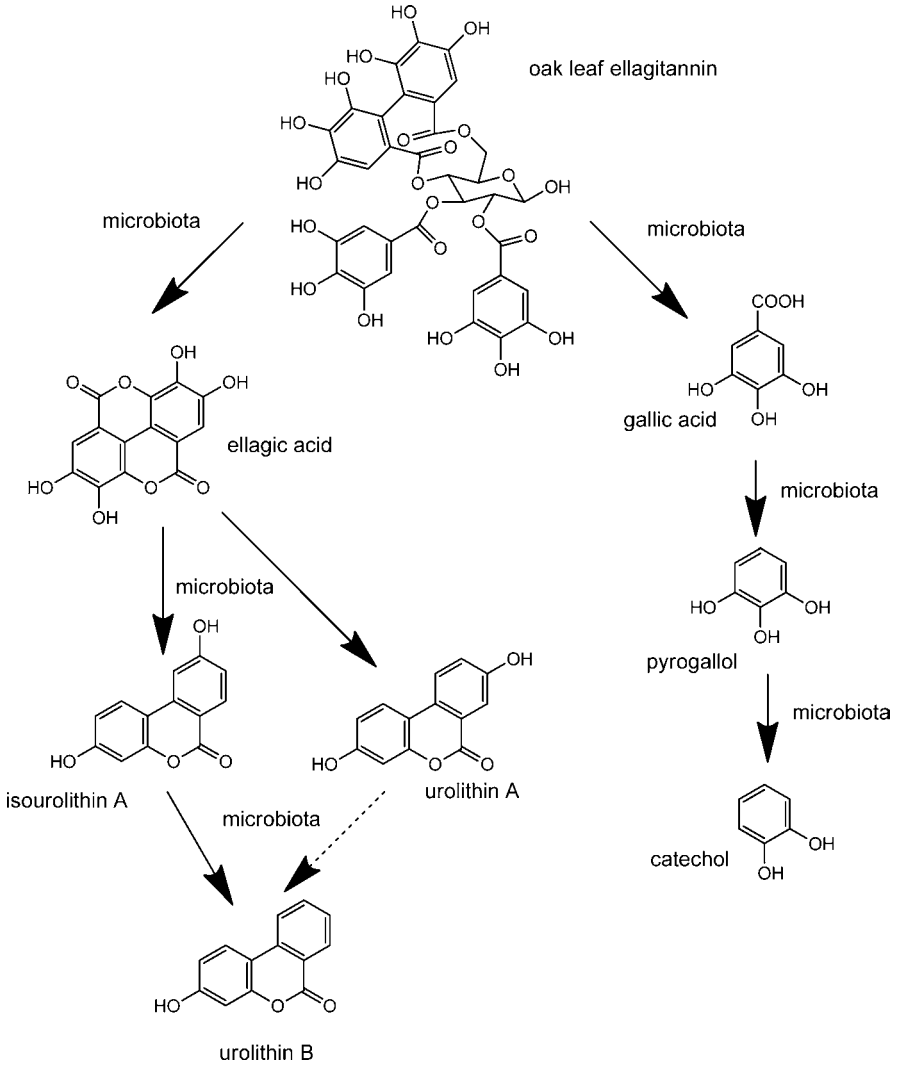

Figure 8. Postulated metabolic pathways of oak leaf ellagitannins in cattle.

significant amounts (Table 2). Only benzoic, phenylacetic, and phenylpropionic acid derivatives were detected as well as small amounts of caffeic, ferulic, and gallic acids and some derivatives. When analyzing the urine samples of the three animals 9 days after oak leaf supply (Figure 7B), catechol, resorcinol, 4-methylcatechol, and 2-methylhydroquinone were detected in significant amounts in addition to the benzoic and phenylacetic derivatives. Hippuric acid and derivatives were also detected (Table 1). Phloroglucinol was not detected in any sample.

The analysis of rumen content by GC−MS (samples collected on day 6 after the oak leaf administration) showed that all the samples had a similar qualitative profile in which all the metabolites detected in urine and feces were detected (**19**−**39**) with the exception of catechol (**15**), resorcinol (**16**), 4-methylcatechol (**17**), and 2-methylhydroquinone (**18**), which were not detected. The different compounds were not quantified due to the nature of the samples and the small amount available.

The analysis of plasma samples collected 9 days after oak leaf intake showed similar patterns in the three animals with benzoic and hydroxycinnamic acid derivatives and hippuric acid with concentrations in the low micromolar range (Table 3). Again, no catechol or resorcinol was detected in the plasma samples.

Monogastric mammals produce mainly urolithin A from ellagitannins and ellagic acid while isourolithin A and urolithin B are seldom found.[20] In human intervention studies, urolithin B is only produced by a limited number of individuals.[10,11,19] In the present study we show that in ruminants, isourolithin A and urolithin B are the main metabolites produced in the rumen (with the limitations of the reduced number of animals studied). This suggests that urolithin B is readily produced from isourolithin A and that production from urolithin A is less favored. This is in agreement with previous reports in which no urolithin B was detected in rats upon oral administration of synthetic urolithin A.[29,30] On the contrary, urolithin A has been reported to be formed from urolithin B in vitro[29,31] and in vivo[29] in a process tentatively assumed to be catalyzed by isoenzymes from the cytochrome P450.

One plausible hypothesis to explain the toxic effect of ellagitannins in cattle, taking into consideration the previous suggestions that the toxic compounds responsible for the toxicosis could be catechol, pyrogallol, and phloroglucinol,[24,25] was that the animals that do not suffer intoxication address their rumen and gut metabolism to produce harmless urolithins, while those that show severe clinical and pathological signs of intoxication produce the potentially toxic small phenolic metabolites (catechol, etc.) instead (Figure 8). These differences

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could be associated with the microbiota that colonizes the gastrointestinal tract of the different animals. This study shows, however, that both urolithins and low molecular weight phenolics were produced in very variable amounts in the different animals. Catechol and small amounts of resorcinol were detected in the urine of all animals. In the fecal samples analyzed, an intense microbial degradation metabolism to yield benzoic, phenylacetic, and phenylpropionic acid metabolites was observed, again with a large variability among the animals studied. This suggests that animals vary in their efficiency to metabolize ellagitannins and this could be linked to the presence of intoxication symptoms. Preliminary results suggest that the metabolic speed of transformation of ellagitannins into urolithin and other phenolic metabolites could be related to the survival of the animals when they ingest oak leaf after a feed restriction period. The large differences found in the rumen metabolism of the ellagitannins in the different animals do suggest that a delay or failure of this metabolism could be related to the initiation of the intoxication, and this could be related to different microbial populations in the rumen, as has been previously reported.[23] Studies with a larger number of animals would be necessary to evaluate the potential implications of ellagitannins metabolism and production of different metabolites on cattle oak leaf intoxication.

These results support the relevance of microbiota in the biological (nutritional, pharmacological, toxicological) effects of dietary constituents, including phenolic compounds. The recent description of three robust gut microbiota clusters (enterotypes)[32] that are not nation- or continent-specific, suggests that dietary polyphenols can have different biological activity, depending on the enterotype. The same situation can also be present in cattle and could be responsible for the different ellagitannin intoxication susceptibility observed in different individuals.

■ ASSOCIATED CONTENT

Supporting Information

Figure 1S (HPLC−PAD chromatogram recorded at 360 nm of the oak leaves phenolic extract) and Table 1S [characterization of flavonols and hydrolyzable tannins (ellagitannins and gallotannins) in the young oak leaves used for the feeding study]. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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