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Use of the pig caecum model to mimic the human intestinal metabolism of hispidulin and related compounds

Samira Labib[1], Sylvia Hummel[1], Elke Richling[1], Hans-Ulrich Humpf[2] and Peter Schreier[1]

[1]Food Chemistry, University of Würzburg, Würzburg, Germany
[2]Food Chemistry, University of Münster, Münster, Germany

Up to now, the metabolism of hispidulin (5,7,4′-trihydroxy-6-methoxyflavone), a potent ligand of the central human benzodiazepine receptor, has not been investigated. To elucidate the metabolism of hispidulin in the large intestine, its biotransformation by the pig caecal microflora was studied. In addition, the efficiency of the pig caecal microflora to degrade galangin (3,5,7-trihydroxyflavone), kaempferol (3,5,7,4′-tetrahydroxyflavone), apigenin (5,7,4′-trihydroxyflavone), and luteolin (5,7,3′,4′-tetrahydroxyflavone) was investigated. Identification of the formed metabolites was performed by high-performance liquid chromatography (HPLC)-diode array detection, HPLC-electrospray ionization-tandem mass spectrometry, and high-resolution gas chromatography-mass spectrometry. The caecal microflora transformed hispidulin to scutellarein (5,6,7,4′-tetrahydroxyflavone), an effective α-glucosidase inhibitor, and 3-(4-hydroxyphenyl)-propionic acid; galangin to phenylacetic acid and phloroglucinol; kaempferol to 4-hydroxyphenylacetic acid, phloroglucinol, and 4-methylphenol; apigenin to 3-(4-hydroxyphenyl)-propionic acid and 3-phenylpropionic acid, and luteolin to 3-(3-hydroxyphenyl)-propionic acid, respectively. To elucidate to what extent different hydroxylation patterns on the B-ring influence the degradation degree of flavonoids, the conversions of galangin and kaempferol as well as that of apigenin and luteolin were compared with those of quercetin (3,5,7,3′,4′-pentahydroxyflavone) and chrysin (5,7-dihydroxyflavone), respectively. Regardless of the flavonoid subclass, the presence of a hydroxy group at the 4′-position seems to be a prerequisite for fast breakdown. An additional hydroxy group at the B-ring did not affect the degradation degree.

Keywords: Flavonoid metabolism / Galangin / Hispidulin / Kaempferol / Pig caecum model

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1 Introduction

Flavonoids are the most common and widely distributed group of plant phenolics, with more than 5000 different compounds described [1]. Depending on structural features, the flavonoids can be subdivided into flavonols, flavanones, flavones, isoflavones, catechins and anthocyanidines. These polyphenolic compounds occur primarily as glycosides in fruits, vegetables and beverages such as red wine and tea. The daily intake of flavonoids is estimated to reach a few hundred milligrams [2]. As dietary supplements rich in flavonoids are increasingly consumed in Western countries, the daily intake can be expected to be even higher.

Various health effects have been proposed for flavonoids, even though they are non-nutritive compounds. A considerable amount of research has been directed towards their activity as antioxidants and radical scavengers, as well as to their anti-mutagenic and anti-inflammatory properties. However, the main interest has been paid to their potential in the prevention of coronary heart disease and cancer [3–8].

Biological properties of flavonoids depend on their bioavailability. To evaluate the bioavailability of flavonoids, it is necessary to know all possible bioactive forms *in vivo*. Flavonoids are extensively metabolized either in tissues, once they are absorbed through the small intestine, or, for the non-absorbed fraction and the compounds re-excreted in the bile, by colonic microorganisms [9]. The products formed can be absorbed in the colon and occur in the plasma. The bacterial metabolites formed may exhibit bio-

Correspondence: Prof. Dr. Peter Schreier, Lehrstuhl für Lebensmittelchemie, Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany
E-mail: schreier@pzlc.uni-wuerzburg.de
Fax: +49-931-888-5484

Abbreviations: DAD, diode array detection; **HRGC**, high-resolution gas chromatography

logical activities that differ from those of the original flavonoids. Thus, it is essential to identify and characterize these products originating from the microbial metabolism.

We have previously reported that the pig caecum model is suitable to simulate the metabolic events taking place in the human large intestine [10]. In this paper, we describe the colonic transformation of the flavone hispidulin (5,7,4′-trihydroxy-6-methoxyflavone) **1**, recently identified as a potent ligand of the central human benzodiazepine receptor, and a rare example of a flavone for which clear-cut *in vivo* effects have been elucidated [11]. In addition, the efficiency of the pig caecal microflora to degrade galangin (3,5,7-trihydroxyflavone) **2**, kaempferol (3,5,7,4′-tetrahydroxyflavone) **3**, apigenin (5,7,4′-trihydroxyflavone) **4**, and luteolin (5,7,3′,4′-tetrahydroxyflavone) **5** is presented. To elucidate to what extent different hydroxylation patterns on the B-ring influence the degradation degree of flavonoids, we compared the microbial conversions of the flavonols **2** and **3** as well as those of the flavones **4** and **5** with that of quercetin (3,5,7,3′,4′-pentahydroxyflavone) **6** and chrysin (5,7-dihydroxyflavone) **7**, respectively [10] (for structures see Fig. 1).

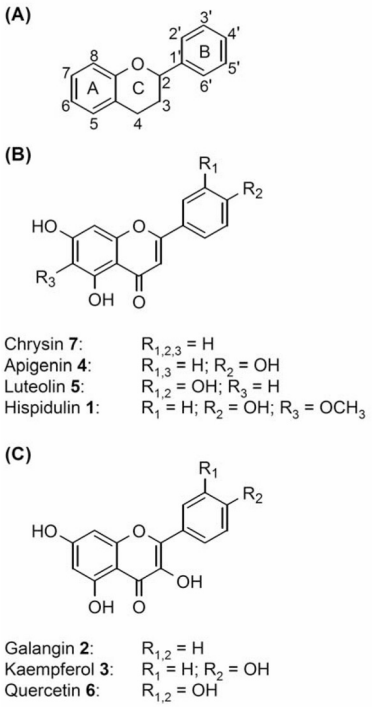

Figure 1. (A) Basic structure and numbering system of flavonoids. (B) Chemical structures of flavones under study. (C) Chemical structures of flavonols under study.

2 Materials and methods

2.1 Chemicals

All chemicals and solvents were of analytical grade. Solvents were distilled before use. Hispidulin **1** was synthesized as described by Kavvadias *et al.* [12]. In brief, it started with 4-benzyloxy-2,3-dimethoxy-6-hydroxyacetophenone and 4-benzyloxybenzoic acid chloride, both prepared in separate routes from 2,4,6-trihydroxyacetophenone and 4-hydroxybenzoic acid, respectively. The benzoyl ester was built from the acetophenone and acid chloride component and rearranged to the corresponding 1,3-diketone under basic conditions. The 1,3-diketone cyclized to the flavone in the presence of acid and heat. In the last step, the protection groups and the labile C-5-methoxy group were removed by reaction with 1 M BCl_3 solution at $-70^{\circ}C$ to yield **1**. UV: λ_{max} = 215, 274, 334 nm; *m/z*: 301 $[M+H]^+$, MS/MS: *m/z*: 301, 286, 258, 168, 140, 121. Scutellarein **1b** was purchased from Extrasynthèse (Genay, France), 3-(4-hydroxyphenyl)-propionic acid **1a**, phloroglucinol **2a**, phenylacetic acid **2b**, 4-hydroxyphenylacetic acid **3a**, 4-methylphenol (p-cresol) **3b** as well as apigenin **4**, and quercetin dihydrate **6** were products from Fluka (Deisenhofen, Germany). Galangin **2**, luteolin **5**, 3-(3-hydroxyphenyl)-propionic acid **5a**, and chrysin **7** were obtained from Lancaster (Frankfurt, Germany); kaempferol **3**, was a product from Roth (Karlsruhe, Germany) and 3-phenylpropionic acid **4a** was purchased from Acros Organic (Geel, Belgium).

2.2 Collection and preparation of inoculum

Caeci were collected from freshly slaughtered healthy German hybrid pigs aged between 3 and 5 months with a live body weight ranging between 110 and 140 kg. The pigs were under strict veterinary monitoring for commercial meat production.

Freshly withdrawn caeci (with a volume ranging between 0.7 and 2.0 L) were immediately ligated, placed in an anaerobic jar containing Anaeorocult® A (Merck, Darmstadt, Germany) to create an anaerobic atmosphere and transported to the laboratory where the anaerobic jar was transferred immediately to an anaerobic chamber (self-constructed) flushed with an N_2/CO_2 gas mixture (80 : 20 v/v). For each experiment aliquots of the content of three or four different caeci (each of 150 mL) were pooled and diluted with the same volume of anaerobic carbonate-phosphate-buffer according to Lebet *et al.* [13] (pH 6.3; 37°C). The caeci contents were briefly homogenized and coarse particles removed by filtration through glass wool. The filtrate was used as inoculum.

2.3 Incubation conditions

Aliquots of 5 mL inoculum were added to each pregassed incubation vessel containing an aliquot of 20 μL from a stock

solution of **1**, **2**, **3**, and **6** (each 125 mM) as well as **4, 5**, and **7** (each 175 mM) in DMSO. The incubation vessels were sealed tightly and incubated at 37°C for 0, 2, 4, 6, 8, 10 and 24 h. To stop the enzymatic reaction the vessels were placed in liquid nitrogen and lyophilized. For controls, samples with 20 μL DMSO and caecal inoculum as well as samples with flavonoids and buffer were prepared and subjected to the procedure described above. All inoculations were performed in triplicate, using two different caeci mixtures.

2.4 Sample preparation

The freeze-dried samples were extracted twice with 2.5 mL 70 or 80% (in the case of hispidulin) methanol in water v/v containing 1% v/v acetic acid. For the extraction of chrysin 100% methanol was used. After extraction, the samples were centrifuged at 5000 × g for 10 min, and supernatants were pooled and filtered (PVDF, 0.45 μm). Controls were treated identically. Aliquots (50 μL) of the extract were injected onto HPLC using the chromatographic system described below.

2.5 HPLC-diode array detection analysis

The HPLC system used was a Hewlett-Packard 1100 HPLC gradient pump and a Hewlett-Packard 1100 photodiode array detector (Waldbronn, Germany), equipped with a Wisp 710b autosampler (Waters, Eschborn, Germany). Data acquisition and evaluation were performed with a Hewlett-Packard Chemstation software. A Eurospher 100-C18 column, 4 × 250 mm, with 5-μm particle size, (Knauer, Germany) was used. The mobile phase consisted of aqueous 0.05% v/v TFA (A) and ACN (B). The gradient applied was 1–99% B in 40 min at a flow rate of 1 mL/min. The peaks were identified by comparison of retention time and absorption spectra (200 to 600 nm) with authentic references. Calibration curves (at the appropriate wavelength according to the absorption maximum of the compounds) were used for quantification.

2.6 Sample preparation for HPLC-ESI-MS, ESI-MS/MS, and HRGC-MS analysis

The molecular mass of the identified flavonoid metabolites was determined by analyzing selected extracts from incubation experiments by HPLC-ESI-MS. To obtain additional structural information a semi-preparative collection of individual compounds was performed by HPLC-diode array detection (DAD) followed by lyophilization. The lyophilizate was dissolved in methanol and analyzed by ESI-MS/MS or GC-MS (in the case of phloroglucinol **2 a** and 4-methylphenol **3 b).**

2.7 HPLC-ESI-MS, ESI-MS/MS analysis

HPLC-ESI-MS was performed with a TSQ 7000 tandem mass spectrometer system equipped with an ESI interface (Finnigan MAT, Bremen, Germany) and an Applied Biosystems 140b pump (BAI, Bensheim, Germany). Data acquisition and evaluation were conducted on a DEC 5000/33 (Digital Equipment, Unterföhring, Germany) using Finnigan MAT ICIS 8.1 software. HPLC chromatographic separation was carried out on a Waters Symmetry C18 column, 2.1 × 150 mm, with 5-μm particle size (Waters, Milford, MA, USA). The mobile phase consisted of aqueous 1% formic acid (A) v/v and ACN (B). The gradient applied was 5–99% B in 30 min at a flow rate of 0.2 mL/min, and 5-μL injection volume. The analysis was performed in negative ionization mode. The spray capillary voltage was set to 3.2 kV, and the temperature of the heated capillary was 200°C. Nitrogen served as both sheath (70 psi) and auxiliary gas (10 U). The mass spectrometer was operated in the full-scan mode, *m/z* 120–650, with total scan duration of 1.0 s. MS/MS experiments were performed at collision energy of 20–40 eV, with argon (2.0 mTorr) serving as collision gas. The obtained molecular ion peaks and product mass spectra were compared with those of authentic references.

2.8 HRGC-MS analysis

High-resolution GC (HRGC)-MS was performed with a Fisons Instruments GC 8000 Series gas chromatograph (Fisons, Egelsbach, Germany) coupled to a Fisons Instruments MD 800 quadrupol mass detector. The GC was equipped with a J&W DB-5 fused silica capillary column (30 m 6 0.25 mm ID; d_f = 0.25 μm; J&W, Folsom, CA, USA). The temperature program was from 60 to 310°C at 5°C/min and held for 10 min at 310°C. Helium was used as carrier gas at a constant pressure of 90 kPa. Injection was carried out using a split injector (1:20) at 250°C. Electron impact mass spectra (EI-MS) were recorded at of 70 eV using a source temperature of 220°C. Identifications were performed by comparison of retention times and mass spectral data of sample constituents with those of authentic reference compounds.

3 Results and discussion

3.1 General remarks

Up to now, the metabolic fate of hispidulin **1** has not been investigated. To mimic the metabolism of hispidulin **1** and related compounds in the large intestine, their biotransformation by the pig caecal microflora was studied. Furthermore, the caecal microflora was also used to investigate the

impact of the chemical structure of selected flavonoids on their metabolization.

3.2 Transformation of hispidulin 1

The pig caecum microflora converted hispidulin **1** (0.5 mM) (retention time, t_R = 23.0 min) almost completely within 24 h of incubation. By means of HPLC-DAD analysis, we detected two metabolites eluting at 14.4 min **1 a** and 19.3 min **1 b**, respectively (data not shown). These peaks were absent in the chromatographic profiles of control samples (flavonoid with buffer and inoculum with DMSO; data not shown). The metabolite eluting at 14.4 min **1 a** was identified as 3-(4-hydroxyphenyl)-propionic acid, its retention time and UV spectrum were identical to those of an authentic reference. The HPLC-ESI-MS (negative mode) spectrum of this metabolite showed the expected molecular ion peak at *m/z* 165. The ESI_{neg}-MS/MS daughter ion pattern was in agreement with that of authentic **1 a**, *i. e.* exhibiting *m/z* of 121, 93 and 59.

The metabolite **1 b** was identified as scutellarein, based on comparison of its retention time and UV spectrum with a commercially available reference. HPLC-ESI-MS (negative mode) analysis confirmed its expected molecular mass (*m/z* 285). In addition, ESI_{neg}-MS/MS analysis led to a pattern of daughter ions that was identical to the authentic reference **1 b**, *i. e.* exhibiting *m/z* of 167, 137 and 95. Structures of **1 a** and **1 b** are shown in Fig. 2.

The time course of hispidulin **1** degradation is depicted in Fig. 3, revealing rapid O-demethylation of **1** to form scutellarein **1 b**. The subsequent degradation of scutellarein **1 b** proceeded very slowly, as only small amounts of 3-(4-hydroxy-phenyl)-propionic acid **1 a** were detectable after 24 h of incubation. The formation of **1 b**, an effective α-glucosidase inhibitor [14], from **1** indicates a demethylase activity of the pig caecum microflora, as previously reported [10]. In addition, similar activity has also been reported for colonic *Eubacterium limosum* in the case of isoflavonoids [15]. Furthermore, Nielson *et al.* [16] have reported that the 6-position was found to be the second most frequent site for demethylation of tangeretin, a 5,6,7,8,4′-pentamethoxyflavone. The formation of **1 a** by subsequent ring cleavage of **1 b** was not surprising, as breakdown of the heterocyclic ring of flavones has been described to be favorized between C-4 and C-5 [17]. However, the at least theoretically expected 1,2,3,5-tetrahydroxybenzene, resulting from A-ring liberation, could not be detected in our study. Heider and Fuchs [18] have reported its rapid transformation via phloroglucinol to form acetyl-CoA and CO_2. Its use as a cosubstrate for pyrogallol degradation by anaerobic bacteria has also been described [18]. Thus, 1,2,3,5-tetrahydroxybenzene was not detected in our experiments, obviously due to low intermediary amounts.

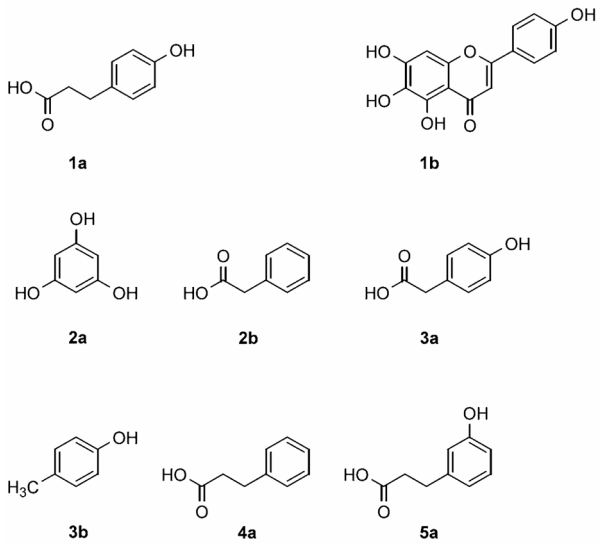

Figure 2. Chemical structures of the metabolites detected in this study (for details see text): 3-(4-hydroxyphenyl)-propionic acid **1 a**, scutellarein **1 b**, phloroglucinol **2 a**, phenylacetic acid **2 b**, 4-hydroxyphenylacetic acid **3 a**, 4-methylphenol **3 b**, 3-phenylpropionic acid **4 a**, 3-(3-hydroxyphenyl)-propionic acid **5 a**.

3.3 Transformation of galangin 2, kaempferol 3 and quercetin 6

The three flavonols **2**, **3**, and **6** (0.5 mM each), which differ only in their hydroxylation patterns on the B-ring (Fig. 1), were inoculated under anoxic conditions with caecal microflora from the same source and the samples were continuously, over 24 h, analyzed by HPLC-DAD. Complete degradation of galangin **2** (t_R = 27.5 min) by the intestinal microflora occurred within 24 h of incubation, yielding one intermediate with a retention time of 7.2 min **2 a** and an end product with a retention time of 17.8 min **2 b**, as detected by HPLC analysis (data not shown). These peaks were not present in the chromatographic profiles of the control samples (flavonoids with buffer; data not shown). Comparison of retention times and UV spectra of both metabolites with those of commercially available references led to their identification as phloroglucinol **2 a** and phenylacetic acid **2 b** (structures in Fig. 2). The identity of **2 a** was further verified by HRGC-MS: retention time and EI-MS spectrum corresponded to those of an authentic reference. Using ESI_{neg}-MS/MS, the pattern of daughter ions of the end product **2 b** was the same as that of an authentic reference, *i. e.* exhibiting *m/z* 91 and 50.

The time course of galangin **2** decomposition is depicted in Fig. 4 A. The concentrations shown are corrected for endogenous levels of the phenolic metabolites or phenolic acids detected at low levels in the control samples (inoculum with

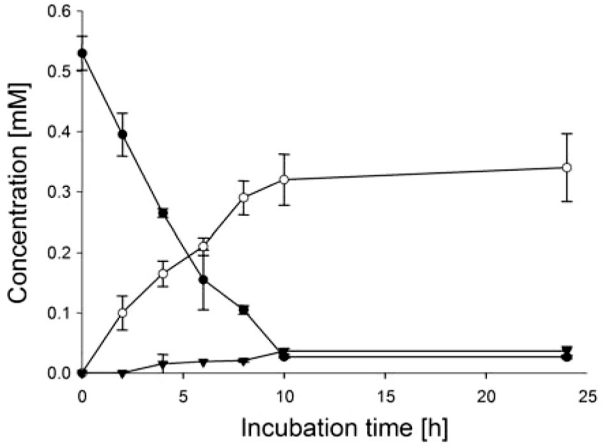

Figure 3. Time course of hispidulin **1** degradation during incubation with pig caecum flora. Hispidulin **1** (●); 3-(4-hydroxyphenyl)-propionic acid **1a** (▼); scutellarein **1b** (○). Data are expressed as mean ± SD.

DMSO; data not shown). If required, this correction was also made in other transformation profiles.

When inoculated with caecal microflora, kaempferol **3** (t_R = 23.1 min) was converted completely within 8 h of incubation. Its degradation was accompanied by the formation of two intermediates and one end product eluting at 7.2 **2a**, 12.4 **3a** and 19.6 min **3b**, respectively, detected by HPLC-DAD analysis (data not shown). These peaks were not present in the chromatographic profiles of the control samples (flavonoids with buffer; data not shown). The time course of kaempferol **3** degradation is shown in Fig. 4B. The metabolite eluting at 7.2 min **2a** was identified as phloroglucinol as described above. The retention times and UV spectra of **3a** and **3b** were identical to those of 4-hydroxyphenylacetic acid and 4-methylphenol, respectively (for structures see Fig. 2). HPLC-MS analysis (negative mode) gave the expected $[M-H]^-$ of *m/z* 151 for **3a**, the pattern of daughter ions for *m/z* 151 $[M-H]^-$ was identical to that of authentic **3a**, *i.e.* exhibiting *m/z* 107, 93 and 79. The identity of 4-methylphenol **3b** was confirmed by HRGC-MS: the EI-MS spectrum of **3b** (*m/z*, %): 108 ($M^{\bullet+}$, 90), 79 (50), 63 (12), 53 (24) was identical to that of the authentic reference.

Quercetin **6** was also completely degraded by the caecal microflora; the metabolites formed were the same as previously reported [10].

As expected, the caecal microflora converted galangin **2** and kaempferol **3** via C-ring cleavage. The A-ring is thereby liberated as phloroglucinol, as postulated for several flavonoid degradation pathways [19–21]. The transient appearance of **2a** in the breakdown of both flavonols (**2** and **3**) is in full agreement with previous microbial studies [10, 19, 20, 22–24]. Furthermore, as reported by others, phloroglucinol being a central intermediate of anaerobic aromatic metabolism, its instant degradation is not surprising [18].

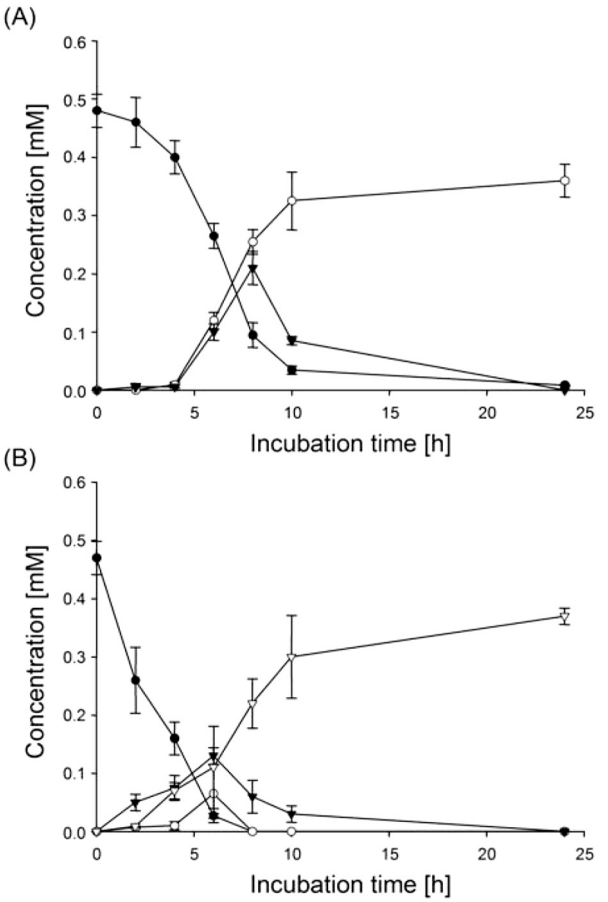

Figure 4. Time course of the degradation of flavonols during incubation with pig caecum flora. (A) Concentrations of galangin **2** (●); phloroglucinol **2a** (▼); phenylacetic acid **2b** (○). (B) Concentrations of kaempferol **3** (●); phloroglucinol **2a** (○); 4-hydroxyphenylacetic acid **3a** (▼); 4-methylphenol **3b** (▽). Data are expressed as mean ± SD.

Phenylacetic acid **2b** and 4-hydroxyphenylacetic acid **3a** were derived from the B-ring of **2** and **3**, respectively. It has already been demonstrated that *Clostridium orbiscindens*, a human colonic bacterium, transformed kaempferol **3** to 4-hydroxyphenylacetic acid **3a** [20]. In addition, incubation of **3** with rat caecal microflora, resulting in the formation of **3a**, has also been reported earlier by Griffiths and Smith [25]. In the present study, the end product of kaempferol **3** degradation was identified as 4-methylphenol **3b**. The concentration of **3b** increased concomitantly with the decrease in the concentration of 4-hydroxyphenylacetic acid **3a** (Fig. 4B). It is therefore conceivable that a subsequent decarboxylation of **3a** led to the formation of **3b**.

It is well known that 4-methylphenol **3b** is formed from tyrosine via 4-hydroxyphenylacetic acid **3a** mainly by anaero-

bic intestinal bacteria [26 – 30]. Recently, Selmer and Andrei [31] have purified and characterized the enzyme responsible for **3b** formation from *C. difficile,* a bacterium that is known to occur in the human gut microbiota. The enzyme was described as highly sensitive towards molecular oxygen and able to decarboxylate only phenylacetates containing a hydroxy group in para-position. Thus, the enzyme decarboxylates p-hydroxyphenylacetic acid **3a** and 3,4-dihydroxyphenylacetic acid to **3b** and 3,4-dihydroxytoluene, respectively, but no decarboxylation occurred when phenylacetic acid **2b** was used [31]. Furthermore, Ward *et al.* [32] have reported the isolation of an anaerobic bacterium from swine faeces, presumably belonging to the genus *Lactobacillus*, which also decarboxylated **3a** to 4-methylphenol **3b**. The isolated bacterium possesses identical specificity of the decarboxylation reaction as the enzyme described above [31].

As to these data, our findings are in agreement with the above-mentioned results. Decarboxylation of phenylacetic acid **2b** was not observed in our study, likely due to the lack of a hydroxy group on the para-position. 4-Hydroxyphenylacetic acid **3a**, in contrast, was rapidly decarboxylated to form 4-methylphenol **3b**. In a previous study, we have shown the formation of 3,4-dihydroxytoluene from 3,4-dihdroxyphenylacetic acid, the result consistent with the mentioned above. Here, we report for the first time a different precursor of **3b**, namely the flavonol kaempferol **3**.

3.4 Transformation of apigenin 4, luteolin 5 and chrysin 7

The three flavones **4**, **5**, and **7** (0.7 mM each), which differ in their hydroxylation patterns on the B-ring (Fig. 1), were also inoculated under anoxic conditions with pig caecal microflora from the same source, and the samples were continuously analyzed by HPLC-DAD for 24 h. The conversion of apigenin **4** (t_R = 23.0 min) yielded two metabolites **1a** and **4a**, exhibiting retention times of 14.4 and 20.1 min, respectively, when analyzed by HPLC-DAD (data not shown). These peaks were not present in the chromatographic profiles of the control samples (flavonoids with buffer; data not shown).

The time course of apigenin **4** degradation is shown in Fig. 5A. Identification of the metabolites was performed by comparing their retention times and UV spectra with those of commercially available 3-(4-hydroxyphenyl)-propionic acid **1a** and 3-phenylpropionic acid **4a**, respectively (for structures see Fig. 2). Using HPLC-ESI_{neg}-MS/MS the identities of **1a** and **4a** were confirmed.

The flavone luteolin **5** (t_R = 20.9 min) was transformed by the intestinal microflora to a single metabolite **5a** eluting at

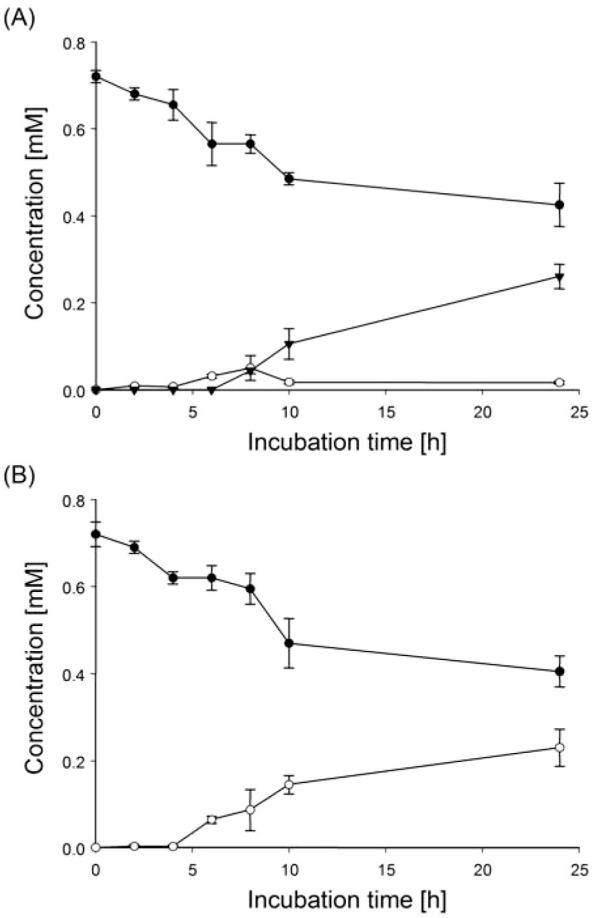

Figure 5. Time course of flavones degradation during incubation with pig caecum flora. (A) Concentrations of apigenin **4** (●); 3-(4-hydroxyphenyl)-propionic acid **1a** (○); 3-phenylpropionic acid **4a** (▼). (B) Concentrations of luteolin **5** (●); 3-(3-hydroxyphenyl)-propionic acid **5a** (●). Data are expressed as mean ± SD.

15 min, as detected by HPLC-DAD. This peak was not present in the chromatographic profiles of the control samples (flavonoids with buffer; data not shown). The time course of luteolin **5** degradation is shown in Fig. 5B. Using the same procedure as for other metabolites, **5a** was identified as 3-(3-hydroxyphenyl)-propionic acid **5a** (for structure see Fig. 2). Confirmation was achieved by HPLC-ESI_{neg}-MS/MS: **5a** and the corresponding reference exhibited the same pattern of daughter ions of *m/z* 165, *i.e. m/z* 121, 119 and 106.

The caecal microflora did not degrade chrysin **7**, as already reported previously [10, 25].

3-(4-Hydroxyphenyl)-propionic acid **1a** has earlier been described as degradation product of apigenin [25] and its further metabolism, resulting in the formation of **4a**, is also well known [10, 33]. The formation of 3-(3,4-dihydroxy-

phenyl)-propionic acid from luteolin **5**, as previously described [23], could not be detected in our study. On the basis of previous experiments, in which the formation of **5a** from 3-(3,4-dihydroxyphenyl)-propionic acid was observed after incubation with human faecal flora [34–36], rapid p-dehydroxylation of the latter has to be considered. In analogy to our observations, immediate p-dehydroxylation has been reported in previous studies [37, 38]. Both flavones were degraded slowly during the incubations (Fig. 5). Accumulation of phloroglucinol **2a**, an expected metabolite by the conversion of **4** and **5**, could not be shown due to its immediate further transformation.

3.5 Comparison of flavonoid degradations

Table 1 summarizes the percentages remained after incubation of flavonoids **2**–**7** with pig caecal microflora at different times. As to flavonols, the microflora was able to convert rapidly kaempferol **3** and quercetin **6** so that, within 4 h of incubation, more than 50% of both flavonols were degraded, and within 10 h of incubation, the substrates disappeared completely. There were nearly no differences between the degradation of kaempferol **3** and quercetin **6**. However, the breakdown of galangin **2**, which lacks any hydroxy group at the B-ring, was quite different. In fact, its degradation proceeded slowly during fermentation in comparison to **3** and **6**, respectively. After 4 h of incubation, about 83% of galangin **2** was still present. Moreover, its complete disappearance, in contrast to **3** and **6**, was achieved only within 24 h of incubation.

The flavonols were selected according to the criterion that the presence of C-3, C-5, and C-7 hydroxy groups in the molecule is a prerequisite for the microbial cleavage [39]. Moreover, the inoculum used in our study should provide an average microflora, due to its preparation by pooling the contents of various caeci from different donors. As the disappearance degree of quercetin **6** (with two hydroxy groups at the B-ring) was very similar to that of kaempferol **3** (with a single hydroxy group at the B-ring), it may be concluded that an additional hydroxy group at the B-ring does not affect the speed of degradation. On the other hand, the slow, compared to **3** and **6**, disappearance of galangin **2** (lacking hydroxy groups at the B-ring), clearly demonstrates that the presence of a hydroxy group at 4′-position enhances the transformation rate of flavonols significantly, when additional hydroxy groups are present at 5- and 7-position. Our finding is consistent with previous observations that hydroxy groups at 5-, 7- and 4′-positions of flavonoids are important structural characteristics for optimal flavonoid degradation by the intestinal microflora [25, 40].

As for flavones (Table 1), both apigenin **4** and luteolin **5** were transformed slowly during incubation, resulting in similar degradation degrees. The concentration of chrysin **7**, in contrast, did not vary during the time course of the experiment. Previously, **7** was recovered to 98% unmetabolized in faeces [41].

As the presence of 5-, 7- and 4′-hydroxy groups in the molecule are reported to be necessary for the microbial breakdown of flavones [17, 25], the substrates were chosen according to this criterion. In accordance with results described above, an additional hydroxy group at the B-ring, as seen in luteolin **5** in comparison to apigenin **4**, did not affect the degradation of flavones. However, comparison of chrysin **7**, which lacks any hydroxylation at the B-ring, with apigenin **4** reveals that the B-ring has to be hydroxylated to enable flavone degradation by the colonic microflora.

From these observations it is apparent that the presence of a hydroxy group in 4′-position at the B-ring , regardless of the flavonoid subclass, seems to be a prerequisite for fast degradation, if additional hydroxy groups are present at the 5- and 7-position. An additional hydroxy group at the B-ring does not affect the degradation.

Since the extent and kinetics of degradation of aglycones also affect the bioavailability of aglycones and their glyco-

Table 1. Percentages remained after incubation of flavonoids with pig caecal microflora at different times[a)]

Flavonoids	% remained after incubation [h][b)]					
	2	4	6	8	10	24
Flavonols						
Galangin **2**	95.8 ± 8.8	83.3 ± 5.9	54.2 ± 5.8	19.8 ± 4.4	7.3 ± 1.5	0
Kaempferol **3**	55.5 ± 13.7	33.3 ± 5.9	5.6 ± 0.9	0	0	0
Quercetin **6**	53.7 ± 1.5	38.9 ± 1.5	18.9 ± 3.0	5.3 ± 1.5	0	0
Flavones						
Chrysin **7**	100.0 ± 3.9	99.9 ± 6.0	98.6 ± 9.9	100.7 ± 1.0	98.6 ± 4.0	99.9 ± 5.9
Apigenin **4**	94.4 ± 1.9	91.0 ± 4.9	78.5 ± 6.9	78.4 ± 1.0	67.4 ± 1.0	59.0 ± 0.9
Luteolin **5**	95.8 ± 1.9	86.1 ± 1.9	86.1 ± 3.9	82.6 ± 4.9	65.3 ± 7.9	56.2 ± 4.9

a) Only flavonoids with identical A-ring are considered.
b) Values are means ± SD.

sides, our results give important clues for the evaluation of the potential bioavailability of flavonoids. Accordingly, it can be suggested that galangin **2** and chrysin **7**, for example, which both lack hydroxylation at the B-ring, may be more bioavailable than rapidly degraded flavonoids, as the microflora degraded them at a slower rate, which gives them a greater opportunity to be absorbed.

A further interpretation of our results (Table 1) reveals that flavonols seem to be more susceptible to breakdown by the intestinal microflora than flavones. Since the only structural difference between both subclasses is a hydroxy group at the position 3 in the heterocyclic C-ring, an association between these structural features and a faster degradation can be assumed. The finding that scutellarein **1 b**, the demethylated product of hispidulin **1**, very slowly degraded during incubation (Fig. 3), is also consistent with our observations. The presence of a hydroxy group at the B-ring enables ring cleavage; however, the degradation is not as fast as in the case of flavonols. These results are in agreement with those of previous studies, which proposed that the C-ring of flavonols is labile for bacterial degradation to yield phenolic acids and ethylbenzene [22, 42]. However, the findings are in disagreement with those of Simons *et al.* [40], who reported that kaempferol **3**, apigenin **4**, and naringenin (5,7,4′ trihydroxyflavanone), the corresponding flavanone of **4** (79 μM each), were extensively degraded when incubated with human faecal microflora. No differences in degradation rates were reported.

Taking into account that the caecal flora differs greatly from the faecal flora [43] and that faecal sampling leads to overestimation of the microbial activity of the caecum [44], the differences between our results and those of others [40] might be explained. In addition, others have described a dependence of the degree of degradation on compound concentrations [33]. Considering that the used flavonoid concentrations in our study were higher than those described by, *e.g.* Simons *et al.* [40], differences of results can also be explained. Finally, Felgines *et al.* [45] have reported, in their experiments established with rats, that after an adaptation to a diet containing naringenin, the rat caecal microflora converted naringenin more rapidly than the rat caecal microflora from non-adapted rats. Therefore, it can not be excluded that because of the frequent occurrence of flavonols in nature compared to flavones, the microflora has adapted to metabolize flavonols faster than flavones.

4 Concluding remarks

Regarding the increasing interest in flavonoids, *e.g.* as dietary supplements, their bacterial transformation to (partly unknown) metabolites has to be considered to prevent the risk of misinterpreting the effects of flavonoids on human health. Not only the activities of the unmodified flavonoids, but also these of their metabolites, have to be assessed. For instance, the metabolite scutellarein **1 b** is an effective α-glucosidase inhibitor, a property that is not known from hispidulin **1**. Alternatively, in addition, other positive effects of **1 b** are also known, such as, *e.g.* its chemopreventive effects on coronary heart disease. On the other hand, the formed metabolites can also possess negative effects on human health, such as, *e.g.* 4-methylphenol [46]. Finally, it is also necessary to take into account that the colonic half-life of the biologically active aglycone is short, as we reported for the flavonols under study. This fact is especially important for interpretation of effects of such flavonoids absorbed only in the large intestine.

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