Mutual interaction of phenolic compounds and microbiota: Metabolism of complex phenolic apigenin *C*- and kaempferol *O*-derivatives by human fecal samples

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ABSTRACT

Human colonic bacteria have an important impact on the biotransformation of flavonoid glycosides and their conversion can result in the formation of bioactive compounds. However, information about the microbial conversion of complex glycosylated flavonoids and the impact on the gut microbiota are still limited. In this study, *in vitro* fermentations with selected flavonoid *O*- and *C*-glycosides and three different fecal samples were performed. As a result, all flavonoid glycosides were metabolized via their aglycones yielding smaller substances. Main metabolites were 3-(4-hydroxyphenyl)propionic acid, 3-phenylpropionic acid, and phenylacetic acid. Differences in the metabolite formation due to different time courses between the donors were determined. Therefore, from all fermentations, the ones with a specific donor were always slower resulting in a lower number of metabolites compared to the others. Exemplarily, tiliroside was totally degraded from 0h (105 ± 13.2 µM) within the first 24h, while in the fermentations with fecal samples from other donors, tiliroside (107 ± 52.7 µM at 0h) was not detected after 7h anymore. In general, fermentation rates of *C*-glycosides were slower compared to the fermentation rates of *O*-glycosides. The *O*-glycoside tiliroside was degraded within 4h while the gut microbiota converted the *C*-glycoside vitexin within 13h. However, significant changes ($p < 0.05$) in the microbiota composition and short chain fatty acid levels as products of carbohydrate fermentation were not detected between incubations with different phenolic compounds. Therefore, microbiota diversity was not affected and a significant prebiotic effect of phenolic compounds cannot be assigned to flavonoid glycosides in food-relevant concentrations.

KEYWORDS

Flavonoid glycosides, metabolism, *in vitro* fermentation, human gut microbiota, short chain fatty acids

INTRODUCTION

Flavonoids are a class of secondary plant metabolites which are found ubiquitously in the plant kingdom. Their chemical structure is characterized by two aromatic rings with at least one hydroxyl group.[1] Flavonoids can be classified into several subgroups depending on the constitution of the heterocyclic C-ring.[2] The chemical structures range from very simple substances to quite complex flavonoids consisting of several phenolic rings with a wide range of substituents. In plants, they are frequently conjugated with different small organic molecules (preferentially sugars and organic acids), affecting their water-solubility and functionality.[3] Only occasionally they are present in plants as non-conjugated flavonoid aglycones.[1] Beside the very frequent *O*-glycosylation of flavonoids, flavonoid glycosides can also occur as *C*-glycosides.[4] The most common sources of flavonoid *C*-glycosides are swiss chard, tomato, lemons, and some cereals such as maize, wheat, and rice.[5, 6]

When consumed with plant foods, flavonoid glycosides are hypothesized to have health beneficial properties.[7] Antimutagenic, anticarcinogenic, antiviral, antibacterial, and antiinflammatory properties have been described.[8-11] Even though studies have mainly focused on the more common *O*-glycosides, it is assumed that *C*-glycosides would have better therapeutic properties due to their enhanced stability over the respective aglycone and *O*-glycosides.[12] But especially for *C*-glycosides, more investigations on resorption, metabolism, and health beneficial effects have to be carried out.[12] However, uptake/bioavailability of flavonoids is a crucial factor for potential bioactivities but this is still discussed controversially.[13] It is highly dependent on the chemical structure and can therefore differ between different compounds. Moreover, bioactivity is influenced by the resorption rate, intestinal, and hepatic metabolism, and the subsequent distribution in the organism.[14] Although different

pathways have been identified as being responsible for an enhanced resorption, it has to be concluded that in most cases resorption rates of intact flavonoids are not very high.[15, 16] Consequently, the most important metabolic transformation of flavonoid glycosides takes place in the colon, where they undergo significant degradation by the gut microbiota.

In the human colon more than 500 different bacterial species are found, whereby the composition of the microbiome is highly diverse and unique for every individual.[7, 17] Some of the bacterial species can catalyze (flavonoid) *O*- and *C*-deglycosylation, demethylation, dehydroxylation, ester cleavage, reduction of carbon-carbon double bond, isomerization, ring fission, and decarboxylation.[18] However, not all bacteria are able to carry out every degradation step.[19] For example, *Enterococcus casseliflavus* is only able to cleave and ferment the sugar moiety from different quercetin-*O*-glycosides to short chain fatty acids (SCFA), while the aglycone is not degraded any further. In contrast, *Eubacterium ramulus* is capable of converting also the aglycone.[20] Additionally, it is possible that some kind of cross-feeding effects due to the degradation of phenolic compounds occur. [21] It means that one strain gives good growth on the primary substrate such as the phenolic compound, which is metabolized to a product being a secondary substrate for another strain. This might enhance growth of further microorganisms and consequently influence composition of the colonic microbiota.[22]

Although the structures of *O*- and *C*-glycosides are very different from a chemical point of view, the conversion of *C*-glucosides also showed a dependence on the presence of specific bacteria for degradation steps such as the cleavage of the C-C bond. For example, *Eubacterium cellulosolvens* is not able to deglycosylate the *C*-

glycosides vitexin, while it is degradable by the intestinal *Lachnospiraceae* strain CG19-1.[23]

When looking at fermentation experiments with fecal samples, it is possible to investigate at least two ways of action: On the one hand, the microbial transformation of flavonoids to bioactive products is of interest, whereas on the other hand, the influence of phenolic compounds and their metabolites on the composition of the gut microbiota is an important aspect, too (two-way phenolic - microbiota interaction).[24] In general, combining metagenomics and metabolomics studies will help to better understand both types of interactions.[25] However, information about the microbial transformation of more complex, highly glycosylated flavonoids, and evidence for their effect on the gut microbiota is still not sufficiently investigated. In some studies, it has been suggested that more complex polyphenols have an even higher effect on the microbiome than simple structures, because a larger variety of potentially bioactive metabolites can be formed.[26] Therefore, such structures might also influence the diversity of the microbiota which is hypothesized to correlate with the health of an individual.[27]

The aim of this study was to investigate the degradation of selected structurally related, highly glycosylated flavonoids and their influence on gut microbiota composition. Special focus was set on the comparative assessment of group specific differences between *O*- and *C*-glycosides. For this purpose, different *in vitro* fermentations with a limited number of human fecal samples were performed. Breakdown products were analyzed using HPLC-ESI-MS/MS analysis. To assess whether phenolic exposure influenced the microbiota, qPCR and Illumina sequencing were carried out to investigate potential compositional changes and SCFA production was determined to detect major functional changes.

MATERIAL AND METHODS

Chemicals. Apigenin (AP, 4',5,7-trihydroxyflavone), kaempferol (K, 3,4',5,7-tetrahydroxyflavone), kaempferol-3,4'-*O*-diglucoside-7-*O*-rhamnoside (K-DG-R), tiliroside (T, kaempferol-3-(6''-trans-*p*-coumaroyl)glucoside), vitexin (V, apigenin-8-*C*-glucoside), and vitexin-2''-*O*-rhamnoside (V-R) were purchased from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany). Vitexin-2''-*O*-glucoside (V-G), and vitexin-2''-*O*-xyloside (V-X) were isolated from swiss chard. The isolation method is described in the supplementary information.

The chemical structures of the substrates used in this study are shown in **Figure 1**. All *O*-glycosides are based on the aglycone structure of kaempferol, whereas the structures of the *C*-glycosides are related to the aglycone apigenin. Both aglycones differ only in a hydroxyl group at C3-position of the basic skeleton. While tiliroside has only one sugar moiety being bound via a C-O bond at the C3-position and further esterification with *p*-coumaric acid, kaempferol-3,4'-*O*-diglucoside-7-*O*-rhamnoside is *O*-glycosylated with three sugar moieties at different positions. In contrast, vitexin is the simplest *C*-glucoside of apigenin, bound via a C-C bond at the C8-position. The different vitexin derivatives have a second sugar moiety at the C2''-position. These bonds are C-O bonds. Consequently, the vitexin derivatives combine *O*- and *C*-glycosidic bonds in one structure.

***In vitro* degradation of phenolic substrates by fecal microbiota.** This study was part of a set of experiments already described by Vollmer, et al. [28] in which three independent *in vitro* fermentations with fecal microbiota were performed. Fecal samples were donated by three different, healthy donors (donor A, B, and C) without a history of gastrointestinal disorders or any antibiotics consumption for at least three month prior to the fermentation experiment. Number of samples and fermentation

144 strategy were similar to related fermentation experiments already described in the
145 literature.[21, 29-31] The preparation of the fecal suspension was conducted according to
146 Vollmer, Schröter, Esders, Farquharson, Neugart, Duncan, Schreiner, Louis, Maul
147 and Rohn [28]. *In vitro* fermentations were performed with eight different flavonoids in
148 triplicate at an initial pH value of 6.5 in a final volume of 10 mL. Based on preliminary
149 experiments with similar levels of carbon source(s) it was estimated that during
150 fermentation the pH drops by 0.5-1 units. The volume of 10 mL consisted of 9.4 mL
151 fermentation medium which contained several minerals, supplements, and
152 carbohydrates to allow the gut bacteria to grow, 100 µL phenolic substrate (final
153 concentration 200 µM, pre-dissolved in DMSO), 14 µL vitamin solution, and 500 µL
154 freshly prepared fecal suspension (0.2% final fecal concentration). The preparation
155 and ingredients of the fermentation media and vitamin solution are described
156 elsewhere.[28]

157 After adding the fecal suspension to the medium, the samples were incubated on a
158 rotator (Stuart SB3, Bibby Scientific, Stone, UK) for 48 h at 37 °C and 25 rpm. Two
159 aliquots of 750 µL were collected initially (0h), and after 3 h, 7 h, 24 h, and 48 h while
160 flushing with CO_2. One aliquot was frozen with liquid nitrogen and stored at -80 °C
161 until HPLC-ESI-MS/MS analysis. The second aliquot was used for investigations of
162 the microbiota and SCFA analyses. For that, the aliquot was separated into
163 supernatant (SCFA analysis) and cell pellet (DNA analysis) by centrifuging.[28]
164 Supernatant was stored at -20 °C until SCFA analysis and cell pellet dissolved in
165 buffer at -80 °C. Incubation parameters of fecal fermentation and sample treatments
166 were already described by Vollmer, Schröter, Esders, Farquharson, Neugart,
167 Duncan, Schreiner, Louis, Maul and Rohn [28].

In addition to fecal suspensions with phenolic substrates, control samples for every fermentation without any substrates were conducted. This was done to determine the metabolite formation caused by compounds endogenously present in the fecal samples. For that, 100 µL DMSO were used instead of the substrate ('control samples I'). For the estimation of the interactions of the substrates with the medium, 100 µL substrate were combined with 9.9 mL medium and 14 µL vitamin solution and were treated the same way as the samples ('control samples II').

The fermentation period in the present study was set to 48 h based on other *in vitro* fermentation experiments on phenolic compounds described in the literature. [19, 32-34] Some of the fermentation experiments ended already after 24 h, but there, mainly simple phenolic substances were investigated. Due to the assumption that the microorganisms need longer for the degradation process of complex phenolic compounds the fermentation period was extended to 48 h.

Sample preparation and HPLC-ESI-MS/MS analysis for quantification. The substrates and their metabolites were extracted with ethyl acetate from 400 µL of the fermentation mixture from every time point. The sample preparation and extraction were carried out according to Vollmer, Schröter, Esders, Farquharson, Neugart, Duncan, Schreiner, Louis, Maul and Rohn [28].

HPLC analyses of the substrates and their metabolites were performed using a 1260 Infinity Series system from Agilent Technologies Deutschland GmbH & Co. KG (Waldbronn, Germany). The system consisted of a binary pump, an online-degasser, an autosampler, and a column oven. The separation of the substances and their metabolites was carried out using a Kinetex 5 u EVO C18 100 A (150 x 2.1 mm) column equipped with an EVO C18 pre-column (both from Phenomenex Inc., Aschaffenburg, Germany). The chromatographic separation took place at 20 °C. The

mobile phase was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) and the following gradient elution was used: 5% B (0-1 min), 11% B (1-2 min), 14% B (2-4 min), 50% B (4-15 min), 95% B (15-18 min), 95% B (18-22 min), 5% B (22-23 min) and 5% B (23-32 min). The flow rate was set to 300 µL/min and the injection volume was 5 µL.

For detecting the substrates and their metabolites, mass analyses were performed in negative ion mode on an API2000 triple quadrupole MS/MS system (AB Sciex Germany GmbH, Darmstadt, Germany) equipped with an ESI interface. The following mass spectrometer settings were used: electrospray voltage = -4500 V, temperature = 450 °C, curtain gas = 1.7 bar, ion source gas 1 = 2.1 bar, ion source gas 2 = 5.2 bar and collisions gas = 0.5 bar (all nitrogen). For each substance, the optimum settings of the declustering potential, entrance potential, collision energy, collision exit potential, and the characteristic fragments were also determined.

For quantification, an external matrix calibration (0-200 µM) for every donor was used (**Supplementary Table 1)**. The matrix consisted of the fermentation medium and the fecal sample. The data obtained were analyzed with the software Analyst® 1.5.2 from AB Sciex Germany GmbH (Darmstadt, Germany).

Beside the substrates used, the following metabolites were included in the method: benzoic acid, 4-hydroxybenzoic acid, caffeic acid, *p*-coumaric acid (*p*-CA), 3-(3,4-dihydroxyphenyl)propionic acid, 3-(3,4-dihydroxyphenyl)acetic acid, 3,4-dihydroxytoluene, ferulic acid, 3-(3-hydroxyphenyl)propionic acid, 3-(4-hydroxyphenyl)propionic acid (4-HPPA), 3-phenylpropionic acid (3-PPA), 3-hydroxyphenylacetic acid (PAA), 4-hydroxyphenylacetic acid, phenylacetic acid (PAA), hippuric acid, and kaempferol-3-*O*-glucoside (K-G).

Analysis of short chain fatty acids. Determination of short chain fatty acids (SCFA) including acetate, propionate, butyrate, iso-butyrate, formate, but also of further organic acids such as lactate, succinate, valerate, and iso-valerate was conducted with 500 µL of the supernatants from the 48 h samples and from one 0 h sample per substrate. The sample preparation was based on the method developed by Richardson, et al. [35] followed by gas chromatography analysis using a Hewlett-Packard gas chromatograph fitted with a fused silica capillary column with helium as a carrier gas. Calculations were done with external standards, 2-ethylbutyrate was used as internal standard. The mean of the SCFA concentration resulting from the fermentations with the different substrates were set in relation to the SCFA concentration from the 'control samples I'.

DNA extractions, fluorimetric DNA quantification and qPCR analysis. DNA extractions were done with 400 µL of the fecal suspension from every donor and with the cell pellets from the 48 h samples using the FastDNA® spin kit for soil (MP Biomedicals, Illkirch, France). Extraction, fluorimetric DNA quantification and qPCR analysis were performed according to the protocol described by Vollmer, Schröter, Esders, Farquharson, Neugart, Duncan, Schreiner, Louis, Maul and Rohn [28]. Universal primers against total bacteria and specific primers against *Bifidobacterium* spp., Bacteroidetes, Ruminococcaceae, Lachnospiraceae, *F. prausnitzii*, *Blautia* spp., and the *Roseburia/Eubacterium rectale* group, were used.[28]

Illumina sequencing of 16S rRNA genes. The Illumina sequencing of selected compounds was performed at the ZIEL – Institute for Food & Health of the TU Munich with DNA extracts of the fecal suspension from every donor (initial composition), the 48 h sample of tiliroside from all three donors, and the 48 h samples of vitexin, vitexin-2''-*O*-rhamnoside and kaempferol-3,4'-*O*-diglucoside-7-*O*-

rhamnoside from donor A and C. The method details are described in the supplementary information. 429,958 initial sequence reads were filtered as described in supplementary information, which resulted in 245,816 final reads (11,698-19,887 per sample). Those were assigned to 151 operational taxonomic units (OTUs) at ≥97% sequence identity (**Supplementary Table 2**). Sequencing data generated during this study are available in the SRA database under SRA accession SRP117249 at http://www.ncbi.nlm.nih.gov/sra/SRP117249.

Statistical analysis. Statistical analysis was performed to investigate whether differences in SCFA levels or in the microbiota composition resulting from the substrate fermentations were significant or not compared to the 'control samples I'. For that, data were analyzed using IBM SPSS Statistics 22 (Ehningen, Germany). All data were tested for normality. With the normal distributed data investigations for significant differences were carried out with an independent t-test. Non-normal distributed values were analyzed with the non-parametric MANN-WHITNEY-U-test. For significance, a confidence level of 95% ($p < 0.05$) was used. In addition, the qPCR, SCFA, and sequence data were summarized by Principal Component Analysis (PCA). All data were standardized before applying PCA. The analysis was performed in R (R Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org). For correlation analysis between qPCR and sequencing data, OTUs were assigned to the corresponding qPCR assays based on their taxonomy (**Supplementary Table 2**).

RESULTS AND DISCUSSION

In the present study, the degradation of selected, structurally related, highly glycosylated flavonoids were determined in three independent *in vitro* fermentations in order to estimate metabolite formation and changes in the microbiota composition. Here, it was of interest to determine if the gut microbiota is able to convert the more complex flavonoids to lower molecular metabolites, or if they are not or only partially degraded. For comparison and as a control, *in vitro* fermentations with the corresponding aglycones were also performed.

In vitro fermentations of the flavonol kaempferol and its *O*-glycosides tiliroside and kaempferol-3,4'-*O*-diglucoside-7-*O*-rhamnoside.

Kaempferol. When starting the fermentation experiments with the comparatively simple aglycone structure kaempferol (K), there was only a slow degradation by the fecal sample from donor A. K was still present at 48 h and only small amounts of the metabolite 3-(4-hydroxyphenyl)propionic acid (4-HPPA) were detected (**Table 1**). However, K was fully metabolized by 7 h and 24 h, respectively, with the fecal sample from donors B and C and 4-HPPA was detected at 7 h at its highest concentration in both cases. During the fermentation with the fecal sample from donor B no further metabolites were present after 24 h. By contrast, the metabolite phenylacetic acid (PAA) was detected in the fermentation with the fecal sample from donor C. In the HPLC-ESI-MS/MS method used, the detector sensitivity for the metabolite 3-phenylpropionic acid (3-PPA) and PAA was rather low. It cannot be judged whether e.g., PAA was only formed to lower amounts by donor B unlike for the other two donors, because responsible microorganisms are missing or the amount was simply lower than the limit of detection (LOD) of the method. Therefore, it is possible that the formation of 3-PPA and PAA could not be detected in the

fermentation B. Nevertheless, the results obtained are in agreement with results described in the literature, where mainly 4-hydroxyphenylacetic acid, the precursor for PAA, which was also detected in the present study, has been described as an important phenolic metabolite (**Figure 2**).[7, 36]

Tiliroside. In all three *in vitro* fermentation experiments, tiliroside (T) was metabolized significantly by the gut microbiota. **Figure 3** displays its degradation steps and metabolite formation. The detected metabolites were kaempferol-3-*O*-glucoside (K-G), kaempferol (K), *p*-coumaric acid (*p*-CA), 3-(4-hydroxyphenyl)propionic acid (4-HPPA), and 3-phenylpropionic acid (3-PPA), whereby the latter was not detected in the fermentation with the fecal sample from donor A. In the fermentations of donor B and C, the bacteria already cleaved the ester bond between the sugar moiety and *p*-CA within short time resulting in the formation of K-G at 0 h, immediately after inoculation with the fecal samples (**Table 1, Figure 3**). An interaction or breakdown due to the fermentation media can be excluded, because K-G was not detected in the 'control samples II' (substrate + medium, data not shown). The detection of K-G leads to the assumption that the microbiota did not mainly first cleave the bond between the kaempferol-moiety and the sugar substituent in T, and T is mostly degraded to its aglycone K via the intermediate product K-G. However, the metabolite K-G was only detected in very low concentrations in fermentation A at 0 h, 3 h, and 7 h. Instead, a significant increase of K and *p*-CA was present within the first 7 h. This may be due to the cleavage of the bond between K and glucose and the release of *p*-CA taking place at the same time intervals. Subsequently, the aglycone was transformed to smaller phenolic acids deriving from the A- and B-ring during the breakdown of the heterocyclic flavonoid C-ring.[25] Furthermore, the microbiota seems also to be able to use the free *p*-CA as a substrateas already described in some

316 studies where different strains were able to convert *p*-CA. As a result, further small
317 metabolites such as 4-HPPA are formed (**Figure 2**).[37, 38] Therefore, the formation of
318 4-HPPA derives from the degradation of the aglycone structure and *p*-CA, being a
319 structural part in the chemical structure T.

320 While K was metabolized only slowly in the fermentation of T with the fecal sample
321 from donor A and was still present in a very high concentration at 48 h, *p*-CA was
322 degraded continuously up to 24 h. This resulted in the formation of 4-HPPA
323 corresponding to the reduction of the double bond, which is a very typical reaction in
324 the microbial transformation of cinnamic acid derivatives.[39] At 48 h, 4-HPPA showed
325 a similar concentration compared to the 24 h samples indicating that no significant
326 further degradation of 4-HPPA was carried out by the microbiota from the fecal
327 sample of donor A. In the fermentations with the fecal samples from donors B and C,
328 4-HPPA was already detected at 7 h at its highest concentration. Subsequently, 4-
329 HPPA was fully metabolized to 3-PPA by a dehydroxylation at the C4-position. This is
330 in accordance with a study published by Scheline [40], who found that the reduction of
331 the double bond by fecal microorganisms greatly exceeds dehydroxylation reactions.
332 Differences between the fermentations B and C can be found in the concentration of
333 the intermediary metabolites *p*-CA and 4-HPPA. Both had their concentration
334 maximum at 7 h. While the concentration of *p*-CA in fermentation B was much lower
335 than the concentration of 4-HPPA, the concentration of both metabolites was almost
336 identical in fermentation C. It is well known, that many anaerobic bacteria are not
337 able to carry out every degradation step and therefore metabolize aromatic
338 compounds not completely.[19] Consequently, it might be possible that the microbiota
339 composition of donor B was better adapted for carrying out reduction reactions than
340 the microbiota of donor C.

Taking the results of the *in vitro* degradation of the reference compound K into account (**Table 1**), only 4-HPPA and PAA were detected. Therefore, it can be assumed that *p*-CA in the fermentations with T was mainly detected because of being a structural part of T. As already mentioned, *p*-CA is also the precursor for the formation of 4-HPPA (**Figure 2**). Therefore, the amount of 4-HPPA in all fermentations with T as substrate was much higher than the concentration of 4-HPPA in the fermentations with the substrate K alone.

Kaempferol-3,4'-O-diglucoside-7-O-rhamnoside. Compared to the fermentation of T, where several metabolites were identified, a lower number of phenolic metabolites was determined in the fermentation of the more complex kaempferol-3,4'-*O*-diglucoside-7-*O*-rhamnoside (K-DG-R, **Table 1**). Due to the fact that K-DG-R itself was not stable in the ion source and therefore, could not be detected with the analytical method used, the degradation was investigated only by the formation and degradation of K-G and K. In the 'control samples II', where the substrate was incubated with the medium, neither K-G nor K were detected (data not shown). Consequently, the formation of the different metabolites resulted only from the microbial degradation of K-DG-R. For quantification, only the metabolite K-G could be included in the method, due to the lack of reference compounds. However, additionally qTOF analyses were carried out and the tentative identification of the metabolites kaempferol-3,4'-*O*-diglucoside, kaempferol-3-*O*-glucoside-7-*O*-rhamnoside, or kaempferol-4'-*O*-glucoside-7-*O*-rhamnoside, and kaempferol-7-*O*-rhamnoside was possible. The metabolites were detected at the same fermentation times as K-G (data not shown). Furthermore, the metabolites K-G (0-7 h), K (3-48 h), and 4-HPPA (48 h) for the fermentation with the fecal sample from donor A and K-G (0-3 h), K (3 h), and PAA (24-48 h) in the fermentations with the fecal sample from

366 donors B and C were detected and quantified. In conclusion, as already shown for
367 the substrate T, K-DG-R was degraded to phenolic acids via the consecutive release
368 of the sugar moieties and the aglycone.

369 In comparison to the reference compound K, similar metabolites were detected. But,
370 in the fermentation B and C of K-DG-R only the phenolic acid PAA but not the
371 metabolite 4-HPPA was present compared to the fermentation of K. It is possible that
372 the formation of PAA went via 4-HPPA which was not detected in the fermentations B
373 and C, probably resulting from the time intervals, when the samples were taken
374 (**Table 1, Figure 2**).

375 ***In vitro* fermentation of the flavone apigenin and its *C*-glycosides vitexin,**
376 **vitexin-2''-*O*-rhamnoside, vitexin-2''-*O*-glucoside and vitexin-2''-*O*-xyloside.** In
377 the *in vitro* fermentations of apigenin (AP) and its *C*-glycosidic derivatives, all three
378 human fecal microbiomes were able to convert the substrates yielding smaller
379 phenolic acids. Analogously to the fermentation of the phenolic compounds
380 mentioned above, differences between the donors in the metabolite formation were
381 determined. Furthermore, the fermentations with the fecal sample from donors B and
382 C showed the formation of smaller molecular metabolites than the fermentation with
383 the fecal sample from donor A. Also, the same metabolites, 4-HPPA, PAA, and 3-
384 PPA were detected, but the intermediate products differed between the initial
385 substrate.

386 *Apigenin*. In the fermentation with the fecal sample from donor A, AP was still
387 detected at 48 h, while in the fermentation with the fecal sample from donors B and
388 C, there were only low concentrations of AP at 0 h. Consequently, as AP was not
389 present in the 'control samples II' (data not shown), an interaction between the
390 substrate and the medium can be excluded, and it appears that it was already

391 partially degraded. In general, the formed metabolites were 4-HPPA (A: 24 h, 48 h; B:
392 7 h, C: 3 h, 7 h) and PAA (A: 48 h; B: 48 h, C: 24 h, 48 h) for all three fermentations
393 and additionally 3-PPA (B: 24 h, 48 h, C: 24 h, 48 h) for the fermentations with the
394 fecal sample from donors B and C (**Table 2**).

395 *Vitexin.* The fermentations with the substrate vitexin (V) showed the same phenolic
396 acid metabolites as the fermentation of AP, with different maxima: 4-HPPA (A: 24 h,
397 48 h; B: 24 h, 48 h), PAA (A: 48 h; B: 24 h, 48 h, C: 24 h, 48 h), and 3-PPA (B: 24 h,
398 48 h; C. 24 h, 48 h) (**Table 2**). V itself was degraded within the first 24 h in the
399 fermentations with the fecal samples from donors B and C but only disappeared after
400 48 h in the fermentation with the fecal sample from donor A. Additionally, the
401 metabolite AP was detected at 48 h in the fermentation with the fecal sample from
402 donor A. Therefore, the degradation of V seems to take place via the formation of the
403 aglycone structure and the glucose moiety was primarily cleaved by the bacteria. Due
404 to faster metabolism, AP was probably not detected in the fermentations with the
405 fecal samples from donors B and C. The metabolites observed are in agreement with
406 descriptions in the literature, where for example the rod-shaped Gram-positive
407 *Lachnospiraceae* strain CG19-1, was able to convert the substrate V.[23] In contrast,
408 two *Lactococcus species* and one *Enterococcus species* were not able to convert V,
409 as described by Kim, et al. [41] .

410 *Vitexin derivatives.* In the fermentations with the different vitexin derivatives, the
411 concentrations of vitexin-2''-*O*-glucoside (V-G), vitexin-2''-*O*-rhamnoside (V-R), and
412 vitexin-2''-*O*-xyloside (V-X) were very low or initial substances not even detectable in
413 the fermentation samples. In contrast to V, those compounds have a second sugar
414 moiety bound which increases their water solubility. It is assumed that the vitexin
415 derivatives still remain in the aqueous fermentation medium when extracting the

substrates and metabolites with ethyl acetate. Due to the fact, that the ‘control samples II’ (substrate + medium) did not show any phenolic breakdown products and substrate concentrations in the ‘control samples II’ and fermentation samples were very similar, the formed metabolites can be attributed to the degradation of the corresponding initial substances (data not shown). Consequently, the degradation of the vitexin derivatives could only be displayed by their metabolite formation.

In all *in vitro* fermentations with the different vitexin derivatives, the degradation went via the intermediate product V indicating that the first step was cleavage of the *O*-glycoside bond by the microbiota. On the basis of the formation of V, it was possible to compare the release of the second glycoside moiety. It can be seen that in fermentation B and C the release of the glucose moiety was quicker than the release of rhamnose, or xylose (**Table 2**). **Figure 4** shows exemplarily the V formation within the *in vitro* fermentation with the fecal sample from donor B for the three different vitexin derivatives investigated. Graphs were normalized to 100%. This effect may be due to the fact that glucoside units are more common in nature and more microorganisms are adapted to utilize glucose linked to secondary plant phenolics than other sugars.[42] Despite the intermediate metabolite V, the main end products of the fermentation of the vitexin derivatives were 4-HPPA, PAA, and only 3-PPA in one case, which is in accordance with the metabolites resulting from the degradation of V (**Table 2**).

The aglycone AP was not detected in the fermentations of the different vitexin derivatives, but similar metabolites were detected compared to the fermentation of pure AP (**Table 2**). Due to the fact, that the metabolite V was present and V itself appears to be degraded to phenolic acids via the intermediate product AP, it was

assumed that the formation of the phenolic acids went via the aglycone structure as well. However, this was not detectable in the present study.

It is recognizable that the recovery rates of the initial substrates in some fermentations (i. e., V-G) were very low compared to the amount of the phenolic substrates in the fermentation mixture. When looking at the 'control samples II', where the phenolic compounds were co-incubated with the medium, it is notably that the concentrations of the phenolic compounds were not higher and very similar compared to the ones in the fermentation mixtures (data not shown). Therefore, one possible reason for the low concentrations is interactions between the compounds and the medium. It is also possible that the compounds precipitate or are adsorbed without metabolism by the microbial biomass, the solid components of the media or the inoculum. Furthermore, due to their different hydrophilicity, it is possible that more hydrophilic substrates still remain in the aqueous medium when extracting with ethyl acetate.

Before starting the fermentation experiments, different extraction procedures were investigated and optimized for an overall mixture of compounds in buffered aqueous media, similar to the one used in the present study, by looking at the recovery rates after the extraction (data not shown). Due to the fact that the substrates and metabolites used in this study show very different polarities and solubilities the methodological approach was a compromise for covering substrates (comparatively more hydrophobic) and metabolites (comparatively more hydrophilic).

qPCR analysis, Illumina sequencing and SCFA production. Overall microbiota composition and activity after 48 h of incubation were assessed by qPCR for different bacterial groups (total bacteria, *Bifidobacterium* spp., Bacteroidetes, Ruminococcaceae, Lachnospiraceae, *F. prausnitzii*, *Blautia* spp., and the

Roseburia/Eubacterium rectale group) and determination of short chain fatty acid production in order to investigate a possible effect of the phenolic substrates on the microbiota. Significant differences ($p < 0.05$) between the 'control samples I' (medium + fecal sample without any substrate) and the different fermentations after 48 h of incubation were not determined for any of the bacterial groups or SCFA (**Supplementary Table 3**). The PCA (**Figure 5**) based on the relative qPCR and SCFA results shows that the different samples, even after 48 h of incubation, clustered by donor and not by the different phenolic substrates used. Therefore, the different phenolic compounds did not have a major effect on the microbiota. Furthermore, it is assumed that the SCFA in this study were mainly formed from the carbohydrates present in the fermentation media and fermenting the sugar moiety of the flavonoid glycosides did not lead to a significant difference because of the quite low concentrations of these compounds in the fermentation mixture.

Additionally, Illumina sequencing was performed with selected samples to investigate whether specific bacteria were affected by the presence of the phenolic substrates that may not have been detected by the qPCR analysis. Correlation between qPCR data and corresponding sequence data overall showed good agreement (**Supplementary Figure 1)**. Small variations are likely due to slight differences in qPCR primer specificity and the presence of un- or misassigned sequences in the sequencing data. **Figure 6** shows the results of the Illumina sequencing at genus level. The distribution on the phylum and family level is displayed in the supporting information (**Supplementary Figures 2 and 3**).

When looking at the microbiota composition, it is recognizable that the composition of the fecal sample from donors B and C were quite similar and in line with a microbiota composition to be found in healthy humans dominated by Firmicutes and

Bacteroidetes.[43] The composition of the fecal sample from donor A on the other hand was very different, which was confirmed by PCA at OTU level (≥97% sequence identity, **Supplementary Figure 4**). In particular, it had a very high abundance of Firmicutes, but low Bacteroidetes (**Supplementary Figure 2**). This initial fecal sample also showed the biggest change in microbiota composition investigated by Illumina sequencing after 48 h of incubation with all substrates (**Supplementary Figure 4**), with a big increase in Bacteroidetes and Proteobacteria at the expense of Firmicutes at the phylum level (**Supplementary Figure 2**). Only a few genera showed a major increase in relative abundance after incubation (*Bacteroides*, *Parabacteroides*, *Clostridium* XIVa, *Acidaminococcus*, *Veillonella*, unknown *Burkholderiales* and *Escherichia/Shigella*; **Figure 6**). Microbiota compositions between samples that were incubated with different phenolic substrates for 48 h were very similar within the donors (**Figure 6, Supplementary Figure 4**). This is in agreement with the results from the qPCR and SCFA analysis. Usually, α-diversity is used to express the mean variation of species to be found in a certain microbiome. In this study, α-diversity was similar in all three fecal samples and remained high after incubation with the fecal sample from donor C, but was lower after incubation with the other two donors, particularly with donor A (**Supplementary Table 4)**. β-Diversity could not be calculated due to the limited number of samples. PCA analysis and a dendogram were used for a visual presentation (**Supplementary Figure 4**).

In a study published by Duda-Chodak [43], it was concluded that flavonoid aglycones may inhibit growth of some intestinal bacteria, consequently leading to a modulation of the whole intestinal microbiome. Especially in the fermentations with the fecal sample from donor A, where the microbiota composition changes most between 0 h and 48 h, the aglycone was always present for longer than in the fermentations with

515 the fecal sample from donors B and C. Consequently, it might be possible that this is
516 a reason for the bigger differences. However, qPCR and SCFA data (**Figure 5**)
517 indicate that the control samples without phenolic substances had a similar
518 microbiota composition and activity. The incubations of fecal microbiota from donor A
519 showed a microbiota shift that may indicate exposure to oxygen as oxygen tolerant
520 bacteria were stimulated (in particular *Escherichia/Shigella*). While this is principally
521 possible, there was no indication that this actually happened in the corresponding
522 incubations. Anaerobic conditions were checked with the addition of resazurin (0.1%)
523 to the fermentation media. Resazurin is a redox indicator that changes from colorless
524 via pink into blue when oxygen is present. In all three fermentations no color change
525 was detected, suggesting that the microbiota shift seen was due to unusual initial
526 composition of fecal sample A rather than an experimental mistake.

527 For decades, it is controversially discussed that manipulation of the composition by
528 prebiotics might help to improve health.[44] A high amount of bifidobacteria in the gut is
529 often associated with health promoting effects. In a review published by Duenas,
530 Munoz-Gonzalez, Cueva, Jimenez-Giron, Sanchez-Patan, Santos-Buelga, Moreno-
531 Arribas and Bartolome [24] studies are described, where a stimulation of the growth of
532 beneficial bacteria, such as bifidobacteria, was caused by polyphenols, thus, exerting
533 prebiotic-like effects. In the present study, an increase in the amount of bifidobacteria
534 between the initial fecal samples and the fermentation samples after 48 h were
535 detected for nearly all substrates tested. However, a significant difference of the
536 amount of bifidobacteria between the ‘control samples l’ and the fermentation
537 samples based on the qPCR results were not detected (**Supplementary Table 3**).
538 So, changes are probably caused by the fermentation media or reaction conditions.

During the fermentation of carbohydrates, where SCFA often result as main products, specific gases are produced, as well. In this context, it could be also possible to cluster the bacteria based on their gas release depending on the specific glycosylated phenolic compounds. For that purpose, gas sensing is an alternative technology for measuring gases from *in vitro* fecal sample fermentation.[45] However, this was not possible to test within this study.

Interindividual comparison of the phenolic conversion rates of the different donors. For all substrates tested, phenolic conversion rate of donor A was slower than the fermentation rates with fecal samples of donors B and C, which were in turn quite similar. For evaluating kinetics of the degradation, D_{50} (degradation$_{50}$) values of all substrates were used. These represent the time point, when 50% of the substrate was totally degraded by the gut microbiota (**Tables 1 and 2**). It was not possible to estimate such values for the metabolites, because their formation and a possible follow-up degradation of a metabolite can take place in parallel. In general, deglycosylation reactions occurred more quickly than the breakdown of the aglycone structures. Consequently, based on the conversion rates, the metabolite profiles and the corresponding time courses differed between the different donors and fermentations, with the fecal samples from donor A showing in general less metabolites. These differences are probably caused by the individual microbial compositions in the microbiota from each donor. In the study published by Justesen, Arrigoni, Larsen and Amado [34], a major decrease of rutin was observed at first after 8 h of incubation. They concluded that it might be possible that other compounds being in the media or feces are more preferred substrates (such as carbohydrates) and are easier to ferment for the bacteria and thus, that the microorganisms probably needed an adaptation period. Moreover, Cassidy and Minihane [46] describe wide

564 interindividual variability in the bioconversion of flavonoids being attributed to specific
565 enterotypes, suggesting that individuals may be either weak or strong flavonoid
566 converters.

567 When comparing the composition of the microbiota after 48 h of incubation with the
568 substrate T in the present study, it is noticeably that Firmicutes were more dominant
569 in the fecal sample from donor B and C. In conjunction with above, the microbial
570 profile of the fermentation A showed more members of the *Escherichia/Shigella*
571 cluster (**Figure 6**). So far, more strains from the Firmicutes have been identified to be
572 responsible for flavonoid conversion than species from Proteobacteria where
573 *Escherichia/Shigella* belongs to.[18] For example, three *Ruminococcaceae* species and
574 different *Lachnospiraceae* species were described in the mentioned review as being
575 part of the degradation of some flavonoid structures, whereby the latter were mainly
576 found to carry out deglycosylation reactions. Looking at the family level of the Illumina
577 sequencing results of the present study (**Supplementary Figure 3**), the amount of
578 *Ruminococcaceae* and *Lachnospiraceae* were much higher in donors B and C than
579 in donor A. Furthermore, some species within the *Lachnospiraceae* family were also
580 identified being able to catalyze reduction reactions, e.g., of *p*-CA.[37, 38] The
581 sequencing data do not allow resolution at species level, but it is possible that donors
582 B and C carry more bacteria that are able to perform deglycosylations and reduction
583 reactions and therefore result in a faster conversion rate than donor A.

584 **Comparison of the degradation of *C*- and *O*-glycosides by human fecal**
585 **microbiota.** When comparing the time courses of the deglycosylations of *O*- and *C*-
586 glycosides, the present study showed that *O*-glycosides are metabolized faster than
587 *C*-glycosides, while the degradation rates of the two aglycone structures are very
588 similar within a fermentation (**Tables 1 and 2**). Looking at the fermentations of T and

589 V with the fecal sample from donor B, exemplarily, the concentration of the *C*-
590 glycoside V remained nearly constant up to 7 h and V was then fully metabolized
591 (**Table 2**), whereas the *O*-glycoside T was already totally degraded within the first 7h
592 (**Table 1, Figure 3B**). Additionally, with regard to the different vitexin derivatives, it
593 was shown that the release of the *O*-glycoside was faster than the degradation of the
594 intermediate product V, where the sugar is bound *C*-glycosidically (**Table 2**). This
595 may be due to the fact that *O*-glycosides occur more frequently in the nature than *C*-
596 glycosides and that microorganisms are more adapted to cleave the C-O-bond
597 providing more binding energy. Furthermore, it can be assumed that after the
598 consumption of *C*- and *O*-glycosides they reach different areas of the gut due to a
599 described enhanced stability of *C*-glycosides over the *O*-glycosides. Based on the
600 different prevailing conditions which are found for several intestinal areas, it might be
601 also possible that *C*- and *O*-glycosides in humans are converted by different
602 microorganisms. Braune and Blaut [23] investigated the potential of the
603 deglycosylations of different flavonoid *C*- and *O*-glycosides by *Eubacterium*
604 *cellulosolvens* which was only able to cleave the *C*-glycoside bond in two out of
605 seven compounds tested. In their study, both aglycones were then not further
606 degraded to phenolic acids. In contrast, the incubation of *E. cellulosolvens* with six
607 different *O*-glycosides showed that the microorganism was able to deglycosylate five
608 substrates.[23]

609 In all *in vitro* fermentations with the different flavonoid *C*- and *O*-glycosides, more
610 than one degradation step was identified. As a consequence, not only
611 deglycosylations occurred, but also a transformation of the aglycones to smaller
612 phenolic compounds took place. It is described in the literature that the hydrolysis of
613 (flavonoid) glycosides to their aglycones results in potentially more bioactive

metabolites compared to the initial compounds. However, a further microbial degradation of the aglycone to smaller metabolites can lead to the formation of more or less active compounds.[47] The fact that microorganisms are often not able to carry out all degradation steps was already described for the small phenolic acid caffeic acid by Peppercorn and Goldman [48], in 1971. This seems to be valid for the degradation of flavonoids as well. For example, in the study published by Braune and Blaut [23], *E. cellulosolvens* was only able to carry out deglycosylation reactions, resulting in the aglycone structure as the only metabolite. However, in the substrates metabolized by *E. cellulosolvens*, the glucose moiety was bound at the C6-position. When glucose was present at the C8-position (e.g., vitexin) degradation did not occur. Braune and Blaut further showed that the intestinal *Lachnospiraceae* strain CG19-1, in contrast to *E. cellulosolvens,* was able to convert six out of the seven tested *C*-glycosides via the aglycone structure to small phenolic acids, indicating deglycosylation, ring fission, and dehydroxylation reactions.[23] Investigations done by Nakamura, et al. [49] on the cleavage of the *C*-glycosyl bond of puerarin (an isoflavone glucoside) by a strain called PUE showed only a conversion to its aglycone daidzein. Smaller phenolic acids were not detected.[49] In this context, it is not clear whether the degradation steps of the substrates used in the present study are carried out by only one or even more microorganisms.

In summary, all substrates used in the present *in vitro* study were converted yielding lower molecular weight phenolic acids. Deglycosylation reactions were carried out by the microbiota first, followed by the further breakdown of the aglycone structure. Comparing the donors with each other, similar metabolites were detected, even though different time courses for metabolite formation were observed. With regard to

638 the microbial composition or activity (SCFA), an influence of the flavonoid glycosides
639 was not detected.

640 When looking at the results of the present study, the degradation of the selected
641 phenolic compounds showed more or less the same course of degradation that
642 resulted in very similar metabolite profiles, as well. Only the fermentation rates were
643 different. Therefore, we did not expect very different degradation pathways and more
644 outcomes when fermenting the substrates with fecal samples of additional
645 volunteers. The degradation pathways might only be significantly different, when
646 using fecal samples of volunteers having a known disorder (e.g., by taking antibiotics)
647 or significantly different dietary habits. It is known that one donor of the present study
648 was a vegetarian, but significant differences in metabolite formation were not
649 detected, at all. Also, seasonable differences might be possible, too, but the main aim
650 of the study was to monitor the degradation profile with regard to the (complex)
651 phenolic compounds selected. Therefore, more fermentations with more fecal
652 samples were not carried out. When comparing with *in vitro* fermentation experiments
653 described in the literature it is obvious that the use of one to four different fecal
654 samples is usual.[21, 29-31]

655 In conclusion, flavonoid glycosides can be metabolized by the human gut
656 microorganisms when consumed in concentrations found in typical diets (~20
657 mg/day), but a prebiotic effect of the phenolic compounds seems not to be
658 achievable. However, in a review by Etxeberria, et al. [50], *in vitro* and *in vivo* studies
659 are described which showed an influence of phenolic compounds on the microbiota
660 composition. The effects were often only significant when the concentrations were
661 artificially high. This does not mean that a certain influence of the microbiome is not
662 possible, as not all species present in the gut can be analyzed comprehensively to

663 date. But, it is also possible that a substrate on its own is not able to influence the gut
664 microbiota and the described effects on the microbiota composition are a result of the
665 interaction of different phenolic compounds and other food components being
666 present in the food matrix such as proteins. Besides other research groups, Ozdal, et
667 al. [51] described that a polyphenol-protein interaction results in changes in the
668 structural, functional, and nutritional properties of both compounds. This interaction is
669 influenced by several parameters such as pH, temperature, and the chemical
670 structure of the compounds. Therefore, interactive effects of the polyphenols with
671 further compounds might influence their metabolism by the microbiota to the
672 individual strength/binding mechanism of the polyphenol-protein interaction, when
673 consuming whole foods.

674 Microbial transformation products might affect microbial composition and might
675 therefore lead to different consequences for the host and its health.[21, 52] Such an
676 effect could be more pronounced after consumption of *C*-glycosides, as their slower
677 cleavage leads to a prolonged exposure to the intact flavonoid. Moreover, an impact
678 on deeper regions of the colon seems to be possible due to the enhanced stability.
679 With regard to diversity, it can be hypothesized that the more complex the substrates
680 (for gastrointestinal fermentation) are, more different metabolites can be formed likely
681 resulting in a higher variation of gut microbial composition. Although this research
682 topic is now studied for more than two decades, a final proof of a health-beneficial
683 effect of isolated phenolic compounds is still missing. In the future, research has to
684 be extended towards synergisms/interactions with other food compounds and all
685 what we would call food matrix.

686

Abbreviations. AP, apigenin; *p*-CA, *p*-coumaric acid; 4-HPPA, 3-(4-hydroxyphenyl)propionic acid; K, kaempferol; K-DG-R, kaempferol-3,4'-*O*-diglucoside-7-*O*-rhamnoside; K-G, kaempferol-3-*O*-glucoside; LOD, limit of detection; PAA, phenylacetic acid; PCA, Principal Component Analysis; 3-PPA, 3-phenylpropionic acid; SCFA, short chain fatty acids; T, tiliroside; V, vitexin; V-G, vitexin-2''-*O*-glucoside; V-R, vitexin-2''-*O*-rhamnoside; V-X, vitexin-2''-*O*-xyloside.

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SUPPORTING INFORMATION

Isolation method of vitexin-2''-*O*-glucoside and vitexin-2''-*O*-xyloside (material and methods), Illumina sequencing of 16S rRNA genes (material and methods), figures about the relative abundance of OTUs grouped at phylum and family level (results), PCA with the sequencing data (results), tables about the SCFA production, microbiota profile and Illumina sequencing operational taxonomic units (results). This material is available free of charge via the Internet at http://pubs.acs.org

REFERENCES

(1) Ross, J. A.; Kasum, C. M., Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Annu Rev Nutr* **2002**, *22*, 19-34.

(2) Beecher, G. R., Overview of dietary flavonoids: Nomenclature, occurrence and intake. *The Journal of Nutrition* **2003**, *133*, 3248s-3254s.

(3) Crozier, A.; Jaganath, I. B.; Clifford, M. N., Dietary phenolics: Chemistry, bioavailability and effects on health. *Natural Product Reports* **2009**, *26*, 1001-43.

(4) de Rijke, E.; Out, P.; Niessen, W. M.; Ariese, F.; Gooijer, C.; Brinkman, U. A., Analytical separation and detection methods for flavonoids. *J Chromatogr A* **2006**, *1112*, 31-63.

(5) Brazier-Hicks, M.; Evans, K. M.; Gershater, M. C.; Puschmann, H.; Steel, P. G.; Edwards, R., The C-Glycosylation of Flavonoids in Cereals. *J Biol Chem* **2009**, *284*, 17926-17934.

(6) Courts, F. L.; Williamson, G., The Occurrence, Fate and Biological Activities of C-glycosyl Flavonoids in the Human Diet. *Crit Rev Food Sci* **2015**, *55*, 1352-1367.

(7) Blaut, M.; Schoefer, L.; Braune, A., Transformation of flavonoids by intestinal microorganisms. *International Journal for Vitamin and Nutrition Research* **2003**, *73*, 79-87.

(8) Wang, H.-K.; Xia, Y.; Yang, Z.-Y.; Morris Natschke, S. L.; Lee, K.-H., Recent Advances in the Discovery and Development of Flavonoids and their Analogues as Antitumor and Anti-HIV Agents. In *Flavonoids in the Living System*, Manthey, J. A.; Buslig, B. S., Eds. Springer US: Boston, MA, 1998; pp 191-225.

(9) Nijveldt, R. J.; van Nood, E.; van Hoorn, D. E. C.; Boelens, P. G.; van Norren, K.; van Leeuwen, P. A. M., Flavonoids: A review of probable mechanisms of action and potential applications. *Am J Clin Nutr* **2001**, *74*, 418-425.

(10) Benavente-Garcia, O.; Castillo, J., Update on uses and properties of Citrus flavonolds: New findings in anticancer, cardiovascular, and anti-inflammatory activity. *J Agric Food Chem* **2008**, *56*, 6185-6205.

(11) Cushnie, T. P. T.; Lamb, A. J., Antimicrobial activity of flavonoids. *Int J Antimicrob Ag* **2005**, *26*, 343-356.

(12) Xiao, J. B.; Capanoglu, E.; Jassbi, A. R.; Miron, A., Advance on the flavonoid C-glycosides and health benefits. *Crit Rev Food Sci* **2016**, *56*, S29-S45.

(13) Williamson, G.; Day, A. J.; Plumb, G. W.; Couteau, D., Human metabolic pathways of dietary flavonoids and cinnamates. *Biochemical Society Transactions* **2000**, *28*, 16-22.

(14) Wiseman, H., The bioavailability of non-nutrient plant factors: dietary flavonoids and phyto-oestrogens. *Proc Nutr Soc* **1999**, *58*, 139-46.

(15) Hollman, P. C. H., Absorption, bioavailability, and metabolism of flavonoids. *Pharm Biol* **2004**, *42*, 74-83.

(16) Hollman, P. C. H.; de Vries, J. H. M.; van Leeuwen, S. D.; Mengelers, M. J. B.; Katan, M. B., Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am J Clin Nutr* **1995**, *62*, 1276-1282.

(17) Russell, W. R.; Hoyles, L.; Flint, H. J.; Dumas, M. E., Colonic bacterial metabolites and human health. *Curr Opin Microbiol* **2013**, *16*, 246-54.

(18) Braune, A.; Blaut, M., Bacterial species involved in the conversion of dietary flavonoids in the human gut. *Gut microbes* **2016**, 1-19.

(19) Ulbrich, K.; Reichardt, N.; Braune, A.; Kroh, L. W.; Blaut, M.; Rohn, S., The microbial degradation of onion flavonol glucosides and their roasting products by the human gut bacteria Eubacterium ramulus and Flavonifractor plautii. *Food Res Int* **2015**, *67*, 349-355.

(20) Schneider, H.; Schwiertz, A.; Collins, M. D.; Blaut, M., Anaerobic transformation of quercetin-3-glucoside by bacteria from the human intestinal tract. *Archives of Microbiology* **1999**, *171*, 81-91.

(21) Parkar, S. G.; Trower, T. M.; Stevenson, D. E., Fecal microbial metabolism of polyphenols and its effects on human gut microbiota. *Anaerobe* **2013**, *23*, 12-19.

(22) Pfeiffer, T.; Bonhoeffer, S., Evolution of cross-feeding in microbial populations. *Am Nat* **2004**, *163*, E126-E135.

(23) Braune, A.; Blaut, M., Intestinal bacterium Eubacterium cellulosolvens deglycosylates flavonoid C- and O-glucosides. *Appl Environ Microbiol* **2012**, *78*, 8151-8153.
(24) Duenas, M.; Munoz-Gonzalez, I.; Cueva, C.; Jimenez-Giron, A.; Sanchez-Patan, F.; Santos-Buelga, C.; Moreno-Arribas, M. V.; Bartolome, B., A survey of modulation of gut microbiota by dietary polyphenols. *Biomed Res Int* **2015**, *2015*, 850902.
(25) Selma, M. V.; Espin, J. C.; Tomas-Barberan, F. A., Interaction between phenolics and gut microbiota: Role in human health. *J Agric Food Chem* **2009**, *57*, 6485-6501.
(26) Gonzalez-Sarrias, A.; Espin, J. C.; Tomas-Barberan, F., Non-extractable polyphenols produce gut microbiota metabolites that persist in circulation and show anti-inflammatory and free radical-scavenging effects. *Trends Food Sci Tech* **2017**.
(27) Le Chatelier, E.; Nielsen, T.; Qin, J. J.; Prifti, E.; Hildebrand, F.; Falony, G.; Almeida, M.; Arumugam, M.; Batto, J. M.; Kennedy, S.; Leonard, P.; Li, J. H.; Burgdorf, K.; Grarup, N.; Jorgensen, T.; Brandslund, I.; Nielsen, H. B.; Juncker, A. S.; Bertalan, M.; Levenez, F.; Pons, N.; Rasmussen, S.; Sunagawa, S.; Tap, J.; Tims, S.; Zoetendal, E. G.; Brunak, S.; Clement, K.; Dore, J.; Kleerebezem, M.; Kristiansen, K.; Renault, P.; Sicheritz-Ponten, T.; de Vos, W. M.; Zucker, J. D.; Raes, J.; Hansen, T.; Bork, P.; Wang, J.; Ehrlich, S. D.; Pedersen, O.; Consortium, M., Richness of human gut microbiome correlates with metabolic markers. *Nature* **2013**, *500*, 541-+.
(28) Vollmer, M.; Schröter, D.; Esders, S.; Farquharson, F. M.; Neugart, S.; Duncan, S. H.; Schreiner, M.; Louis, P.; Maul, R.; Rohn, S., Chlorogenic acid versus amaranth's caffeoylisocitric acid - Gut microbial degradation of caffeic acid derivatives. *Food Res Int* **2017**, *100*, 375-384.
(29) Sanchez-Patan, F.; Cueva, C.; Monagas, M.; Walton, G. E.; Gibson, G. R.; Quintanilla-Lopez, J. E.; Lebron-Aguilar, R.; Martin-Alvarez, P. J.; Moreno-Arribas, M. V.; Bartolome, B., In vitro fermentation of a red wine extract by human gut microbiota: changes in microbial groups and formation of phenolic metabolites. *J Agric Food Chem* **2012**, *60*, 2136-47.
(30) Mills, C. E.; Tzounis, X.; Oruna-Concha, M. J.; Mottram, D. S.; Gibson, G. R.; Spencer, J. P. E., In vitro colonic metabolism of coffee and chlorogenic acid results in selective changes in human faecal microbiota growth. *Brit J Nutr* **2015**, *113*, 1220-1227.
(31) Aura, A. M.; O'Leary, K. A.; Williamson, G.; Ojala, M.; Bailey, M.; Puupponen-Pimia, R.; Nuutila, A. M.; Oksman-Caldentey, K. M.; Poutanen, K., Quercetin derivatives are deconjugated and converted to hydroxyphenylacetic acids but not methylated by human fecal flora in vitro. *J Agric Food Chem* **2002**, *50*, 1725-30.
(32) Gonthier, M. P.; Remesy, C.; Scalbert, A.; Cheynier, V.; Souquet, J. M.; Poutanen, K.; Aura, A. M., Microbial metabolism of caffeic acid and its esters chlorogenic and caftaric acids by human faecal microbiota in vitro. *Biomed Pharmacother* **2006**, *60*, 536-540.
(33) Rechner, A. R.; Smith, M. A.; Kuhnle, G.; Gibson, G. R.; Debnam, E. S.; Srai, S. K. S.; Moore, K. P.; Rice-Evans, C. A., Colonic metabolism of dietary polyphenols: Influence of structure on microbial fermentation products. *Free Radical Bio Med* **2004**, *36*, 212-225.
(34) Justesen, U.; Arrigoni, E.; Larsen, B. R.; Amado, R., Degradation of flavonoid glycosides and aglycones during in vitro fermentation with human faecal flora. *Lebensm-Wiss Technol* **2000**, *33*, 424-430.
(35) Richardson, A. J.; Calder, A. G.; Stewart, C. S.; Smith, A., Simultaneous Determination of Volatile and Non-Volatile Acidic Fermentation Products of Anaerobes by Capillary Gas-Chromatography. *Lett Appl Microbiol* **1989**, *9*, 5-8.
(36) Labib, S.; Hummel, S.; Richling, E.; Humpf, H. U.; Schreier, P., Use of the pig caecum model to mimic the human intestinal metabolism of hispidulin and related compounds. *Molecular nutrition & food research* **2006**, *50*, 78-86.
(37) Chamkha, M.; Garcia, J. L.; Labat, M., Metabolism of cinnamic acids by some Clostridiales and emendation of the descriptions of Clostridium aerotolerans, Clostridium celerecrescens and Clostridium xylanolyticum. *Int J Syst Evol Micr* **2001**, *51*, 2105-2111.
(38) Chamkha, M.; Labat, M.; Patel, B. K. C.; Garcia, J. L., Isolation of a cinnamic acid-metabolizing Clostridium glycolicum strain from oil mill wastewaters and emendation of the species description. *Int J Syst Evol Micr* **2001**, *51*, 2049-2054.

(39) Soleim, H. A.; Scheline, R. R., Metabolism of xenobiotics by strains of intestinal bacteria. *Acta Pharmacol Tox* **1972**, *31*, 471-480.
(40) Scheline, R. R., Metabolism of phenolic acids by the rat intestinal microflora. *Acta Pharmacol Tox* **1968**, *26*, 189-205.
(41) Kim, M.; Lee, J.; Han, J., Deglycosylation of isoflavone C-glycosides by newly isolated human intestinal bacteria. *J Sci Food Agric* **2015**, *95*, 1925-1931.
(42) Heim, K. E.; Tagliaferro, A. R.; Bobilya, D. J., Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. *The Journal of Nutritional Biochemistry* **2002**, *13*, 572-584.
(43) Duda-Chodak, A., The inhibitory effect of polyphenols on human gut microbiota. *J Physiol Pharmacol* **2012**, *63*, 497-503.
(44) Kataoka, K., The intestinal microbiota and its role in human health and disease. *J Med Investig* **2016**, *63*, 27-37.
(45) Kalantar-Zadeh, K.; Yao, C. K.; Berean, K. J.; Ha, N.; Ou, J. Z.; Ward, S. A.; Pillai, N.; Hill, J.; Cottrell, J. J.; Dunshea, F. R.; McSweeney, C.; Muir, J. G.; Gibson, P. R., Intestinal Gas Capsules: A Proof-of-Concept Demonstration. *Gastroenterology* **2016**, *150*, 37-39.
(46) Cassidy, A.; Minihane, A. M., The role of metabolism (and the microbiome) in defining the clinical efficacy of dietary flavonoids. *Am J Clin Nutr* **2017**, *105*, 10-22.
(47) Lampe, J. W.; Chang, J. L., Interindividual differences in phytochemical metabolism and disposition. *Semin Cancer Biol* **2007**, *17*, 347-353.
(48) Peppercorn, M. A.; Goldman, P., Caffeic acid metabolism by bacteria of the human gastrointestinal tract. *J Bacteriol* **1971**, *108*, 996-1000.
(49) Nakamura, K.; Nishihata, T.; Jin, J. S.; Ma, C. M.; Komatsu, K.; Iwashima, M.; Hattori, M., The C-Glucosyl Bond of Puerarin Was Cleaved Hydrolytically by a Human Intestinal Bacterium Strain PUE to Yield Its Aglycone Daidzein and an Intact Glucose. *Chem Pharm Bull (Tokyo)* **2011**, *59*, 23-27.
(50) Etxeberria, U.; Fernandez-Quintela, A.; Milagro, F. I.; Aguirre, L.; Martinez, J. A.; Portillo, M. P., Impact of polyphenols and polyphenol-rich dietary sources on gut microbiota composition. *J Agric Food Chem* **2013**, *61*, 9517-9533.
(51) Ozdal, T.; Capanoglu, E.; Altay, F., A review on protein-phenolic interactions and associated changes. *Food Res Int* **2013**, *51*, 954-970.
(52) Cardona, F.; Andres-Lacueva, C.; Tulipani, S.; Tinahones, F. J.; Queipo-Ortuno, M. I., Benefits of polyphenols on gut microbiota and implications in human health. *The Journal of Nutritional Biochemistry* **2013**, *24*, 1415-22.

LIST OF FIGURE CAPTIONS

Figure 1: Structures of the compounds used for the *in vitro* fermentations.

Figure 2: Overview of the degradation pattern of the kaempferol-*O*-derivates und apigenin-*C*-derivatives used in the in vitro study (AP, apigenin; *p*-CA, *p*-coumaric acid; Glc, glucose; 4-HPAA, 3-(4-hydroxyphenyl)acetic acid; 4-HPPA, 3-(4-hydroxyphenyl)propionic acid; K, kaempferol; K-DG-R, kaempferol-3,4'-O-diglucoside-7-O-rhamnoside; K-G, kaempferol-3-O-glucoside; PAA, phenylacetic acid; 3-PPA, 3-phenylpropionic acid; Rha, rhamnose; T, tiliroside; V, vitexin; V-G, vitexin-2''-O-glucoside; V-R, vitexin-2''-O-rhamnoside; V-X, vitexin-2''-O-xyloside; - - - > = pathway is not totally identified).

Figure 3: *In vitro* degradation and metabolite formation of tiliroside with three different donors (A, B, and C). Data represent the mean and standard deviation of triplicates (T, tiliroside; K-G, kaempferol-3-*O*-glucoside; K, kaempferol; 4-HPPA, 3-(4-hydroxyphenyl)propionic acid; *p*-CA, *p*-coumaric acid; 3-PPA, 3-phenylpropionic acid).

Figure 4: Comparison of vitexin formation within the *in vitro* fermentations with different vitexin derivatives of donor B**.** Data are normalized to 100%.

Figure 5: Principal component analysis with the percentage data for net SCFA and production microbiota composition (qPCR) after 48 h of incubation. Colors are coded by different donor (donor A: black, donor B: red, donor C: blue).

Figure 6: Relative abundance of OTUs obtained by Illumina sequencing of the three different initial fecal samples (donor A, B and C) and after 48 h of incubation with different substrates. OTUs are grouped together at the genus level.

TABLES

Table 1: Concentration of the substrates and metabolites within the *in vitro* degradation of K, T, and K-DG-R with the three different donors (A, B, and C). Data represent the mean and standard deviation of triplicates (D_{50}, time point when 50% of the substrate was degraded).

		Concentration [µM]					D_{50}
Compound	**Donor**	**0 h**	**3 h**	**7 h**	**24 h**	**48 h**	
Kaempferol	A	232 ± 16.0	201 ± 15.8	179 ± 51.4	170 ± 8.32	34.0 ± 20.7	33 h 32 min
	B	68.9 ± 13.4	61.1 ± 32.9	<LOD	<LOD	<LOD	4 h 45 min
	C	27.5 ± 5.31	16.9 ± 4.53	8.28 ± 11.8	<LOD	<LOD	4 h 28 min
K metabolites							
4-HPPA	A	<LOD	<LOD	<LOD	<LOD	32.8 ± 0.0100	
	B	<LOD	<LOD	19.4*	5.97*	<LOD	
	C	<LOD	<LOD	13.8 ± 5.91**	<LOD	<LOD	
PAA	A	<LOD	<LOD	<LOD	<LOD	<LOD	
	B	<LOD	<LOD	<LOD	<LOD	<LOD	
	C	<LOD	<LOD	<LOD	13.7 ± 4.91	21.1 ± 18.6	
Tiliroside	A	105 ± 13.2	84.4 ± 6.65	54.6 ± 13.0	<LOD	<LOD	7 h 17 min
	B	183 ± 48.5	125 ± 35.1	<LOD	<LOD	<LOD	4 h 4 min
	C	107 ± 52.7	75.7 ± 38.2	<LOD	<LOD	<LOD	3 h
T metabolites							
K-G	A	5.85 ± 2.41	7.16 ± 0.522	8.91 ± 2.62	<LOD	<LOD	
	B	91.4 ± 28.1	22.1 ± 10.5	<LOD	<LOD	<LOD	
	C	31.7 ± 6.36	16.8 ± 3.82	<LOD	<LOD	<LOD	
K	A	32.3 ± 17.7	95.3 ± 21.2	145 ± 19.9	89.6 ± 0.719	79.4 ± 27.2	
	B	5.24 ± 0.176	39.8 ± 8.89	<LOD	<LOD	<LOD	
	C	4.53 ± 2.33	8.70 ± 4.45	<LOD	<LOD	<LOD	
p-CA	A	19.5 ± 2.61	31.3 ± 12.2	123 ± 19.5	17.1 ± 24.4	3.02 ± 0.776	
	B	3.97 ± 0.453	5.86 ± 1.46	12.7 ± 8.36	<LOD	<LOD	
	C	8.14 ± 1.56	19.4 ± 4.35	49.1 ± 9.71	<LOD	<LOD	

4-HPPA	A	<LOD	<LOD	<LOD	123 ± 3.56	121 ± 12.4
	B	<LOD	<LOD	99.9 ± 53.2	<LOD	<LOD
	C	<LOD	<LOD	46.9 ± 19.1	<LOD	<LOD
3-PPA	A	<LOD	<LOD	<LOD	<LOD	<LOD
	B	<LOD	<LOD	<LOD	27.6 ± 26.8**	5.36 ± 9.28**
	C	<LOD	<LOD	<LOD	91.8 ± 13.3**	38.0 ± 65.8**
K-DG-R	A	<LOD	<LOD	<LOD	<LOD	<LOD
	B	<LOD	<LOD	<LOD	<LOD	<LOD
	C	<LOD	<LOD	<LOD	<LOD	<LOD
K-DG-R metabolites						
K-G	A	8.77 ± 1.94	36.6 ± 13.2	10.3 ± 1.17	<LOD	<LOD
	B	35.8 ± 6.55	88.7 ± 61.2	<LOD	<LOD	<LOD
	C	3.92 ± 0.976	30.7 ± 24.9	<LOD	<LOD	<LOD
K	A	13.0 ± 7.57	131 ± 12.1	170 ± 14.4	151 ± 40.8	6.60 ± 1.65
	B	<LOD	54.9 ± 0.701	<LOD	<LOD	<LOD
	C	<LOD	13.3 ± 5.25	<LOD	<LOD	<LOD
4-HPPA	A	<LOD	<LOD	<LOD	<LOD	33.5 ± 2.88
	B	<LOD	<LOD	<LOD	<LOD	<LOD
	C	<LOD	<LOD	<LOD	<LOD	<LOD
PAA	A	<LOD	<LOD	<LOD	<LOD	<LOD
	B	<LOD	<LOD	<LOD	21.8 ± 23.4	15.5 ± 19.9
	C	<LOD	<LOD	<LOD	3.05 ± 7.74	18.3 ± 10.1

<LOD (limit of detection)
* the metabolite was only detected in one sample out of the triplicate fermentation
** the metabolite was only detected in two samples out of the triplicate fermentation

Table 2: Concentrations of the substrates and metabolites within the *in vitro* degradation of AP, V, V-G, V-R and V-X with the three different donors (A, B, and C). Data represent the mean and standard deviation of triplicates (D_{50}, time point when 50% of the substrate was degraded).

		Concentration [µM]					D_{50}
Compound	**Donor**	**0 h**	**3 h**	**7 h**	**24 h**	**48 h**	
Apigenin	A	106 ± 16.0	97.9 ± 7.57	98.6 ± 11.7	65.4 ± 26.9	34.0 ± 20.7	33 h 29 min
	B	22.9 ± 14.5	15.4 ± 3.02	<LOD	<LOD	<LOD	4 h 35 min
	C	24.1 ± 2.66	25.3 ± 6.55	<LOD	<LOD	<LOD	5 h 6 min
AP metabolites							
4-HPPA	A	<LOD	<LOD	<LOD	38.7 ± 9.56	173 ± 55.5	
	B	<LOD	<LOD	187 ± 66.2	<LOD	<LOD	
	C	<LOD	32.9 ± 18.6	124 ± 7.95	<LOD	<LOD	
3-PPA	A	<LOD	<LOD	<LOD	<LOD	<LOD	
	B	<LOD	<LOD	<LOD	8.01 ± 1.55	49.8 ± 35.1	
	C	<LOD	<LOD	<LOD	143 ± 24.1	214 ± 39.4**	
PAA	A	<LOD	<LOD	<LOD	<LOD	13.8 ± 12.1	
	B	<LOD	<LOD	<LOD	<LOD	24.4 ± 21.3	
	C	<LOD	<LOD	<LOD	52.4 ± 14.9	50.4 ± 7.64	
Vitexin	A	113 ± 23.1	117 ± 27.3	108 ± 11.6	112 ± 1.04	<LOD	35 h 52 min
	B	147 ± 21.9	96.6 ± 31.1	115 ± 17.8	<LOD	<LOD	13 h 8 min
	C	131 ± 4.53	121*	128 ± 4.85	<LOD	<LOD	15 h 17 min
V metabolites							
AP	A	<LOD	<LOD	<LOD	<LOD	39.4 ± 30.5	
	B	<LOD	<LOD	<LOD	<LOD	<LOD	
	C	<LOD	<LOD	<LOD	<LOD	<LOD	
4-HPPA	A	<LOD	<LOD	<LOD	22.6 ± 0.961	102 ± 32.6	
	B	<LOD	<LOD	<LOD	123 ± 6.76	80.2 ± 17.9	
	C	<LOD	<LOD	<LOD	<LOD	<LOD	
3-PPA	A	<LOD	<LOD	<LOD	<LOD	<LOD	
	B	<LOD	<LOD	<LOD	41.1 ± 27.0**	25.7 ± 4.90*	

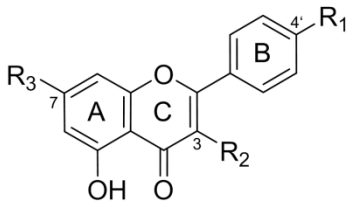
	C	<LOD	<LOD	<LOD	131 ± 30.7	142 ± 35.9	
PAA	A	<LOD	<LOD	6.70*	<LOD	10.9 ± 4.79	
	B	<LOD	<LOD	<LOD	43.8 ± 10.2	47.0 ± 10.2	
	C	<LOD	<LOD	<LOD	47.9 ± 19.8	47.3 ± 9.75	
Vitexin-glucoside	A	1.84 ± 0.342	2.02 ± 0.652	2.34 ± 0.519	1.22 ± 0.765	0.769 ± 0.152	27 h 50 min
	B	0.500 ± 0.200	<LOD	<LOD	<LOD	<LOD	1 h 29 min
	C	<LOD	<LOD	<LOD	<LOD	<LOD	-
V-G metabolites							
V	A	<LOD	0.351 ± 0.0137	0.714 ± 0.127	1.29 ± 0.612	<LOD	
	B	<LOD	7.02 ± 1.01	3.52 ± 2.09	<LOD	<LOD	
	C	0.824 ± 0.0560	2.29 ± 0.578	2.45 ± 1.23	<LOD	<LOD	
4-HPPA	A	<LOD	<LOD	<LOD	25.9*	37.5 ± 14.4**	
	B	<LOD	<LOD	14.0 ± 3.79**	<LOD	<LOD	
	C	<LOD	<LOD	<LOD	<LOD	<LOD	
PAA	A	<LOD	<LOD	<LOD	<LOD	7.37 ± 1.30**	
	B	<LOD	<LOD	<LOD	38.3 ± 10.0	32.2 ± 3.6	
	C	<LOD	<LOD	.n.d	35.1 ± 29.0	19.6 ± 1.41	
Vitexin-rhamnoside	A	8.43 ± 0.623	8.95 ± 1.38	12.3 ± 3.46	13.7 ± 1.66	<LOD	36 h 3 min
	B	12.0 ± 5.17	9.71 ± 0.424	4.65 ± 0.443	<LOD	<LOD	5 h 55 min
	C	5.58 ± 1.43	7.51 ± 1.40	10.3 ± 0.550	<LOD	<LOD	15 h 29 min
V-R metabolites							
V	A	<LOD	<LOD	2.29 ± 0.851	10.7 ± 7.45	<LOD	
	B	<LOD	1.65 ± 0.415	68.8 ± 19.8	<LOD	<LOD	
	C	0.845 ± 0.154	1.41 ± 0.475	7.82 ± 1.30	0.748 ± 0.063	<LOD	
AP	A	<LOD	<LOD	<LOD	<LOD	28.2*	
	B	<LOD	<LOD	<LOD	<LOD	<LOD	
	C	<LOD	<LOD	<LOD	18.8*	16.6*	
4-HPPA	A	<LOD	<LOD	<LOD	23.2 ± 0**	130.8 ± 48.1	
	B	<LOD	<LOD	<LOD	230 ± 25.7	169 ± 59.9	
	C	<LOD	<LOD	<LOD	29.2*	18.0*	
3-PPA	A	<LOD	<LOD	<LOD	<LOD	<LOD	
	B	<LOD	<LOD	<LOD	<LOD	<LOD	
	C	<LOD	<LOD	<LOD	<LOD	236*	
PAA	A	<LOD	<LOD	4.84*	<LOD	127*	
	B	<LOD	<LOD	<LOD	52.4 ± 7.21	68.6 ± 22.5	
	C	<LOD	<LOD	<LOD	16.0 ± 11.8	41.4 ± 34.2	

Vitexin-xyloside* **	A	3.99 ± 0.934	2.94 ± 0.194	3.88 ± 0.314	<LOD	<LOD	15 h 16 min
	B	3.23 ± 0.962	3.48 ± 0.367	4.62 ± 0.559	<LOD	<LOD	15 h 36 min
	C	1.37 ± 0.336	1.19 ± 0.218	<LOD	<LOD	<LOD	4 h 39 min
V-X metabolites							
V	A	1.60 ± 0.284	6.20 ± 0.593	14.2 ± 2.05	57.5 ± 5.07	<LOD	
	B	<LOD	0.746 ± 0.093	6.96 ± 2.07	<LOD	<LOD	
	C	0.949 ± 0.034	2.81 ± 1.03	14.7 ± 5.78	0.710 ± 0.008	<LOD	
4-HPPA	A	<LOD	<LOD	<LOD	24.6*	60.9 ± 7.46	
	B	<LOD	<LOD	<LOD	36.8 ± 9.47**	32.7 ± 30.2**	
	C	<LOD	<LOD	<LOD	<LOD	<LOD	
PAA	A	<LOD	<LOD	<LOD	<LOD	9.41 ± 2.47	
	B	<LOD	<LOD	<LOD	54.0 ± 10.1	65.4 ± 20.7	
	C	<LOD	<LOD	<LOD	42.2 ± 13.0	38.9 ± 13.3	

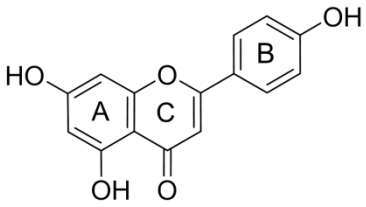
<LOD (limit of detection)
* the metabolite was only detected in one sample out of the triplicate fermentation
** the metabolite was only detected in two samples out of the triplicate fermentation
*** Vitexin-xyloside was quantified via Vitexin-rhamnoside

FIGURE GRAPHICS

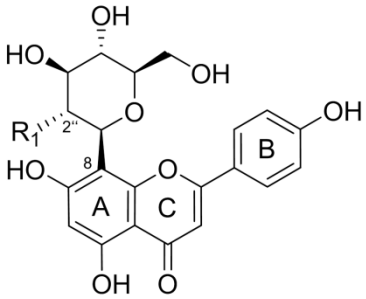
Figure 1



Kaempferol: R_1 & R_2 & R_3 = OH
Kaempferol-3,4'-*O*-diglucoside-7-*O*-rhamnoside: R_1 & R_2 = glucosyl, R_3 = rhamnosyl
Tiliroside: R_1 = OH, R_2 = glucosyl-6''-trans-*p*-coumaroyl, R_3 = OH



Apigenin



Vitexin: R_1 = OH
Vitexin-2''-*O*-glucoside: R_1 = glucosyl
Vitexin-2''-*O*-rhamnoside: R_1 = rhamnosyl
Vitexin-2''-*O*-xyloside: R_1 = xylosyl

Figure 2

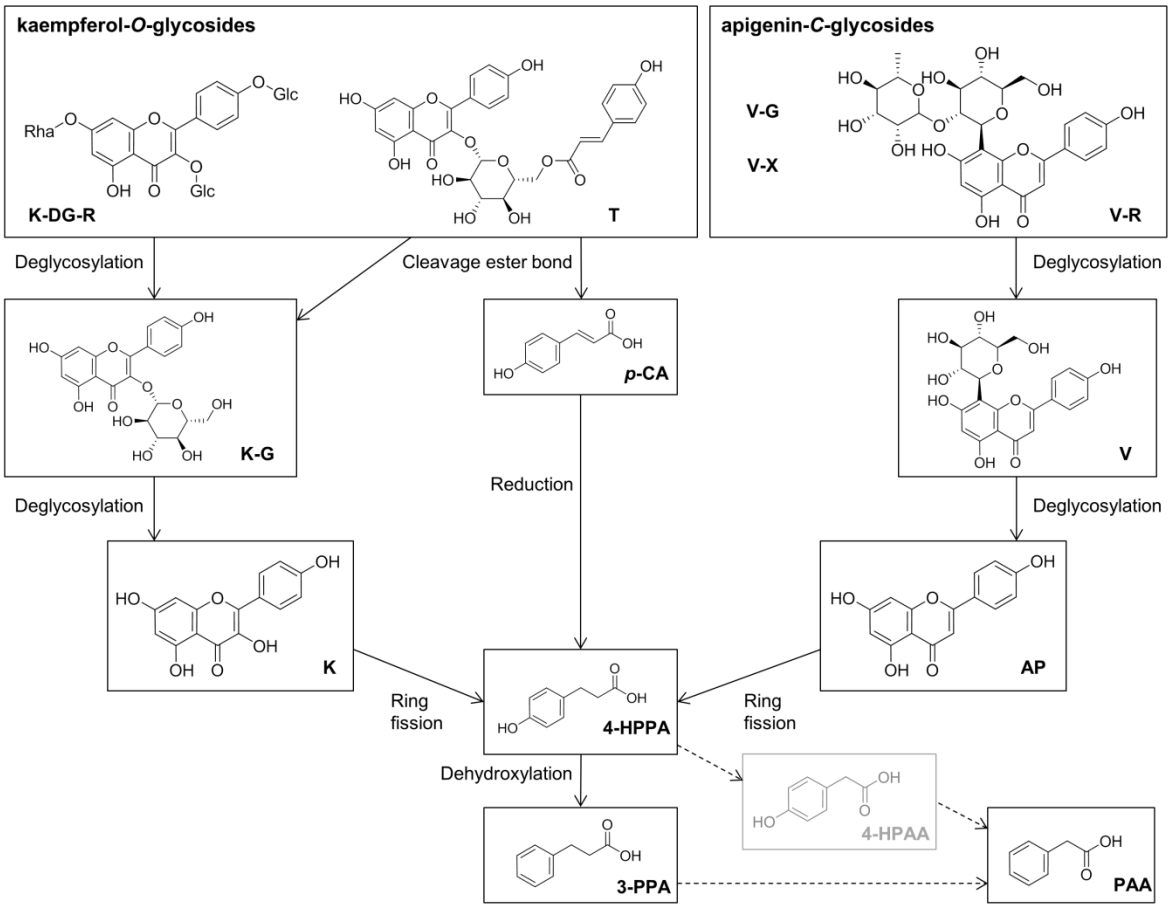

kaempferol-O-glycosides
K-DG-R
T
apigenin-C-glycosides
V-G
V-X
V-R
Deglycosylation
Cleavage ester bond
Deglycosylation
K-G
p-CA
V
Reduction
Deglycosylation
Deglycosylation
K
AP
Ring fission
Ring fission
4-HPPA
Dehydroxylation
4-HPAA
3-PPA
PAA

Figure 3

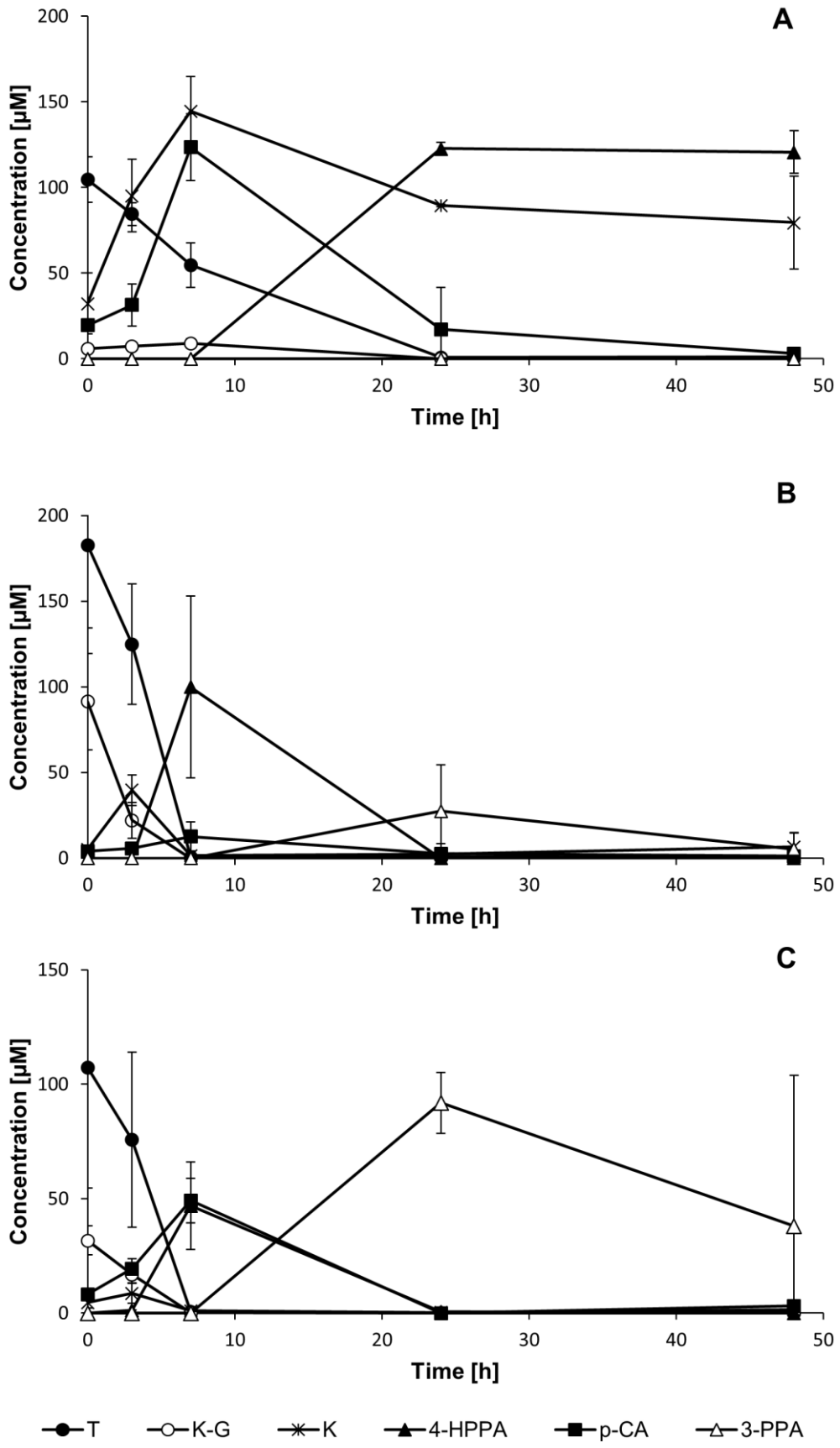

A
B
C
Concentration [µM]
Time [h]
0
10
20
30
40
50
0
50
100
150
200
T
K-G
K
4-HPPA
p-CA
3-PPA

Figure 4

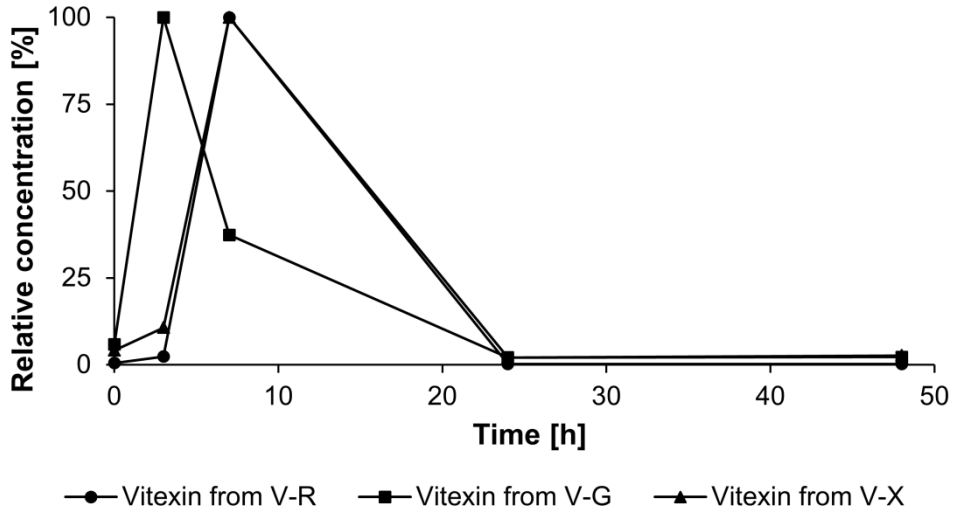

Relative concentration [%]
100
75
50
25
0
0
10
20
30
40
50
Time [h]
Vitexin from V-R
Vitexin from V-G
Vitexin from V-X

Figure 5

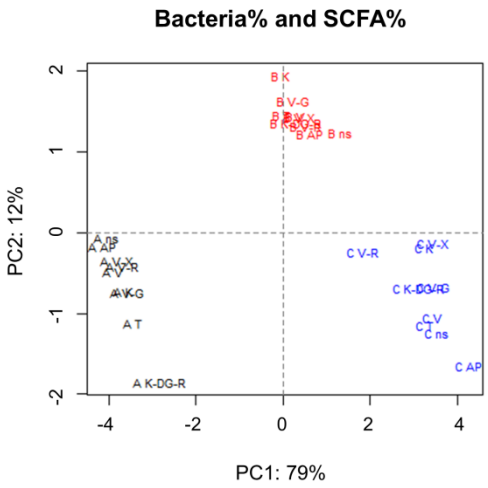

Bacteria% and SCFA%
PC2: 12%
PC1: 79%
B K
B V-G
B AP
B ns
C V-R
C V-X
C K-DG-R
C V
C T
C ns
C AP
A T
A K-DG-R
-4
-2
0
2
4
2
1
0
-1
-2

Figure 6

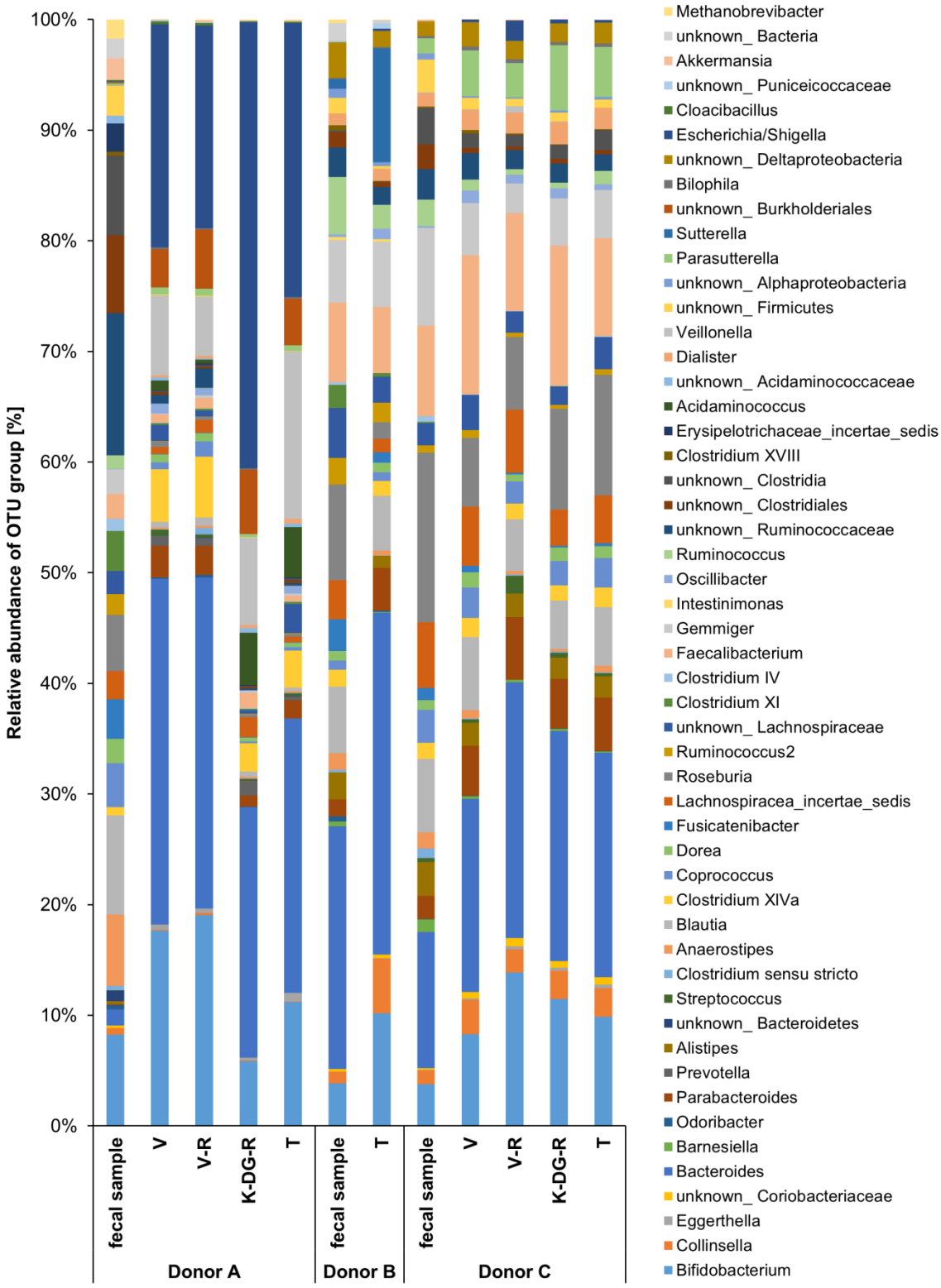

Relative abundance of OTU group [%]
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
fecal sample
V
V-R
K-DG-R
T
fecal sample
T
fecal sample
V
V-R
K-DG-R
T
Donor A
Donor B
Donor C
Methanobrevibacter
unknown_ Bacteria
Akkermansia
unknown_ Puniceicoccaceae
Cloacibacillus
Escherichia/Shigella
unknown_ Deltaproteobacteria
Bilophila
unknown_ Burkholderiales
Sutterella
Parasutterella
unknown_ Alphaproteobacteria
unknown_ Firmicutes
Veillonella
Dialister
unknown_ Acidaminococcaceae
Acidaminococcus
Erysipelotrichaceae_incertae_sedis
Clostridium XVIII
unknown_ Clostridia
unknown_ Clostridiales
unknown_ Ruminococcaceae
Ruminococcus
Oscillibacter
Intestinimonas
Gemmiger
Faecalibacterium
Clostridium IV
Clostridium XI
unknown_ Lachnospiraceae
Ruminococcus2
Roseburia
Lachnospiracea_incertae_sedis
Fusicatenibacter
Dorea
Coprococcus
Clostridium XlVa
Blautia
Anaerostipes
Clostridium sensu stricto
Streptococcus
unknown_ Bacteroidetes
Alistipes
Prevotella
Parabacteroides
Odoribacter
Barnesiella
Bacteroides
unknown_ Coriobacteriaceae
Eggerthella
Collinsella
Bifidobacterium

TABLE OF CONTENTS