

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

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16 **ABSTRACT**

17 Human colonic bacteria have an important impact on the biotransformation of  
18 flavonoid glycosides and their conversion can result in the formation of bioactive  
19 compounds. However, information about the microbial conversion of complex  
20 glycosylated flavonoids and the impact on the gut microbiota are still limited. In this  
21 study, *in vitro* fermentations with selected flavonoid O- and C-glycosides and three  
22 different fecal samples were performed. As a result, all flavonoid glycosides were  
23 metabolized via their aglycones yielding smaller substances. Main metabolites were  
24 3-(4-hydroxyphenyl)propionic acid, 3-phenylpropionic acid, and phenylacetic acid.  
25 Differences in the metabolite formation due to different time courses between the  
26 donors were determined. Therefore, from all fermentations, the ones with a specific  
27 donor were always slower resulting in a lower number of metabolites compared to the  
28 others. Exemplarily, tiliroside was totally degraded from 0h ( $105 \pm 13.2 \mu\text{M}$ ) within the  
29 first 24h, while in the fermentations with fecal samples from other donors, tiliroside  
30 ( $107 \pm 52.7 \mu\text{M}$  at 0h) was not detected after 7h anymore. In general, fermentation  
31 rates of C-glycosides were slower compared to the fermentation rates of O-  
32 glycosides. The O-glycoside tiliroside was degraded within 4h while the gut  
33 microbiota converted the C-glycoside vitexin within 13h. However, significant  
34 changes ( $p < 0.05$ ) in the microbiota composition and short chain fatty acid levels as  
35 products of carbohydrate fermentation were not detected between incubations with  
36 different phenolic compounds. Therefore, microbiota diversity was not affected and a  
37 significant prebiotic effect of phenolic compounds cannot be assigned to flavonoid  
38 glycosides in food-relevant concentrations.

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41 **KEYWORDS**

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

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## 45 INTRODUCTION

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

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

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

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

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

94 glycosides vitexin, while it is degradable by the intestinal *Lachnospiraceae* strain  
95 CG19-1.<sup>23</sup>

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

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

## 119 MATERIAL AND METHODS

120 **Chemicals.** Apigenin (AP, 4',5,7-trihydroxyflavone), kaempferol (K, 3,4',5,7-  
121 tetrahydroxyflavone), kaempferol-3,4'-O-diglucoside-7-O-rhamnoside (K-DG-R),  
122 tiliroside (T, kaempferol-3-(6"-trans-*p*-coumaroyl)glucoside), vitexin (V, apigenin-8-C-  
123 glucoside), and vitexin-2''-O-rhamnoside (V-R) were purchased from Phytolab GmbH  
124 & Co. KG (Vestenbergsgreuth, Germany). Vitexin-2''-O-glucoside (V-G), and vitexin-  
125 2''-O-xyloside (V-X) were isolated from swiss chard. The isolation method is  
126 described in the supplementary information.

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

138 ***In vitro* degradation of phenolic substrates by fecal microbiota.** This study was  
139 part of a set of experiments already described by Vollmer, et al. <sup>28</sup> in which three  
140 independent *in vitro* fermentations with fecal microbiota were performed. Fecal  
141 samples were donated by three different, healthy donors (donor A, B, and C) without  
142 a history of gastrointestinal disorders or any antibiotics consumption for at least three  
143 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.<sup>21, 29-31</sup> The preparation of the fecal suspension was conducted according to  
146 Vollmer, Schröter, Esders, Farquharson, Neugart, Duncan, Schreiner, Louis, Maul  
147 and Rohn<sup>28</sup>. *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.<sup>28</sup>

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<sub>2</sub>. 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.<sup>28</sup>  
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<sup>28</sup>.



168 In addition to fecal suspensions with phenolic substrates, control samples for every  
169 fermentation without any substrates were conducted. This was done to determine the  
170 metabolite formation caused by compounds endogenously present in the fecal  
171 samples. For that, 100 µL DMSO were used instead of the substrate ('control  
172 samples I'). For the estimation of the interactions of the substrates with the medium,  
173 100 µL substrate were combined with 9.9 mL medium and 14 µL vitamin solution and  
174 were treated the same way as the samples ('control samples II').

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

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

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

193 mobile phase was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile  
194 (B) and the following gradient elution was used: 5% B (0-1 min), 11% B (1-2 min),  
195 14% B (2-4 min), 50% B (4-15 min), 95% B (15-18 min), 95% B (18-22 min), 5% B  
196 (22-23 min) and 5% B (23-32 min). The flow rate was set to 300  $\mu$ L/min and the  
197 injection volume was 5  $\mu$ L.

198 For detecting the substrates and their metabolites, mass analyses were performed in  
199 negative ion mode on an API2000 triple quadrupole MS/MS system (AB Sciex  
200 Germany GmbH, Darmstadt, Germany) equipped with an ESI interface. The following  
201 mass spectrometer settings were used: electrospray voltage = -4500 V, temperature  
202 = 450  $^{\circ}$ C, curtain gas = 1.7 bar, ion source gas 1 = 2.1 bar, ion source gas 2 = 5.2  
203 bar and collisions gas = 0.5 bar (all nitrogen). For each substance, the optimum  
204 settings of the declustering potential, entrance potential, collision energy, collision  
205 exit potential, and the characteristic fragments were also determined.

206 For quantification, an external matrix calibration (0-200  $\mu$ M) for every donor was used  
207 (**Supplementary Table 1**). The matrix consisted of the fermentation medium and the  
208 fecal sample. The data obtained were analyzed with the software Analyst<sup>®</sup> 1.5.2 from  
209 AB Sciex Germany GmbH (Darmstadt, Germany).

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

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

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

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

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

249 **Statistical analysis.** Statistical analysis was performed to investigate whether  
250 differences in SCFA levels or in the microbiota composition resulting from the  
251 substrate fermentations were significant or not compared to the 'control samples I'.  
252 For that, data were analyzed using IBM SPSS Statistics 22 (Ehningen, Germany). All  
253 data were tested for normality. With the normal distributed data investigations for  
254 significant differences were carried out with an independent t-test. Non-normal  
255 distributed values were analyzed with the non-parametric MANN-WHITNEY-U-test. For  
256 significance, a confidence level of 95% ( $p < 0.05$ ) was used. In addition, the qPCR,  
257 SCFA, and sequence data were summarized by Principal Component Analysis  
258 (PCA). All data were standardized before applying PCA. The analysis was performed  
259 in R (R Core Team (2012). R: A language and environment for statistical computing.  
260 R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org>).  
261 For correlation analysis between qPCR and sequencing data, OTUs were assigned  
262 to the corresponding qPCR assays based on their taxonomy (**Supplementary Table**  
263 **2**).

264

265

## 266 RESULTS AND DISCUSSION

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

### 274 ***In vitro* fermentations of the flavonol kaempferol and its O-glycosides tiliroside** 275 **and kaempferol-3,4'-O-diglucoside-7-O-rhamnoside.**

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

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

295 Tiliroside. In all three *in vitro* fermentation experiments, tiliroside (T) was metabolized  
296 significantly by the gut microbiota. **Figure 3** displays its degradation steps and  
297 metabolite formation. The detected metabolites were kaempferol-3-*O*-glucoside (K-  
298 G), kaempferol (K), *p*-coumaric acid (*p*-CA), 3-(4-hydroxyphenyl)propionic acid (4-  
299 HPPA), and 3-phenylpropionic acid (3-PPA), whereby the latter was not detected in  
300 the fermentation with the fecal sample from donor A. In the fermentations of donor B  
301 and C, the bacteria already cleaved the ester bond between the sugar moiety and *p*-  
302 CA within short time resulting in the formation of K-G at 0 h, immediately after  
303 inoculation with the fecal samples (**Table 1, Figure 3**). An interaction or breakdown  
304 due to the fermentation media can be excluded, because K-G was not detected in  
305 the 'control samples II' (substrate + medium, data not shown). The detection of K-G  
306 leads to the assumption that the microbiota did not mainly first cleave the bond  
307 between the kaempferol-moiety and the sugar substituent in T, and T is mostly  
308 degraded to its aglycone K via the intermediate product K-G. However, the  
309 metabolite K-G was only detected in very low concentrations in fermentation A at 0 h,  
310 3 h, and 7 h. Instead, a significant increase of K and *p*-CA was present within the first  
311 7 h. This may be due to the cleavage of the bond between K and glucose and the  
312 release of *p*-CA taking place at the same time intervals. Subsequently, the aglycone  
313 was transformed to smaller phenolic acids deriving from the A- and B-ring during the  
314 breakdown of the heterocyclic flavonoid C-ring.<sup>25</sup> Furthermore, the microbiota seems  
315 also to be able to use the free *p*-CA as a substrate as 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**).<sup>37, 38</sup> 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.<sup>39</sup> 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<sup>40</sup>, 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.<sup>19</sup> 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.

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

348 *Kaempferol-3,4'-O-diglucoside-7-O-rhamnoside*. Compared to the fermentation of T,  
349 where several metabolites were identified, a lower number of phenolic metabolites  
350 was determined in the fermentation of the more complex kaempferol-3,4'-O-  
351 diglucoside-7-O-rhamnoside (K-DG-R, **Table 1**). Due to the fact that K-DG-R itself  
352 was not stable in the ion source and therefore, could not be detected with the  
353 analytical method used, the degradation was investigated only by the formation and  
354 degradation of K-G and K. In the 'control samples II', where the substrate was  
355 incubated with the medium, neither K-G nor K were detected (data not shown).  
356 Consequently, the formation of the different metabolites resulted only from the  
357 microbial degradation of K-DG-R. For quantification, only the metabolite K-G could be  
358 included in the method, due to the lack of reference compounds. However,  
359 additionally qTOF analyses were carried out and the tentative identification of the  
360 metabolites kaempferol-3,4'-O-diglucoside, kaempferol-3-O-glucoside-7-O-  
361 rhamnoside, or kaempferol-4'-O-glucoside-7-O-rhamnoside, and kaempferol-7-O-  
362 rhamnoside was possible. The metabolites were detected at the same fermentation  
363 times as K-G (data not shown). Furthermore, the metabolites K-G (0-7 h), K (3-48 h),  
364 and 4-HPPA (48 h) for the fermentation with the fecal sample from donor A and K-G  
365 (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.<sup>23</sup> In contrast,  
408 two *Lactococcus species* and one *Enterococcus species* were not able to convert V,  
409 as described by Kim, et al.<sup>41</sup> .

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

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

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

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

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

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

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

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

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

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

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

490 Bacteroidetes.<sup>43</sup> The composition of the fecal sample from donor A on the other hand  
491 was very different, which was confirmed by PCA at OTU level ( $\geq 97\%$  sequence  
492 identity, **Supplementary Figure 4**). In particular, it had a very high abundance of  
493 Firmicutes, but low Bacteroidetes (**Supplementary Figure 2**). This initial fecal  
494 sample also showed the biggest change in microbiota composition investigated by  
495 Illumina sequencing after 48 h of incubation with all substrates (**Supplementary**  
496 **Figure 4**), with a big increase in Bacteroidetes and Proteobacteria at the expense of  
497 Firmicutes at the phylum level (**Supplementary Figure 2**). Only a few genera  
498 showed a major increase in relative abundance after incubation (*Bacteroides*,  
499 *Parabacteroides*, *Clostridium* XIVa, *Acidaminococcus*, *Veillonella*, unknown  
500 *Burkholderiales* and *Escherichia/Shigella*; **Figure 6**). Microbiota compositions  
501 between samples that were incubated with different phenolic substrates for 48 h were  
502 very similar within the donors (**Figure 6, Supplementary Figure 4**). This is in  
503 agreement with the results from the qPCR and SCFA analysis. Usually,  $\alpha$ -diversity is  
504 used to express the mean variation of species to be found in a certain microbiome. In  
505 this study,  $\alpha$ -diversity was similar in all three fecal samples and remained high after  
506 incubation with the fecal sample from donor C, but was lower after incubation with the  
507 other two donors, particularly with donor A (**Supplementary Table 4**).  $\beta$ -Diversity  
508 could not be calculated due to the limited number of samples. PCA analysis and a  
509 dendrogram were used for a visual presentation (**Supplementary Figure 4**).

510 In a study published by Duda-Chodak<sup>43</sup>, it was concluded that flavonoid aglycones  
511 may inhibit growth of some intestinal bacteria, consequently leading to a modulation  
512 of the whole intestinal microbiome. Especially in the fermentations with the fecal  
513 sample from donor A, where the microbiota composition changes most between 0 h  
514 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.<sup>44</sup> 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 <sup>24</sup> 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 I' 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.

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

545 **Interindividual comparison of the phenolic conversion rates of the different**  
546 **donors.** For all substrates tested, phenolic conversion rate of donor A was slower  
547 than the fermentation rates with fecal samples of donors B and C, which were in turn  
548 quite similar. For evaluating kinetics of the degradation, D<sub>50</sub> (degradation<sub>50</sub>) values of  
549 all substrates were used. These represent the time point, when 50% of the substrate  
550 was totally degraded by the gut microbiota (**Tables 1 and 2**). It was not possible to  
551 estimate such values for the metabolites, because their formation and a possible  
552 follow-up degradation of a metabolite can take place in parallel. In general,  
553 deglycosylation reactions occurred more quickly than the breakdown of the aglycone  
554 structures. Consequently, based on the conversion rates, the metabolite profiles and  
555 the corresponding time courses differed between the different donors and  
556 fermentations, with the fecal samples from donor A showing in general less  
557 metabolites. These differences are probably caused by the individual microbial  
558 compositions in the microbiota from each donor. In the study published by Justesen,  
559 Arrigoni, Larsen and Amado<sup>34</sup>, a major decrease of rutin was observed at first after 8  
560 h of incubation. They concluded that it might be possible that other compounds being  
561 in the media or feces are more preferred substrates (such as carbohydrates) and are  
562 easier to ferment for the bacteria and thus, that the microorganisms probably needed  
563 an adaptation period. Moreover, Cassidy and Minihane<sup>46</sup> 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.<sup>18</sup> 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.<sup>37, 38</sup> 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 <sup>23</sup> 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.<sup>23</sup>

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

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

633 In summary, all substrates used in the present *in vitro* study were converted yielding  
634 lower molecular weight phenolic acids. Deglycosylation reactions were carried out by  
635 the microbiota first, followed by the further breakdown of the aglycone structure.  
636 Comparing the donors with each other, similar metabolites were detected, even  
637 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.<sup>21, 29-31</sup>

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.<sup>50</sup>, *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.<sup>51</sup> 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.<sup>21, 52</sup> 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

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

693

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698

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## 702 **SUPPORTING INFORMATION**

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

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844

845 **LIST OF FIGURE CAPTIONS**

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

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

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

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

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

865 **Figure 6:** Relative abundance of OTUs obtained by Illumina sequencing of the three  
866 different initial fecal samples (donor A, B and C) and after 48 h of incubation with  
867 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).

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

Compound	Donor	Concentration [μM]					D <sub>50</sub>
		0 h	3 h	7 h	24 h	48 h	
<b>Apigenin</b>	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
<i>AP metabolites</i>							
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	
<b>Vitexin</b>	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
<i>V metabolites</i>							
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*	

PAA	C	<LOD	<LOD	<LOD	131 ± 30.7	142 ± 35.9	
	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	
<b>Vitexin-glucoside</b>	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	-
<i>V-G metabolites</i>							
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	
<b>Vitexin-rhamnoside</b>	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
<i>V-R metabolites</i>							
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	

<b>Vitexin-xyloside***</b>	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
<i>V-X metabolites</i>							
	V						
4-HPPA	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	
PAA	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)

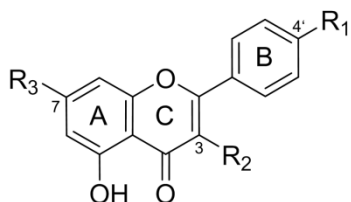
\* the metabolite was only detected in one sample out of the triplicate fermentation

\*\* the metabolite was only detected in two samples out of the triplicate fermentation

\*\*\* Vitexin-xyloside was quantified via Vitexin-rhamnoside

## FIGURE GRAPHICS

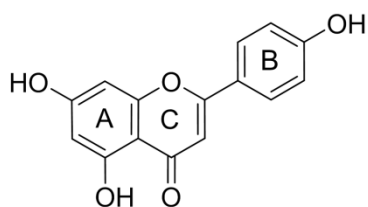
Figure 1



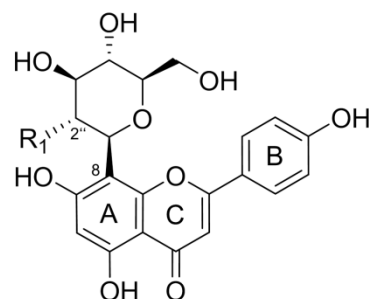
Kaempferol: R<sub>1</sub> & R<sub>2</sub> & R<sub>3</sub> = OH

Kaempferol-3,4'-O-diglucoside-7-O-rhamnoside: R<sub>1</sub> & R<sub>2</sub> = glucosyl, R<sub>3</sub> = rhamnosyl

Tiliroside: R<sub>1</sub> = OH, R<sub>2</sub> = glucosyl-6''-trans-*p*-coumaroyl, R<sub>3</sub> = OH



Apigenin



Vitexin: R<sub>1</sub> = OH

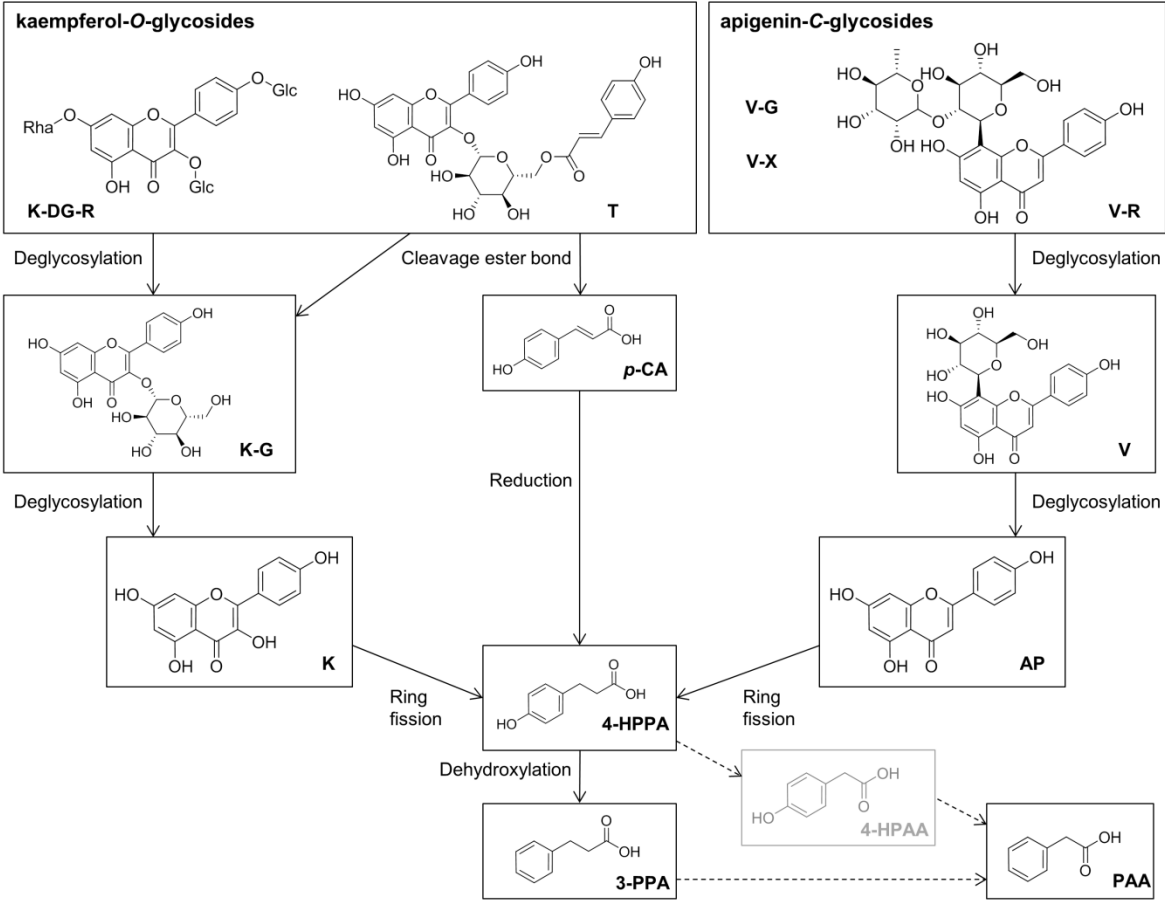
Vitexin-2''-O-glucoside: R<sub>1</sub> = glucosyl

Vitexin-2''-O-rhamnoside: R<sub>1</sub> = rhamnosyl

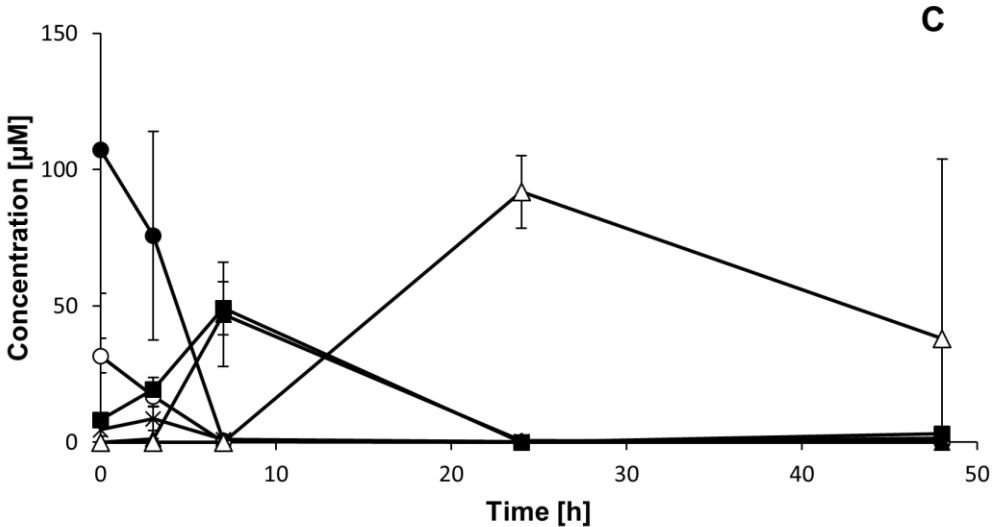
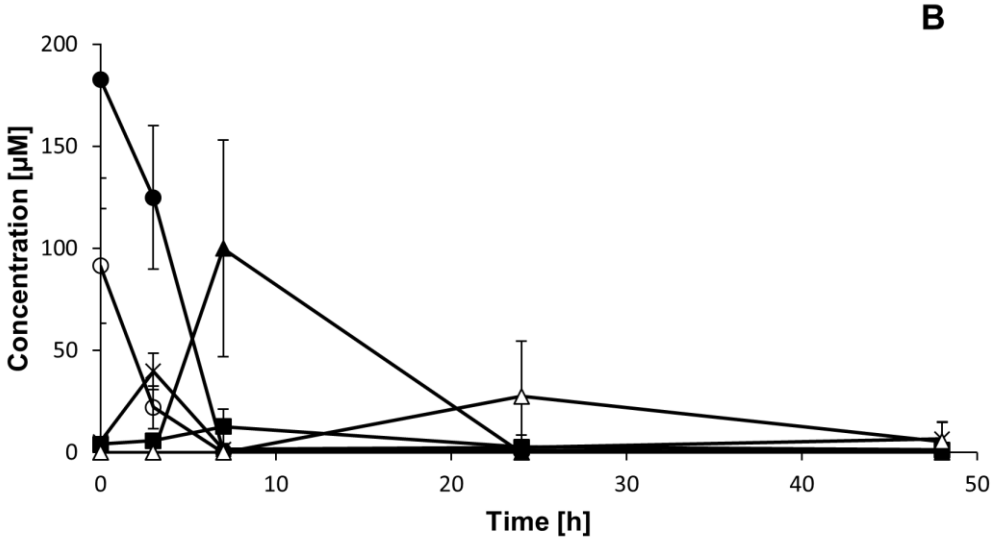
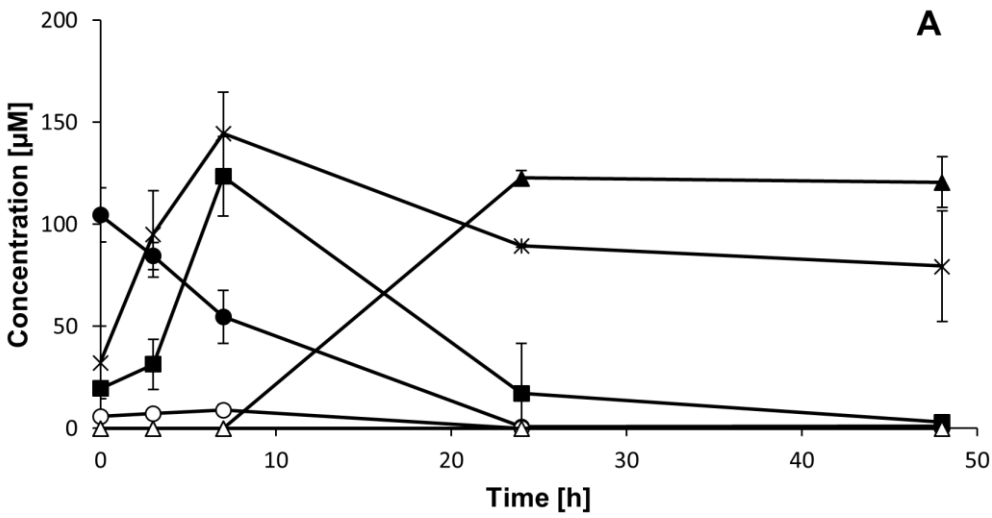
Vitexin-2''-O-xyloside: R<sub>1</sub> = xylosyl



**Figure 2**



**Figure 3**



● T    ○ K-G    \* K    ▲ 4-HPPA    ■ p-CA    △ 3-PPA

Figure 4

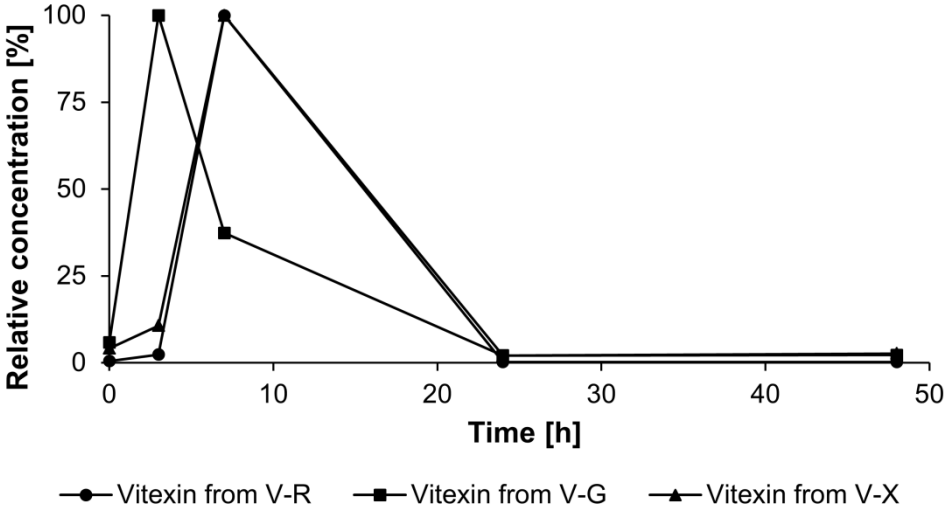
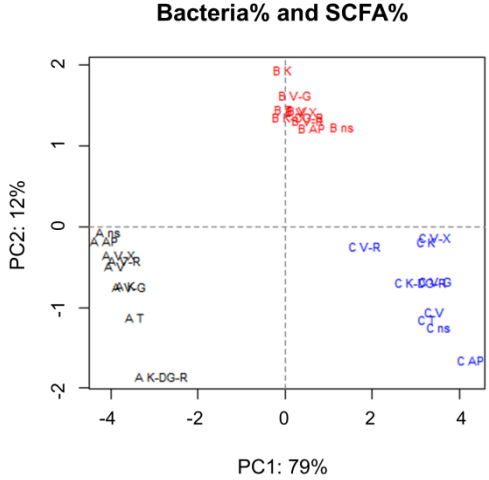
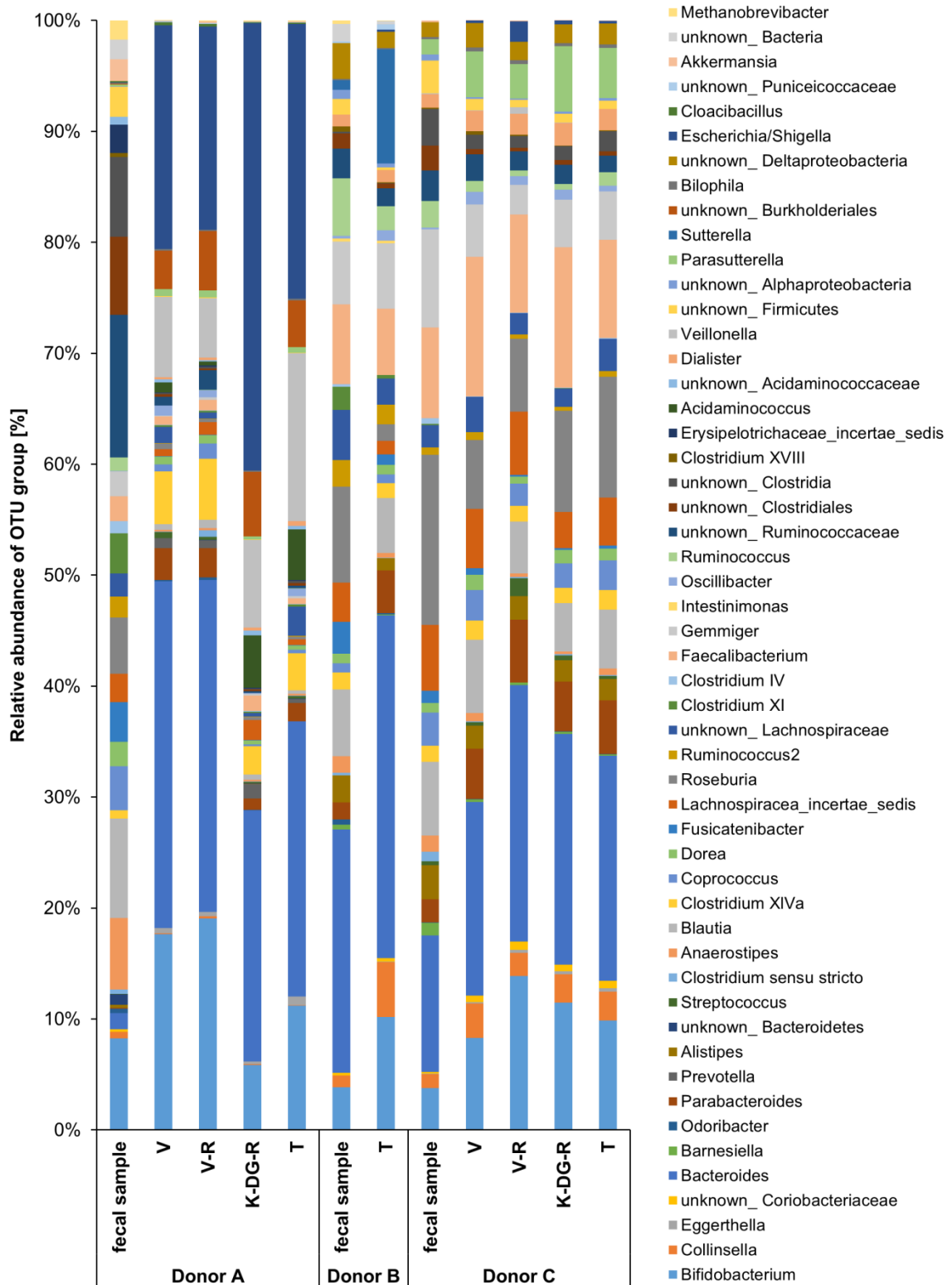


Figure 5



**Figure 6**



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