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The pig caecum model: A suitable tool to study the intestinal metabolism of flavonoids

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Pig caecum was used under anaerobic conditions to metabolize flavonoids from several classes, *i. e.*, chrysin **1**, naringenin **2,** quercetin **3**, and hesperetin **4**. Whereas chrysin **1** was not converted by the pig intestinal flora under the experimental conditions used, naringenin **2** was transformed to 3-(4-hydroxyphenyl)-propionic acid and 3-phenylpropionic acid. Quercetin **3** was metabolized to phloroglucinol, 3,4-dihydroxyphenylacetic acid, and 3,4-dihydroxytoluene. Hesperetin **4** was degraded *via* eriodictyol to 3-(3-hydroxyphenyl)-propionic acid and phloroglucinol. Structural elucidation of the formed metabolites was performed by high-performance liquid chromatography – diode array detection (HPLC – DAD) as well as HPLC-electrospray ionization – mass spectrometry (ESI-MS (MS)) and high resolution gas chromatography-mass spectrometry (HRGC-MS) analyses. The time course of microbial conversion of **2** – **4** was determined by HPLC-DAD analysis, revealing slow degradation of **2** and rapid transformation of **3** and **4**. The results lead to the conclusion that the pig caecum model is a suitable *ex vivo* model for studying the intestinal degradation of flavonoids.

Keywords: Flavonoid metabolism / Pig caecum model / Polyphenols

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1 Introduction

Flavonoids are a widely distributed group of polyphenols present in the diet. More than 4000 different flavonoids are identified to date. They are categorized as flavonols, flavanones, flavones, isoflavones, catechins, and anthocyanidins. Most flavonoids are present in the diet as glycosides from plants. Dietary sources of flavonoids are fruits, green vegetables, green and black tea, cocoa, chocolate, grape juice and red wine as well as onions [1]. 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 much higher. Within the last ten years the chemical-analytical, as well as biochemical focus on polyphenols, has increased rapidly [3]. For instance, several studies show the ability of polyphenols to protect from degenerative diseases [4, 5]. Especially the antioxidative potential of polyphenols and the protective effect to prevent cardiovascular diseases have been studied intensively [6 – 9]. Anti-carcinogenic, anti-inflammatory, and anti-parasitic effects are described as well [3,10].

To evaluate the bioavailability of flavonoids, it is necessary to know all possible bioactive forms *in vivo*. It is known that flavonoids consumed as glycosides are deglycosylated in the small intestine by enzymes to the aglycone [11]. This process is followed, in part, by degradation of the flavonoid by colonic microorganisms [11, 12]. The formed products can be absorbed in the colon and appear in the plasma. This part of flavonoid metabolism is very important, *inter alia*, for the evaluation of the antioxidant potential of flavonoids in the human diet.

Not much is known about the degradation products of flavonoids. Flavonoids which are not absorbed in the stomach or small intestine are carried to the colon. Even absorbed flavonoids can reach the colon as glucuronides when they are excreted *via* the bile. The colon contains about 10^{12} microorganisms per gram [1]. They are involved in deglycosylation and degradation of dietary phenols. For example, quercetin-3-*O*-rhamnoglucoside and quercetin-3-*O*-glucoside are hydrolyzed by gut microflora to quercetin by organisms such as *Baceroides distasonis* (exhibiting α-rhamnosidase and β-glucosidase activity) [1]. Deglycosylation of quercetin, which occurs in onions and apples mainly in glycosylated form, has been demonstrated recently by Walle *et al.* [13] in the small intestine. Colonic *Eubacterium ramulus* trans-

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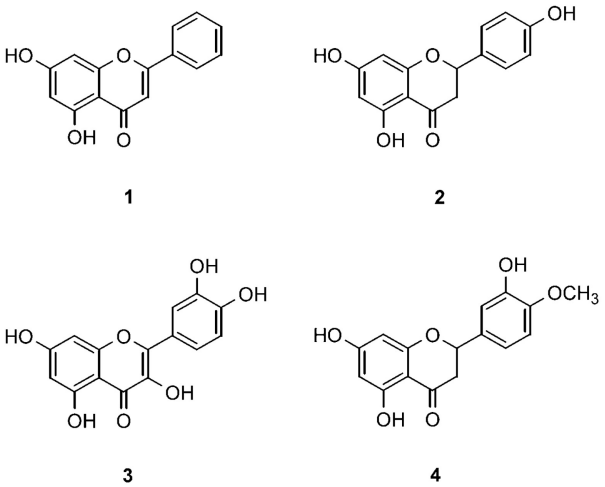
Abbreviations: DAD, diode array detection; **HRGC**, high resolution gas-chromatography; **ESI**, electrospray

forms quercetin-3-*O*-glucoside into 3,4-dihydroxyphenylacetic acid, acetate, and butyrate with the formation of quercetin and phloroglucinol as intermediates [12]. Luteolin (3′,4′,5,7-tetrahydroxyflavone) was reported to be degraded to 3-(3,4-dihydroxyphenyl)-propionic acid by *E. ramulus* as well [14]. In addition, Rechner *et al.* [11] have reported phenolic compounds such as hydroxyphenyl valerolactones and hydroxyphenylpropionic acids as degradation products of flavan-3-ols. Finally, studies performed with human fecal microflora have shown the excretion of benzoic, phenylacetic, phenylpropionic, and phenylvaleric acids in urine [15].

Physiological similarities between man and pig make this animal an excellent model for human in several research areas. Thus, many years ago, pig has been reported as a well-recognized experimental animal in studies concerning the nutrition of human beings [16]. Later, the pig caecum model was proposed to test colon targeting drug delivery [17]. Recently, pig caecum has been applied in metabolic studies of xenobiotics [18]. In our group, the first experiments with the pig intestinal microflora were performed by Seefelder [19]. Thus, it was interesting to assess its suitability for metabolic studies of flavonoids of several classes. In this paper, we report the degradation of chrysin **1**, naringenin **2**, quercetin **3**, and hesperetin **4** by pig caecum intestinal flora (Scheme 1).



Scheme 1. Chemical structures of chrysin **1**, naringenin **2**, quercetin **3**, and hesperetin **4**.

2 Materials and methods

2.1 Chemicals

All chemicals and solvents were of analytical grade. Solvents were distilled before use. Chrysin **1** was purchased from Lancaster (Frankfurt, Germany), naringenin **2** was from Roth (Karlsruhe, Germany), quercetin dihydrate **3** was a Fluka (Deisenhofen, Germany) product, hesperetin **4** was from Roth, phloroglucinol **3a** and 3-(4-hydroxyphenyl)-propionic acid **2a** were purchased form Fluka, hydrocinnamic acid **2b** was obtained from Acros Organic (Geel, Belgium), 3,4-dihydroxyphenylacetic acid **3b** was a Fluka product, 3,4-dihydroxytoluene **3c** was from Merck (Darmstadt, Germany), eriodictyol **4a** was from Roth and 3-(3-hydroxyphenyl)-propionic acid **4b** was purchased from Lancaster.

2.2 Preparation of inoculum

Freshly withdrawn caeci (under control obtained from slaughterhouse) from healthy pigs were immediately ligated, placed in an anaerobic jar containig Anaeorocult® A (Merck) 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 a N_2/CO_2 gas mixture (80 : 20; v/v). For each experiment (**1** – **4**) aliquots of the content of three different caeci (each of 150 mL) were pooled and diluted with the same volume of anaerobic carbonate-phosphate-buffer according to Lebet *et al.* [20] (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** – **4** (250 mM each) in dimethylsulfoxide. 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 dimethylsulfoxide and caecal inoculum as well as samples with flavonoids and buffer were prepared and subjected to the procedure described above. All experiments were performed in triplicate.

2.4 Sample preparation

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

2.5 HPLC-diode array detection (DAD) 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 Hewlett-Packard ChemStation software. An 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) trifluoroacetic acid (A) and acetonitrile (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 UV spectra (200 – 400 nm) with authentic references. Calibration curves (at the appropriate wavelengths 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-electrospray ionization-mass spectrometry (ESI-MS). To obtain additional structural information, semipreparative collection of individual compounds was performed by HPLC-DAD followed by lyophilization. The lyophilizate was dissolved in methanol and analyzed by collision-induced dissociation tandem mass spectrometry (ESI-MS/MS) or GC-MS (in the case of phloroglucinol **3a**).

2.7 HPLC-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 separations were 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% (v/v) formic acid (A) and acetonitrile (B). The gradient applied was 5 – 99% B in 30 min at a flow rate of 0.2 mL/min, and 20 μL injection volume. The analysis was performed in negative ionization mode. The spray capillary voltage was set to 3.5 kV, and the temperature of the heated capillary was 200°C. Nitrogen served both as sheath (70 psi) and auxiliary gas (10 units). The mass spectrometer was operated in the full-scan mode, *m/z* 120 – 650, with a total scan duration of 1.0 s. MS/MS experiments were performed at a collision energy of 20 – 40 eV, with argon (2.0 mTorr) serving as collision gas. The obtained molecular ion peaks and mass spectra were compared to those of references.

2.8 HRGC-MS

HRGC-MS was performed with a Fisons Instruments GC 8000 Series gas chromatograph (Fisons, Egelsbach, Germany) coupled to a Fisons Instruments MD 800 quadrupole mass detector. The GC was equipped with a J&W DB-5 fused-silica capillary column (30 m × 0.25 mm ID; d_f = 0.25 μm; J&W, Folsom, CA, USA; temperature program: from 60°C 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. The electron impact mass spectra (EI-MS) were recorded with an ionization voltage of 70 eV and a source temperature of 220°C. Identification was performed by comparison of mass spectral data of sample constituents with those of authentic reference compounds.

3 Results and discussion

In order to mimic the metabolism of chrysin **1**, naringenin **2**, quercetin **3**, and hesperetin **4** in the colon, the content of pig caecum was used as inoculum. Compounds **1** – **4** were incubated under anaerobic conditions with the inoculum over 24 h and the samples were continuously analyzed by HPLC-DAD, HPLC-ESI-MS (MS), and HRGC-MS.

3.1 Chrysin 1

The flavone chrysin **1**, which lacks hydroxylation on the B-ring, was not degraded. No metabolites were formed during the incubation of **1** with the microflora under the experimental conditions used. This finding is in full agreement with the results obtained previously by Griffiths and Smith [21] in their incubation experiments carried out with rat caecal microflora.

3.2 Naringenin 2

The pig caecum microflora converted naringenin **2** (1 mM) slowly, as considerable amounts of intact naringenin **2** were still detected after 24 h of incubation. The degradation of **2** was accompanied by the formation of two metabolites eluting at 14.4 min **2a** and 20.1 min **2b**, respectively, using HPLC-DAD detection (Fig. 1). These peaks were absent in the chromatographic profile of control samples (flavonoid with buffer and inoculum with dimethylsulfoxide; data not

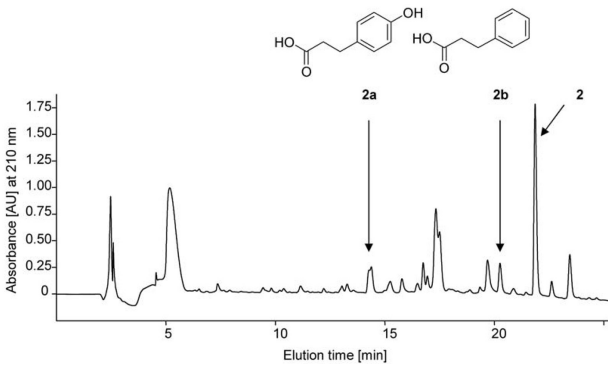


Figure 1. HPLC elution profile of naringenin **2** after 24 h of incubation with pig caecum flora under anaerobic conditions. **2a**: 3-(4-hydroxyphenyl)-propionic acid; **2b**: 3-phenylpropionic acid. Unlabeled peaks are caecum constituents.

shown). Comparison of the retention times and UV spectra of the metabolites **2a** and **2b** with authentic reference substances revealed their identity with 3-(4-hydroxyphenyl)-propionic acid and 3-phenylpropionic acid, respectively (data not shown). The expected molecular masses $[M–H^+]^-$ of *m/z* 165 for **2a** and *m/z* 149 for **2b** were confirmed by HPLC-ESI-MS in negative mode. Besides the retention times, UV spectra, and molecular masses, ESI-MS/MS led to the same pattern of daughter ions of *m/z* 165 and *m/z* 149 as the authentic references **2a** and **2b**, *i.e.*, exhibiting *m/z* 121, 93, 59 for **2a** and *m/z* 105, 77 for **2b**.

As shown in Fig. 2, naringenin **2** was slowly degraded, with significant levels still present after 24 h of incubation. The first metabolite **2a** was detected after 6 h of incubation, indicating an apparent ring fission. The second metabolite **2b** was detectable after 8 h; the amounts of both metabolites increased slowly with incubation. Extension of incubation time to 48 h led to a further decrease of naringenin **2**, a decrease of **2a** and further increase of **2b** (data not shown), indicating dehydroxylation of **2a** to form 3-phenylpropionic acid **2b**.

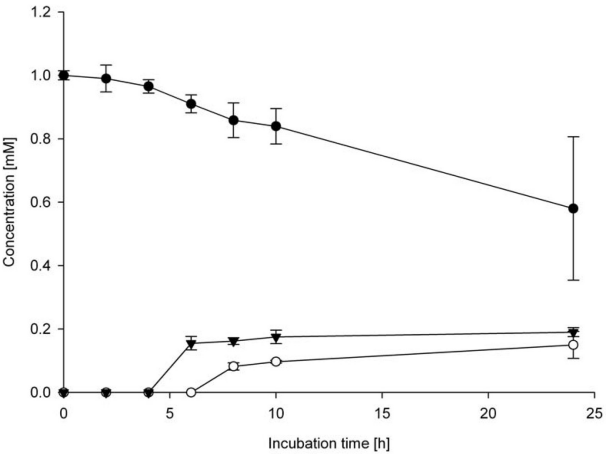


Figure 2. Time course of naringenin **2** degradation during incubation with pig caecum flora. Naringenin **2** (●); 3-(4-hydroxyphenyl)-propionic acid **2a** (▼); 3-phenylpropionic acid **2b** (○). Data are expressed as mean ± SD.

3-(4-Hydroxyphenyl)-propionic acid **2a** has previously found to be metabolized to **2b** by human intestinal bacteria [22]. The other results obtained in our experiments were in good agreement with data provided in the literature [21, 23, 24]. Recently, Rechner *et al.* [23] reported the formation of **2a** and **2b** from naringin (naringenin-7-rhamnoglucoside) after incubation with human fecal flora. Incubation of naringin with rat caecal flora resulting in the formation of **2a** and (intact) naringenin **2** has been reported earlier by Griffiths and Smith [21]. The expected phloroglucinol, formed by ring fission of **2** [24, 25], could not be detected in our study, probably owing to its low intermediary amounts.

3.3 Quercetin 3

Complete degradation of quercetin (1 mM) by the intestinal bacteria occurred after 24 h of incubation, resulting in three metabolites with retention times of 7.2, 10.4, and 15.3 min, respectively, using HPLC-DAD detection (Fig. 3). These peaks were not present in the chromatographic profiles of control samples (data not shown). The metabolite eluting at 7.2 min was identified as phloroglucinol **3a**; its retention time and UV spectrum were identical with that of an authentic reference. The identity of **3a** was further verfied by HRGC-MS; retention time and EI-MS spectrum corresponded to that of authentic **3a**. The metabolite eluting at 10.2 min was identified as 3,4-dihydroxyphenylacetic acid **3b** by comparing retention time and UV spectrum with that of an authentic reference. The HPLC-ESI-MS spectrum (negative mode) of this metabolite showed the expected molecular ion peak at *m/z* 167. The ESI-MS/MS daughter ion pattern was in agreement with that of authentic **3b**, *i.e.*,

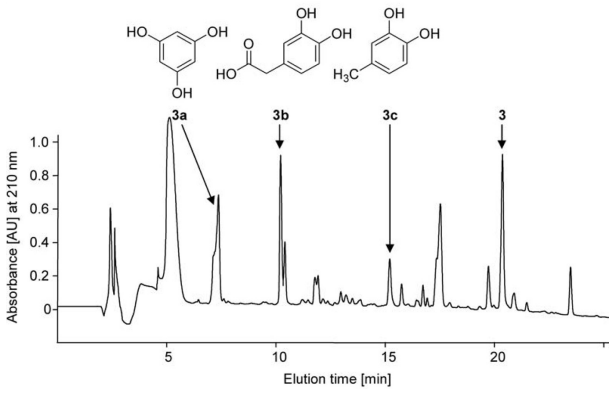


Figure 3. HPLC elution profile of quercetin **3** after 8 h of incubation with pig caecum flora under anaerobic conditions. **3a**: phloroglucinol; **3b**: 3,4-dihydroxyphenylacetic acid; **3c**: 3,4-dihydroxytoluene. Unlabeled peaks are caecum constituents.

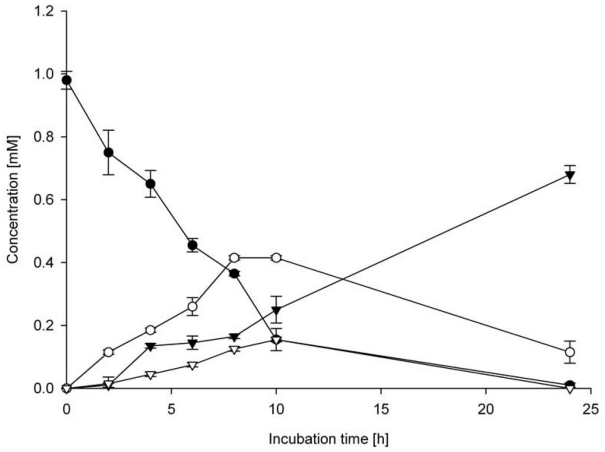


Figure 4. Time course of quercetin **3** degradation during incubation with pig caecum flora. (●) Quercetin **3**; (▽) phloroglucinol **3a**; (○) 3,4-dihydroxyphenylacetic acid **3b**; (▼) 3,4-dihydroxytoluene **3c**. Data are expressed as mean ± SD.

exhibiting *m/z* of 123, 108, 105, 95. The compound eluting at 15.2 min was identified as 3,4-dihydroxytoluene **3c**; its retention time and UV spectrum corresponded to that of an authentic reference. The HPLC-MS analysis (in negative mode) of this metabolite showed the expected $[M–H^+]^-$ of *m/z* 123. Using ESI-MS/MS the pattern of daughter ions of *m/z* 123 $[M–H^+]^-$ was the same as that for an authentic reference, *i. e.,* exhibiting *m/z* of 108, 105, 95.

As shown in Fig. 4, the amount of quercetin **3** decreased continuously during the incubation with the pig caecal flora; after 24 h **3** was completely degraded. The metabolites **3a** and **3b** were already formed within the first 2 h of incubation, indicating an apparent ring fission of quercetin. The concentration of **3a** and **3b** increased during the time of incubation, reaching a maximum of approximately 0.15 mM and 0.4 mM, respectively, after 10 h of incubation. Whereas small amounts of **3b** (approximately 0.1 mM) were still present after 24 h of incubation, **3a** decomposed completely during the course of incubation. The third metabolite, 3,4-dihydroxytoluene **3c,** was detected for the first time after 4 h of incubation; its concentration increased concomitantly with the decrease in concentration of **3b** and reached a maximum (around 0.68 mM) after 24 h of incubation.

The transient appearance of phloroglucinol **3a** is in full agreement with previous microbial studies [12, 14, 26 – 28]. Analogously, the acid **3b** has been recognized as a specific metabolite of the colonic transformation of quercetin (or quercetin glycosides) in microbiological investigations or human intervention studies [14, 29 – 32]. 3,4-Dihydroxytoluene **3c** has also been described as a degradation product of quercetin after incubation of rutin or quercetin with human fecal flora [23]. Additionally, the formation of **3c** from quercetin glycosides after their oral administration in humans has been reported [31, 32].

3.4 Hesperetin 4

The flavanone **4** (1 mM) was almost completely degraded by intestinal microflora within 24 h. *Via* HPLC analysis three metabolites were detectable, eluting at 7.2, 14.9, and 19.2 min, respectively (Fig. 5). These peaks were not present in the chromatographic profiles of control samples (data not shown). The metabolite eluting at 7.2 min was identified as phloroglucinol **3a** as described above. The retention time and UV spectra of the metabolites eluting at 19.2 and 14.9 min were identical with those of eriodictyol **4a** and 3-(3-hydroxyphenyl)-propionic acid **4b**, respectively. HPLC-MS analysis (in negative mode) gave the expected $[M–H^+]^-$ of *m/z* 287 and *m/z* 165 for **4a** and **4b**, respectively. Using ESI-MS/MS, the pattern of daughter ions for *m/z* 287 $[M–H^+]^-$ and *m/z* 165 $[M–H^+]^-$ were the same as that of authentic **4a** and **4b**, *i. e.,* exhibiting *m/z* 151, 135, 107 and *m/z* 121, 119, 106, respectively.

As shown in Fig. 6, the amount of **4** decreased rapidly during incubation with intestinal bacteria to end up at approximately 0.01 mM after 10 h. The first occurring metabolite **4a**, the demethylation product of **4**, was formed during the first 2 h of incubation and reached a maximum concentration at about 0.3 mM after 8 h. 3-(3-Hydroxyphenyl)-propionic acid **4b** was detected after 4 h of incubation, its amount increased concomitantly with the decrease in concentration of the first occurring metabolite eriodictyol **4a**, and reached a maximum concentration (around 0.6 mM) after 24 h of incubation, indicating dehydroxylation and ring fission of **4a**. The degradation of **4** was accompanied by the transient formation of **3a** at lower concentration (data not shown).

Based on the structural identification of the metabolites and the time course of hesperetin conversion, we assume that **4** was transformed as follows: Initially, *O*-demethylation of hesperetin **4** led to the formation of the intermediate **4a**.

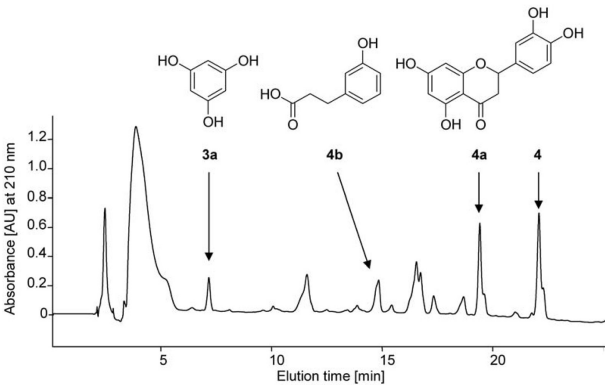


Figure 5. HPLC elution profile of hesperetin **4** after 8 h of incubation with pig caecum flora under anaerobic conditions. **4a**: eriodictyol; **3a**: phloroglucinol, **4b**: 3-(3-hydroxyphenyl)-propionic acid. Unlabeled peaks are caecum constituents.

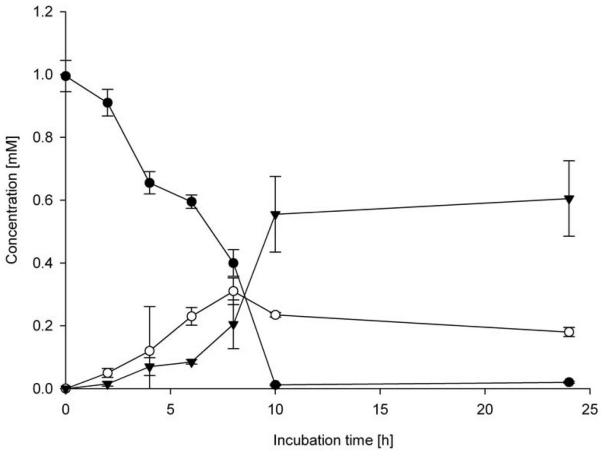


Figure 6. Time course of hesperetin **4** degradation during incubation with pig caecum flora. (●) Hesperetin **4**; (○) eriodictyol **4a**; (▼) 3-(3-hydroxyphenyl)-propionic acid **4b**; phloroglucinol **3a** not shown (≤ 0.03 mM). Data are expressed as mean ± SD.

This finding indicates a demethylase activity of the pig caecum microflora as recently reported for *Eubacterium limosum* in the case of isoflavonoids [33]. Furthermore, since it is known that the presence of a free hydroxyl group at C-4′ in the B ring is necessary for ring fission [21, 34], the formation of eriodictyol **4a** is explained. The subsequent ring fission of **4a** resulted in the formation of phloroglucinol **3a** and 3-(3-hydroxyphenyl)-propionic acid **4b**. This acid is known to be a colonic metabolite of hesperetin [11, 35]; in earlier studies, the formation of **4b** from hesperetin and eriodictyol, respectively, was reported after oral ingestion by rats [36]. In addition, Honohan *et al.* [37] also observed the formation of **4b** after incubation of **4** with rat caecal flora.

The formation of 3,4-dihydroxyphenylpropionic acid **4c** from **4a**, as previously described by others [14, 27], could not be detected in our study. On the basis of previous experiments, in which the formation of **4b** from **4c** was observed after incubation with human faecal flora [11, 35, 38], rapid *p*-dehydroxylation of **4c** has to be considered. Analogous to our observations, immediate *p*-dehydroxylation was reported in previous studies [34, 39]. In a few of our experiments, subsequent dehydroxylation of the acid **4b** to form 3-phenylpropionic acid **2b** was observed (data not shown). Such differences in the composition of microbial fermentation end products have also been described in the literature [23].

4 Concluding remarks

Most of the *in vitro* studies on the metabolic fate of flavonoids have been performed using human fecal bacteria (diluted to physiological caecal concentration) or rat caecal contents. Since the digestive systems of pigs and humans have a high degree of similarity, pig caecum should provide a ready access to obtain valuable information about processes in the human intestinal tract. Our results clearly show that, in fact, the pig caecum model is excellently suited for the examination of the metabolism of flavonoids in human colon.

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