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Deconjugation and Degradation of Flavonol Glycosides by Pig Cecal Microbiota Characterized by Fluorescence in Situ Hybridization (FISH)

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As the bioavailability of flavonoids is influenced by intestinal metabolism, we have investigated the microbial deconjugation and degradation of several flavonols and flavonol glycosides using the pig cecum in vitro model system developed in our group. For this model system the microbiota was directly isolated from the cecal lumen of freshly slaughtered pigs. The characterization of the cecal microbiota by fluorescence in situ hybridization (FISH) with 16S rRNA-based oligonucleotide probes confirmed the suitability of the model system for studying intestinal metabolism by the human microbiota. We have investigated the microbial degradation of quercetin-3-*O*-β-D-rutinoside **1**, quercetin-3-*O*-β-D-glucopyranoside **2**, quercetin-4′-*O*-β-D-glucopyranoside **3**, quercetin-3-*O*-β-D-galactopyranoside **4**, quercetin-3-*O*-β-D-rhamnopyranoside **5**, quercetin-3-*O*-[α-L-dirhamnopyranosyl-(1→2)-(1→6)-β-D-glucopyranoside **6**, kaempferol-3-*O*-[α-L-dirhamnopyranosyl-(1→2)-(1→6)-β-D-glucopyranoside **7**, apigenin **8**, apigenin-8-*C*-glucoside (vitexin) **9**, and feruloyl-*O*-β-D-glucopyranoside **10** (100 μM), representing flavonoids with different aglycones, sugar moieties, and types of glycosidic bonds. The degradation rate was monitored using HPLC-DAD. The flavonol *O*-glycosides under study were almost completely metabolized by the intestinal microbiota within 20 min and 4 h depending on the sugar moiety and the type of glycosidic bond. The degradation rates of the quercetin monoglycosides showed a clear dependency on the hydroxyl pattern of the sugar moiety. The degradation of **2** with all hydroxyl groups of the glucose in the equatorial position was the fastest. The intestinal metabolism of di- and trisaccharides was much slower compared to the monoglycosides. The structure of the aglycone has not much influence on the intestinal metabolism; however, the type of glycosidic bond (*C*- or *O*-glycoside) has substantial influence on the degradation rate. The liberated aglycones were completely metabolized within 8 h. Phenolic compounds such as 3,4-dihydroxyphenylacetic acid **12**, 4-hydroxyphenylacetic acid **13**, and phloroglucinol **18** were detected by GC-MS as main degradation products.

KEYWORDS: Flavonoids; degradation; gastrointestinal tract; microbiota; intestinal metabolism; pig cecum; FISH

INTRODUCTION

Flavonoids are secondary plant metabolites and mainly found in higher plants, for example, fruits, vegetables, or tea. Data about the daily intake of flavonoids vary from 20 mg/day to 1 g/day (*1–3*) depending on the different food patterns. With the exception of flavanols, most flavonoids occur in plants as glycosides. The linkage of different sugar moieties stabilizes the aglycones and increases their water solubility (*4, 5*). Many studies have shown that flavonoids are potent antioxidants and radical scavengers in different model systems (*6–9*). Due to these properties it is assumed that they have protective effects on cardiovascular diseases and certain forms of cancer (*10, 11*). A requirement for these physiological effects, except from some local effects, would be the absorption of the flavonoids in the gastrointestinal (GI) tract. Several studies have shown that many of the aglycones and some of the monoglycosides are already absorbed in the ileum, but more complex flavonoid glycosides cannot be absorbed by the small intestine and reach the colon (*12–15*). Even if flavonoids are absorbed, they reach again the bile and GI tract as the rapidly formed glucuronides or sulfates of flavonoids, which are again substrates for the intestinal microbiota. Recent studies have investigated the microbial degradation of flavonoids in the large intestine using

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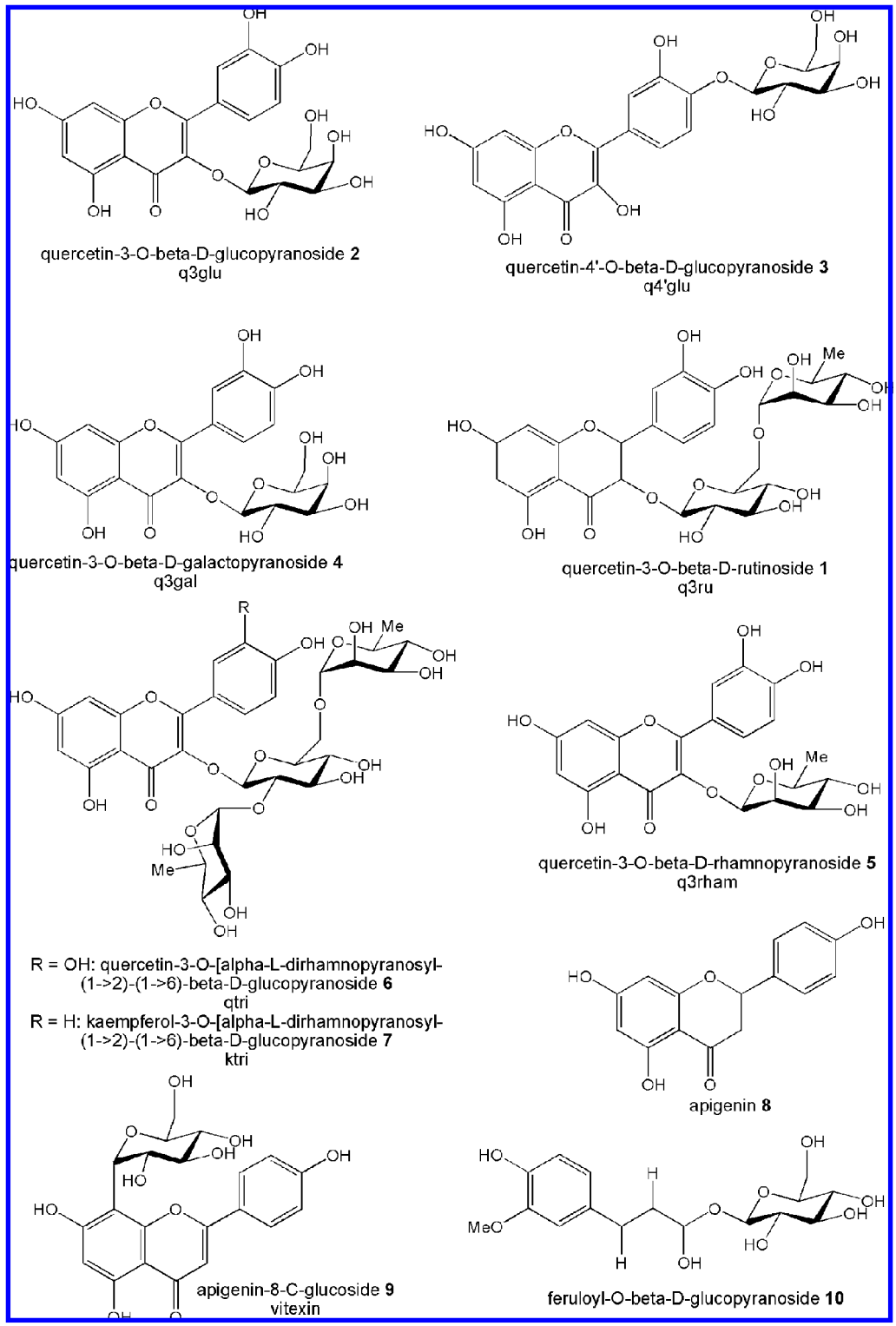

Figure 1. Structures of the investigated flavonoids.

single bacterium species (*16, 17*), mixed culture models (*18*), human stool specimen (*19*), or pig inoculum (*20–22*). They reported an extensive metabolism of these compounds by the gut microbiota, especially hydrolysis of glycosidic bonds and even ring scissions. In the present study, we have investigated the microbial degradation of flavonoids with different sugar moieties, aglycones, and types of glycosidic bonds (**Figure 1**). Special attention was paid toward coherences between different sugar moieties and the degradation rate.

For studying the metabolism of flavonoids by the intestinal microbiota, we have established a new in vitro model system in our group (*20, 21*) for which the intestinal microbiota is directly isolated from the cecum of freshly slaughtered pigs produced by biodynamic farming. The pig more closely resembles the human than any other nonprimate mammalian species because of the similarities in digestive anatomy, physiology, and nutrition (*23–25*). The cecum as the connection between the small and large intestine contains more than 400 bacterial species, most of them strict anaerobes (*26, 27*). Besides the proximal colon the cecum is described as the segment with the highest fermentation rate (*28*). Owing to sampling difficulties microbial incubation studies often are performed rather with

human stool samples. This experimental design has many disadvantages: human stool samples do not represent the microbiota in the middle intestine due to a more anaerobic atmosphere and a lower luminal pH in the proximal than in the distal colon, which affect the growth of bacteria in the intestine markedly (*29, 30*). Furthermore, sampling of the stool specimen under strict anaerobic conditions represents another critical step. To demonstrate that the applied model system is a suitable tool for studying human intestinal metabolism, we further characterized the pig cecal microbiota by fluorescence in situ hybridization (FISH). Five oligonucleotide probes were applied for the detection of bacterial 16S rRNAs of the genus *Bifidobacterium* and of different phylogenetic clusters of *Bacteroides* spp., *Eubacterium* spp., and *Clostridium* spp. Total bacterial counts in the cecal samples were enumerated using a *Bacteria* domain-specific probe.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased in p.A. quality. 3-(4-Hydroxyphenyl)propionic acid **11**, 3,4-dihydroxyphenylacetic acid (DOPAC) **12**, 4-hydroxyphenylacetic acid **13**, ferulic acid **14**, and quercetin-3-*O*-*β*-D-rhamnopyranoside (q3rham) **5** were purchased from Sigma-Aldrich (Steinheim, Germany). Apigenin **8**, vitexin **9**, kaempferol **15**, kaempferol-3-*O*-*β*-D-glucopyranoside (k3glu) **16**, kaempferol-3-*O*-*β*-D-rutinoside (k3ru) **17**, quercetin-3-*O*-*β*-D-glucopyranoside (q3glu) **2**, quercetin-4′-*O*-*β*-D-glucopyranoside (q4′glu) **3**, and quercetin-3-*O*-*β*-D-galactopyranoside (q3gal) **4** were provided from Roth (Karlsruhe, Germany). *N*,*O*-Bis(trimethylsilyl)acetamide was obtained from Fluka (Buchs, Switzerland). Phloroglucinol **18** was ordered from Merck (Darmstadt, Germany). Quercetin **19** and quercetin-3-*O*-*β*-D-rutinoside (q3ru) **1** were purchased from Extrasynthese (Genay, France). The feruloyl-*O*-*β*-D-glucopyranoside (fglu) **10** was isolated by Thorsten Bernsmann (Institute of Food Chemistry, University of Münster, Germany) from spinach (*31*).

Quercetin-3-*O*-[α-L-dirhamnopyranosyl-(1→2)-(1→6)-*β*-D-glucopyranoside (qtri) **6** and kaempferol-3-*O*-[α-L-dirhamnopyranosyl-(1→2)-(1→6)-*β*-D-glucopyranoside (ktri) **7** were isolated by Bernd Schwarz (Institute of Food Chemistry, Muenster, Germany) from red currants (*32*).

Oligonucleotide probes for FISH were synthesized and purified by Thermo Electron (Ulm, Germany). Lysozyme was provided from Roth, and the mounting medium Vectashield H-1000 was purchased from Alexis (Grünberg, Germany).

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).

General Methods. Stock solutions (1 mM) of the analytes were prepared as follows: the flavonol glycosides (1 mM) q3glu **2**, q3gal **4**, q3rham **5**, q3ru **1**, qtri **6**, and ktri **7**, the flavone glycoside vitexin **9**, and the hydroxycinnamic acid glycoside fglu **10** were dissolved in methanol/water (70:30, v/v); apigenin **8** was dissolved in methanol/water (70:30, v/v) containing 10% dimethyl sulfoxide (DMSO).

Preparation of Inoculum. The pigs (German Landrace or Angler Sattel × Pietrain) from which the ceca were obtained were bred by biodynamic farming. They were 8–12 months old and weighed 120−150 kg. They were fed 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 transported 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 (Sekuroka-Glove Bag, Roth) flushed with CO_2 to retain an anaerobic atmosphere. The inocula of each cecum were isolated and suspended in the same volume (v/v) of 0.15 M PBS (pH 6.4) containing a 0.0125% (v/v) trace element solution (*20, 21*). All solutions, buffers, and vessels were flushed with a mixture of N_2 and CO_2 (5:1, v/v) before use. Removal of large particles from the 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 fresh inoculum as described before. The inactivated fecal suspensions were used as control for chemical degradation and matrix effects. Each incubation experiment was performed in duplicate determinations with three different ceca.

For fluorescence in situ hybridization (FISH) the contents of the ceca were diluted 1:10 (v/v) in the supplemented 0.15 M PBS described above. Cell fixation and storage was modified according to ref *33*. After a washing step with PBS (pH 7.4) (*34*), 0.5 mL of each cecal suspension was fixed with fresh, cold paraformaldehyde solution (4% in PBS) for 16 h at 4 °C. The cells were pelleted by centrifugation (15000*g*, 5 min, 4 °C), washed with PBS to remove residual fixative, pelleted again, and resuspended in 0.25 mL of PBS containing 1% Tween 80. The same volume of cold absolute ethanol was added to these stocks, and further dilutions using PBS containing 1% Tween 80 [1:500 until 1:4000 (v/v)] were made. All samples were formed in duplicate. The fixed cells were stored at −20 °C until further use.

Enumeration of Bacteria by FISH. The fixed cecal suspensions were processed, and FISH analysis was performed with the oligonucleotide probes Eub338 (*33*), Lab158 (*35*), Erec482 (*34*), Bac303 (*36*), Bif164 (*37*), and Lowgc2P (*34*) as described by these authors, with some modifications. Probe Eub338 was labeled at its 5′ end with fluorescein; all other probes were coupled with carbocyanin (CY3). For hybridization 10 μL of the fixed cell suspensions was smeared on adhesive microscope slides (Roth) and dried for 15 min at 45 °C. After dehydrating in a graded ethanol series [50, 75, and 96% ethanol/H_2O (v/v)], cells were treated with 20 μL of lysozyme (1 mg/mL in 1 M Tris-HCl, pH 7.2) to allow permeabilization of the Gram-positive bacteria cell membranes (20 min, 37 °C). The slides were washed (30 s) and dehydrated in ethanol/H_2O again as described before. Twenty microliters of hybridization buffer [0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, 0.1% SDS (w/v)] containing 2.5 ng/μL of the fluorescently labeled probe were added to the cell smears. The cells were incubated at 50 °C (46 °C for probe Lowgc2P) for 16 h in a buffer-saturated hybridization chamber. Subsequently, slides were rinsed in wash solution (hybridization buffer without SDS) for 30 min at 50 °C (48 °C for probe Lowgc2P), air-dried, and mounted in Vectashield H-1000. Hybridized cells were enumerated visually with a Zeiss Standard 16 epifluorescence microscope (Jena, Germany) with a PlanNeofluar100 objective, an HBO50W mercury lamp, and light excitation filter sets 09 and 15. All fixed cell dilutions were counted in duplicate with a minimum of 15 microscopic fields per assay. Dry weights were determined by lyophilizing a precisely weighed portion of each cecal sample in duplicate.

Flavonol (Glycoside) Incubation Experiments. The preparation of the incubation experiments was performed in the glovebag under anaerobic conditions; 0.1 mL of the stock solution of the analytes was added to 0.9 mL of vital or sterilized inoculum filtrate in a 2 mL Eppendorf cap (Eppendorf, Hamburg, Germany). Aliquots of both sterilized and active inoculum filtrate were used as matrix blanks. The maximum methanol concentration in the incubation experiments reached 7% (v/v), which did not affect the bacterial vitality as was shown by measuring the α-D-galactosidase activity (*20*). In the case of **8** the final DMSO concentration was 1% (v/v) and did not affect the bacterial microbiota. The sealed vials were placed in an incubator (37 °C) for 20 min, 40 min, and 1, 1.5, 2, 4, 6, 8, and 24 h. The microbial metabolism was stopped by freezing the vessels at −80 °C.

Sample Preparation and Analysis. *HPLC-DAD Analysis.* The frozen samples were thawed quickly in a water bath. The samples were diluted immediately with methanol (1:1, v/v) to abandon any microbial degradation and to extract the analytes. After shaking, the samples were centrifuged at 12000*g* for 20 min (4 °C). The samples were stored on ice until a sterile filtration of the supernatants was performed (GHP Acrodisc, 0.2 μm, Pall Life Sciences, Dreieich, Germany). Aliquots of the inoculum filtrate 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 a Jasco PU-2089 low-pressure gradient HPLC pump (Jasco, Gross-Umstadt, Germany) with acetonitrile as solvent A and 2% (v/v) acetic acid as solvent B. For the separation of the single-compound incubation experiments the following gradient at room temperature was used:

0 min, 95% solvent B; 25 min, 45% solvent B; 30 min, 10% solvent B; 34 min, 95% solvent B; 35 min, 95% solvent B. The flow rate was 1 mL/min. Filtrates of bacterial inoculum suspension with a mixture of compounds were separated using the following gradient: 0 min, 85% solvent B; 10 min, 83% solvent B; 15 min, 83% solvent B; 20 min, 45% solvent B; 24 min, 10% solvent B; 25 min, 10% solvent B; 29 min, 85% solvent B; 30 min, 85% solvent B. The flow rate was 1 mL/min, and the column temperature was 45 °C. For injection (40 μL), a Jasco autosampler (AS-2057 Plus) was used. A Jasco diode array detector (MP-2010 Plus) was applied for peak detection over the wavelength range of 220–600 nm. Acquisition of the data was carried out with the software Borwin-PDA 1.5 (Jasco). The identification of the compounds was achieved by comparison of the UV spectra and the retention times with those of 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 0.9967 to 0.9999. The recovery for most of the compounds under study ranged from 85 to 102%. The recovery was determined by spiking sterilized inoculum samples with the corresponding compounds (12.5–125 μM). The recovery could not be determined for **6** and **7** due to the limited amounts of these compounds. Each sample was injected at least three times. The following wavelengths were monitored for quantitative analysis: 360 nm for all flavonols **1**–**7** and **15**–**17** except apigenin **8** and vitexin **9**, which were determined at 330 nm.

GC-MS Analysis. Aliquots (300 μL) of the inoculum filtrates (see HPLC-DAD Analysis) were supplemented with 1,3-dihydroxybenzene (end concentration = 20 μM) as internal standard and dried under a nitrogen stream. The residues were derivatized with 150 μL of *N*,*O*-bis(trimethylsilyl)-acetamide at 55 °C for 20 min. After centrifugation (12000*g*, 10 min), the supernatants were injected into the GC-MS system. Electron impact (EI) gas chromatography–mass spectrometry (GC-MS) data were collected with a HP6890 series gas chromatograph and HP5973 mass spectrometer (Hewlett-Packard/Agilent, Böblingen, Germany). Data were analyzed by Chemstation software (Agilent). 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. The steady rise of 4 °C/min was chosen until 260 °C and then the rise was increased to 15 °C/min to 320 °C, which was held isothermally 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).

RESULTS AND DISCUSSION

Characterization of the Pig Cecal Microbiota by FISH. The mammalian intestinal microbiota is a complex ecosystem involved in nutrition and health. In the past few years molecular tools have made it possible to study the composition of the intestinal inhabitants in a culture-independent way based on the detection of bacterial rRNAs (*38, 39*). For quantitative analysis of the pig cecal microbiota we applied 16S rRNA-based oligonucleotide probes in FISH. The specificity of the probes was adjusted from domain (Eub338), phylogenetic cluster (Lab158, Erec482, Bac303, and Lowgc2P), to genus (Bif164). The total hybridizable bacteria obtained with the universal probe Eub338 were, on average for all animals, $(1.6 \pm 0.3) \times 10^{11}$ cells/g (dry weight). Results of all cluster- and genus-specific probes were calculated as percentage to Eub338, which is specific for virtually all bacteria (*33*) (**Figure 2**). The Lab158 probe captured species of the genera *Lactobacillus*, *Leuconostoc*, and *Enterococcus* and reflected 19 ± 7% of the total bacteria.

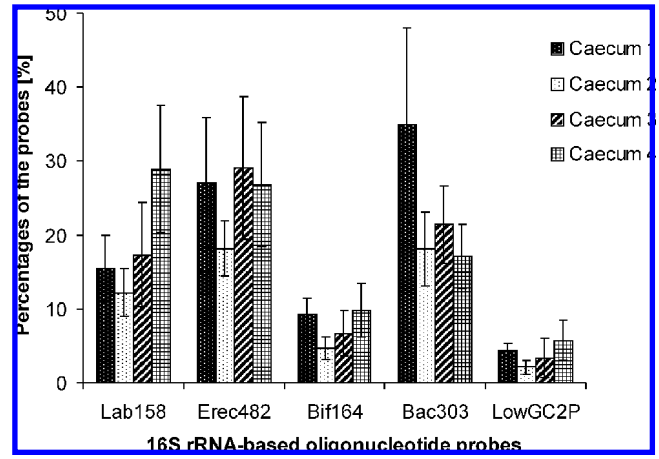

Figure 2. Relative proportions of the *Lactobacillus*/*Leuconostoc* and *Enterococcus* spp. group (Lab158), the cluster of *Clostridium coccoides*/*Eubacterium rectale* (Erec482), *Bifidobacterium* spp. (Bif 164), the *Bacteroides*/*Prevotella* spp. cluster (Bac303), and the species of the *Fusobacterium prausnitzii*/*Eubacterium* spp. cluster (Lowgc2P) in the pig cecal microbiota in relation to the *Bacteria*-universal probe Eub338 (100%) detected by FISH.

These microaerophils represented besides the anaerobic cluster of *Clostridium coccoides*/*Eubacterium rectale* (probe Erec482) and *Bacteroides*/*Prevotella* (probe Bac303) one main group of the pig cecal microbiota. Counts enumerated with the probe Erec482 were on average 25 ± 5%; the Bac303 probe detected a mean number of 23 ± 8%. Less important was the presence of Bifidobacteria, which is typical for mammals after weaning. The Bif164 probe counted on average 8 ± 2%. Likewise, species of the *Fusobacterium prausnitzii*/*Eubacterium* spp. cluster represented with 4 ± 1% (probe Lowgc2P) a minor group. With the set of one genus-specific and four cluster-specific probes used in this study, we detected 55–91% of the total bacteria microbiota counted with the Eub338 probe in the pig ceca. The presented results indicate an individual heterogeneity in the composition of the cecal microbiota, although the applied pigs were fed the same basal diet. Genotypic factors are held responsible for these differences (*40*). Only a few authors have described the composition of the human cecal microbiota (*30*), owing to sampling difficulties and the need to maintain anaerobic conditions by using culture-dependent methods. Human stool samples have been the common target for determinative 16S rRNA-based oligonucleotide probes in recent studies. In consideration of the different ecological conditions in the cecum and the distal colon, these studies give comparable results to the pig cecal microbiota (*34, 41*). Anaerobic bacteria were detected in human feces with 22% (*41*) and 29% (*34*) for species from the *Clostridium coccoides*/*Eubacterium rectale* cluster and 21% (*34*) and 28% (*41*) for representatives of the *Bacteroides*/*Prevotella* group in relation to the total counts measured by the probe Eub338. Likewise, *Bifidobacteria* [4% (*34*) and 5% (*41*)] and species of the *Fusobacterium prausnitzii*/*Eubacterium* spp. cluster [11% (*41*) and 12% (*34*)] belong to the minor groups of the fecal microbiota. Against that, the importance of the members detected by the Lab158 probe differs with <0.01% (*41*) greatly between human stool specimen and pig cecal contents. Because of its different physiological conditions in the stomach the pig has a higher colonization with lactic acid bacteria in the upper gastrointestinal tract than humans (*42*). On the other hand, relative quantification of the human stool and cecal microbial populations achieved by dot blot hybridization using the probe Lab158 showed with 23%

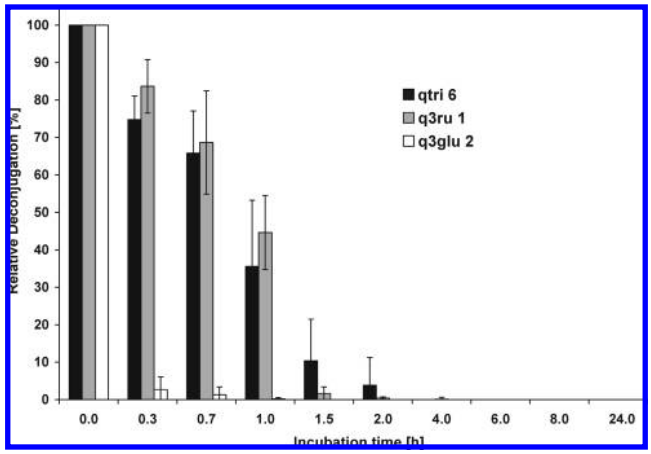

Figure 3. Relative proportions (percent) of the monoglycosides q3rham **5**, q3gal **4**, and q3glu **2** in active cecal suspension with reference to that in sterilized inoculum ($n = 3$, mean ± SD; measured by HPLC-DAD at 360 nm).

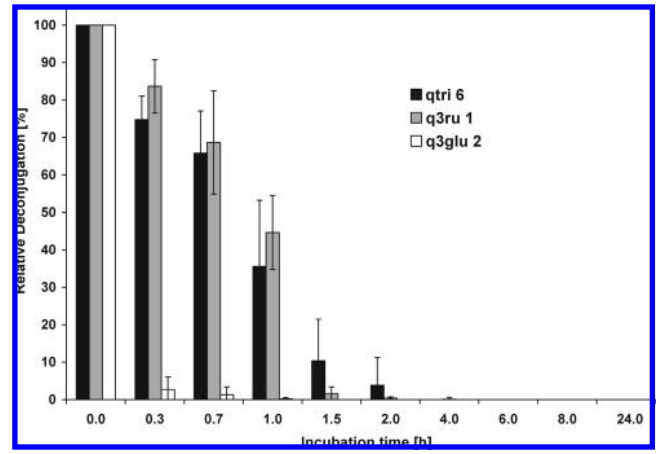

Figure 4. Relative proportions (percent) of the quercetin glycosides qtri **6**, q3ru **1** and q3glu **2** in active cecal suspension with reference to that in sterilized inoculum ($n = 3$, mean ± SD; measured by HPLC-DAD at 360 nm).

much higher rRNA proportions in human cecal contents than in stool specimen (7%) (*30*). The study of Marteau et al. confirms that the cecal flora differs greatly from the fecal microbiota. Therefore, human stool specimens could not present the microbial metabolism in the upper intestine in a correct way. Furthermore, the microbial composition is influenced by nutritive factors. Mammals, as the applied pigs, consuming a vegetarian and fiber-rich diet show higher levels of *Lactobacillus* and less *Bacteroides* than animals or humans preferring a meat-based diet (*43*). In conclusion, the presented pig cecal model system shows a corresponding composition of the main bacterial groups compared to the human intestinal microbiota considering phenotypic and genotypic varieties, which are also common in humans. Therefore, the pig cecum model is an excellent tool for studying the intestinal metabolism of food ingredients by the human microbiota.

Microbial Degradation of Quercetin Monoglycosides Dependent of the Sugar Moiety. To investigate the microbial degradation of quercetin monoglycosides, q3glu **2**, q3gal **4**, and q3rham **5** were incubated with a bacterial suspension of the pig cecum. The intent of this assay was to study a correlation between the hydroxyl pattern of the different sugar moieties (glucose, galactose, rhamnose) and the degradation rate of the corresponding quercetin monoglycosides. **Figure 3** shows the decreasing amounts of the quercetin monoglycosides **2**, **4**, and **5** during the incubation (24 h) with the gut microbiota.

The degradation rates of the quercetin monoglycosides **2**, **4**, and **5** showed a clear dependency on the hydroxyl pattern of the sugar moiety. The degradation of q3glu **2**, with all hydroxyl groups of the glucose in equatorial position, was the fastest, followed by q3gal **4**, which has the hydroxyl group on C4 in axial position. The q3rham **5**, bearing the desoxy sugar moiety, showed the slowest degradation rate. During the metabolism of the quercetin-3-*O*-*β*-D-glycosides **2**, **4**, and **5** an increasing amount of the aglycone quercetin **19** was detectable with the highest concentration in the case of **2** due to the fastest hydrolysis. In the first 20 min of incubation, q3glu **2** was nearly completely degraded; in contrast, only 54.0 ± 9.8% of q3gal **4** and only 84.1 ± 7.8% of q3rham **5** were hydrolyzed by the gut microbiota. After 90 min, the degradation of q3gal **4** was almost 100% (**Figure 3**). The full metabolism of q3rham **5** was visible after an incubation time of 4 h. In the incubation samples of all three quercetin-3-*O*-*β*-D-monoglycosides **2**, **4**, and **5** the liberated aglycone quercetin **19** was further metabolized by the microbiota. The quercetin degradation products phloroglucinol **18** and DOPAC **12** were detectable via GC-MS after silylation. Alternative ring fission products were not detectable via HPLC-DAD and GC-MS. The liberated aglycone quercetin **19** was almost completely metabolized after 8 h. These data are not shown here because the degradation of quercetin **19** using the pig cecum model has been already described in detail by Keppler et al. (*46*).

Microbial Degradation of Quercetin Glycosides Depending on the Size of the Sugar Moiety. To compare the microbial metabolism of quercetin glycosides with sugar moieties of different sizes, q3glu **2**, q3ru **1**, and qtri **6** were incubated with a bacterial inoculum suspension. These compounds are used as examples of flavonol monoglycosides (q3glu **2**), diglycosides (q3ru **1**), and triglycosides (qtri **6**). Because the sugar moieties of the glycosides **1**, **2**, and **6** were linked over a *β*-glycosidic bonded D-glucopyranoside to the C3-position of quercetin **19**, it can be expected that the deglycosylation is catalyzed by *β*-glucosidases. **Figure 4** shows the declining concentrations of the three quercetin glycosides **1**, **2**, and **6** due to the microbial degradation.

As seen before, the q3glu **2** was nearly completely degraded within 20 min. The hydrolysis rates of the diglycoside **1** and the triglycoside **6** are almost identical. Both were fully hydrolyzed after 4 h of incubation time. The different degradation rates of the mono- **2** and both the di- and triglycosides **1** and **6** indicated a steric hindrance of the latter. In contrast to this, the similar degradation rates of the di- **1** and the triglycoside **6** are unexpected. Regarding the detected metabolites of the di- **1** and triglycosides **6**, the effect becomes clear. The sugar moiety of the diglycoside **5** was hydrolyzed as intact disaccharide, and the aglycone **19** was directly liberated, whereas the trisaccharide **6** was partially cleaved before the hydrolysis of the sugar moiety from quercetin **19**. The proposed degradation pathway of trisaccharides **6** and **7** is shown in **Figure 5**. In the case of **6** we could detect two metabolites: the quercetin-3-*O*-*β*-D-isorutinoside (iso-q3ru) **20** [retention time (t_R) 12.8 min] and the q3ru **1** (t_R 13.6 min), but not the deglycosylation product q3glu **2**. The same metabolism was observed in the case of the ktri **7**. Obviously, one of the α-L-rhamnopyranosides has to be split off by the corresponding enzyme (cleaving position and pathways 1 and 2, **Figure 5**) before the remaining disaccharide is hydrolyzed. We conclude from these results that the steric demand for the *β*-glucosidase seems to be inappropriate to

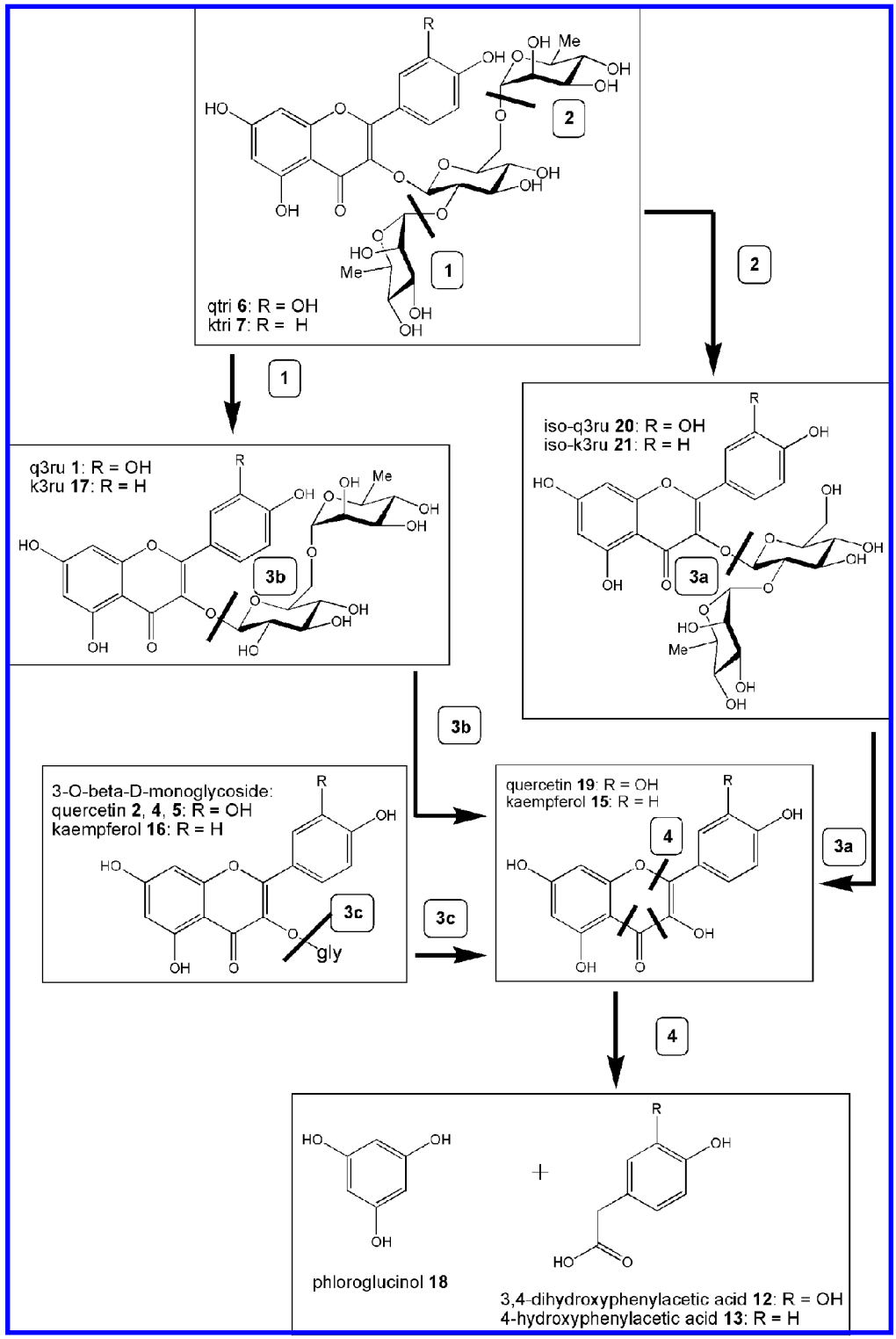

Figure 5. Proposed degradation pathway of the flavonol triglycosides **6** and **7** and of flavonol monoglycosides (numbers in circles indicate cleavage position and pathway).

directly hydrolyze the trisaccharide as a whole. Consequently, an α-rhamnosidase cleaves the trisaccharide, leading to the diglycosides **1** and **20** (cleaving position and pathways 1 and 2, **Figure 5**). Then a *β*-glucosidase hydrolyzes the diglycosidic moiety from the aglycone as described before (cleaving position and pathways 3a and 3b, **Figure 5**). There is no hint for cleaving a further rhamnopyranoside from the diglycoside. This indicates that the cleavage of disaccharides, as less preferred substrates for *β*-glucosidases, is the limiting step. These observations correspond with our knowledge of the composition of the gut microbiota with different bacteria and therefore a wide variety of enzyme activities. Common bacteria species in the gut have the necessary enzyme activities to cleave *β*-D-glucosidic bonds (*44*). In contrast to that, the fission of other glycosidic linkages, for example, the *C*-glycosidic or α-L-rhamnosidic bonds, require special enzymes. These are exhibited either only by a few of the bacteria species or in low concentrations. For instance, Aura et al. determined the rhamnosidase and glucosidase activities in the feces of humans using *p*-nitrophenol-*β*-D-glucopyranoside and *p*-nitrophenol-α-L-rhamnopyranoside as substrates for the

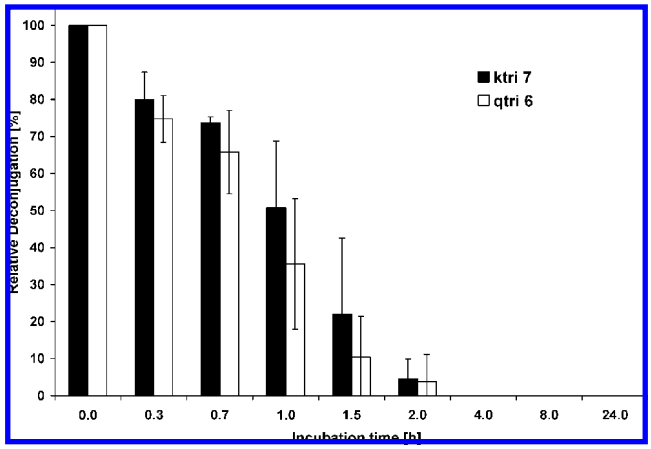

Figure 6. Relative proportions (percent) of the triglycosides qtri **6** and ktri **7** in active cecal suspension with reference to that in sterilized inoculum ($n = 3$, mean ± SD; measured by HPLC-DAD at 360 nm).

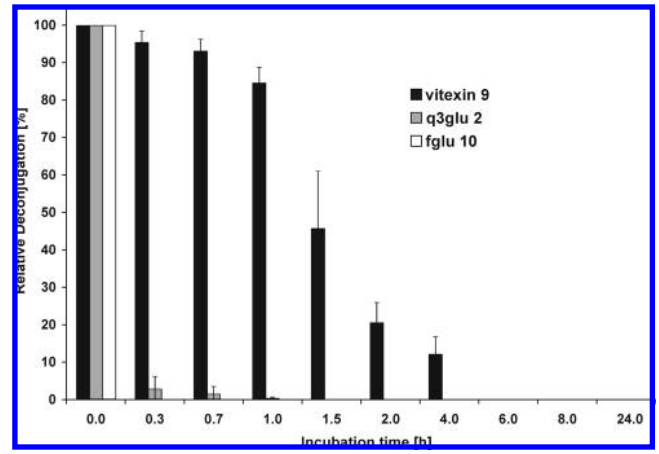

Figure 7. Relative proportions (percent) of vitexin **9**, q3glu **2**, and fglu **10** in active cecal suspension with reference to that in sterilized inoculum ($n = 3$, mean ± SD; measured by HPLC-DAD at 360 nm).

corresponding enzymes. They determined a β-D-glucosidase activity nearly 3 times higher than the α-L-rhamnosidase activity (*45*). In some other studies the authors observed also a difference in the microbial degradation rate of various flavonoid glycosides depending on the sugar moiety. For example, they reported that the degradation of the quercetin diglucoside quercetin-3-*O*-β-D-rutinoside (q3ru **1**) proceeds more slowly than that of its monoglucoside quercetin-3-*O*-β-D-glucoside (q3glu **2**) (*19, 46*).

In the degradation experiments from all three quercetin glycosides **1**, **2**, and **6** we could detect the metabolites from the degradation of the aglycone quercetin **19**, phloroglucinol **18**, and DOPAC **12** by GC-MS as described before. Furthermore, in the incubation experiments of ktri **7** we were also able to detect the corresponding metabolites from the aglycone kaempferol **15**, phloroglucinol **18**, and 4-hydroxyphenylacetic acid **13**. Alternative ring fission products were not detectable by HPLC-DAD and GC-MS. The liberated aglycones **15** and **19** were completely metabolized within 8 h (data not shown).

Microbial Degradation of Flavonol Triglycosides Depending on the Kind of Aglycone. To evaluate the influence of the aglycone structure on the microbial degradation rate, we incubated the flavonol triglycosides **6** and **7** showing a different hydroxyl pattern of the aglycone with a bacterial inoculum suspension. As can be seen from **Figure 6**, the microbial degradations of the two flavonol triglycosides **6** and **7** are in close resemblance. After an incubation time of 1 h, the degradation reached nearly 50%, and within 4 h of incubation both triglycosides **6** and **7** were fully hydrolyzed. Therefore, we came to the conclusion that, in accordance with Simons et al., the variation of the structure within a subgroup has no influence on the microbial degradation rate if the 5,7,4′-hydroxyl pattern is still complete (*47*). This is in agreement with our recent results. Keppler et al. demonstrated that the influence of the structure of the aglycone has no influence on the microbial degradation rate of flavonol glycosides even within different subgroups as flavonols and anthocyanins. For these studies, the microbial degradation of q3ru **1** and the cyanidin-3-*O*-[-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (c3ru) was investigated (*20, 46*).

Microbial Degradation of Polyphenol Monoglycosides Depending on the Type of Glycosidic Bond. Due to the variations of the enzyme activities in the gut microbiota as described above, we studied the influence of the type of glycosidic bond on the degradation rate using q3glu **2** with a common β-D-*O*-glucosidic bond, vitexin **9** representing a *C*-glucoside, and fglu **10** as example for an ester binding. In this experiment only compounds with the same sugar were used, as we have shown that the kind of sugar moiety has a considerable effect on the degradation rate. In **Figure 7** the microbial degradation of the polyphenol glycosides **2**, **9**, **10** is summarized.

The q3glu **2** was completely deglycosylated within 1 h of incubation as seen before. The hydrolysis of the ester fglu **10** was much faster. Already after 20 min of incubation, no fglu **10** could be detected via HPLC-DAD. After 1 h of incubation, 84.7 ± 4.2% of the vitexin **9** was still detectable. Finally, it was completely metabolized between 4 and 6 h of incubation time. These results indicate that the type of glycosidic bond has a substantial influence on the degradation rate of flavonoid glycosides. In fact, the metabolism of a *C*-glycosidic bond seems to be much slower than the hydrolysis of an *O*-glycosidic bond. The degradation of the ester-linked glycoside shows the fastest degradation rate from all three examined compounds.

Microbial Degradation of Flavonol Triglycosides Depending on the Kind of Binding Site. After studying the influence of different saccharides bound to flavonoids, we were also interested if the binding site of the sugar moiety to the aglycone has any influence on the microbial degradation. For this investigation we chose the two quercetin glucosides q3glu **2** and q4'glu **3** as model compounds, unfortunately there is no quercetin monoglucoside commercially available with its O-glycoside bound to the A-Ring.

The microbial metabolism of the two quercetin glucosides **2** and **3** was in close resemblance. After an incubation time of 20 min, the degradation reached nearly 98%, and within 1 h of incubation both triglycosides **2** and **3** were fully hydrolyzed (**Figure 8**). This result is again a clear indication for the sugar moiety being the most critical factor in the microbial metabolism. There is no significant difference for degradation rates of both flavonol glucosides regardless if the sugar is bound to the C-ring (**2**) or the B-ring (**3**), which was expected from previous findings (*20*). The quercetin aglycone **19** liberated in these experiments was further metabolized to mainly DOPAC **12** and 3,4-dihydroxyphenylacetic acid **13** as already discussed above.

Microbial Degradation of Polyphenol Glycosides in the Presence of Other Flavonoids. To simulate the degradation conditions in real samples, a standard mixture of five different flavonoids, each 100 μM, was incubated with a bacterial inoculum suspension. We used vitexin **9** as representative for

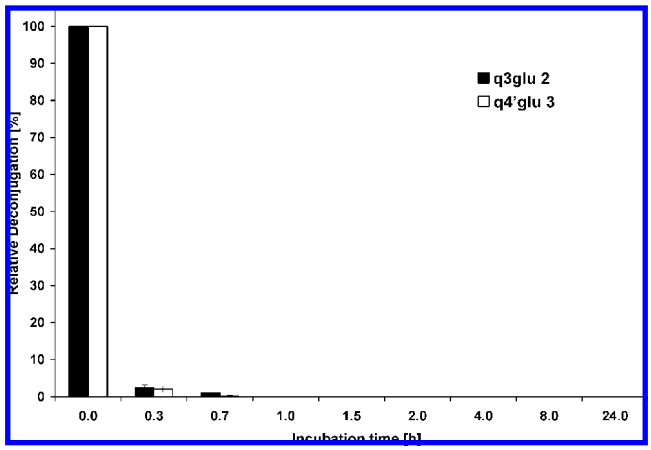

Figure 8. Relative proportions (percent) of q3glu **2** and q4′glu **3** in active cecal suspension with reference to that in sterilized inoculum ($n = 3$, mean ± SD; measured by HPLC-DAD at 330 and 360 nm).

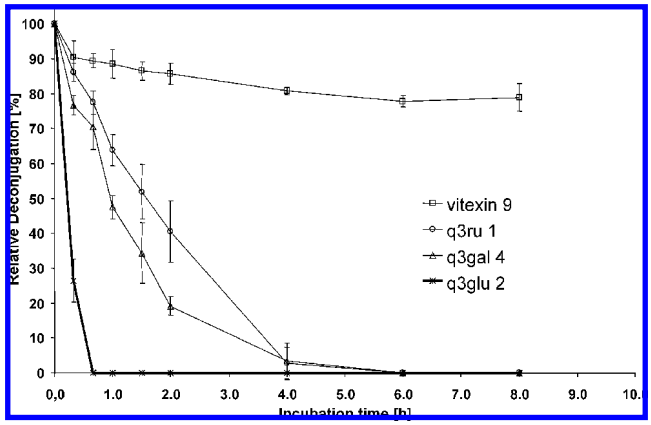

Figure 9. Relative proportions (percent) of a mixture of vitexin **9**, q3ru **1**, q3glu **2** and q3gal **4** in active cecal suspension with reference to that in sterilized inoculum ($n = 3$, mean ± SD; measured by HPLC-DAD at 330 and 360 nm).

C-monoglycosides and q3ru **1** for the large group of *O*-oligoglycosides. The isomers q3glu **2** and q3gal **4** were chosen representing two *O*-monoglycosides with different sugar moieties. The choice for the aglycone quercetin **19** was motivated by the question of how the bacterial hydrolysis of quercetin glycosides affects the metabolism of the aglycone itself. We expected for the four glycosides **1**, **2**, **4**, and **9** a degradation behavior comparable to the results of the single-compound incubation experiments. **Figure 9** shows the degradation profiles of the four flavonoid glycosides **1**, **2**, **4**, and **9**. For the three *O*-glycosides **1**, **2**, and **4** a degradation similar to the single-compound incubation experiments was noticed. In the observed concentration span, the presence of other flavonoid glycosides seemed to have no influence on the degradation at all, even if the rates slightly decrease due to the larger total amount of flavonoids. Only the *C*-glycoside **9** was much more slowly metabolized compared to the single-compound incubation experiment (**Figure 7**), suggesting that the bacteria first metabolize easy accessible energy sources. After 8 h of incubation with inoculum suspension, only 11.7 ± 2.9% of the vitexin **9** was degraded. In **Figure 10** the degradation profile of quercetin **19** during the microbial metabolism of the flavonoid mixture is shown. As expected, the quercetin concentration increased during the first hour of incubation due to the hydrolysis of the quercetin glycosides to a maximum of 165.0 ± 36.9 μM. This concentration remained almost constant for the next 3 h

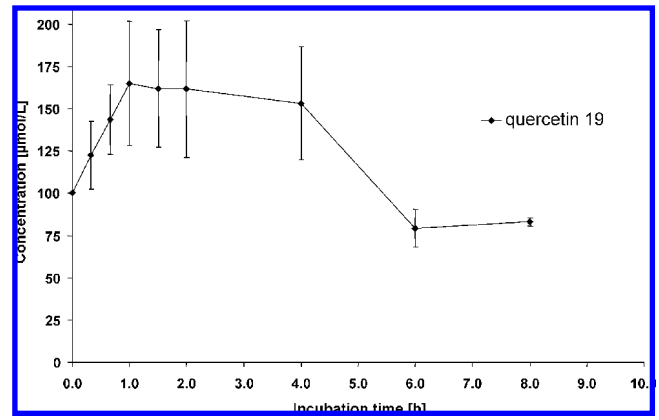

Figure 10. Concentration profile of quercetin **19** (8 h of incubation with pig cecum inoculum filtrate, quantified by HPLC-DAD at 360 nm, $n = 3$, mean ± SD).

of incubation until the microbial degradation of the aglycone **19** became apparent after 4 h. This was the time point at which the quercetin *O*-glycosides **1**, **2**, and **4** were degraded almost totally.

Concluding Remarks. We have shown that both the sugar moiety and the type of glycosidic binding affect the microbial degradation of flavonoids. The degradation rates of the quercetin monoglycosides **2**, **4**, and **5** showed a clear dependency on the hydroxyl pattern of the sugar moiety. The degradation of q3glu **2**, with all hydroxyl groups of the glucose in equatorial position, was the fastest. Compared to the monoglycosides, the intestinal metabolism of quercetin di- and triglycosides **1** and **6** was much slower. As reported before, the structure of the aglycone has not much influence on the intestinal metabolism, as can be seen from the similar degradation rates of the quercetin and kaempferol triglycosides qtri **6** and ktri **7**. The proposed degradation pathway of **6** and **7** is based on the identification of flavonol isorutinoside **20** and **21** and flavonol rutinosides **5** and **17** as intestinal metabolites via LC-MS/MS. These findings indicate that the intestinal enzyme system is not able to directly cleave the trisaccharide; one of the α-L-rhamnopyranosides has to be split off before the remaining disaccharide is hydrolyzed.

Furthermore, we could show that the type of glycosidic bond has a great influence on the degradation rate. The intestinal metabolism of the ester fglu **10** was 3 times faster compared to that of the β-glycoside **2**. In contrast, the degradation of the *C*-glucoside vitexin **9** was 5 times slower compared to that of **2**. Finally, to simulate the degradation conditions in real samples, a standard mixture of five different flavonoids (**1**, **2**, **4**, and **9**) was incubated with a bacterial inoculum suspension. Interestingly, most of the compounds showed a similar degradation rate compared to the incubation experiments using the single compound except for vitexin, which was much more slowly metabolized.

In summary, we could demonstrate that flavonol *O*-glycosides are almost completely metabolized by the intestinal microbiota within 20 min and 4 h depending on the sugar moiety and the type of glycosidic bond. The liberated aglycones were completely metabolized within 8 h to phenolic degradation products such as 3,4-dihydroxyphenylacetic acid **12**, 4-hydroxyphenylacetic acid **13**, and phloroglucinol **18**, which might be responsible for the observed antioxidative activities described in the literature. The degradation of the *C*-glycoside vitexin was much slower and even slower in the presence of other flavonoids, indicating that the bacteria first metabolize easily accessible energy sources.

The characterization of the cecal microbiota by FISH with 16S rRNA-based oligonucleotide probes detecting species of the genus *Bifidobacterium* and of different phylogenetic clusters of *Bacteroides* spp., *Eubacterium* spp., and *Clostridium* spp. confirmed the suitability of the pig cecal model system for studying intestinal metabolism by the human microbiota.

ABBREVIATIONS USED

FISH, fluorescence in situ hybridization; DMSO, dimethyl sulfoxide; DOPAC, 3,4-dihydroxyphenylacetic acid; fglu, feruloyl-*O*-*β*-D-glucopyranoside; ktri, kaempferol-3-*O*-[α-L-dirhamnopyranosyl-(1→2)-(1→6)-*β*-D-glucopyranoside; q3gal, quercetin-3-*O*-*β*-D-galactoside; q3glu, quercetin-3-*O*-*β*-D-glucoside; q3rham, quercetin-3-*O*-*β*-D-rhamnoside; q3ru, quercetin-3-*O*-*β*-D-rutinoside; q4′glu, quercetin-4′-*O*-*β*-D-glucoside; qtri, quercetin-3-*O*-[α-L-dirhamnopyranosyl-(1→2)-(1→6)-*β*-D-glucopyranoside; t_R, retention time.

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