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Degradation and Metabolism of Catechin, Epigallocatechin-3-gallate (EGCG), and Related Compounds by the Intestinal Microbiota in the Pig Cecum Model

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As intestinal metabolism strongly influences the bioavailability of flavonoids, this study investigated the microbial deconjugation and degradation of the most common flavan-3-ols using the pig cecum in vitro model system developed in the authors' group. The microbial degradation of (+)-/(−)-catechin, (−)-epicatechin, (−)-gallocatechin, (−)-epigallocatechin, (−)-gallocatechin gallate, (−)-epigallocatechin gallate, procyanidin B2, and gallic acid was investigated under anaerobic physiological conditions in single incubation experiments and as a mixture. Incubation was done with the microbiota from three different animals in repeat determinations (n = 6). The flavan-3-ols under study were almost completely metabolized by the intestinal microbiota within 4−8 h. No difference was observed for catechin enantiomers. In addition to monomeric flavonoids, procyanidins are also metabolized by the intestinal microbiota as shown for procyanidin B2. The arising hydroxylated phenolcarboxylic acids are similar for all tested substances. These small phenolic degradation products might be responsible for the observed antioxidative activities described in the literature.

KEYWORDS: Flavonoids; flavan-3-ols; catechin; EGCG; procyanidin B2; degradation; gastrointestinal tract; tea; microbiota; intestinal metabolism; pig cecum; bioavailability

INTRODUCTION

Flavonoids are secondary plant metabolites mainly found in higher plants, for example, fruits, vegetables, or tea (*1*). Data about the daily intake of flavonoids vary from 20 mg/day to 1 g/day (*2*−*4*) depending on different dietary habits. Flavan-3-ols mainly occur as free aglycones, as galloyl esters or as oligomers, which makes them unique in the group of flavonoids (*5*). Many studies have shown that flavonoids are potent antioxidants and radical scavengers in different model systems (*6*). Due to these properties it is assumed that they have protective effects against cardiovascular diseases and certain forms of cancer (*7*). Requirement for these physiological effects, except from some local effects, would be the absorption of the flavonoids in the gastrointestinal tract. Several studies indicate that flavan-3-ols are partially absorbed in the ileum but are subject to microbial metabolism at the same time, especially if more complex flavonoids are present (*8*−*11*). Recent studies have investigated the microbial degradation of flavonoids in the large intestine using single bacteria species (*12*, *13*), mixed culture models (*14*), human stool samples (*15*), or pig inoculum (*16*, *17*). The latter ex vivo model developed in our group is based on the intestinal microbiota isolated from the cecum of freshly slaughtered pigs. This pig cecum model has been described in detail in the literature (*16*, *17*). Furthermore, studies using fluorescence in situ hybridization (FISH) revealed that the composition of the pig cecum microbiota is similar to that of humans for most bacterial species (*18*).

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In animal studies an extensive metabolism of flavonoids was reported, especially methylation and ring scissions (*19*, *20*). In comparison with in vitro systems with one or only a few bacterial species, the importance of a versatile mixture of microorganisms became clear. However, in most animal studies, fecal samples, which would be good sources for data on the metabolism of microbiota, have not been analyzed (*21*, *22*). Between 8% (mol/mol) (*10*) and 40% (mol/mol) (*9*) of unmetabolized flavan-3-ol monomers and metabolites are excreted with the urine within 24 h, and no accumulation could be proven in any organ or tissue (*8*). Therefore, the fate of at least 60% (mol/mol) is not considered and was our impulse for research. The aim of the present study was to investigate the microbial degradation of flavan-3-ols with different stereochemistries, hydroxylation patterns, and types of bound moieties (see **Figure 1**). Special attention was paid to arising metabolites. For this the intestinal degradation and metabolism of (+)-catechin, (−)-catechin, DL-catechin, (−)-epicatechin, (−)-gallocatechin, (−)-epigallocatechin, (−)-gallocatechin gallate, (−)-epigallocatechin gallate, procyanidin B2, and gallic acid was investigated using the pig cecum model. These compounds represent the most common flavan-3-ols and derivatives in food (*3*, *23*). The compounds were studied in single incubation experiments as well as in a mixture with other glycosides of the flavonol and flavone type.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased in p.A. quality. 3,4-Dihydroxyphenylpropionic acid (3,4-hppa), 3- and 4-hydroxyphenylacetic acid (3-/4-hpaa), (−)-catechin, (−)-gallocatechin gallate ((−)-gcg), and (−)-epigallocatechin gallate ((−)-egcg) were purchased from Sigma-Aldrich

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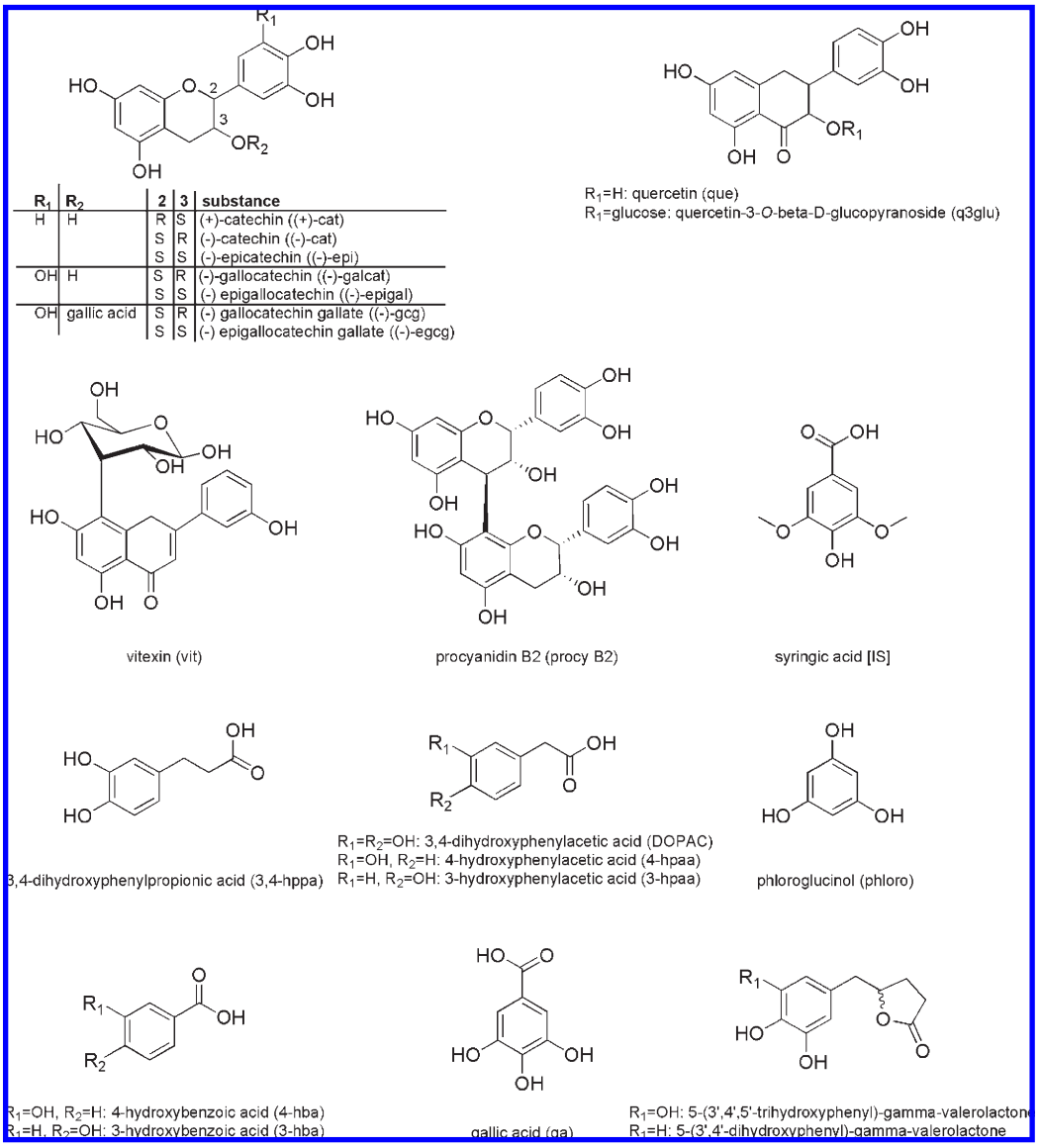

Figure 1. Chemical structures of the used flavonoids and their degradation products.

(Steinheim, Germany). DL-Catechin, (+)-catechin ((+)-cat), (−)-epicatechin ((−)-epi), (−)-gallocatechin ((−)-galcat), vitexin (vit), quercetin-3-*O*-*β*-D-glucopyranoside (q3glu), quercetin (que), and 3,4-dihydroxyphenylacetic acid (DOPAC) were provided from Roth (Karlsruhe, Germany). Gallic acid (ga) and *N*,*O*-bis(trimethylsilyl)acetamide (BSA) were obtained from Fluka (Buchs, Switzerland). Phloroglucinol (phloro) and 3- and 4-hydroxybenzoic acid (3-/4-hba) were ordered from Merck (Darmstadt, Germany). (−)-Epigallocatechin ((−)-epigal) was purchased from Extrasynthese (Genay, France). Procyanidin B2 (procy B2) was kindly provided by Prof. Dr. A. Hensel (Institute for Pharmaceutical Biology and Phytochemistry, University of Muenster).

All other chemicals were obtained, in p.A. quality, from Merck and Sigma-Aldrich. The water was demineralized by a Milli-Q Gradient A10-System (Millipore, Schwalbach, Germany).

Methods. *Stock Solutions*. Stock solutions (1 mM) of the analytes were prepared in methanol. Stock solutions of gallic acid were prepared in methanol but with different concentrations, given in the text.

Preparation of Inoculum. The pigs (German Landrace or Angler Sattel × Pietrain) from which the ceca were obtained were raised by biodynamic farming. They were 10−12 months old and weighed 120−150 kg. They were fed with a basal diet composed of rye, spelt, linseed, lentil, corn, millet, and rice enhanced with clover silage.

The ceca were ligated and culled during slaughtering and then handled under anaerobic conditions using an anaerobic jar containing Anaerocult A (Merck). Furthermore, all preparations and experiments with the inoculum were performed in an anaerobic chamber flushed with CO_2 to retain an anaerobic atmosphere. The inocula of each cecum were isolated and suspended in the same volume (v/v) 0.15 M PBS (pH 6.4) containing a trace element solution 0.0125% (v/v) (*16*). All solutions, buffers, and vessels were flushed with a mixture of N_2 and CO_2 (5:1; v/v) before use, which causes a pH shift of the buffer to 6.2. Removal of large particles from inoculum suspension was accomplished by filtration through a coarsely meshed net. Filtrates were used for the incubation experiments. An aliquot of inoculum was inactivated by sterilization at 121 °C for 15 min at 1.1 bar (AMB240 autoclave, Astell, Kent, U.K.) and treated like the active inoculum as described before. The sterilized fecal suspensions were used as control for chemical degradation and matrix effects. Likewise, reagent samples without fecal suspensions and blank samples with feces alone were examined. Each incubation experiment was performed in duplicate with three different ceca.

Inoculum suspension is used in microscale batches: 0.1 mL of the stock solution of the analytes was added to 0.9 mL active or sterilized inoculum suspension in 2 mL Eppendorf caps (Eppendorf, Hamburg, Germany). Aliquots of both sterilized and active inoculum suspensions were used as matrix blanks. The maximum methanol concentration in the incubation experiments reached 10% (v/v), which did not affect the bacterial viability. The sealed vials were placed in an incubator (37 °C) for 20 or 40 min or 1, 2, 4, or 8 h. The microbial metabolism was stopped by deep freezing the vessels at −80 °C.

Sample Preparation and Analysis

(*a*) *HPLC-DAD and -FLD Analyses*. The frozen samples were thawed quickly in a water bath. The samples were diluted immediately with an equal amount of methanol/hydrochloric acid (99:1; v/v) with or without 2% (w/v) sodium dodecyl sulfate (SDS) to prevent any further microbial degradation and to extract the analytes. After 15 min of ultrasonic treatment, the samples were centrifuged at 12000*g* for 15 min. Aliquots of the supernatant were used for HPLC analysis. The compounds were separated on an analytical Eurospher 100 column (250 × 4.6 mm i.d., 5 μm; Knauer, Berlin, Germany) using a binary gradient generated by an Agilent/HP 1100 low-pressure gradient HPLC pump (G1311A) (Agilent/HP, Böblingen, Germany) with methanol as solvent A and 0.1% (v/v) formic acid as solvent B. For the separation of the incubation experiments the following gradient at room temperature was used: 0 min, 95% (v/v) solvent B; 25 min, 10% (v/v) solvent B; 27 min, 0% (v/v) solvent B; 29 min, 0% (v/v) solvent B; 29.50 min, 95% (v/v) solvent B, equilibrating the column for a further 3 min. The flow rate was 1 mL/min. For injection (10 μL), an Agilent/HP 1100 autosampler (G1329A) was used. An Agilent/HP 1100 diode array detector (G1315A) was applied as detector monitoring the wavelength range of 200−800 nm, followed by an Agilent/HP 1100 fluorescence detector (G1321A), if fluorescence occurred. Acquisition of the data was carried out with the ChemStation software (A.08.03) (Agilent/HP). The identification of the compounds was achieved by comparison of UV/vis spectra and retention times with authentic references. Concentrations were calculated using calibration curves ranging from 0 to 150 μM for all substances. The calibration curves were linear in this range for all compounds with correlation coefficients ranging from 0.9924 to 0.9999 The recovery was determined by spiking blank cecal samples with the compounds under study followed by the extraction method described above. The recovery ranged from 78 to 102% (mol/mol). Each sample was injected at least three times. The following wavelengths were monitored for quantitative analysis: 280 and 360 nm for all flavan-3-ols, respectively. For the nongalloylated flavan-3-ols the emission was measured at 330 nm after excitation at 290 nm; for procyanidin B2 the emission was measured at 310 nm after excitation at 270 nm.

(*b*) *GC-MS Analysis*. To aliquots (420 μL) of the inoculum suspensions (see above) syringic acid was added as internal standard (IS) with a final concentration of 25 μM. These were dried using a vacuum concentrator BA-VC 300 H (H. Saur, Reutlingen, Germany) at 40 °C and 40 mbar. The residues were derivatized with 150 μL of BSA at 55 °C for 20 min and then diluted with 150 μL of trichlorotrifluoroethane. After centrifugation (4000*g*, 2 min, only for cloudy samples), the supernatants were injected into the gas chromatography−mass spectrometry system (GC-MS) with electron impact ionization (EI). Data were collected with an Agilent/HP 6890 chromatograph (G1530A) and Agilent/HP 5973 mass spectrometer (Agilent/HP, Böblingen, Germany) equipped with a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland). Data were analyzed by ChemStation software (B.01.00) (Agilent/HP). Separation of the analytes was performed on a 60 m × 0.25 mm i.d. CP-SIL 8 CB column (Chrompack/Varian, Darmstadt, Germany) using 1 mL/min helium as carrier gas. The injector temperature was set at 280 °C, whereas the injection volume was 1 μL splitless. The initial column temperature was 160 °C and was held for 1 min. A steady rise of 4 °C/min was chosen to 200 °C and increased to 15 °C/min to 320 °C, which was held for 10 min. The transfer line was heated at 320 °C. The mass spectrometer was operated in the electron impact mode (EI, 70 eV electron energy) with a source temperature of 230 °C and a quadrupole temperature of 150 °C. Mass spectra were acquired in the full-scan mode ranging from *m*/*z* 40 to 800 with a scan rate of 2.0 scans/s. Signals were identified by comparison of the retention times and mass spectra with silylated authentic references and a mass spectra library (NIST, Gaithersburg, MD).

(*c*) *Statistics*. Student's *t* test was used to test if the amount of applied compounds was significantly changed in time, if production of hydroxylated phenolcarboxylic acids took part, and if there were significant differences in recovery after extraction. A probability of <0.05 was considered to be significant. Error bars are depicted as mean ± SD. Statistical analysis was carried out with Excel 2003 (Microsoft Corp., Redmond, WA) and OriginPro 7G (Microcal Software, Northampton, MA).

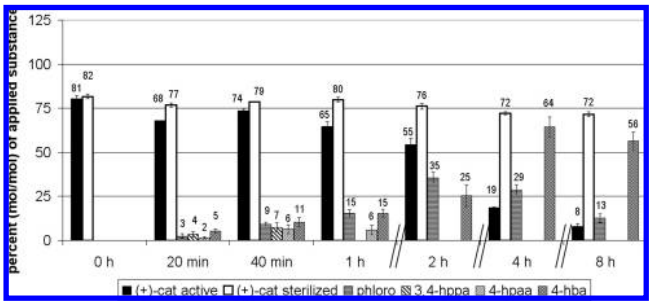

Figure 2. Relative proportions (% (mol/mol)) of (+)-catechin ((+)-cat) and its microbial metabolites phloroglucinol (phloro), 3,4-dihydroxyphenylpropionic acid (3,4-hppa), 4-hydroxyphenylacetic acid (4-hpaa), and 4-hydroxybenzoic acid (4-hba) in active cecal suspension in reference to that in sterilized inoculum (n = 6, mean ± SD; measured by HPLC-UVD/-FLD and GC-MS).

RESULTS

Microbial Degradation of Catechin and Epicatechin. *Studying Epimers*. As representative examples of flavan-3-ols the intestinal metabolism of the epimers (+)-catechin and (−)-epicatechin was studied as a continuation of our recent work dealing with flavonols and flavones such as quercetin or apigenin (*18*, *24*).

The first experiments with catechin showed an unexpected effect as the degradation curves for (+)-catechin in active and sterilized inoculum suspensions were nearly identical. After 20 min, neither in the active nor in the sterilized inoculum suspension was any (+)-catechin detectable (data not shown). A clear difference was observed only for occurring metabolites, as there were none in the sterilized samples but an expected pattern of metabolites in the active inoculum suspension. This clearly indicated that an intestinal metabolism has occurred, although catechin was not detectable. For this reason the protocol was slightly modified and sodium dodecyl sulfate (SDS, 2% (w/v)) was added to the extraction solution (*25*, *26*). This detergent solves non-covalent bonds between the flavan-3-ols and presumably protein moieties from chyme or bacterial membranes. Results obtained with the modified extraction procedure are shown in **Figure 2**. In detail (+)-catechin ((+)-cat) was essentially stable during 8 h of incubation time in the sterilized suspension. During intestinal degradation 3,4-dihydroxyphenylpropionic acid (3,4-hppa) is the main intermediate metabolite with 4 ± 2% (mol/mol) after 20 min and 7 ± 3% (mol/mol) after 40 min. However, 3,4-dihydroxyphenylpropionic acid is further metabolized by the microbiota and is not detectable after 1 h of incubation. Release of 4-hydroxybenzoic acid (4-hba) was nearly linear to the time point of 2 h with 25 ± 6% (mol/mol). This amount rose to 64 ± 6% (mol/mol) after 4 h and was not reduced significantly until 8 h (see **Figure 2**). 3-Hydroxylated compounds were detectable in only traces and in only two of three ceca. An increase of phloroglucinol was observed; within 60 min 15 ± 3% (mol/mol) of the applied flavan-3-ol was liberated. The maximum of 35 ± 3% (mol/mol) was reached after 2 h; thereafter, a degradation of this intermediate metabolite prevailed over generation from (+)-catechin. As can be seen in **Figure 3** the incubation of (−)-epicatechin ((−)-epi) yielded comparable results, and therefore no differences in the microbial degradation between epimers were detectable.

Studying Enantiomers. We were interested to see if microorganisms in the gut differentiate between both enantiomers. For this purpose (+)-catechin, (−)-catechin, and DL-catechin were incubated with different aliquots from the same three ceca. No

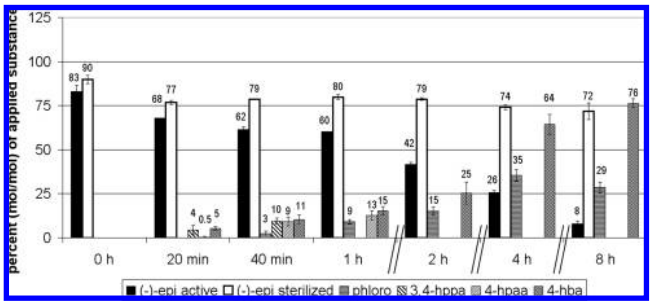

Figure 3. Relative proportions (% (mol/mol)) of (−)-epicatechin ((−)-epi) and its microbial metabolites phloroglucinol (phloro), 3,4-dihydroxyphenylpropionic acid (3,4-hppa), 4-hydroxyphenylacetic acid (4-hpaa), and 4-hydroxybenzoic acid (4-hba) in active cecal suspension in reference to that in sterilized inoculum (n = 6, mean ± SD; measured by HPLC-UVD/-FLD and GC-MS).

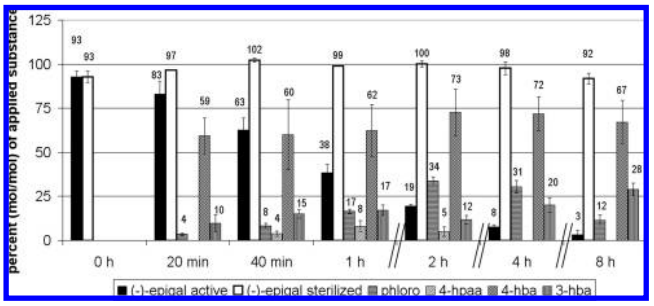

Figure 4. Relative proportions (% (mol/mol)) of (−)-epigallocatechin ((−)-epigal) and its microbial metabolites phloroglucinol (phloro), 4-phenylacetic acid (4-hpaa), and 3- and 4-hydroxybenzoic acid (3-hba, 4-hba) in active cecal suspension in reference to that in sterilized inoculum (n = 6, mean ± SD; measured by HPLC-UVD/-FLD and GC-MS).

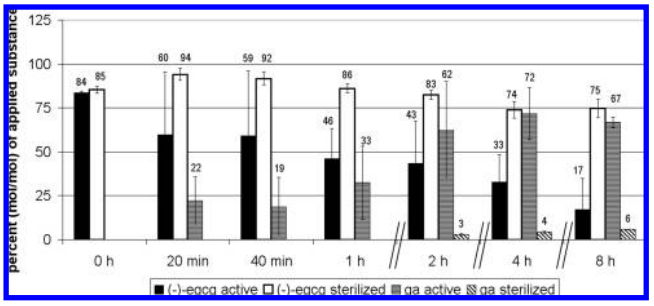

Figure 5. Relative proportions (% (mol/mol)) of (−)-epigallocatechin gallate (−)-egcg) and its microbial metabolite gallic acid (ga) in active cecal suspension in reference to that in sterilized inoculum (n = 6, mean ± SD; measured by HPLC-UVD/-FLD).

significantly different results were achieved for one of the three incubation experiments (data not shown).

Microbial Degradation of (−)-Gallocatechin and (−)-Epigallocatechin. Compared to (+)-catechin and (−)-epicatechin the occurrence of a broader variety of microbial metabolites caused by the additional hydroxyl group at position C2′ (see **Figure 1**) was expected. Within 1 h 62 ± 5% (mol/mol) of (−)-epigallocatechin ((−)-epigal) was degraded (see **Figure 4**), which is slightly faster compared to (+)-catechin (see **Figure 2**). Within the following 7 h of incubation time the metabolic rate decreased and the flavonoid was degraded to 3 ± 2% (mol/mol) of the applied amount.

Intermediate metabolites such as 3,4-dihydroxyphenylpropionic (3,4-hppa), 3-dihydroxyphenylpropionic (3-hppa), 4-dihydroxyphenylpropionic (4-hppa), and 3,4-dihydroxyphenylacetic acid (DOPAC) as well as 3-hydroxyphenylacetic (3-hpaa) acid were detectable only in traces until a time point between 2 and 4 h (for structures see **Figure 1**).

The main degradation product of the hydroxyphenylpropionic acids is 4-hydroxyphenylacetic acid (4-hpaa). This occurs between time points of 40 min and 2 h in amounts up to 8 ± 3% (mol/mol). The major intestinal metabolite, 4-hydroxybenzoic acid (4-hba), clearly dominates with 59 ± 11% (mol/mol) for (−)-epigallocatechin after 20 min (see **Figure 4**). These amounts varied only in a narrow interval until the end of incubation time. The corresponding 3-hydroxybenzoic acid (3-hba) is measurable only in quantities up to 28 ± 4% (mol/mol) after 8 h in these samples. If the final concentrations of 3- and 4-hydroxybenzoic acid are summed, they account for at least 95 ± 12% (mol/mol) of the flavan-3-ol. The ratio between the 3- and 4-hydroxyphenylacetic acids (3-/4-hpaa) as precursors for the hydroxybenzoic acids lies in the same magnitude, even if the total amount is much lower (data not shown). Phloroglucinol is liberated similarly compared to the incubation experiments with catechins described above with a maximum amount of 34 ± 3% (mol/mol) after 2 h of incubation. The ongoing metabolism of phloroglucinol leads then to a decrease of this compound (see **Figure 4**).

For (−)-gallocatechin the degradation was nearly 50% (mol/mol) in the first 60 min and around 92% (mol/mol) after 8 h of incubation, and the pattern and concentrations of metabolites were comparable, too (data not shown).

Microbial Degradation of (−)-Gallocatechin Gallate and (−)-Epigallocatechin Gallate. As expected, the ester bonds of the gallate derivatives were not stable against microbial degradation; possibly this is the reason why nongalloylated compounds seem to be more bioavailable (*27*). Both (−)-gallocatechin gallate ((−)-gcg) and (−)-epigallocatechin gallate ((−)-egcg) were hydrolyzed.

As a representative example, only the degradation of (−)-epigallocatechin gallate is shown in **Figure 5**. Within the first hour of incubation, around 33 ± 20% (mol/mol) of gallic acid (ga) was detectable (see **Figure 5**). In the following 3 h the gallic acid concentration increased to a maximum of 72 ± 15% (mol/mol). Within the next 4 h the concentration remained in the same range and no degradation was observed. A small amount (ca. 6% (mol/mol)) of gallic acid was also detectable in the sterilized control samples after 8 h of incubation because of a chemical degradation, which has to be taken into account for the active samples, too.

In previous studies gallic acid from anthocyanin degradation was subject to further metabolism, but the arising amounts were much lower (*16*). In contrast to these data we did not observe abundant degradation of gallic acid in our experiments. When 100 μM gallic acid was present, no degradation took place. To study this in more detail incubation experiments with gallic acid concentrations of 10−100 μM were performed. When interindividual differences are taken into account, amounts up to 30−40 μM gallic acid were degraded by intestinal microbiota. Higher doses of gallic acid were not metabolized by the microbiota (data not shown).

Microbial Degradation of Procyanidin B2. Interestingly, unlike in the above-mentioned experiments with the *C*-glycoside vitexin, which showed a different degradation behavior (*18*), procyanidin B2 (procy B2) shows similar properties compared to most of the other compounds (see **Figure 6**, procy B2 active combined). Within 2 h 61 ± 48% (mol/mol) of procyanidin B2 was degraded. Again the degradation rate lowers, and after 8 h the tested substance has nearly totally disappeared. Among the three different ceca large quantitative differences in the metabolism of this dimer can be found as can be seen in **Figure 6**. Existing

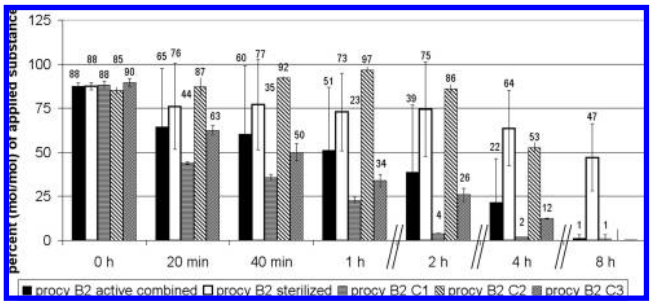

Figure 6. Relative proportions (% (mol/mol)) of procyanidin B2 in active cecal suspension (procy B2 active combined ($n = 6$)) in reference to that in sterilized inoculum (procy B2 sterilized ($n = 6$)) and the single incubation experiments (procy B2 C1−3) (mean ± SD; measured by HPLC-UVD/-FLD).

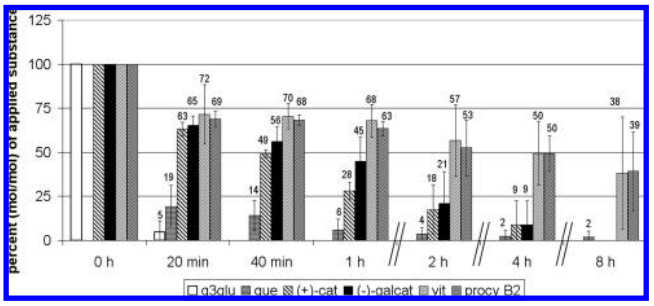

Figure 7. Relative proportions (% (mol/mol)) of a mixture of five flavonoids, quercetin-3-*O*-β-D-glucopyranoside (q3glu), (+)-catechin ((+)-cat), (−)-gallocatechin ((−)-galcat), vitexin (vit), and procyanidin B2 (procy B2), in active cecal suspension with the metabolite quercetin (que) liberated from q3glu ($n = 6$, mean ± SD; measured by HPLC-UVD/-FLD). Data are normalized for a recovery of 100% (mol/mol) at 0 h.

interindividual differences between different ceca lead to the observation of two donor individuals (see **Figure 6**, procy B2 C1 and procy B2 C3), which have a microbiota able to degrade procyanidin B2 directly and nearly totally within 4 h, but also with remarkable differences between each other. The microbiota of the third cecum (see **Figure 6**, procy B2 C2) degraded procyanidin B2 much more slowly up to a time point of 4 h, after which 53 ± 3% (mol/mol) was still available. All control compounds having been co-incubated with the same ceca were degraded as expected with a small standard deviation. For this reason the collected data clearly show the influence of different sources of the microbiota reflecting interindividual differences. Degradation of procyanidin B2 did not process via cleavage into flavan-3-ol monomers, which could not be detected at any time point. This is in accordance with Donovan et al., who found no monomers studying the metabolism of procyanidin B3 and a grapeseed extract containing several procyanidins. Even if monomers are formed inside the microorganism, they should be detected by using SDS in the extraction solvent as this detergent also leads to the disintegration of microbial cell membranes. Loss of procyanidin B2 in sterilized inoculum suspension is much stronger than for the monomers. After 8 h, nearly 50% (mol/mol) of the applied concentration (100 μM) disappeared (see **Figure 6**, procy B2 sterilized) without formation of any metabolites. This can be explained by a binding of procyanidin B2 to proteins, which is supported by the observation that more complex flavonoids interfere more strongly with proteins than small ones (*28*, *29*).

Formation of hydroxylated phenolcarboxylic acids and phloroglucinol is in the same magnitude compared to monomers, taking into account that per mole of procyanidin B2, 2 mol of metabolites are formed. The main metabolite is again 4-hydroxybenzoic acid (4-hba) with an amount of 85 ± 30% (mol/mol) after 8 h of incubation (data not shown). In addition, an unknown metabolite was detectable in the chromatograms at different time points. However, the structure could not be elucidated due to limited amounts of compound.

Microbial Degradation of a Mixture of Five Flavonoids. As there is always a mixture of different flavonoids in real samples, we studied the microbial degradation of flavonoids under more realistic conditions. For this a mixture of five different flavonoids (each 100 μM) was incubated with a bacterial inoculum suspension. **Figure 7** shows the degradation profiles of the five flavonoid substances.

Quercetin-3-*O*-β-D-glucopyranoside (q3glu) is degraded very quickly; after 20 min, < 10% (mol/mol) is detectable. Therefore, 19 ± 11% (mol/mol) of the aglycone quercetin is liberated, which is subject to further microbial degradation. These results fit well to our previous findings in single and combined degradation studies (*18*). (+)-Catechin ((+)-cat) is degraded slightly more quickly than in the single incubation experiment (see **Figures 2** and **7**), and (−)-gallocatechin ((−)-galcat) is broken down equally compared to the single incubation experiments. Arising hydroxylated phenolcarboxylic acids occur in the same pattern and in comparable amounts to the single incubation experiments, which leads to a potent pool of absorbable antioxidative substances as 300−500 μmol/L phenolic compounds is available between time points of 1−2 h (see **Table 1**).

As expected, the strongest influence on the microbial degradation of a mixture of flavonoids was observed for the *C*-glycoside vitexin (vit) as well as for procyanidin B2 (procy B2). These two C−C-linked compounds behave similarly as they were degraded slowly compared to other tested flavonoids. They resisted much longer and are available for exhibiting local effects in the gut. In the case of procyanidin B2, intestinal metabolism is slower, with only 47 ± 16% (mol/mol) degradation within 2 h, compared to 61 ± 36% in the single incubation experiments (see **Figures 6** and **7**). At the time point of 8 h, degradation of procyanidin B2 is nearly complete if incubated alone, but 38 ± 23% is left in the incubation experiment with the mixture of five substances. This means a time shift in degradation of nearly 6 h, in which higher amounts of polyphenolic substances are present for uptake or for local effects.

DISCUSSION

In the experiments described above we studied the intestinal metabolism of several flavan-3-ol derivatives in microorganisms directly isolated from the cecum of freshly slaughtered pigs. Metabolism of flavan-3-ol monomers takes part via ring fission in the C-ring with phloroglucinol and hydroxylated phenolcarboxylic acids being liberated. Both types of compounds were further degraded.

Flavonoid−Protein Complexes. The gastrointestinal tract contains several sources for proteins, such as food constituents, digestive enzymes, or proteins from epithelial or microbial cells. Formation of complexes between flavonoids and protein moieties is well-known and is discussed as the critical step for the loss of nutritional value (*26*, *30*) or reduced intestinal absorption of noncovalent flavonoid−protein complexes as shown with Caco-2 cells (*31*). Considering the methodical difficulties leading to equal degradation curves for the active and the sterilized inoculum suspension as described above for catechin, we assumed binding of these substances with matrix compounds as none of the tested substances disappeared in reagent samples without fecal content. Results from our experiments show that protein-bound

or -complexed flavan-3-ols could not only act as antioxidants and radical scavengers (*26*) but are still available for the degradation by the intestinal microbiota. However, only the use of SDS in the extraction solvent led to the recovery of catechin or epicatechin from the inoculum suspension. The ability of microorganisms to degrade flavan-3-ol−protein complexes with covalent bonds in the gastrointestinal tract is also of interest, but no experiments have been done until now. Also, the building mechanisms for these compounds are not fully cleared yet (*29*, *32*, *33*).

Degradation of Flavan-3-ols. For (+)-catechin (see **Figure 2**), its enantiomer (−)-catechin, and the racemic mixture DL-catechin microbial degradation is equal. There are no differences in concentration−time profiles or resulting metabolites, indicating that the microbiota does not distinguish between these isomers. This is interesting as Donovan et al. described differences in the intestinal uptake depending on the stereochemistry (*34*).

For the diastereomer (−)-epicatechin also no significant difference compared with catechin can be found (see **Figures 2** and **3**). The small quantitative differences between (+)-catechin and (−)-epicatechin for degradation after 2 or 4 h, for example, are caused by interindividual differences resulting from the ceca of different animals.

The microbial degradations of derivatives bearing an additional hydroxy group, the diastereomers (−)-gallocatechin and (−)-epigallocatechin, are comparable to one another, although the pattern of hydroxylated phenolcarboxylic acid liberated from the B-ring moiety is wider due to the additional hydroxy group, leading to 3- or 3,4-dihydroxylated metabolites (see **Figure 4**). Measured values for hydroxybenzoic acids (3-/4-hba, see **Figure 4**) showed larger standard deviations than in other experiments and are quite high compared to degraded flavan-3-ols. A possible explanation might be that the benzoic acid derivatives are formed by microbial degradation of other compounds present in the cecal samples. However, the analysis of blank samples did not give hints for the formation of high concentrations of benzoic acid derivatives.

The accumulation of hydroxybenzoic acids is an interesting aspect. In vivo hydroxybenzoic acids are taken up quickly through the ileal epithelium by monocarboxylic acid transporters (MCT), which cannot be mimicked in our model. This leads to low amounts that cannot profitably be utilized for microbial metabolism, which might be the reason why there are no microorganisms present that are able to degrade hydroxybenzoic acids. However, the increasing amounts of benzoic acid derivatives did not have any influence on the metabolic activity of the microbiota, which could be shown by co-incubation of 3- or 4-hydroxybenzoic acid with well-studied model substances such as quercetin-3-*O*-β-D-glucopyranoside (data not shown) or with the incubation of a mixture of compounds (see below).

Degradation of Galloylated Compounds. As the galloylated derivatives (−)-gallocatechin gallate and (−)-epigallocatechin gallate are incubated with inoculum suspension, the ester bonds are cleaved rapidly, supporting previous results obtained in our group (*18*). The microbial degradation of both liberated gallocatechin epimers was analogous to the degradation profiles shown above for the non-esterified flavan-3-ols (see **Figure 4**). However, due to the ester cleavage in the first step the degradation was slower, and for this reason the concentration of liberated hydroxylated phenolcarboxylic acids was approximately 20% (mol/mol) lower at the time points from 1 to 4 h. Another interesting point is the large standard deviation of the results from three combined ceca. Whereas repeat determination of the same cecum leads to very similar findings and small standard deviations, the interindividual differences are immense. However, this nicely reflects the in vivo situation as similar interindividual differences are expected for humans, which can also be seen for procyanidin B2.

Table 1. Concentrations (Micromoles per Liter) of Phenolcarboxylic Acids Liberated from the Intestinal Degradation of a Mixture of Five Flavonoids [Phloroglucinol (phloro), the Phenylpropionic Acids with Hydroxylation at C3 and/or C4 (sum hppa), the Phenylacetic Acids with Hydroxylation at C3 and/or C4 (sum hpaa), the Hydroxybenzoic Acids with Hydroxylation at C3 or C4 (sum hba)] in Active Cecal Suspension (n = 6, Mean ± SD; Measured by GC-MS)[a]

time	phloro	sum hppa	sum hpaa	sum hba
20 min	16 ± 10	8 ± 5	nd	nd
40 min	40 ± 8	26 ± 15	76 ± 18	93 ± 7
1 h	43 ± 7	18 ± 9	52 ± 27	273 ± 17
2 h	203 ± 15	3 ± 2	18 ± 3	384 ± 21
4 h	185 ± 18	nd	7 ± 4	409 ± 15
8 h	67 ± 11	nd	nd	358 ± 19

[a] nd, not detectable.

The liberated gallic acid influences the microbial metabolism dose dependently. If amounts between 30 and 40 μM or lower are present, gallic acid is degraded by the microbiota. If amounts rise above these quantities, gallic acid constricts the degradation of flavan-3-ols (see **Figures 4** and **5**). A likely explanation is the dose-dependent antimicrobial effect of gallic acid (*35*). Meselhy et al. described not only interindividual differences but even species determinant differences of the ability of microbiota between rats and men, which questions the use of rodents as model organisms in this case (*15*).

Degradation of Compounds Bearing C−C Linkages. We observed large differences for the degradation of procyanidin B2 between the three different ceca (see **Figure 6**). These interindividual differences are well-known and normally were overcome by pooling of samples. The metabolic profile of the microbiota is unique for each human individual and is influenced strongly by nutrition, host−microorganism cross talk, and interaction between different microorganisms (*36*−*38*). For this reason a single incubation experiment with later combination of the data is preferred, because this approach reflects the in vivo situation much better.

In addition to the single incubation experiments a mixture of five flavonoids was tested to analyze the influence on the microbial metabolism. The results show that substances with C−C linkages such as the *C*-glucoside vitexin or the dimer procyanidin B2 are more slowly degraded in this incubation experiment. This is explainable for two reasons. At first, the total applied amount of flavonoids is higher, but as the results for (+)-catechin and (−)-gallocatechin show, the intestinal microbiota still has the ability for a fast degradation. From these results and the degradation of quercetin-3-*O*-β-D-glucopyranoside within 20 min, we conclude that the microbiota is not overburdened by the flavonoid mixture. Second, bacteria degrade these compounds for their own metabolism and predominantly use easily accessible energy sources first, which explains the faster degradation of less complex substances. By longer presence of procyanidin B2 and vitexin, they are potentially more available for intestinal uptake, but the possibility is still controversially discussed (*9*, *22*, *39*); otherwise, they may have local effects in the gut (*26*).

Degradation products of the flavonoids are different phenolic compounds, for example, phloroglucinol and hydrolylated phenylacetic acid. Many studies showed their good absorption in the gut and the possible antioxidative properties in the blood (*11*). Our experiments show a large possible pool (see **Table 1**) of these compounds that can be generated from the microbiota and may be absorbed via MCT or can exhibit local effects.

Comparison with Similar Studies. Besides differences in quantitative results between metabolism studies performed with flavan-3-ol monomers, dimers, and oligomers, two aspects are acknowledged: (1) bioavailability of intact polyphenolic substances is low and (2) metabolites found are different hydroxylated phenolcarboxylic acids. These are generated by the intestinal microbiota as shown in this as well as in different other studies (*15*, *40*−*43*).

The data observed for hydroxylated phenolcarboxylic acids in incubation experiments with flavan-3-ol monomers are qualitatively in accordance with studies from other groups working with rodents (*19*) and human stool samples (*42*). Differences exist with regard to phenylvalerolactones, which are discussed below. Quantitatively different results between our experiments and those from other groups can be explained by two points. First, mostly stool samples with a different metabolic ability or composition of microbiota according to oxygen influence by passage through the lower gastrointestinal tract are used. Second, values vary between different organisms as well as between individuals as their nutrition behavior varies. Data for the degradation of flavan-3-ols by the intestinal microbiota are limited, because the analysis of fecal samples during animal experiments is not done routinely or is limited to a few substances (*9*, *20*).

A study on humans ingesting grapeseed extract with mainly flavan-3-ol monomers and dimers reported urinary excretion of several hydroxylated phenolcarboxylic acids generated by microbiota (*44*). Unfortunately, fecal samples have not been analyzed in this study. However, these data clearly show that generated metabolites can be taken up, which is yet unclear for intact nonmonomeric flavonoids.

Phenylvalerolactones, or corresponding hydroxylated phenylvaleric acids, which are often discussed as key metabolites (*15*,*40*−*42*), could not be found in our experiments. Neither experiments with authentic references of (−)-5-(3′4′,5′-trihydroxyphenyl)-γ-valerolactone and (−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (*45*) nor the search of typical *m*/*z* ratios leads to detection of these compounds in incubation experiments performed in this study. Several influences on microorganisms, or a combination of them, could possibly account for this phenomenon. In studies using whole organisms, aerotolerant microorganisms from lower parts of the gut are present (*40*, *46*, *47*), and this is inevitably also the case for examinations done with stool samples (*15*). Third, the chosen pH differs depending on the experimental design. Often conditions are used that match the conditions in the colon (*15*,*41*) and not in the cecum, where the pH is between 5.9 and 7.2 with a weighted average at 6.3. This may lead to qualitative and quantitative differences in the microbiota or induce pathways in microorganisms.

In summary, the flavan-3-ols under study were almost completely metabolized by the intestinal microbiota within 4 and 8 h. We conclude that the small phenolic degradation products such as 3,4-dihydroxyphenylpropionic acid (3,4-hppa), 4-hydroxyphenylacetic acid (4-hpaa), 3-/4-hydroxybenzoic acid (3-/4-hba), and phloroglucinol (phloro), which are formed as the main metabolites during the intestinal metabolism of flavan-3-ols, might be responsible for the observed antioxidative activities described in the literature. However, it is unclear which substances are responsible for certain effects and whether these hydroxylated phenolcarboxylic acids are taken up in quantities high enough to be responsible for health-related systemic effects.

ABBREVIATIONS USED

(+)-cat, (+)-catechin; BSA, *N*,*O*-bis(trimethylsilyl)acetamide; DOPAC, 3,4-dihydroxyphenylacetic acid; (−)-egcg, (−)-epigallocatechin gallate; EI, electron impact; (−)-epi, (−)-epicatechin; (−)-epigal; (−)-epigallocatechin; FISH, fluorescence in situ hybridization; (−)-galcat, (−)-gallocatechin; glu, glucose; hba, hydroxybenzoic acid; hpaa, hydroxyphenylacetic acid; 3,4-hppa, 3,4-dihydroxyphenylpropionic acid; i.d., inner diameter; IS, internal standard; MCT, monocarboxylic acid transporter; nd, not detectable; phloro, phloroglucinol; procy B2, procyanidin B2; q3glu, quercetin-3-*O*-β-D-glucopyranoside; que, quercetin; SD, standard deviation; SDS, sodium dodecyl sulfate; UV/vis, ultraviolet/visible; v/v, volume to volume; vit, vitexin; w/v, weight to volume.

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