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Metabolism of quercetin and rutin by the pig caecal microflora prepared by freeze-preservation

Katrin Keppler, Eva-Maria Hein and Hans-Ulrich Humpf

Institut für Lebensmittelchemie, Westfälische Wilhelms-Universität Münster, Münster, Germany

Several studies confirm a protection of cardiovascular diseases and certain forms of cancer by dietary flavonoid intake. The bioavailability of flavonoids is influenced by the metabolism of the microflora in the intestine. Using a new *in vitro* model system the deglycosylation of the flavonol rutin and the degradation of its aglycone quercetin were investigated by using fresh pig caecal inocula in comparison to inocula prepared before by freeze-preservation between 6 wk and 5 months. The incubation experiments led to the same pattern of phenolic degradation products in comparable amounts in both preparations using HPLC-DAD and GC-flame ionization detection (GC-FID) or GC-MS detection within 24 – 48 h of incubation. With the preservation of the microbial vitality and the metabolic efficiency by freeze-preparation over several months the experimental design of microbial metabolism studies will be independent in time and locality.

Keywords: Caecal microflora / Freeze-preservation / Metabolism / Quercetin / Rutin

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1 Introduction

With the observation of the low cardiovascular mortality rate in Mediterranean populations, known as French Paradox [1, 2] there has been an increasing interest in phenolic phytochemicals such as phenolic acids and flavonoids due to their strong antioxidative and radical scavenging properties [3, 4] which are related to their anticarcinogenic [5], antiatherosclerotic [6, 7] and antiinflammatoric [8, 9] effects in different model systems. Epidemiologic studies confirm a protection against cardiovascular diseases and certain forms of cancer by dietary flavonoid intake [10]. However, physiological effects of these polyphenols depend on both their respective intake and their bioavailability. Dietary intake of polyphenols shows a high variability due to individual food preferences [11]. However, the total polyphenol intake has been estimated at up to 1 g/day in people who eat several servings of fruits and vegetables *per* day [12]. The bioavailability is influenced by the chemical structure of the compounds themselves, the food matrix, digestive factors such as pH and enzymes, intestinal absorption followed by conjugation reactions in the intestinal and hepatic tissues and at last by the rate of biliary and urinary excretion of these metabolites [13, 14]. Polyphenols that are not absorbed in the upper small intestine or secreted as conjugates *via* the bile without enterohepatic recirculation reach the terminal ileum and caecum. There, the microbial metabolism plays an important role in the bioavailability of phenolic phytochemicals. After the oral ingestion of flavonoids, degradation products such as aglycones and phenolic acids are identified in colonic contents, urine and plasma, confirming that the microflora has an enormous hydrolytic potential and even ring scission properties [15–17]. Thus, the metabolism by the gut microflora has a major effect on tissue exposure to the origin polyphenols. Furthermore, plasma concentrations and urinary excretion of microbial metabolites can be higher than those of the orally ingested compounds [18, 19]. Polyhydroxy derivatives of aromatic acids have been studied with comparable results for their antioxidant activity and free radical scavenging capacity [3, 20, 21].

The caecum as the connection between the small and large intestine contains more than 400 bacterial species, most of them as strict anaerobes [22, 23]. The constant flow of ileostomy effluent and the partial reflux from the ascendic colon into the caecum [24] offer environmental conditions which generate the high species variety. Fermentation studies with single bacteria species [25, 26] or a mixed culture model [27] represent only a small section of this complex microbial metabolism. Furthermore, incubations with

Correspondence: Professor Dr. Hans-Ulrich Humpf, Institut für Lebensmittelchemie, Westfälische Wilhelms-Universität Münster, Corrensstrasse 45, 48149 Münster, Germany
E-mail: humpf@uni-muenster.de
Fax: +49-251-8333396

Abbreviations: DOPAC, 3,4-dihydroxyphenylacetic acid; **FID,** flame ionization detector; **PHG,** phloroglucinol; **PNP,** *p*-nitrophenol; **PNPα-gal,** *p*-nitrophenyl α-D-galactopyranoside

human faecal samples [28–30] do not represent the microflora 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 [31–33]. Recently, we presented a metabolic study of anthocyanins [34] based on a new *in vitro* model system [35, 36] for which the gut microflora is directly isolated from the caecum of freshly slaughtered pigs produced by biodynamic agriculture. The pig resembles the human in more ways than any other nonprimate mammalian species because of the similarities in digestive anatomy, physiology and nutrition [37–39]. The incubation experiments with the naturally composed microflora could be performed easily under strict anaerobic conditions on a microscale, so that in the meantime, this model system has been adopted by another research group [40].

One disadvantage of this model system is the fact that the microflora isolated from freshly slaughtered pigs is only useful for one set of experiments. In order to overcome this problem we extended this model system in the present study by freeze-preservation of the caecal inocula. For freezing we placed an aliquot of the fresh caecal content with an O_2-free headspace into a deep-freeze cabinet (−80°C). Glycerol with a final concentration of 20% w/w was added to the inocula as a cryoprotectant. We investigated the microbial metabolism of the flavonol quercetin **1** and its 3-β-D-*O*-rutinoside **2** (rutin), both with fresh inocula and inocula prepared by freeze-preservation. The hydrolysis of the β-glycosidic bond of rutin **2** and the decay of the aglycone quercetin **1** were measured during the incubation time. The resulting degradation products of quercetin **1** phloroglucinol (PHG) **3** and 3,4-dihydroxyphenylacetic acid (DOPAC) **4** were identified and quantified. Vitality and metabolic efficiency of the microflora were also studied by the microbial marker enzyme α-D-galactosidase in both preparations.

2 Materials and methods

2.1 Chemicals

Quercetin dihydrate **1** and quercetin 3–*O*-α-*L*-rhamnopyranosyl-(1→6)-β-D-glucopyranoside trihydrate **2** (rutin) were purchased from Extrasynthese (Genay, France); **3** (PHG), **4** (DOPAC), *p*-nitrophenyl α-D-galactopyranoside (PNP α-gal) and *p*-nitrophenol (PNP) were provided by Sigma-Aldrich (Taufkirchen, Germany) and 3,5-dimethoxy-4-hydroxybenzoic acid **IS** (syringic acid) was purchased from Roth (Karlsruhe, Germany). General names and abbreviations are used in the manuscript because it is more practical. Solvents for HPLC as well as all other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich of in gradient or reagent grade quality. Water was purified with a Milli-Q Gradient A10 (Millipore, Schwalbach, Germany) system.

2.2 Preparation of fresh inoculum

Animals (female German Landrace, Angler Sattel × Pietrain; age, 8–12 months; weight, 90–120 kg) were obtained from a commercial herd produced by biodynamic agriculture (Gut Wewel, Senden, Germany). The basal diet contained a mixture of rye, spelt, linseed, lentil, corn, millet and rice meal enhanced with clover silage. The caeca were isolated and prepared from freshly slaughtered pigs as described previously [34]. All buffers, solutions and vessels were flushed with a mixture of N_2 and CO_2 (5:1 v/v) before use. To remove larger particles, the inoculum suspensions were filtered through a coarse material net. The filtrates were used for the incubation experiments. An aliquot of the filtrates was inactivated by sterilization at 121°C for 15 min at 1.1 bar (AMB240 autoclave, Astell, Kent, UK). The inactivated caecal suspensions were used as control for chemical degradation and matrix effects. Each incubation experiment was performed with three different caeca in duplicate.

2.3 Preparation of inoculum for freeze-preservation

As described for the fresh caeca, each isolated inoculum was suspended in the same volume (w/w) of supplemented PBS (pH 6.4) [34]. For freeze-preservation glycerol was added as a cryoprotectant with a final concentration of 20% w/w in the inoculum preparation. The inocula were incubated for 15 min at room temperature and then stored in a deep-freeze cabinet at −80°C between 6 wk and 6 months. For the incubation experiments the frozen inocula were thawed quickly in a water bath at 37°C. The samples were centrifuged immediately at 5.000 × *g* for 20 min (4°C) to remove the freezing buffer. The pellets were suspended in fresh supplemented PBS without glycerol and filtered through a material net as described for the fresh inocula. After 15 min of re-equilibration at 37°C the filtrates were used for the incubation experiments. Deglycosylation of PNP α-gal was performed with three caeca frozen for 3 months and one selected caecum frozen for 2, 4 and 5 months. The same caecum was used for the degradation of quercetin **1**. For the metabolism of rutin **2**, three caeca with different duration of freezing (6 wk to 6 months) were used. Each incubation experiment was performed in duplicate.

2.4 Incubation experiments

Stock solutions of the analytes (each 10 mM) were prepared: quercetin **1** and rutin **2** were dissolved in methanol; PHG **3** and DOPAC **4** were dissolved in methanol/water (7:3 v/v); PNP α-gal was dissolved in methanol/water (1:1 v/v). The preparation of the incubation experiments was carried out in the glove bag maintaining anaerobiosis. From

the stock solutions 0.1 mL was added to 0.9 mL of nonsterilized or sterilized inoculum filtrate in a 2 mL plastic vial. Aliquots of the nonsterilized inoculum filtrate with solvent of the referring stock solution were prepared as matrix blank samples. The vital and matrix blank samples were prepared from the fresh or the formerly frozen inocula filtrates. The maximum methanol concentration in the incubation experiments reached 10% v/v, which did not have any negative effect on the bacterial vitality (data not shown). For each incubation time a separate vial was used. The sealed vials were placed in an incubator (37°C) for 20 min, 1, 4, 8, 24 and 48 h. The microbial metabolism was stopped by placing the vessels in a deep-freeze cabinet at −80°C.

2.5 Sample preparation and analysis

2.5.1 α-D-Galactosidase activity

The frozen samples (prepared as described in Section 2.4) were thawed quickly in a water bath at 37°C. The samples were centrifuged immediately at $15.000 \times g$ for 20 min (4°C). Aliquots of the supernatants were diluted with 0.5 M KOH (1:20) in a 96-well microplate. The absorbance of each well was measured with a microplate reader (FLUOstar OPTIMA, BMG LabTechnologies, Jena, Germany) at 405 nm, calculating the released PNP using a standard calibration curve in the range of 0.1–15 µg/mL. Because of the matrix background absorption, active inoculum without substrate was applied as blank.

2.5.2 HPLC-DAD quantification of 1–4

The frozen samples were thawed quickly in a water bath at 37°C. For inactivation and extraction 1.1 mL methanol was added immediately. After shaking, the samples were centrifuged at $15.000 \times g$ for 20 min (4°C). Supernatants were filtered (GHP Ascrodisc 0.2 µm, Pall Life Sciences, Ann Arbor, USA), and aliquots of the filtrate were injected (4.2 µL for quercetin **1** and rutin **2**, 42 µL for PHG **3** and DOPAC **4**) into the HPLC. The compounds were separated on an analytical Eurospher 100 column (250 × 4.6 mm id, 5 µm; Knauer, Berlin, Germany) using a binary gradient delivered by a Jasco PU-2089 low-pressure gradient HPLC pump (Jasco, Groß-Umstadt, Germany) with ACN as solvent A and 2% v/v acetic acid as solvent B for quercetin **1** and rutin **2**: 0 min, 22% A; 3 min, 22% A; 8 min, 80% A; 15 min, 22% A; 20 min, 22% A. The binary gradient was modified for PHG **3** and DOPAC **4** as following: 0 min, 1% A; 3 min, 1% A; 20 min, 35% A; 25 min, 90% A; 28 min, 90 A; 33 min, 1% A; 35 min, 1% A. The flow rate was 1 mL/min. For injection a Jasco autosampler AS-2057 Plus was used. A Jasco DAD MP-2010 Plus was applied over the wavelength range of 220–600 nm for peak detection. Data acquisition was carried out with the software Borwin-PDA 1.5 (Jasco). Compounds were identified by comparison of the UV spectra and the retention times t_r (quercetin **1**, t_r = 8.3 min; rutin **2**, t_r = 5.8 min; PHG **3**, t_r = 6.9 min; DOPAC **4**, t_r = 13.6 min) with authentic references. Concentrations were calculated using calibration curves ranging from 0.005 to 0.12 mM for quercetin **1** and rutin **2** and 0.1–1.0 mM for PHG **3** and DOPAC **4**. The following wavelengths were monitored for quantitative analysis: 360 nm for **1** and **2**, 265 nm for **3** and 275 nm for **4**.

2.5.3 GC-FID quantification of 3 and 4

Aliquots of the HPLC filtrates were supplemented with syringic acid (final concentration: 0.25 mM) as internal standard and dried under a stream of nitrogen. The residues were derivatized with *N*,*O*-bis(trimethylsilyl)acetamide (BSA) for 30 min at 55°C. The same volume of trichlorotrifluoroethane (TCTFE) was added to the BSA derivatization mixture. After centrifugation ($15.000 \times g$, 10 min) the supernatants were injected into the GC-FID system. Data were acquired on an HP6890 series gas chromatograph coupled with an FID (Hewlett Packard/Agilent, Böblingen, Germany). Data acquisition was carried out with the Chemstation software (Agilent). Chromatographic separation was performed on a 30 m × 0.32 mm id fused silica, 0.25 µm crosslinked 5% methylphenyl silicone coating Hewlett Packard Scientific HP-5 column (Agilent) using 1.3 mL/min hydrogen as carrier gas. For injection, a Hewlett Packard autosampler HP7683 was used. The injector temperature was set at 330°C, the injection volume was 1 µL splitless. The column temperature was held initially at 100°C for 1 min, then programmed at 4°C/min to 170°C, then with 15°C/min to 320°C, which was held isothermally for 10 min. The detector was heated at 340°C. Air (450 mL/min) and hydrogen (40 mL/min) made up the FID flame using nitrogen as makeup gas. Signals were identified by comparison of the retention times t_r (PHG **3**, t_r = 16.93 min; DOPAC **4**, t_r = 21.18 min; syringic acid **IS**, t_r = 22.10 min) with silylated authentic reference compounds. Concentrations were calculated using calibration curves ranging from 0.0625 to 0.75 mM for **3** and **4**.

2.5.4 GC-MS identification of 3 and 4

Aliquots of the silylated GC-FID solutions were diluted with toluene 1:5 v/v and injected into the GC-MS system. Electron impact (EI) GC-MS data were acquired on an HP6890 series gas chromatograph and HP5973 mass spectrometer (Agilent). Data acquisition was carried out with the Chemstation software (Agilent). Chromatographic separation was performed in a 60 m × 0.25 mm id fused silica, 0.25 µm crosslinked 5% methylphenyl silicone coating Chrompack CP-Sil 8 CB column (Agilent) using 1 mL/min helium as carrier gas. For injection, an autosampler COMBI PAL (CTC Analytics, Zwingen, Schweiz) was used. The injector temperature was set at 280°C, and the injection volume was 1 µL (split 9.3:1). The column temperature was held initially

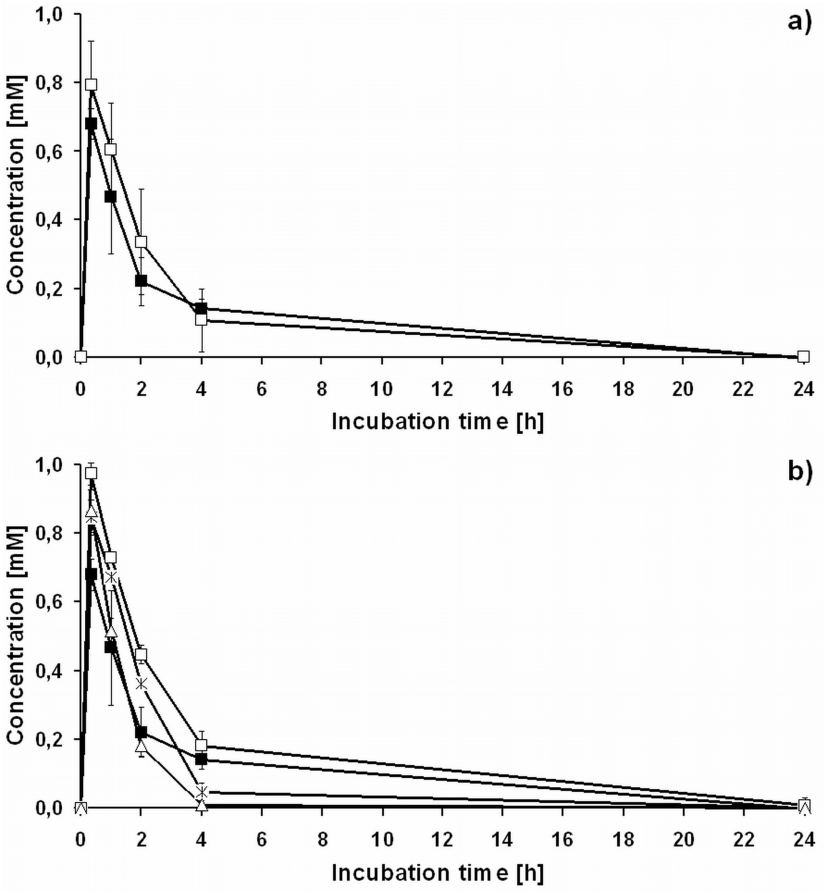

Figure 1. Time-dependent release of PNP after incubation of PNP α-gal (1 mM) with (a) fresh inocula (■) and inocula prepared by freeze-preservation for 3 months at −80°C (□) (n_{caeca} = 3, mean± SD) and (b) fresh inocula (■), (n_{caeca} = 3, mean ± SD) and one selected inoculum prepared by freeze-preservation for 2 (*), 4 (□) and 5 (△) months at −80°C (n = 2, mean ± SD). Nonsterilized inoculum filtrate without substrate was measured as blank for matrix background absorption. Absorption was measured at 405 nm with a microplate reader.

at 100°C for 1 min, then programmed at 4°C/min to 260°C, then with 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 the quadrupol heated at 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. The signals were identified by comparison of the retention times t_r (syringic acid **IS**, t_r = 31.9 min; PHG **3**, t_r = 25.0 min; DOPAC **4**, t_r = 30.09 min; quercetin **1**, t_r = 51.3 min) and mass spectral data with silylated authentic reference compounds and a mass spectra library (NIST, Gaithersburg, USA).

3 Results and discussion

3.1 Assay of the α-D-galactosidase activity

The enzyme α-D-galactosidase is an exoglycosidase, which is exhibited by the intestinal microflora and not by the pig or human digestive system [41, 42]. Therefore, it is an excellent tool to assay the enzymatic activity of the intestinal microflora. We studied the activity of the microbial marker enzyme α-D-galactosidase to control the vitality and metabolic efficiency of the microorganisms from fresh caecal inocula and inocula after freeze-preservation.

The assay was performed using PNP α-gal as substrate. The bacterial α-D-galactosidase hydrolysed the substrate into the corresponding sugar and PNP which is measured photometrically. Figure 1a shows the release of PNP after incubation of PNP α-gal with representive fresh inocula and freeze-preserved inocula for 3 months. PNP was measured after 20 min of incubation with 0.68 ± 0.05 mM (n_{caeca} = 3, mean ± SD) in the fresh inocula. From this data point the amount of PNP decreased due to its further metabolism by the microflora. For this reason the theoretical maximum value (1 mM) of PNP could not be traced. A more special enzymatic composition is necessary for aromatic ring scission. Therefore, the degradation of PNP was also a marker

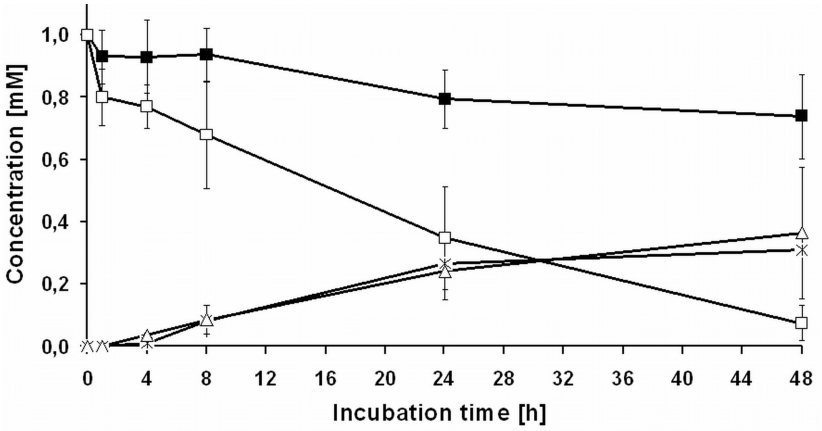

Figure 2. Time–concentration degradation-profiles of quercetin **1** (measured by HPLC-DAD at 360 nm) and release of phenolic degradation products **3** (*) and **4** (△) (measured by HPLC-DAD at 265 nm for **3** and 275 nm for **4** or GC-FID) after incubation of **1** with fresh inoculum (□). Sterilized inoculum filtrates (■) were used for measuring the chemical stability of **1** (n_{caeca} = 3, mean ± SD).

for the vitality and metabolic efficiency of the microflora. The freeze-preserved inocula gave a similar graph with even a higher value of PNP (0.79 ± 0.12 mM; n_{caeca} = 3, mean ± SD) after 20 min of incubation suggesting that the α-D-galactosidase activity is higher compared to the fresh inocula. However, this is also an indication that in the case of the freeze-preserved inocula less bacteria were able to further metabolize PNP, immediately leading to a higher concentration of PNP. Loss of bacteria by removal of the freezing buffer is the main explanation for the lower ring cleavage properties at the starting point. The degradation curve of PNP followed the one with the fresh inocula and after 4 h of incubation there were no differences between the two inocula preparations.

Furthermore, we assayed the enzymatic activity in one selected freeze-preserved inoculum between 2 and 5 months in comparison to the fresh inocula described before (Fig. 1b). By regarding only one caecum we wanted to confirm the preservation of the vitality and microbial efficiency without host-dependent deviations. As seen before, higher amounts of PNP were obtained at the maximum after 20 min of incubation (0.85 ± 0.05, 0.97 ± 0.03, 0.87 ± 0.06 mM after 2, 4 and 5 months of freeze-preservation, respectively; n = 2, mean ± SD) followed by a rapid degradation of PNP. Remarkably, PNP decreased in the inocula frozen for the longest time more rapidly than in the other preparations. So, the metabolism of PNP α-gal seemed to be independent for a period of 5 months of freeze-preservation.

3.2 Metabolism of flavonols 1 and 2

Quercetin **1** and rutin **2** were selected for the incubation experiments because they occur in many beverages and servings of fruits and vegetables: red wine, tea infusions, onions, apples and others [11, 43]. For the degradation experiments quercetin **1** and rutin **2** were incubated at 1 mM for 48 and 24 h, respectively, with the nonsterilized fresh or freeze-preserved inocula. The chemical stability under physiological conditions in the intestine (37°C, reductive milieu, pH 6.4) was determined by the incubation of **1** and **2** with the sterilized inoculum filtrate. Samples were analysed by HPLC-DAD from 1 to 24 h (48 h for **1**). Flavonols **1** and **2** and phenolic degradation products PHG **3** and DOPAC **4** were identified by comparison of HPLC retention times and UV/VIS spectra with authentic reference compounds. HPLC-DAD detection was used for the quantification of **1** and **2**; PHG **3** and DOPAC **4** were quantified using HPLC-DAD or GC-FID with syringic acid **IS** as internal standard. Aliquots of the GC-FID sample preparations were measured additionally by GC-MS to confirm the identity of **3** and **4**. Nonsterilized inoculum aliquots without substrate were also analysed by HPLC-DAD, GC-FID and GC-MS as matrix blank (for details see Section 2.5).

3.2.1 Metabolism of quercetin 1

Figure 2 shows the decreasing amounts of quercetin **1** and increasing amounts of the phenolic degradation products PHG **3** and DOPAC **4** using the fresh inocula filtrates. Quercetin **1** was metabolized slowly retaining 0.075 ± 0.057 mM (n_{caeca} = 3; mean ± SD) after 48 h of incubation. PHG **3** and DOPAC **4** were detectable as ring scission products primarily after 4 h of incubation. They increased in close resemblance until 48 h with 0.311 ± 0.150 mM for **3** and 0.364 ± 0.210 mM for **4**, confirming the results of Braune *et al.* [44] that PHG **3** and DOPAC **4** are the main microbial metabolites of quercetin (Fig. 3). Although Braune *et al.* used *Eubacterium ramulus* as a single species for their incubation experiments, the complex caecal microflora used in

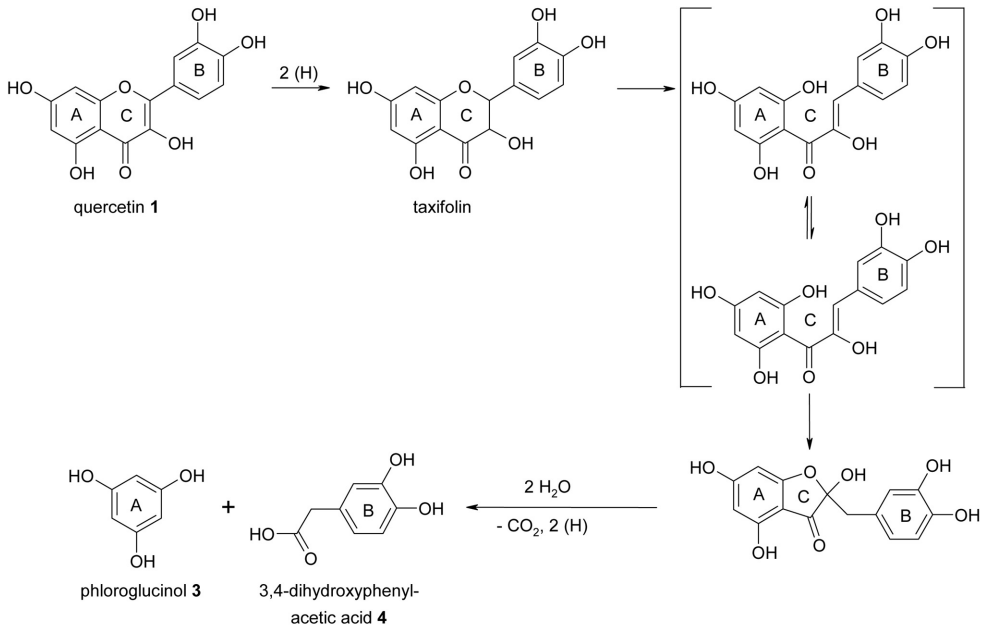

Figure 3. Proposed pathway of the degradation of quercetin **1** to PHG **3** and DOPAC **4** by *E. ramulus* modified according to Braune *et al.* [42].

our experiments led surprisingly not to another pattern of key degradation metabolites. Therefore, we suggest that *E. ramulus* is a common inhabitant of the pig gastrointestinal tract as found in human [45], or cleavage of quercetin to PHG **3** and DOPAC **4** is a common way in microbial metabolism as the degradation pathway of other intestinal bacterial species such as *Eubacterium oxidoreducens* and *Clostridium orbiscindens* confirms [46, 47]. 3,4-Dihydroxytoluene, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3-(3-hydroxyphenyl)-propionic acid and 3-hydroxyphenylacetic acid were not detectable by GC-MS in our incubation experiments as described by other authors using pig caecum contents [41] or human faecal samples [48–50]. We assume that the dissimilar pattern of degradation products could be ascribed to the variety of bacterial composition in the different research materials. Figure 4 shows the GC-MS chromatograms of a quercetin sample incubated with fresh inoculum and an inoculum blank within 24 h of incubation. However, the measured amounts of PHG **3** and DOPAC **4** could not explain the starting concentration of quercetin **1** (1 mM). In further incubation experiments we showed that the phenolic degradation products **3** and **4** were degraded by the microbial metabolism in a moderate way (data not shown) as we described previously for the phenolic acids from the decay of anthocyans [34]. The chemical stability of quercetin **1** in the sterilized inoculum filtrate is also shown in Fig. 2 (■). Amounts decreased slowly throughout the incubation time with a recovery of 0.735 ± 0.135 mM at 48 h. PHG **3**, DOPAC **4** or other phenolic degradation products could not be detected by GC-MS suggesting that quercetin was reduced by matrix effects such as irreversible protein-binding and redox reactions.

In continuation to the α-galactosidase assay (see Section 3.1; Fig. 1b), we incubated quercetin **1** with the microflora prepared from the selected caecum which was preserved by freezing between 2 and 5 months excluding host-dependent deviations. Figure 5 shows the degradation of quercetin **1** by the freeze-preserved inocula in comparison to the representive fresh caeca presented above. Due to the loss of bacteria after removing the freezing buffer, amounts of quercetin **1** decreased more slowly at the starting point in the inocula prepared by freeze-preservation. During the incubation time, bacteria were able to propagate and the metabolic efficiency increased continuously, very similar to the fresh inocula. However, regarding the results of quercetin **1**, cleavage of the complex aromatic ring system was not as independent from the duration of freeze-preservation as presented by the degradation of PNP before. After 48 h of incubation only 0.019 ± 0.011 mM ($n = 2$; mean ± SD) and 0.030 ± 0.004 mM quercetin **1** was traceable in the inocula preparation preserved for 2 and 4 months at −80°C, respectively, but preserving the inoculum for 5 months by freezing a loss in the metabolic efficiency was visible. Still, 0.257 ± 0.005 mM of quercetin was detectable after 48 h of incubation.

The degradation products PHG **3** and DOPAC **4** could be measured in all preparations throughout the incubation time. Interestingly, the amounts of **4** were half of those for **3**

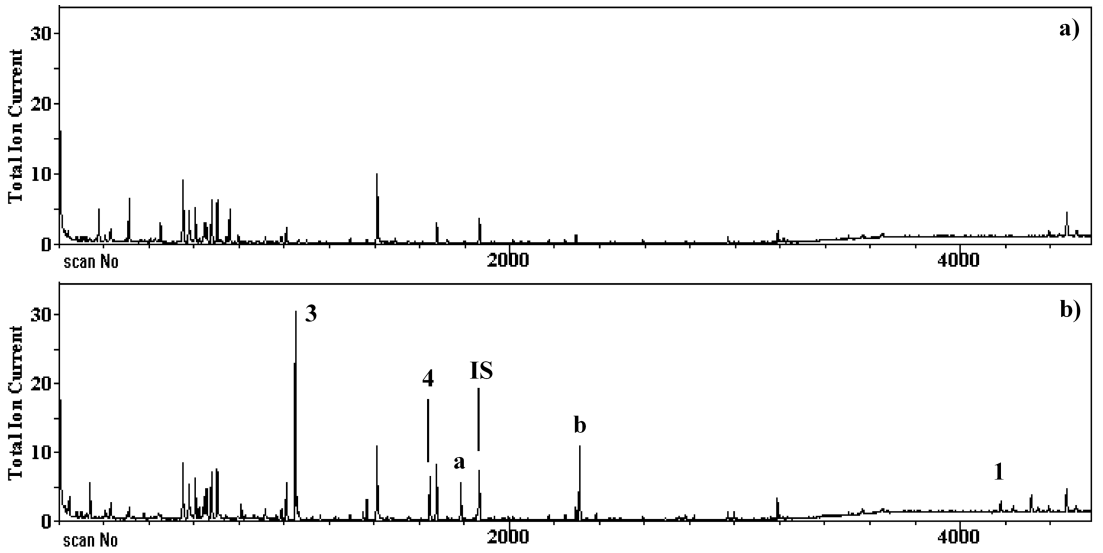

Figure 4. Representative GC-MS chromatograms (monitored as total ion chromatogram (TIC)) after incubation of (a) inoculum blank and (b) quercetin **1** (1 mM) with fresh inoculum filtrate for 24 h. PHG **3** and DOPAC **4** could be detected as degradation products of quercetin **1** only in (b); internal standard **IS**: syringic acid. Unlabelled peaks are inoculum constituents; peaks **a** and **b** with more intensity in (b) were identified as glucose **a** and palmitin acid **b** with mass spectra library NIST (all samples were silylated as described in Section 2. 5. 3).

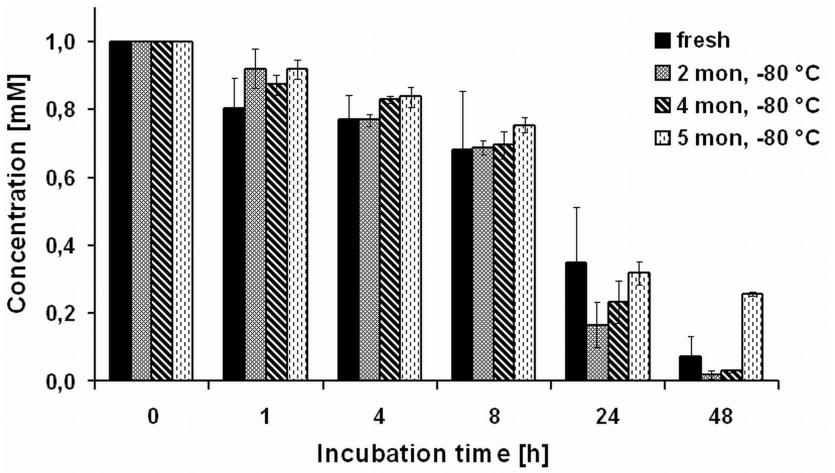

Figure 5. Time–concentration degradation-profiles of quercetin **1** (measured by HPLC-DAD at 360 nm) after incubation with fresh inoculum (n_{caeca} = 3, mean ± SD) or one selected inoculum prepared by freeze-preservation for 2, 4 or 5 months at −80°C (n = 2, mean ± SD).

after 48 h of incubation, suggesting that the acid was a preferred energy source for the bacteria prepared before by freeze-preservation (data not shown). In conclusion, quercetin **1** was metabolized similarly by fresh inocula and inocula prepared by freeze-preservation at −80°C between 2 and 4 months within 48 h of incubation.

3.2.2 Metabolism of rutin 2

Whereas certain flavonoid monoglucosides can be rapidly deglycosylated by cell-free extracts of the human small intestine, rutin **2** is not a substrate for human β-glucosidases [51]. The sugar moiety is an important determinant for the flavonoid absorption as the pharmacokinetic data of rutin **2** bioavailability in human volunteers have confirmed [52]. The aglycone quercetin **1** is absorbed from the caecum and colon after deglycosylation of rutin **2** by the microflora.

Figure 6 shows HPLC chromatograms of the rutin **2** deglycosylation within 24 h using the fresh inoculum filtrate. The decreasing amounts of rutin **2** and the increasing amounts of its aglycone quercetin **1** are demonstrated in

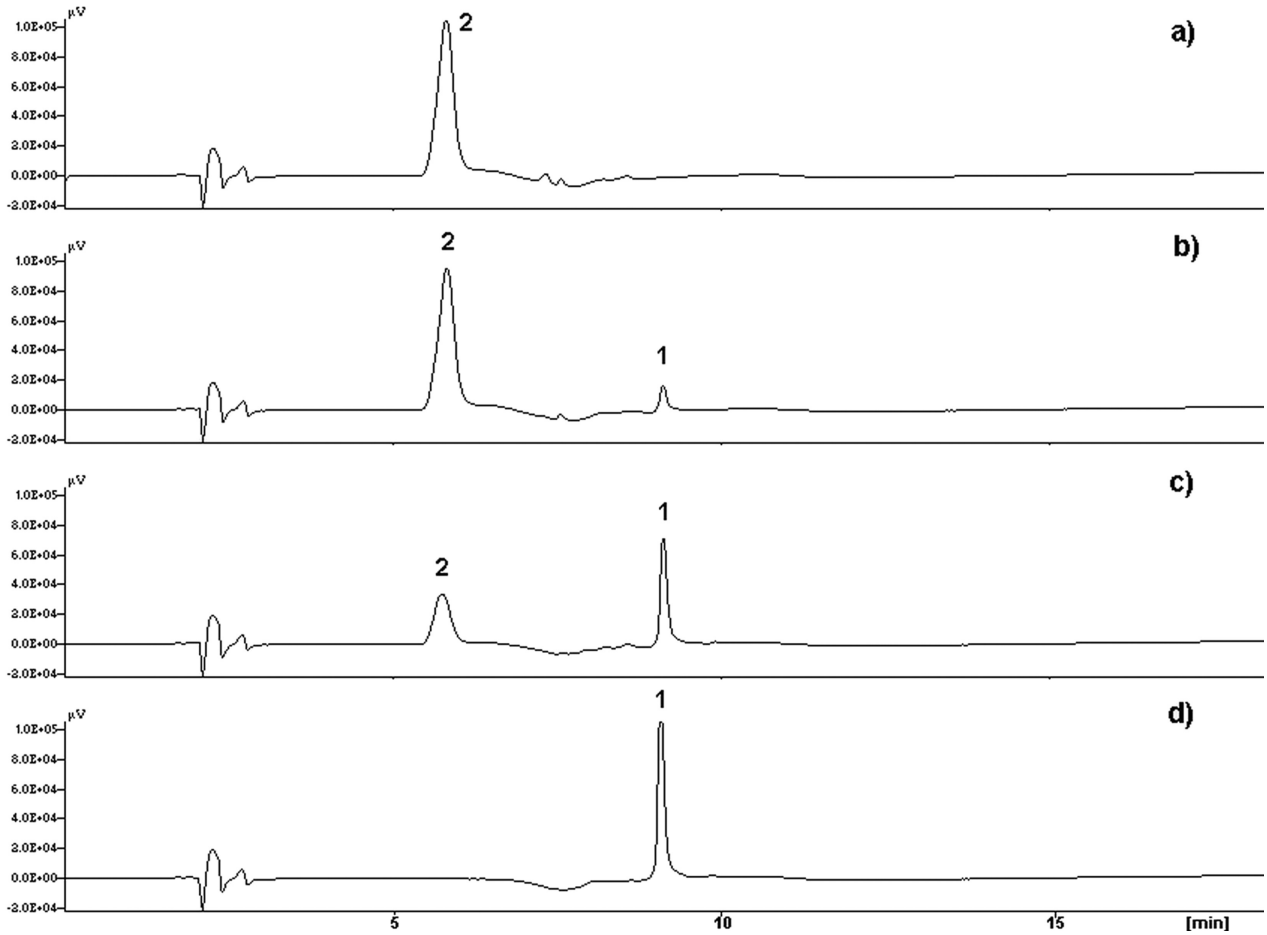

Figure 6. HPLC chromatograms of rutin **2** (1 mM) deglycosylation after (a) incubation with sterilized inoculum for 1 h; (b) incubation with fresh inoculum for 1 h; (c) incubation with fresh inoculum for 8 h; (d) incubation with fresh inoculum for 24 h and release of quercetin **1** (HPLC chromatograms were monitored at 360 nm).

Fig. 7. We also measured the phenolic degradation products PHG **3** and DOPAC **4** resulting from the ring scission of quercetin **1**. After 24 h of incubation, rutin **2** was completely deglycosylated leading to considerable concentrations of quercetin **1** (0.400 ± 0.228 mM; $n_{caeca} = 3$, mean ± SD), PHG **3** (0.162 ± 0.079 mM) and DOPAC **4** (0.155 ± 0.102 mM). As we described previously, flavonoids linked to a rhamnoglucoside moiety are hydrolysed more slowly than mono or diglucosidic derivatives [34] independent of the released aglycone [53]. The decreased rate of rutin deglycosylation indicates a steric hindrance of the β-glucosidase because of the complex sugar moiety rhamnoglucoside. As already mentioned for the chemical stability of quercetin **1**, the applied concentration of rutin **2** (1 mM) was reduced by matrix effects with detectable amounts of 0.756 ± 0.037 and 0.619 ± 0.081 mM after 1 and 24 h of incubation in the sterilized inocula filtrates, respectively. Quercetin **1**, PHG **3** and DOPAC **4** were not detected when sterilized inocula were used.

We studied the deglycosylation of rutin **2** by the microflora prepared from different caeca and preserved by freezing between 6 wk and 6 months. As the cleavage of the sugar moiety is the first but not the critical step in the microbial flavonoid metabolism, we wanted to include host-dependent deviations. This experimental design was chosen to prove the caecal model system as a reproducing tool in the deglycosylation of polyphenols independent of the host and its caecum preparation by freezing. Figure 8 shows the degradation of rutin **2** by the freeze-preserved inocula in comparison to the representive fresh caeca presented above. Deglycosylation of rutin **2** in the freeze-preserved caeca followed the degradation rate of the fresh inocula within their SD. After 24 h of incubation rutin **2** was hydrolysed completely in all preparations leading to the same metabolite patterns **1**, **3** and **4** with comparable amounts (data not shown) as described above. The fresh inocula resembled host-dependent deviations as well as the freeze-preserved inocula in addition to a different duration of freezing.

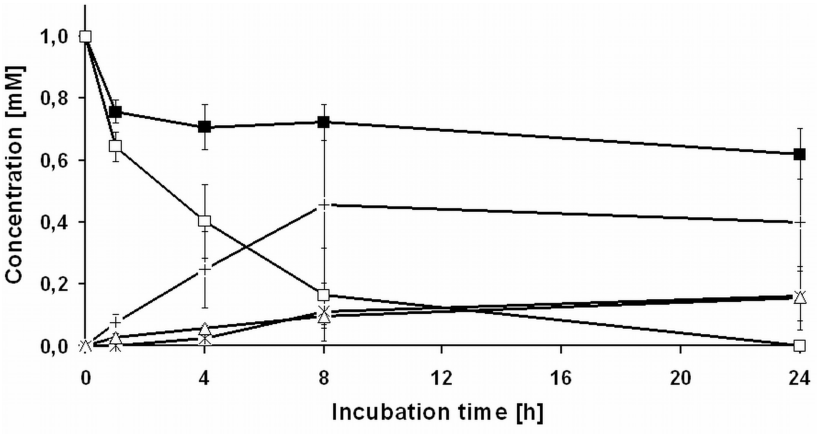

Figure 7. Time–concentration degradation-profiles of rutin **2** and release of quercetin **1** (+) (measured by HPLC-DAD at 360 nm), and phenolic degradation products **3** (*) and **4** (△) (measured by HPLC-DAD at 265 nm for **3** and 275 nm for **4** or GC-FID) after incubation of **2** with fresh inoculum (▫). Sterilized inoculum filtrates (■) were used for measuring the chemical stability of **2** (n_{caeca} = 3, mean ± SD).

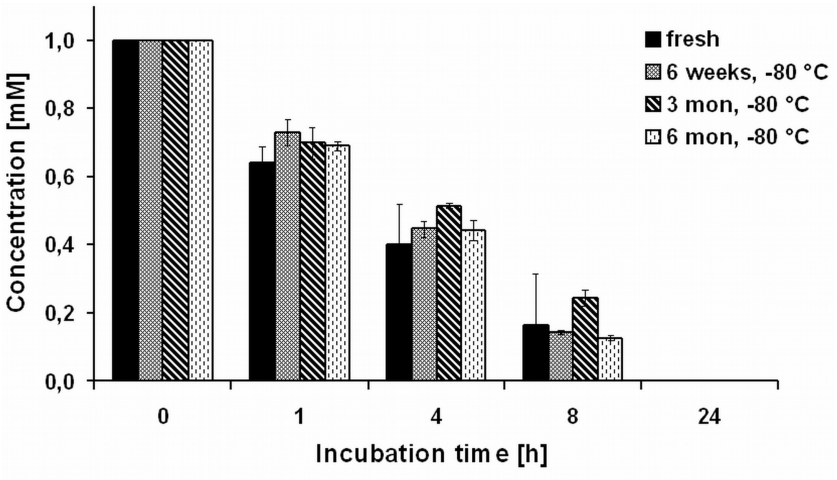

Figure 8. Time–concentration degradation-profiles of rutin **2** (measured by HPLC-DAD at 360 nm) after incubation with fresh inoculum (n_{caeca} = 3, mean ± SD) or inocula prepared by freeze-preservation for 6 wk, 3 or 6 months at −80°C (n = 2, mean ± SD).

4 Concluding remarks

Metabolism by the gut microflora is one important factor for the bioavailability of phenolic phytochemicals. We have developed an *in vitro* model system for which the microflora is directly isolated from the caecum of freshly slaughtered pigs. The preservation of the naturally composed microflora under strict anaerobic conditions gave a better reproducibility of the *in vivo* conditions than the incubation with single bacterial species, mixed cultures or faecal samples used elsewhere. In this study we extended the model system by freeze-preservation of the caecal inocula over several months for economic and experimental design reasons: the research laboratory will be independent of the slaughterhouse in time and locality and the inocula with an equally composed microflora will be available over several months so that a multitude of compounds and replicate samples could be incubated with an equally composed microflora. We proved the vitality and the metabolic efficiency of the freeze-preserved microflora by the bacterial marker enzyme α-galactosidase and the degradation of quercetin **1** and its glycoside rutin **2**. The deglycosylation of PNP α-gal and rutin **2** was not affected adversely by the freeze-preservation (2–6 months at −80°C) in comparison to the fresh inocula. Neither the duration of freezing nor host-dependent deviations as in the case of rutin influenced the results in both preparations at the end point of the incubation. However, regarding the results of quercetin **1**, the cleavage of the complex aromatic ring system was not independent of the duration of freeze-preservation. For one caecum, the metabolic efficiency of the ring scission diminished slightly within 5 months of freezing. However, the incubation of quercetin **1** with the freeze-preserved inocula led to the same pattern of degradation products PHG **3** and DOPAC **4** as with the fresh inocula.

In conclusion, quercetin **1** and rutin **2** were metabolized very similarly by fresh inocula and inocula prepared by freeze-preservation for at least 4–5 months at −80°C. The incubation experiments led to the same pattern of phenolic degradation products in comparable amounts. We confirmed that vitality and metabolic efficiency were preserved in the freeze-preserved inocula similar to the fresh inocula. Therefore, freeze-preservation is an helpful tool in studying the metabolism of polyphenols in detail over several months under equalized conditions.

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