In vivo and in vitro metabolism of trans-resveratrol by human gut microbiota

Lisa M Bode, Diana Bunzel, Melanie Huch, Gyu-Sung Cho, Denise Ruhland, Mirko Bunzel, Achim Bub, Charles MAP Franz, and Sabine E Kulling

ABSTRACT

Background: Strong interindividual differences in the microbial conversion of some dietary polyphenols have been reported. In-depth studies of trans-resveratrol metabolism by human gut microbiota, however, are lacking, and only one bacterial metabolite, namely dihydroresveratrol, has been described.

Objective: The aim of this study was to elucidate interindividual differences in trans-resveratrol metabolism by human gut microbiota and to identify bacterial strains involved.

Design: In the first part of the study, in vitro fermentation experiments were performed with feces samples from 7 healthy volunteers, and metabolite formation was measured by liquid chromatography–ultraviolet/visible (UV/Vis)–mass spectrometry (MS)/MS detection. Microbial diversities in 3 feces samples were analyzed by high-throughput pyrosequencing and quantitative real-time polymerase chain reaction. In addition, trans-resveratrol conversion experiments were conducted with selected fecal bacterial strains in pure culture. The second part of the study was a controlled intervention study with 12 healthy volunteers. After a washout period, all of the subjects received a one-time oral dose of 0.5 mg trans-resveratrol/kg body weight in the form of a grapevine-shoot supplement, and 24-h urine samples were analyzed by liquid chromatography–UV/Vis–MS/MS.

Results: Besides dihydroresveratrol, 2 previously unknown bacterial trans-resveratrol metabolites were identified in vitro and in vivo: 3,4′-dihydroxy-trans-stilbene and 3,4′-dihydroxybibenzyl (lunularin). Their formation, however, varied among the volunteers. Two strains, Slackia equolifaciens and Adlercreutzia equolifaciens, were identified as dihydroresveratrol producers. Gut bacteria able to produce dehydroxylated metabolites could, however, not be identified.

Conclusions: trans-Resveratrol metabolism by human gut microbiota shows pronounced interindividual differences, which should be taken into account during investigation of health-related effects of this stilbene. This trial was registered at the German Clinical Trials Register as DRKS00004311, Universal Trial Number (WHO) UTN: U1111-1133-4621.

INTRODUCTION

Resveratrol is a dietary polyphenol found in a variety of foods and plants, such as red wine, grapes, peanuts, pistachios, berries, and Itadori tea (Reynoutria japonica) (1). A multitude of studies have shown various functions of this stilbene, such as antioxidant and antiinflammatory effects (2–4), the ability to block human platelet aggregation and eicosanoid synthesis (5, 6), agonistic effects on estrogen receptor function (7), and inhibiting effects on COX-2 enzyme activity (8). Resveratrol was also reported to mimic the beneficial effects of dietary restriction, extending the life span of mice and other species (9, 10). Because of its presence in red wine, resveratrol research has focused on cardioprotective effects related to moderate wine consumption (11), and the underlying mechanisms possible were recently reviewed (12, 13). In addition, resveratrol may act as a cancer chemopreventive agent (14). However, some in vivo and in vitro data that were obtained are contradictory (15, 16).

Despite the vast amount of data that have become available over the past 2 decades, the physiologic effects of resveratrol and their underlying mechanisms are not yet fully understood. This may be partially attributable to gaps that still exist in our knowledge of the metabolic fate of resveratrol. Resveratrol is rapidly absorbed and metabolized in humans (17). However, it is important to fully elucidate the metabolic route of resveratrol, because its bioconversion can lead to metabolites with significantly different chemical and biological activities. This may affect the health-related effects of resveratrol. Thus, it is crucial to understand the extent of the conversion and the nature of the metabolites produced, because either bioactivation or inactivation may occur. Numerous studies have provided information about the metabolism of resveratrol via intestinal and hepatic conjugation reactions (17–19). It is also known that intestinal bacteria are able to convert resveratrol to dihydro-
resveratrol, which, at least partially, is absorbed and further metabolized to conjugated forms that can be excreted in urine (17, 18, 20, 21). In-depth studies of resveratrol bioconversion by human gut bacteria, however, are still lacking.

The microbial conversion of a range of dietary phenolics has been well described (22). Bacterial transformation reactions include ring fission, reduction, demethylation, decarboxylation, and dehydroxylation (22–24). It is important to point out that there can be significant interindividual differences in microbial transformations, as in the case of the production of either equol or O-demethylangolensin and dihydrodaidzein from the isoflavone daidzein (25–27). Given the complex metabolism of other polyphenols, we hypothesized that unknown microbial resveratrol metabolites other than dihydroresveratrol may exist. Detailed in vitro and in vivo experiments were performed to identify new metabolites, to elucidate interindividual routes of resveratrol conversion by the human gut microbiota, and to identify the bacterial species involved.

SUBJECTS AND METHODS

Bacterial strains, culture conditions, growth media, and chemicals

The bacterial strains used in this study were obtained from culture collections or isolated from human feces and identified by sequencing the almost complete 16S ribosomal RNA (rRNA4) gene (Table 1). *Adlercreutzia equolificiens*, *Bifidobacterium pseudocatenulatum*, *Eggerthella lenta*, *Faecalibacterium prausnitzii*, and *Slackia equolificiens* were grown anaerobically under N₂ atmosphere in Hungates in DSM 339 (German collection of microorganisms and cell cultures; Deutsche Sammlung von Mikroorganismen und Zellkulturen, www.dsmz.de). *Clostridium scindens* and *Slackia isoflavoniconvertens* were grown in the same medium, to which 1% glucose or 1% arginine was added, respectively. *Eubacterium rectale* and *Bacteroides plebeius* were grown in modified PYG medium (DSM medium 104). *Lactococcus garvieae* was grown in de Man/Rogosa/Sharpe medium. *Peptinophilus harei*, *Finnoglobina magna*, *Bifidobacterium animalis*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium bifidum*, and *Bifidobacterium* spp. MRL-F 45, 48, and 49 were grown in modified tryptone soy broth (Oxoid) containing the following (g/L): yeast extract, 5; zinc sulfate, 0.25; Tween 80, 1.0; magnesium chloride, 0.5; calcium chloride, 0.15; and glucose, 12.0. *Bacteroides ovatus*, *Bacteroides vulgatus*, *Citrobacter murliniae*, *Escherichia coli*, *Enterococcus faecium*, and *Lactobacillus salivarius* were grown in anaerobic broth containing the following (g/L): proteose peptone, 1; peptone from pancreatic meat digest, 9; sodium chloride, 3; sodium dihydrogen phosphate dihydrate, 2; meat extract, 3; yeast extract, 4; r(+)-glucose, 6; cysteine mono hydrochloride, 0.25; Tween 80, 0.5; salt solution B, 2.5; and L-cystine, 0.25 (pH 7.0). Salt solution B contained the following (g/L): magnesium sulfate heptahydrate, 40; ferrous sulfate heptahydrate, 2; sodium chloride, 2; and manganese sulfate dihydrate, 1.34. Bacteria in this medium were grown anaerobically under N₂/CO₂ atmosphere (80:20, by vol) in Hungate tubes. Fresh overnight cultures were used to inoculate the respective media containing trans-resveratrol. All bacteria were generally grown stationary at 37°C for up to 96 h.

β-Glucuronidase (from bovine liver) and sulfatase (from *Helix pomatia*) were purchased from Sigma Aldrich. All solvents used for liquid chromatography (LC) analysis were of HPLC grade. *trans-Resveratrol* was purchased from Sigma Aldrich at a purity of 99% as determined by gas chromatography, dihydroresveratrol was bought from Santa Cruz Biotechnology, and 3,5-dihydroxy-*trans*-stilbene (pinoresinol) was from Apin Chemicals Ltd. All other chemicals used were of analytic grade.

**Grapevine-shoot extract**

The grapevine-shoot extract Vineatrol, hereafter referred to as “grapevine-shoot extract,” was kindly provided by Breko GmbH (Bremen, Germany) and Actchem (Montauban, France), who co-developed and produced this product. The extract contained 7.7% *trans*-resveratrol as well as other stilbene mono- and oligomers [14.6% *e*-viniferin, 3.4% amelopsin A, 1.8% hopeaphenol, 0.6% *trans*-piceatannol, 1.6% r-2-viniferin (vitisin A), 2.5% miyabenol C, 2.5% r-viniferin (vitisin B), and 2.4% *iso-trans*-e-viniferin]. VineatrolSOL, hereafter referred to as “colloid formulation,” is a liquid mixture of edible oils and food-approved emulsifiers containing 10% dissolved Vineatrol. On contact with an aqueous phase, the mixture spontaneously self-assembles into colloidal droplets containing solubilized Vineatrol.

**Collection of human fecal samples**

Fecal samples were donated by 7 healthy volunteers (5 women and 2 men) aged 26–54 y, who consumed a nonspecified Western diet. They had no history of gastrointestinal disease or any chronic disease, were nonsmokers, and had not used antibiotics in the 3 mo before sample collection. Female volunteers were neither pregnant nor lactating. To conduct fermentation experiments in duplicate, each volunteer donated 2 fecal samples. Donation times were kept 1–4 wk apart. The fecal samples were stored at 4°C and were further processed within 1 h of donation. Anoxic conditions were maintained by using the AnaeroGen Compact System (Oxoid).

**Fermentation with human fecal cultures**

Preparation of fecal suspensions and subsequent culturing experiments were conducted under anoxic conditions in an anaerobic work station (MK3 Anaerobic Workstation; Don Whitley) at 37°C, which provided a gas atmosphere of N₂/CO₂/H₂ (80:10:10, by vol). Aliquots (7.5 g) of fecal samples were suspended in 22.5 g modified brain heart broth medium (Merck) (37 g brain heart broth, 0.5 g L-cystine monohydrochloride, and 1 mg resazurin sodium salt in 1 L distilled water; pH 7.4–7.8) and mixed for 10 min at 37°C and 180 rpm. To remove larger particulates, the suspensions were centrifuged for 10 min at 285 × g at room temperature. The supernatant fluid was diluted with modified brain heart broth medium to produce a fecal suspension at a final concentration of 5% (by vol).
Table I

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1 DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; MRI, Max Rubner-Institut.

Aliquots of fecal suspensions were transferred to 50-mL glass bottles and a 32-mmol/L solution of trans-resveratrol in methanol was added. The final concentrations of trans-resveratrol and methanol were 80 μmol/L and 0.25%, respectively. Duplicate cultures were prepared in parallel from each fecal suspension. In addition, 2 controls were used, one without fecal suspension and one without resveratrol. The batches were incubated at 37°C for 48 h with stirring at 220 rpm. Samples (1 mL) were collected at 0, 2, 4, 6, 8, 24, and 48 h, and bacterial cells were removed by centrifugation for 10 min at 4°C and 8600 × g. The supernatant fluid was collected and stored at −20°C until further analysis.

Human intervention study subjects and design

Twelve nonsmoking men aged 19–28 y participated in this study. They were in good health as determined by a screening history and medical examination. The BMI (in kg/m²) of subjects ranged between 20 and 26. The study protocol was approved by the Ethical Committee of the Landesärztekammer Baden-Württemberg, Germany (F-2010-094). All participants had given their written consent before the study.

In this study, resveratrol was administered in the form of a colloid formulation. The dose of the supplement was standardized on the basis of the trans-resveratrol content and was calculated as 0.5 mg trans-resveratrol/kg body weight.

Subjects remained at the Human Nutrition Unit of the Max Rubner-Institut (Karlsruhe, Germany) during the 24 h of the intervention. The intervention period started with a single oral ingestion of a hard gelatin capsule containing the colloid formulation with a glass of water and a standardized breakfast. Urine was collected before (spot urine) and 0–24 h after the intake of the colloid formulation. Urine samples were collected on ice in dark bottles. The volume of urine collected was measured, and aliquots were stored at −80°C until further analysis.

Sample clean-up for LC analyses

A 200-μL sample of the supernatant fluid, obtained in fermentation experiments with human fecal suspensions, was extracted 3 times with 500 μL ethyl acetate containing 5% isopropanol and 5% 1-butanol with the use of trans-piceatannol as internal standard at a final concentration of 20 μmol/L. The combined organic layers that were extracted were dried under nitrogen and redissolved in 200 μL of 0.1% aqueous formic acid/acetonitrile/methanol (90:5:5, by vol). After membrane filtration (polytetrafluoroethylene, 0.2 μm), the samples were analyzed by LC–ultraviolet/visible (UV/Vis) and LC–mass spectrometry (MS).

Urinary resveratrol and its metabolites were analyzed after enzymatic hydrolysis of the conjugates with a mixture of β-glucuronidase and sulfatase and extraction as follows: 500 μL...
of the urine sample was diluted with 330 μL sodium acetate buffer (0.1 mol/L; pH 5.0). After the addition of 10 μL internal standard solution (20 μmol/L [13C6]resveratrol), 80 μL β-glucuronidase (25 U/μL), and 80 μL sulfatase (1.875 U/μL), the samples were incubated for 90 min at 37°C and were subsequently cooled on crushed ice. Further clean-up was performed by solid-phase extraction on Strata X cartridges (Phenomenex) as follows: solid-phase extraction columns were preconditioned with 1 mL methanol and 1 mL of 0.1% formic acid. The hydrolysate was loaded and the column was washed with 2 mL of 0.1% formic acid and 2 mL of 5% methanol. Finally, resveratrol and its metabolites were eluted with 2 mL methanol. The eluate was dried under a stream of nitrogen and was reconstituted with 100 μL of 0.1% aqueous formic acid/acetonic/methanol (90:5:5, by vol). After membrane filtration (polytetrafluoroethylene, 0.2 μm), the samples were analyzed by LC-UV/Vis and LC-MS.

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

Chromatography was carried out on a Phenomenex C18 Kinetex column (100 × 4.6 mm, particle size 2.6 μm) with a 4.6-mm internal diameter C18 SecurityGuard ULTRA (Phenomenex) by using 0.1% formic acid and methanol/acetone (1:1, by vol) as eluents A and B, respectively. The binary gradient was run at a flow rate of 0.6 mL/min as follows: 0–2 min isocratic at 15% B, 2–11 min from 15% to 23% B, 11–20.5 min isocratic at 23% B, 20.5–45 min from 23% to 100% B, 45–48 min isocratic at 100% B, 48–52 min from 100% to 15% B, and 52–60 min isocratic with initial conditions.

The LC analyses were performed on 2 systems. The first system was a QTrap 5500 mass spectrometer (AB Sciex) equipped with an electrospray ionization (ESI) source and an LC system (Shimadzu), which consisted of a controller (CBM-20A), a degasser (DGU-20A5), 2 pumps (LC-30AD), an autosampler (1290 Sampler G4226A), a column oven (1290 TCC G1316C), and a DAD (1290 DAD G4226A), a column oven (1290 TCC G1316C), and a DAD (1290 DAD G4212A). The LC-MS system was controlled by using the software program Analyst TF 1.5.1. HPLC conditions were the same as described above. The DuoSpray source was operated in negative ESI mode using the following source parameters: curtain gas, 50 psi; ion spray voltage floating, −4500 V; ion source gas-1, 70 psi; ion source gas-2, 70 psi; and temperature, 600°C. The declustering potential was −80 V. The TOF-MS experiments were performed with a scan range from m/z 50 to m/z 1000, an accumulation time of 250 ms, and a collision energy voltage of −10 V. The TOF-MS/MS experiments were done with the use of a collision energy voltage of −40 V, a collision energy spread of 10 V, and an accumulation time of 150 ms. Nitrogen was used as collision gas. LC-TOF-MS data were analyzed by using the software Peak View 1.1.1 (AB Sciex).

In fecal cultures, resveratrol and microbial metabolites were quantified by ultraviolet detection on the basis of calibration curves obtained with trans-resveratrol (3.34–214 μmol/L) and dihydroresveratrol (3.41–218 μmol/L) measured at 307 and 280 nm, respectively, by using trans-piceatannol as internal standard. Because of the lack of reference compounds, lunularin and 3,4′-dihydroxy-trans-stilbene concentrations were measured semiquantitatively by using calibration curves of dihydroresveratrol and trans-resveratrol, respectively. Lunularin and dihydroresveratrol show similar ultraviolet spectra (data not shown), and the same is true for 3,4′-dihydroxy-trans-stilbene and trans-resveratrol (data not shown), which is why this semiquantitative approach was deemed the best approximation possible.

In urine samples, trans-resveratrol and dihydroresveratrol were quantified with MS detection deploying automatically optimized multiple reaction monitoring transitions: for resveratrol, the first transition (quantifier) was m/z 227.0 – m/z 143.0, and the qualifier was m/z 227.0 – m/z 185.0. The quantifier for dihydroresveratrol was m/z 229.0 – m/z 123.1, and the qualifier was m/z 229.0 – m/z 91.1. For calibration, different concentrations of trans-resveratrol and dihydroresveratrol were added to 500 μL of an independent pool of blank urine from 4 female volunteers. Sample clean-up was carried out as described for the intervention study samples. Calibration curves were constructed from the peak area of the first multiple reaction monitoring transition compared with the concentration with linear least squares regression calculation. trans-[13C6]resveratrol was used as internal standard with m/z 233.0 – m/z 149.0 and m/z 233.0 – m/z 191.1 as quantifier and qualifier, respectively. Concentrations of 3,4′-dihydroxy-trans-stilbene and lunularin were measured with ultraviolet detection via an external matrix calibration with trans-resveratrol and dihydroresveratrol, respectively, which were added to the pool of blank urine.

**Isolation of lunularin and nuclear magnetic resonance analyses**

Upscaled fermentation batches (20 mL) using fecal sample A were carried out for 48 h as described above. Lunularin was
extracted by using an upscaled version of the analytic procedure described for the fecal culture samples and was purified by semipreparative HPLC for subsequent nuclear magnetic resonance (NMR) analysis. The LC system consisted of an L-6220 intelligent pump (Merek Hitachi), a CTO-10A column oven (Shimadzu), and an L-4250 UV/Vis detector (Merek Hitachi). Aliquots (up to 300 μL) of the extract were injected onto a Phenomenex C18 110A Gemini column (250 × 10 mm, particle size 5 μm), and 0.1% formic acid and methanol/acetonitrile (1:1, by vol) were used as eluents A and B, respectively. A binary gradient was run at 6 mL/min and 45°C as follows: 0–1 min from 15% to 20% B, 1–10 min from 20% to 25% B, 10–14 min from 23% to 25% B, 14–17 min from 25% to 27% B, 17–21 min from 27% to 32% B, 21–24 min from 32% to 77% B, 24–28 min from 77% to 87% B, 28–31 min from 87% to 92% B, 31–35 min from 92% to 100% B, 35–40 min isocratic at 100% B, and 40–42 min from 100% to 15% B. Chromatograms were recorded at 280 nm by using the software KromaSystem 2000 version 1.80 (Bio-Tek Kontron Instruments). Several runs were performed, and the fraction containing lunularin was collected, pooled, and dried under nitrogen. Before NMR analysis, the sample was dried again by using a high-vacuum line.

NMR experiments were carried out on a 700-MHz Avance spectrometer (Bruker) equipped with a 5-mm TXI (Triple Resonance Inverse) proton-enhanced cryoprobe. Structure identification was performed by using various 1- and 2-dimensional NMR experiments [1H, H,H correlation spectroscopy, heteronuclear single quantum coherence (HSQC), and heteronuclear multiple-bond correlation]. Carbon data were taken from the 2-dimensional experiments HSQC and heteronuclear multiple-bond correlation. Lunularin was measured in acetone-d6. Chemical shifts (δ) were referenced to the central solvent signals (δH 2.05 and δC 29.84 ppm) (28); lunularin (M3; 3,4'-dihydroxybibenzyl): H2, 6.69 ppm [1H, broad doublet (bd), J = 1.9 Hz]; H4, 6.64 ppm [1H, doublet of doublet (dd), J = 8.1 Hz, 1.9 Hz]; H5, 7.07 ppm [1H, dd (pseudotriplet), J = 8.1 Hz, 7.8 Hz]; H6, 6.68 ppm (1H, bd, J = 7.8 Hz); H7, 2.78 ppm (from HSQC); H8, 2.78 ppm (from HSQC); H2′/H6′, 7.04 ppm (2H, bd, J = 8.4 Hz); H3′/H5′, 6.73 ppm (2H, bd, J = 8.4 Hz); C1, 144.2 ppm; C2, 115.9 ppm; C3, 158.2 ppm; C4, 113.3 ppm; C5, 129.8 ppm; C6, 120.2 ppm; C7, 38.7 ppm; C8, 37.4 ppm; C1′, 133.5 ppm; C2′/C6′, 129.9 ppm; C3′/C5′, 115.6 ppm; and C4′, 156.3 ppm. Chemical shifts for H7 and H8 were taken from the HSQC experiment because of an impurity (water) that interfered with those signals.

Isolation of DNA from feces samples and 454 high-throughput pyrosequencing of 16S rRNA genes

Bacterial DNA was isolated from 3 selected fecal samples by using a modified RBB+C (repeated bead beating plus column) method as described previously (29). Only 3 fecal samples were analyzed, because these stemmed from 3 volunteers who had different routes of trans-resveratrol metabolism. Because of the known large differences in composition of the gastrointestinal microbiota between individuals, this reductionist approach was used to determine whether differences observed in the routes of trans-resveratrol metabolism could be linked to specific differences in the microbiota composition of these individuals. DNA quality was confirmed by running a 2-μL aliquot of DNA solution on an 0.8% agarose gel for 1 h at 100 V. Isolated DNA samples were kept at −20°C until further analysis.

Microbial composition and diversity were characterized by polymerase chain reaction (PCR) amplification of the V3 and V4 variable regions of the bacterial 16S rRNA genes by using the 347F and 803R primers described by Nossa et al (30) (Table 2) followed by 454 high-throughput pyrosequencing. Forward and reverse primers were tagged with unique 4-mer barcode labels (Table 2) at the 5′ end to allow multiple samples to be included on one sequencing plate. An individual primer set was used for each of the volunteer’s fecal sample (347-F01 + 803-R-01, 347-F03 + 803-R-03, and 347-F04 and 803-R-04, respectively). The PCR was carried out in a final volume of 50 μL containing 2.5 μL of each primer (10 pmol/μL) and 25 μL of master mix, which included Phusion polymerase (New England Biolabs) and 10 μL template DNA (10 ng/μL) in milliQ water. PCR conditions were as follows: an initial cycle of 180 s at 94°C, followed by 32 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 45 s and a final elongation step at 72°C for 300 s.

Six PCR batches were run for each fecal sample. 16S rRNA gene PCR products were pooled and purified for sequencing by phenol chloroform extraction and ethanol precipitation by standard methods (31). The sample was resuspended in 45 μL and run on a 0.8% agarose gel to extract a single 16S rRNA gene PCR product band. The QIAEX II Gel Extraction Kit (Qiagen) was used to extract DNA from agarose gel. Sequencing of the 16S rRNA genes was performed on a 454 GS FLX Titanium Pyrosequencing System (Roche) at GATC Biotech (Konstanz, Germany). Pyrosequencing data were analyzed by using the metagenomic analysis server MG-Rast (http://metagenomics.anl.gov). Sequences that failed quality-control standards were excluded.

Isolation of RNA from fecal cultures and reverse transcription into cDNA

Quantitative PCR was used to enumerate bacteria in fecal cultures. To enumerate viable bacteria only, cDNA instead of DNA was used for quantitative real-time PCR (qRT-PCR). RNA was isolated from fecal cultures in the fermentation experiments with trans-resveratrol described above by using the Illustra RNAspin Mini Isolation Kit (GE Health Care). The presence of contaminating genomic DNA was detected after RNAse digestion of the RNA sample followed by amplification of the 16S rRNA housekeeping gene by using 16Sfw and 16Srev primers (5′-TCA TGA TTT ACA TTT GAG TG-3′ and 5′-GAC CAT GGC GTC CAA GTT GTT-3′), respectively. Therefore, the DNA digestion was repeated with DNase from the DNA-free kit (Ambion; Life Technologies). RNA quality was confirmed by high-throughput sequencing of 16S rRNA genes of fecal microbiota from 3 human volunteers.

TABLE 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence7</th>
<th>Bar code sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>347-F-01</td>
<td>5′-GGAGGCACTTCRGGAT-3′</td>
<td>5′-AGAC-3′</td>
</tr>
<tr>
<td>803-R-01</td>
<td>5′-CTACCRGGTGATCAATCC-3′</td>
<td>5′-ACAG-3′</td>
</tr>
<tr>
<td>347-F-03</td>
<td>5′-GGAGGCACTTCRGGAT-3′</td>
<td>5′-ACAG-3′</td>
</tr>
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<td>803-R-03</td>
<td>5′-CTACCRGGTGATCAATCC-3′</td>
<td>5′-ACAG-3′</td>
</tr>
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<td>347-F-04</td>
<td>5′-GGAGGCACTTCRGGAT-3′</td>
<td>5′-AAGT-3′</td>
</tr>
<tr>
<td>803-R-04</td>
<td>5′-CTACCRGGTGATCAATCC-3′</td>
<td>5′-AAGT-3′</td>
</tr>
</tbody>
</table>

7 From Nossa et al (30); R = A or G. rRNA, ribosomal RNA.
running a 5-μL aliquot of RNA solution and 10 μL RNA sample loading buffer on a 1.5% agarose gel for 1 h at 100 V.

RNA was transcribed into cDNA by using reverse transcriptase from the iScript cDNA Synthesis Kit (Bio-Rad Laboratory GmbH). Transcription was carried out in a final volume of 50 μL containing 10 μL of 5× iScript reaction mix, 25 μL iScript reverse transcriptase, and 5 μL RNA template in nuclease free water. cDNA synthesis conditions were as follows: 5 min at 25°C, followed by 30 min at 42°C and 5 min at 85°C.

qRT-PCR of bacterial groups in fecal cultures

cDNA from dominant groups of fecal bacteria was quantified with qRT-PCR by using iQ SYBR Green Supermix (Bio-Rad) and primers as summarized in Table 3. qRT-PCR assays were performed in 96-well plates on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). A cDNA template of each fecal sample was analyzed in triplicate. Each qRT-PCR assay was carried out in a final volume of 20 μL containing 10 μL iQ SYBR Green Supermix, 0.2 μL of each primer (10 pmol/μL), and 3 μL cDNA template in nuclease free water. PCR conditions were as follows: an initial 3 min at 95°C, followed by 39 cycles of 95°C for 10 s, 20 s at primer-specific annealing temperatures (Table 3), and 20 s at 72°C. Standard curves were determined with DNA isolated from selected bacterial strains: DNA from Lactobacillus plantarum, Lactobacillus fermentum, and Lactobacillus gasseri strains used for Lactobacillaceae; DNA from E. faecalis was used for Enterococcaceae; DNA from B. bifidum was used for Bifidobacteriaceae; and DNA from E. coli was used for Enterobacteriaceae. For Firmicutes and Bacteroidetes, DNA from Bacillus subtilis and Bacteroides uniformis was used, respectively. DNA was isolated by using the method described by Pitcher et al (38).

Incubation of trans-resveratrol with human intestinal bacteria

The various bacterial strains obtained from culture collections or isolated from human feces and identified by 16S rRNA gene sequencing (Table 1) were used in in vitro experiments to investigate their capacity to metabolize trans-resveratrol. trans-Resveratrol (23 μmol/L) was incubated with the intestinal bacterial strains in 9-mL cultures at 37°C by using the specific culture media used for the different stains indicated above. One-milliliter samples were collected after 0, 48, 72, and 96 h and centrifuged for 10 min at 13,000 × g, and supernatant fluid was kept at −20°C until LC analysis as described above. As negative controls, uninoculated media either with or without trans-resveratrol and inoculated media without resveratrol were incubated under the same conditions.

RESULTS

In vitro metabolism of trans-resveratrol by human fecal microbiota

In vitro experiments were conducted with fecal cultures from 7 volunteers, all of whom metabolized trans-resveratrol. Although the rate of metabolism differed between fecal cultures, trans-resveratrol was completely degraded after 2 to 24 h of fermentation (Figure 1, A–D). In most 0-h samples of the fecal cultures, trans-resveratrol recoveries were observed to be lower than the 80 μmol/L applied, which may be due to resveratrol adsorption to bacteria or other feces constituents. The extent of this effect was dependent on the feces samples used. However, after 48 h of fermentation the total metabolite concentration either reached or was close to the 80 μmol/L, which indicated that the amount of trans-resveratrol subjected to the culture was either fully or largely recovered in the form of metabolites. Overall, 3 compounds, marked as M1, M2, and M3, were detected by LC-UV/Vis (Figure 2, A and B) that were absent in control samples without trans-resveratrol and were therefore identified as microbial metabolites of the stilbene. All of them eluted later than trans-resveratrol, which indicated its conversion to more hydrophobic compounds. One of these was identified as the previously reported (17, 18, 20) colonic resveratrol metabolite dihydroresveratrol (M1; Figure 2A), as was confirmed by using a reference compound. In addition, 2 unknown metabolites were detected at 29.9 and 30.2 min, respectively, and were designated as M2 and M3 (Figure 2B).

TABLE 3

Table entries represent the percentage of the metabolites detected in the control samples without resveratrol and with trans-resveratrol and were therefore identified as microbial metabolites of the stilbene. All of them eluted later than trans-resveratrol, which indicated its conversion to more hydrophobic compounds. One of these was identified as the previously reported (17, 18, 20) colonic resveratrol metabolite dihydroresveratrol (M1; Figure 2A), as was confirmed by using a reference compound. In addition, 2 unknown metabolites were detected at 29.9 and 30.2 min, respectively, and were designated as M2 and M3 (Figure 2B).

Table 3

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Target group</th>
<th>Annealing temperature</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BifidgrpF</td>
<td>Bifidobacterium spp.</td>
<td>56°C</td>
<td>Rinttila et al, 2004 (32)</td>
</tr>
<tr>
<td>BifidgrpR</td>
<td>Bacteroidetes</td>
<td>60°C</td>
<td>Guo et al, 2008 (33)</td>
</tr>
<tr>
<td>Bact934F</td>
<td>Enterococcus spp.</td>
<td>54°C</td>
<td>Rinttila et al, 2004 (32)</td>
</tr>
<tr>
<td>Bact1060R</td>
<td>Enterobacteriaceae</td>
<td>54°C</td>
<td>Malinen et al, 2003 (34)</td>
</tr>
<tr>
<td>EntgrpF</td>
<td>Enterobacteriaceae</td>
<td>54°C</td>
<td>Malinen et al, 2003 (34)</td>
</tr>
<tr>
<td>EntgrpR</td>
<td>Enterobacteriaceae</td>
<td>54°C</td>
<td>Malinen et al, 2003 (34)</td>
</tr>
<tr>
<td>EmbaC</td>
<td>Eubacteria</td>
<td>54°C</td>
<td>Lee et al, 1996 (35)</td>
</tr>
<tr>
<td>EmbaR</td>
<td>Firmicutes</td>
<td>60°C</td>
<td>Armougom et al, 2009 (36)</td>
</tr>
<tr>
<td>FirmicateF</td>
<td>Lactobacillus (Weissella)</td>
<td>54°C</td>
<td>Walter et al, 2001 (37)</td>
</tr>
<tr>
<td>FirmicateR</td>
<td>Leuconostoc group</td>
<td>54°C</td>
<td>Walter et al, 2001 (37)</td>
</tr>
</tbody>
</table>

Y = C or T; R = A or G.
Dihydroresveratrol and compound M3 were major endproducts of fermentation, each produced in fecal cultures from 6 of the 7 volunteers, compound M2, if at all, was only found in small amounts and thus appeared to be a minor product or intermediate in the in vitro metabolism of trans-resveratrol by the human gut microbiota (Table 4).

Structural identification of M2 and M3 was carried out by means of ultraviolet spectra, MS and MS/MS experiments, and NMR spectroscopy. With maxima at 318, 305, and 245 nm, the ultraviolet spectrum of M2 resembled the trans-resveratrol spectrum (data not shown), which suggested a structurally related compound. The presence of the maximum at 318 nm furthermore indicated that the trans-ethylene bridge was conserved. More detailed structural information was obtained by HPLC-ESI-MS experiments conducted in negative mode. The quasi molecular ion [M-H]− m/z 211 of M2 showed a difference of −16 Da compared with trans-resveratrol (m/z 227) (Figure 3, A and B), which indicated the cleavage of a hydroxyl group by the fecal microbiota. This assumption was further strengthened by exact mass measurements applying HPLC coupled with high-resolution hybrid quadrupole/TOF-MS. Indeed, exact masses of the molecular ion [M-H]− and all fragment ions showed little error when compared with the theoretical values for the sum formula C14H11O2−. The mass error in MS mode was 4.0 ppm, and the intensity-weighted average error for the MS/MS data (19 ions) was 2.0 ppm. In addition, the measured isotope distribution of [M-H]− was in good agreement with the one calculated for C14H11O2− (data not shown). Further study of the MS/MS spectrum also strongly suggested M2 to be 3,4′-dihydroxy-trans-stilbene: characteristic fragment ions were m/z 169, m/z 143, m/z 117, m/z 119, m/z 93, and m/z 92 (Figure 3B).

In analogy with the negative ESI-MS/MS spectrum of trans-resveratrol (18, 39) (Figure 3A), the neutral loss of CH=COH (42 Da) from [M-H]− m/z 211 led to the fragment ion m/z 169. This anion further fragmented to yield the even electron anion m/z 143 via the neutral loss of CH=CH (26 Da). While trans-resveratrol characteristically shows a second neutral loss of CH=COH (18, 39), this could not be observed for M2, which

FIGURE 1. Conversion of trans-resveratrol during in vitro batch fermentation with human fecal suspensions. Human fecal cultures consisted of 5% human fecal suspensions in brain heart broth medium containing 80 μmol trans-resveratrol/L and were incubated for 48 h at 37°C under anaerobic conditions. The values shown are arithmetic means (±SD) of 2 independent experiments, each conducted in duplicate (see Collection of human fecal samples and Fermentation with human fecal cultures). Curves are shown only for metabolites that were detectable in a fecal culture. Values below the limit of quantitation are illustrated as 0 μmol/L. Incubations representing different routes of trans-resveratrol metabolism are shown. A: Lunularin (M3) production (fecal sample A). B: Dihydroresveratrol (M1) production (fecal sample F) and mixed production of dihydroresveratrol and lunularin with the 2 subgroups: dihydroresveratrol present as endproduct (fecal sample G) (C) and dihydroresveratrol produced as intermediate (fecal sample D) (D). 3,4′-Dihydroxy-trans-stilbene (M2) was detected only as a minor metabolite or intermediate in some samples. Fecal cultures B, C, and E also followed route 3 (data not shown). Because of the lack of reference compounds, lunularin and 3,4′-dihydroxy-trans-stilbene concentrations were measured semiquantitatively by using calibration curves of dihydroresveratrol and trans-resveratrol, respectively.
and the phenoxide anion (m/z 93), which have been described for molecules containing monohydroxylated phenolic units (40, 41).

In addition, acetylene and ethylene phenoxide anions (m/z 117 and m/z 119, respectively) were present, which are also known as characteristic fragments of trans-resveratrol (18). Finally, the commercially available reference compound pinosylvin (3,5-dihydroxystilbene), which equals C4'-dehydroxylated trans-resveratrol, showed a different chromatographic behavior than metabolite M2 when analyzed by HPLC (data not shown). This again indicates that M2 was formed by a dehydroxylation of the resorcin moiety of trans-resveratrol.

Whereas M2 was detected only as a minor metabolite in some samples, M3, when produced, was a major microbial transformation product. Its ultraviolet spectrum was similar to the spectrum of dihydroresveratrol, which suggests that the ethylene bridge had been reduced (data not shown). However, M3 eluted much later than dihydroresveratrol in reversed-phase HPLC; thus, it was more hydrophilic. In HPLC-ESI-MS analysis, the quasi-molecular ion [M-H]^- m/z 213 was detected (Figure 3C). This mass, along with its ultraviolet spectrum and retention behavior, strongly suggested that M3 results from a combined reduction of the ethylene carbon-carbon double bond and a dehydroxylation of trans-resveratrol by the fecal microbiota. Exact masses of [M-H]^- and its characteristic fragments determined by TOF-MS were in good agreement with the theoretical values for C14H13O2^-, showing ~0.5- and 2.8-ppm mass errors in the MS and MS/MS modes, respectively. The measured isotope pattern of [M-H]^- was also in good agreement with the theoretical distribution of C14H13O2^- (data not shown). Assuming the dehydroxylation again appeared at the resorcin moiety, M3 would be a molecule composed of 2 monohydroxylated phenolic units connected via an ethyl bridge. This was confirmed by the MS/MS spectrum of M3, which showed only 2 characteristic fragment ions, the odd electron ion m/z 106, and the even electron ion m/z 107 in almost equal intensities (Figure 3C). The fragmentation pattern can be described as a 2-step process: the heterolytic cleavage of the molecule across the ethyl bridge and the neutral loss of C,H,O (106 Da) leading to the methyl phenoxide ion m/z 107, followed by a radical β-elimination of H- resulting in the distonic methyl phenoxide radical ion m/z

![FIGURE 2. Exemplary liquid chromatography/ultraviolet chromatograms of in vitro fecal cultures with 80 μmol trans-resveratrol/L. The chromatograms obtained after 0 h (gray line) and 8 h (black line) of fermentation with fecal cultures F (A) and A (B) are shown. Metabolites produced are designated as M1 (dihydroresveratrol), M2 (trans-3,4'-dihydroxystilbene), and M3 (lunularin) according to their order of elution. Please note that chromatogram 2B is a snapshot taken after 8 h, which illustrates the formation of M2. Information on metabolite concentrations is given in Table 4.](image)

**Table 4**

Maximal concentration of metabolites detected within 48 h of fermentation of 80 μmol trans-resveratrol/L with human fecal samples

<table>
<thead>
<tr>
<th>Fecal sample</th>
<th>M1: dihydroresveratrol</th>
<th>M2: 3,4'-dihydroxy-trans-stilbene</th>
<th>M3: lunularin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ND</td>
<td>11.1 ± 2.2^4 (8 h)</td>
<td>79.8 ± 17.5 (48 h)</td>
</tr>
<tr>
<td>B</td>
<td>55.9 ± 4.9 (2 h)</td>
<td>ND</td>
<td>21.1 ± 5.2 (48 h)</td>
</tr>
<tr>
<td>C</td>
<td>14.2 ± 7.9 (6 h)</td>
<td>&lt;LOQ^4 (2–48 h)</td>
<td>59.9 ± 19.0 (24 h)</td>
</tr>
<tr>
<td>D</td>
<td>17.9 ± 6.6 (8 h)</td>
<td>&lt;LOQ (2–8 h)</td>
<td>57.7 ± 11.3 (48 h)</td>
</tr>
<tr>
<td>E</td>
<td>38.5 ± 23.4 (2 h)</td>
<td>&lt;LOQ (4–48 h)</td>
<td>58.3 ± 8.4 (8 h)</td>
</tr>
<tr>
<td>F</td>
<td>86.9 ± 32.1 (24 h)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G</td>
<td>78.9 ± 15.3 (2 h)</td>
<td>ND</td>
<td>38.3 ± 11.5 (48 h)</td>
</tr>
</tbody>
</table>

^1LOQ, limit of quantitation; ND, not detected.

^2Samples were collected after 0, 2, 4, 6, 8, 24, and 48 h and were analyzed by HPLC-ultraviolet/visible. Because of the lack of reference compounds, lunularin and 3,4'-dihydroxy-trans-stilbene concentrations were measured semiquantitatively by using calibration curves for dihydroresveratrol and trans-resveratrol, respectively.

^3Arithmetic mean (±SD) of 2 independent experiments each conducted in duplicate (all such values).

^4LOQ = 3.3 μmol/L.
106. The latter fragmentation has been shown for ortho-, meta-, and para-methylphenol (42). A neutral loss of 106 Da and a subsequent H· elimination can also be observed for dihydroresveratrol (Figure 3D). In sum, MS data indicated M3 to be 3,4'-dihydroxybibenzyl, which is also known as lunularin (43).
To confirm the structure proposed by MS/MS experiments, an upscaled batch fermentation using fecal sample A, was run for 48 h, and the major metabolite M3 was purified by preparative HPLC. Subsequent 1-dimensional and 2-dimensional NMR experiments confirmed the structure elucidated by the MS experiments.

Thus, comprehensive structural analyses performed in this study led to the identification of 3,4′-dihydroxy-trans-stilbene (M2) and lunularin (M3) as microbial metabolites (Figure 4). These compounds have, to the best of our knowledge, not been reported as resveratrol metabolites in the literature before.

Strong interindividual differences in fermentation patterns and in the metabolites produced were observed (Figure 1, A–D). The microbiota from 6 of 7 fecal cultures converted trans-resveratrol into dihydroresveratrol, which thus far has been the only known resveratrol metabolite produced by gut bacteria (17, 18, 20). The same number of fecal cultures produced the newly identified metabolite lunularin (Table 4). In view of the major metabolites produced, microbial trans-resveratrol metabolism can thus proceed by 1 of 3 different routes: 1) lunularin production (Figure 1A), 2) dihydroresveratrol production (Figure 1B), and 3) mixed production of dihydroresveratrol and lunularin. The latter may be further divided into 2 subgroups: dihydroresveratrol present as endproduct (Figure 1C) and dihydroresveratrol produced as intermediate (Figure 1D). The metabolic fate of trans-resveratrol in 1 of 7 fecal cultures followed route 1, in another it followed route 2, whereas in the remaining 5 it followed route 3. The newly identified minor metabolite 3,4′-dihydroxy-trans-stilbene was observed to occur in 4 fecal cultures, but only fecal culture A had amounts above the limit of quantification (Table 4). Interestingly, none of the cultures producing 3,4′-dihydroxy-trans-stilbene contained dihydroresveratrol as an endproduct.

**Bacterial diversity of human feces samples used in this study**

Fecal samples from 3 of the volunteers who donated feces for the investigation of the metabolic fate of trans-resveratrol in vitro (see Collection of human fecal samples) were also analyzed for their bacterial diversity by high-throughput 16S rRNA gene sequencing. Samples included one lunularin producer (fecal sample A; Figure 5A), one mixed dihydroresveratrol/lunularin producer (fecal sample G; Figure 5B), and one dihydroresveratrol producer (fecal sample F; Figure 5C). Data on bacterial diversity in the feces by this metagenomics approach were analyzed at the phylum, family, and genus levels. Similar to previous reports (44–46), *Firmicutes* (53.7–72.4%) and *Bacteroidetes* (11.3–18.4%) were the most dominant phyla in the fecal samples analyzed in this study. The bacterial diversity in fecal sample A was noticeably different from that of samples F and G, which showed a lower relative abundance of sequences from *Firmicutes* and higher abundances of *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, and *Cyanobacteria*, the latter 2 of which each accounted for only <1% of the sequences in fecal samples F and G. Furthermore, the microbiota from fecal sample A showed a higher incidence in numbers of *Enterobacteriaceae* (6.5%) when compared with fecal samples F and G (3.6% and 2.8%, respectively; data not shown). It is interesting to note that the microbial consortium from fecal sample A was the only one that did not produce any dihydroresveratrol from trans-resveratrol in vitro.

*Bacteroidetes* comprised mainly (between 82 and 93%) *Bacteroides* spp. in all fecal samples, with the microbiota of fecal sample A showing considerably higher percentages of *Bacteroides* spp. than samples F and G (16.7% compared with 9.7–10.6%). *Actinobacteria* were mainly represented by *Bifidobacterium* spp. and were present in much higher proportions in fecal sample A than in the other 2 samples (7.6% compared with 0.3–1.3%). In addition, the microbiota of fecal sample A also showed a higher incidence of bacteria of the *Coriobacteriaceae* family (0.36% compared with 0.13% and 0.05% in samples F and G, respectively), which together with the *Bifidobacteriaceae* family belongs to the phylum *Actinobacteria* (data not shown).

Interindividual differences in fecal microbiota were also analyzed by qRT-PCR (Figure 6, A–C) by using primers (Table 3) that target the main bacterial groups, ie, *Firmicutes*, *Bacteroidetes*, lactobacilli, enterobacteria, bifidobacteria, and enterococci. qRT-PCR enumeration of all *Eubacteria* showed that bacteria were present in fecal samples from all 3 volunteers at ~1 × 10^{11} to 1 × 10^{12} colony forming units (CFU)/g, which is consistent with other reports on the numbers of eubacteria in

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**FIGURE 4.** Structures of trans-resveratrol and metabolites formed from trans-resveratrol by human gut microbiota.
Human feces (44, 45). Firmicutes and Bacteroidetes were the most numerous bacteria present at concentrations $>1 \times 10^{10}$ CFU/g, which agrees well with the metagenomics data, indicating that these bacteria clearly represent the majority of sequences. Fecal sample A showed $\sim 1 \times 10^8$ CFU/g enterobacteria (Figure 6A), which was $\sim 1$ log higher than enterobacterial counts for fecal samples G and F (Figure 6, B and C, respectively); this was confirmed by the metagenomics data.

**FIGURE 5.** Bacterial diversity in selected fecal samples as determined by 454 high-throughput pyrosequencing of the bacterial 16S rRNA gene. Microbial composition was characterized by amplification of V3 and V4 variable regions of 16S rRNA genes by polymerase chain reaction using the 347F and 803R primers described by Nossa et al (30) followed by 454 high-throughput pyrosequencing. Relative abundances of sequences are illustrated on the phylum and genus levels. A: Fecal sample A (lunularin producer); B: fecal sample G (mixed lunularin/dihydroresveratrol producer); C: fecal sample F (dihydroresveratrol producer). rRNA, ribosomal RNA.
which showed that sample A contained ~6% entrobacterial sequences that were higher than the ~3% of sequences in fecal samples F and G (data not shown). Bifidobacteria occurred at ~1 × 10^9 CFU/g in fecal samples A and G and were noticeably lower in sample F, which contained only ~1 × 10^7 CFU/g. Fecal sample F also contained a noticeably lower concentration of lactobacilli (~1 × 10^8 CFU/g) than did fecal samples G and A (~1 × 10^7 and 1 × 10^6 CFU/g, respectively). A corresponding difference in the incidence of 16S rRNA gene sequences of bacteria belonging to this family could, however, not be determined from the metagenomics sequence analysis, in which *Lactobacillaceae* occurred at incidences from 1.1% (fecal sample G) to 3.2% (fecal sample F) and 3.3% (fecal sample A).

In vivo metabolism of *trans*-resveratrol by human gut microbiota

A separate in vivo study was conducted with 12 healthy volunteers to confirm the production of the 3 microbial metabolites detected in vitro. Because dihydroresveratrol had already been identified as a microbial *trans*-resveratrol metabolite in humans, we were particularly interested in lunularin and 3,4′-dihydroxy-*trans*-stilbene as potential metabolites in vivo.

In our study, volunteers were given an oral dose of 0.5 mg *trans*-resveratrol/kg body weight in the form of a grapevine-shoot extract. Within the following 24 h, 19.6–41.1% of the *trans*-resveratrol administered was recovered in urine samples as *trans*-resveratrol (following β-glucuronidase and sulfatase treatment), which showed that a significant amount of the stilbene was absorbed before transformation by intestinal bacteria (Figure 7). However, a substantial proportion (8.1–62.7%) of the *trans*-resveratrol was metabolized by the gastrointestinal microbiota. All 3 microbial metabolites found in the in vitro model were also detected in the urine samples, the 2 major metabolites being dihydroresveratrol and lunularin, which was in good agreement with the in vitro results. Whereas dihydroresveratrol was a main metabolite in 9 humans, lunularin was a main microbial conversion product in 3 of the volunteers. In addition, 3,4′-dihydroxy-*trans*-stilbene was detected in urine samples of 3 humans, all of whom also produced lunularin. One volunteer produced both lunularin and dihydroresveratrol as main metabolites, whereas another one only produced low amounts of each and a slightly higher amount of 3,4′-dihydroxy-*trans*-stilbene. Thus, we could not only confirm the relevance of the 2 new metabolites in vivo but also demonstrate that the interindividual routes of microbial *trans*-resveratrol conversion described above held true under in vivo conditions: 1) lunularin production, 2) dihydroresveratrol production, and 3) mixed production of dihydroresveratrol and lunularin (Figure 7). Compared with the in vitro results, 3,4′-dihydroxy-*trans*-stilbene, when produced, was found in slightly higher proportions, which might be explained by the limitation of further metabolism by gut microbiota because of its absorption by the intestinal epithelium. Overall, the extent of urinary excretion (with and without microbial metabolism) of *trans*-resveratrol ranged from 36.3% to 82.9% of the oral dose given, which indicated pronounced interindividual differences.

Human intestinal bacteria strains capable of metabolizing *trans*-resveratrol

Selected fecal bacterial strains (Table 1) were tested for their ability to metabolize *trans*-resveratrol when grown in pure culture for up to 96 h. *E. faecium*, *F. magnus*, *L. salivarius*, *P. harei*, *B. pseudocatenulatum*, *B. vulgatus*, *B. ovatus*, *C. scindens*, *E. lenta* strains, and *E. coli* were each isolated from the human fecal samples used in this study. In addition, *S. equolifaciens*, *S. isoflavoniconvertens*, *B. plebeius*, *C. scindens*, *E. rectale*, *F. prausnitzii*, and *A. equolifaciens* were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, whereas *L. garvieae*, *B. animalis*, *B. infants*, *B. bifidum*, and *B. longum* were strains obtained from the MRI culture collection or isolated from a commercial probiotic preparation. Of these, only *S. equolifaciens* and *A. equolifaciens* were able to metabolize *trans*-resveratrol to dihydroresveratrol as determined by LC-UV/Vis; no other resveratrol metabolites could be detected.
However, _A. equolifaciens_ produced dihydroresveratrol only in trace amounts, whereas _S. equolifaciens_ fully converted trans-resveratrol within 96 h of fermentation (data not shown).

**DISCUSSION**

This study presented a comprehensive investigation of the role of human intestinal microbiota and its diversity in resveratrol metabolism combining in vivo and in vitro experiments. Our data show that gut bacteria readily convert trans-resveratrol and therefore largely contribute to its metabolic fate in humans: after administering an oral dose of a colloid formulation, 8.1–62.7% of the resveratrol given was recovered in urine as microbial metabolites. The overall recovery of resveratrol and its metabolites in 24-h urine ranged from 36.3% to 82.9% of the oral dose, which is in good agreement with the results of Walle et al (17), who reported that 53.4–84.9% of the radioactivity was recovered in urine after the intake of single oral doses of $[^{14}C]$ resveratrol.

This study yet again demonstrated how the gut microbiota can have a great effect on the bioconversion of dietary constituents. Whereas in some humans the main microbial product formed was the previously reported dihydroresveratrol (17, 18), it was the newly identified lunularin, or a combination of both, in others. A similar effect of interindividual differences is known for the metabolism of daidzein, whereas, in some humans, S(–)-equol is the main microbial metabolite, O-demethyldaidzein or dihydrodaidzein is the main metabolite in others (25, 27). The different resveratrol metabolites identified in this study may have physiologic effects that are significantly different from those of resveratrol but have yet to be elucidated. S-Equol shows biologic properties that exceed those of the parent daidzein, such as a higher binding affinity to the estrogen receptor $\beta$ (46) and the ability to bind $5\alpha$-dihydrotestosterone (47). The formation of equol is therefore believed to play a key role in the health benefits associated with isoflavone intake (46, 47).

The different resveratrol metabolites identified in this study may also have physiologic effects that are significantly different from those of resveratrol but have yet to be elucidated. So far, dihydroresveratrol is described to stimulate the proliferation of the hormone-sensitive breast cancer cell line MCF-7 at concentrations in the picomolar range, at which trans-resveratrol is inactive (48). The interindividual differences of _trans_-resveratrol metabolism observed in our study was closely related to microbial diversity. Fecal sample A, which was the only one tested that did not produce any dihydroresveratrol from _trans_-resveratrol, was noticeably different from samples F and G, which indicates that the primers for qRT-PCR for the lactobacilli are not specific only for the genus _Lactobacillus_, but can also amplify other genera belonging to the family _Lactobacillaceae_, eg, _Weissella_ and _Leuconostoc_ (37). For the other main bacterial groups, however, the metagenomics data agreed well with the quantitative PCR.

Actinobacteria were mainly represented by _Bifidobacterium_ spp. and were also found in noticeably higher proportions in fecal sample A than in the other 2 samples (7.6% compared with 0.3–1.3%). However, in pure culture, none of the _Bifidobacteria_ spp. tested were able to metabolize _trans_-resveratrol.

To the best of our knowledge this is the first report of 2 new resveratrol metabolites produced by intestinal bacteria, namely lunularin and 3,4‘-dihydroxy-trans-stilbene. However, the human gut microbiota possesses an impressive array of enzymes to use and convert dietary constituents, and reduction and dehydroxylation reactions are not uncommon. Dihydroresveratrol has already been described as a resveratrol metabolite (17, 18, 20), and the reduction of the carbon-carbon double bond by gut microbiota has also been shown for other dietary compounds such as isoflavones (23) or hydroxycinnamates (49). Cleavage of hydroxyl groups, for example, plays a role in the microbial transformation of compounds such as lignans, phenolic acids, and bile acids (49–53). It is important to note that the dehydroxylation of resveratrol, as shown in our study, occurred at the resorcin moiety, ie, 1 of 2 _meta_-positioned hydroxyl groups was eliminated. To our knowledge, dehydroxylation has thus far been observed only for monohydroxyl- or for _ortho_-dihydroxy phenolic moieties, eg, during transformation of ferulic acids or lignans with human intestinal bacteria (49, 51, 52, 54).

In this study, we screened a range of gut bacteria for their ability to metabolize resveratrol in pure culture, some of which are known to metabolize other phenolics (55–57). Among the tested bacteria, only _S. equolifaciens_ and _A. equolifaciens_, which both belong to _Coriobacteriaceae_, produced dihydroresveratrol. This finding is in good agreement with a study by Jung et al (21) who identified _E. lenta_ ATCC 43055, which also belongs to _Coriobacteriaceae_ as a dihydroresveratrol producer. It thus appears that genera of this family are notably involved in _trans_-resveratrol metabolism. The production of dihydroresveratrol by _S. equolifaciens_ and _A. equolifaciens_ has not been reported thus far. However, Jung et al (21) further showed that _B. uniformis_ ATCC 8492 is able to produce dihydroresveratrol (21), and this strain, and possibly other _Bacteroides_ spp., may also play a role in the bacterial metabolism of resveratrol.

Although _E. lenta_ species are also known for lignan metabolism, which includes dehydroxylation of 2,3-bis(3,4-dihydroxybenzyl)butene-1,4-diol derived from secoisolariciresinol and resulting in enterodiol (50, 51, 58), we could not demonstrate their ability to dehydroxylate _trans_-resveratrol in anaerobic incubation experiments using 3 different _E. lenta_ strains (Table 1) (data not shown). _trans_-Resveratrol contains _meta_-instead of _ortho_-positioned hydroxyl groups, which are present in polyphenols that have been shown to be dehydroxylated by gut bacteria, eg, 2,3-bis(3,4-dihydroxybenzyl)butene-1,4-diol in lignan metabolism. More studies are needed to identify bacterial strains involved in the formation of dehydroxylated metabolites. Our results indicate that lunularin may be formed in 2 different ways: 1) via dehydroxylation of the intermediate dihydroresveratrol or 2) via reduction of the intermediate 3,4‘-dihydroxy-trans-stilbene. Both routes may be performed by individual microorganisms and/or they rely on the presence of a diversity of bacteria, eg, _coriobacteria_ and other yet unidentified bacteria, which need further investigation.

In conclusion, we identified 3 microbial _trans_-resveratrol metabolites, 2 of which were previously unknown and we also
identified 2 intestinal bacterial species that were previously not known to produce dihydroresveratrol. In addition, we showed that there can be pronounced interindividual differences in resveratrol conversion by human gut microbiota. Our intervention study showed that all of the bacterial metabolites identified in vitro can be absorbed in the gut and are excreted in urine, the extent of which, however, varies interindividually. There was high agreement between our in vitro and in vivo experiments, even though the results were obtained with a different set of volunteers. Over the past decade there has been enormous public and scientific interest in resveratrol because of its proposed health-promoting effects, which, nonetheless, are far from being fully understood. Future studies should take dihydroresveratrol and the newly identified dehydroxylated metabolites into account when investigating resveratrol absorption and metabolism as well as their physiologic effects in humans.

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