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# Conversion of Dehydrodiferulic Acids by Human Intestinal Microbiota

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Plant cell wall associated dehydrodiferulic acids (DFA) are abundant components of cereal insoluble dietary fibers ingested by humans. The ability of human intestinal microbiota to convert DFA was studied in vitro by incubating 8-*O*-4- and 5-5-coupled DFA with fecal suspensions. 8-*O*-4-DFA was completely degraded by the intestinal microbiota of the majority of donors, yielding homovanillic acid, 3-(3,4-dihydroxyphenyl)propionic acid, and 3,4-dihydroxyphenylacetic acid as the main metabolites. The transient formation of ferulic acid and presumably 3-(3-hydroxy-4-methoxyphenyl)pyruvic acid suggests an initial cleavage of the ether bond. In contrast to 8-*O*-4-DFA, the 5-5-coupled DFA was not cleaved into monomers by any of the fecal suspensions. Only the side chains were hydrogenated and the methoxy groups were demethylated. The cleavage of DFA by human intestinal microbiota, which depended on their coupling type, may affect both the bioavailability of DFA and the degradability of DFA-coupled fiber in the gut.

**KEYWORDS: Diferulic acids; diferulate; ferulic acid; 8-*O*-4-dehydrodiferulic acid; 5-5-dehydrodiferulic acid; human intestinal microbiota; conversion**

## INTRODUCTION

Plant cell wall associated dehydrodiferulic acids (DFA) are abundant components of insoluble cereal dietary fiber. For example, an intake of 10 g of wheat bran provides approximately 10 mg of DFA (*1*). The highest amount of DFA within cereal grains studied was found in the insoluble fiber fraction of maize amounting to 12.6 mg of DFA/g (*2*). Next to their occurrence in monocotyledons, DFA are also cell-wall components of dicotyledons, such as carrot and sugar beet (*3, 4*). The ferulate dehydrodimers are formed via radical coupling of two monomeric ferulates (*5*) that are ester-linked to polymers in the plant cell wall (*6*). Thus, by cross-linking polysaccharides to each other or to lignin-like polymers, ferulate dimerization provides stability to the cell wall but may inhibit intestinal fiber degradability by host and microbial enzymes (*7, 8*).

The consumption of high-fiber diets has been implicated in the prevention of colorectal cancer (*9, 10*), but this issue remains controversial (*11*). In particular, the intake of insoluble dietary fiber was reported to be inversely associated with colon cancer risk (*12*). However, protection could also be attributed to other nutrients contained in high-fiber diets or phenolic compounds associated with or bound to dietary fiber (*13*). Thus, hydroxycinnamic acids such as ferulic acid and DFA have antioxidant and chemoprotective effects in vitro and in vivo (*14*−*17*). After intake, both mucosal and bacterial esterases are supposed to release fiber-bound hydroxycinnamates such as DFA not only from low molecular weight (model) compounds but also from cereal brans into the gut lumen, which then are potentially absorbed (*18*−*20*). However, there are no reports on the absorption of DFA in humans, indicating that the bulk of dimers is further metabolized by intestinal microbiota or excreted in feces.

In contrast to microbial hydrolysis of the ester bonds, knowledge of the potential breakdown and further metabolism of DFA by intestinal bacteria is very limited. The rates of conversion of differently coupled DFA model compounds by rumen and human intestinal microbiota in vitro were found to be low (*21*). So far, microbial metabolites resulting from DFA conversion have not been described.

In this study, we demonstrate the ability of human gut microbiota to transform two selected DFA regioisomers, 5-5-DFA and 8-*O*-4-DFA, and identify their microbial metabolites.

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## MATERIALS AND METHODS

**Chemicals.** 8-*O*-4-DFA and 5-5-DFA were preparatively isolated from maize bran by gel permeation chromatography following saponification (*22*). Dihydroferulic acid-5-5-dihydroferulic acid and dihydroferulic acid were synthesized by catalytic hydrogenation as briefly described: Ferulic acid or 5-5-DFA was dissolved in methanol. Palladium on activated carbon was carefully added. A balloon filled with hydrogen was cautiously capped on the flask, and the mixture was stirred at room temperature overnight. The catalyst was carefully filtered off, and the product solution was dried. Hydrogenation was verified by $^{1}$H and $^{13}$C NMR (360 MHz Bruker DRX-360 instrument). 3-(4-Hydroxy-3-methoxyphenyl)pyruvic acid was obtained from TCI Europe (Zwijndrecht, Belgium). Ferulic acid, caffeic acid, homovanillic acid, 3-(3,4-dihydroxyphenyl)propionic acid, and 3,4-dihydroxyphenylacetic acid were purchased from Fluka (Deisenhofen, Germany), and 3-(3-hydroxyphenyl)propionic acid was obtained from Alfa Aesar (Karlsruhe, Germany).

**Conversion Experiments with Human Fecal Slurries.** Fecal samples were collected from seven healthy human volunteers (three males and four females) with a mean age of 34 years (range of 25−52 years), who consumed an unspecified Western diet and had not taken antibiotics in the 6 months prior to the study. Freshly voided feces were kept under anoxic conditions using AnaeroGen sachets (Oxoid, Hampshire, U.K.) and stored at 4 °C for a maximum of 1 h before processing. Fecal suspensions were prepared at room temperature in an anaerobic glovebox (MACS Anaerobic Workstation, Don Whitley Scientific Ltd., Shipley, U.K.) with a gas atmosphere of $N_2/CO_2/H_2$ (80:10:10%, v/v/v) using a defined bicarbonate-buffered medium (medium B) (*23*) supplemented with 10 mM glucose. An aliquot of 1 g of feces was transferred into a 16 mL tube fitted with a butyl rubber stopper and a screw cap (Hungate tube) containing 10 mL of medium B and subsequently homogenized by vigorous shaking.

For conversion experiments, aliquots of 500 μL of the fecal suspension were used to inoculate 16 mL Hungate tubes gassed with $N_2/CO_2$ (80:20%, v/v) and containing each 10 mL of medium B supplemented with 8-*O*-4-DFA, 5-5-DFA, or 3-(4-hydroxy-3-methoxyphenyl)pyruvic acid at a final concentration of 190 μM. DFA and 3-(4-hydroxy-3-methoxyphenyl)pyruvic acid were dissolved in dimethyl sulfoxide (DMSO), and 50 μL of the respective stock solution was added to the medium with a syringe. For control, the compounds were incubated without fecal inoculum (addition of 500 μL of medium B). In addition, the fecal slurries were incubated in the absence of the compounds (addition of 50 μL of DMSO). The tubes were incubated at 37 °C in a culture tube rotator at 6 rpm (SC1, Stuart Scientific, Redhill, U.K.). At the times indicated, 500 μL samples were withdrawn by using a syringe and stored at −20 °C. The assigned microbial metabolites were exclusively detected following the incubation of 8-*O*-4-DFA, 5-5-DFA, and 3-(3-hydroxy-4-methoxyphenyl)pyruvic acid, respectively, with the fecal slurries. None of these metabolites was observed in the absence of either the compounds or microbiota.

**Sample Preparation for HPLC Analysis.** Aliquots of samples from the conversion experiments with 8-*O*-4-DFA and 3-(4-hydroxy-3-methoxyphenyl)pyruvic acid were centrifuged (12000*g*, 5 min), and 112.5 μL of the supernatant was acidified by adding 2.5 μL of aqueous 4 M HCl. After centrifugation (12000*g*, 5 min), 102 μL of the resulting supernatant was applied to the HPLC system. Whereas the recoveries of 8-*O*-4-DFA, 3-(3-hydroxy-4-methoxyphenyl)pyruvic acid, and their metabolites were maximal when the acidified supernatants were analyzed directly, 5-5-DFA and its metabolites were recovered most efficiently by extraction with ethyl acetate. For analysis of samples from the conversion experiments with 5-5-DFA, aliquots of 200 μL were extracted twice with 400 μL each of ethyl acetate after the addition of 5 μL of aqueous 2 M HCl. The combined organic layers were dried by vacuum centrifugation (RC 10.22; Jouan, Saint-Nazaire, France), and the residue was dissolved in 40 μL of 70% aqueous methanol (v/v). After centrifugation (12000*g*, 5 min), 20 μL of the resulting supernatant was applied to the HPLC system.

**HPLC Analysis.** DFA and aromatic metabolites were determined using an HPLC system with a photodiode array detector (Summit HPLC Analytical System, Dionex, Idstein, Germany). The HPLC system was equipped with a pump P680A LPG-4, an autosampler ASI-100T, a thermostated column compartment TCC-100 with eluent preconditioner, a photodiode array detector UVD 340U, and a reversed-phase $C_{18}$ column (LiChroCART 250-4 LiChrospher 100 RP-18, 5 μm; 250 × 4 mm i.d.; Merck, Darmstadt, Germany). The column temperature was maintained at 50 °C. Aqueous 0.1% trifluoroacetic acid (v/v, solvent system A) and methanol (solvent system B) served as the mobile phase in a gradient mode with a flow rate of 1 mL/min. Detection was at 280 nm. For analysis of 8-*O*-4-DFA, 3-(4-hydroxy-3-methoxyphenyl)pyruvic acid, and their metabolites, the following gradient was applied: B from 5 to 30% in 20 min, from 30 to 50% in 5 min, from 50 to 80% in 10 min, and from 80 to 100% in 4 min. For analysis of 5-5-DFA and its metabolites, the following gradient was used: B from 5 to 52.5% in 70 min and from 52.5 to 100% in 5 min. If applicable, compounds were identified by their retention times and UV spectra ($\lambda = 200-400$ nm) in comparison to reference substances. External calibration curves were used for quantification.

**Sample Preparation for GC-MS Analysis.** To gather additional structural information, aliquots of some samples from the conversion experiments were also investigated by GC-MS. For this purpose, sample aliquots were centrifuged (12000*g*, 5 min), and the supernatant (200 μL) was lyophilized (Alpha 2-4, Christ, Osterode, Germany). The residues were redissolved in water, acidified to pH 2.0, and extracted twice with ethyl acetate. The combined organic layers were dried in a reacti-vial under a gentle stream of nitrogen and used for derivatization.

**Isolation of Metabolites for GC-MS Analysis.** Some metabolites could not be identified by HPLC analysis and comparison with standard reference compounds. To identify these metabolites, additional GC-MS experiments were necessary. Unidentified metabolites were isolated from samples of the conversion experiments. Samples of 102 μL each prepared as described above were run on the HPLC system as described above. Fractions containing the metabolites of interest were manually collected, pooled, and dried by vacuum centrifugation. Samples were transferred into reacti-vials by sequentially using ethyl acetate and acetone. After drying, the samples were derivatized as described below.

**GC-MS Analysis.** Samples were silylated by using bis(trimethylsilyl)trifluoroacetamide (BSTFA) (40 μL) and pyridine (10 μL); however, in some cases the amounts of reagents were equally reduced. Silylation was carried out for 30 min at 60 °C in sealed reacti-vials.

Over the time of the study, two different GC-MS systems were used. System 1 used a Trace 2000 ThermoQuest GC coupled to a PolarisQ ion trap (Thermo Scientific, Dreieich, Germany). Samples were injected in the splitless mode (injector temperature = 300 °C). Separation was carried out on a 30 m × 0.32 mm i.d., 0.25 μm film, HP-5-MS fused silica capillary column (Hewlett-Packard, Waldbronn, Germany). Compounds were characterized by their electron impact mass data (70 eV, scan range *m*/*z* 50−800). He (1 mL/min) was used as a carrier gas. GC conditions were as follows: initial column temperature, 150 °C, ramped at 3 °C/min to 250 °C, ramped at 30 °C/min or at 4 °C/min to 300 °C, held for 30 min.

System 2 used a 2010 GC coupled to a QP2010 quadrupole mass spectrometer (Shimadzu, Columbia, MD). Samples were injected using a split ratio of 10 (injector temperature = 250 °C). Separation was carried out on a 30 m × 0.25 mm i.d., 0.25 μm film, SHR5XLB capillary column (Shimadzu). Compounds were characterized by their electron impact mass data (70 eV, scan range *m*/*z* 45−900). He (0.85 mL/min) was used as carrier gas. GC conditions were as follows: initial column temperature, 150 °C, held for 1 min, ramped at 10 °C/min to 310 °C, held for 25 min.

## RESULTS

**Conversion of 8-*O*-4-DFA by Human Intestinal Microbiota.** Fecal suspensions of seven human donors were tested for their ability to transform 8-*O*-4-DFA. Whereas the compound was stable during incubation in medium without an inoculum, 8-*O*-4-DFA was completely converted by six of seven fecal slurries within 15−40 h of incubation (**Table 1**). After 35 h, no major changes in the metabolite patterns were observed for most of the fecal suspensions. The complete conversion of 8-*O*-

**Table 1.** Conversion of 8-*O*-4-Dehydrodiferulic Acid (8-*O*-4-DFA), 5-5-Dehydrodiferulic Acid (5-5-DFA), and 3-(4-Hydroxy-3-methoxyphenyl)pyruvic Acid (HMPP) by Human Fecal Suspensions (FS) within 164 h of Anoxic Incubation

| | period of complete conversion (h) | | |
|---|---|---|---|
| FS | 8-*O*-4-DFA | 5-5-DFA | HMPP |
| 1 | 15 | nc[a] | nd[b] |
| 2 | 35 | nc | 48 |
| 3 | 25 | nc | 48 |
| 4 | 40 | nc | nd |
| 5 | 25 | 40 | nd |
| 6 | nc | nc | nd |
| 7 | 25 | nc | nd |

[a] Not completed. [b] Not determined.

4-DFA [recovery at 0 h (± SD), 159 ± 2 $\mu$M] as performed by the six fecal slurries resulted in cleavage of the dimer and formation of a similar pattern of phenolic acids, which were identified by comparison to standard reference compounds using HPLC-DAD and when necessary by GC-MS. Ferulic acid, dihydroferulic acid, 3-(3,4-dihydroxyphenyl)propionic acid, homovanillic acid, and 3,4-dihydroxyphenylacetic acid were detected in all of the incubations, whereas 3-(4-hydroxy-3-methoxyphenyl)pyruvic acid, caffeic acid, and 3-(3-hydroxyphenyl)propionic acid were each observed in incubations with only three to four of the fecal slurries (for chemical structures see **Figure 2**). The main metabolites occurring at maximal concentrations of >80 $\mu$M in the course of 8-*O*-4-DFA conversion by the majority of fecal slurries were 3-(3,4-dihydroxyphenyl)propionic acid, homovanillic acid, and 3,4-dihydroxyphenylacetic acid. These metabolites, together with 3-(3-hydroxyphenyl)propionic acid, also represented the final products of 8-*O*-4-DFA conversion under the conditions used. Major differences between incubations with fecal slurries of individual donors were observed with regard to metabolite kinetics. The time course of 8-*O*-4-DFA conversion by a representative fecal slurry forming the complete spectrum of metabolites (FS 5) is depicted in **Figure 1A**.

The fecal microbiota of one of seven human subjects only partially transformed 8-*O*-4-DFA. After 58 h of incubation with this fecal suspension (FS 6), approximately half of the 8-*O*-4-DFA was converted and no further transformation was observed (**Figure 1B**). From 8-*O*-4-DFA was formed one main product showing a spectrum [$\lambda_{max}$ 218, 238, 290(S), 327 nm] nearly identical to that of the parental compound [$\lambda_{max}$ 218, 235, 290(S), 326 nm] but eluting earlier using RP-HPLC. Further characterization of the metabolite by GC-MS indicated the loss of a methyl group. The characteristic ions of the parental, silylated 8-*O*-4-DFA with $m/z$ 602 (molecular ion), 484 (M − TMSOH − CO), and 394 (M − 2 TMSOH − CO) were shifted toward the ions with $m/z$ 660 (molecular ion), 542, and 452. The mass difference of 58 is explained by the loss of the methyl group, creating another hydroxyl group, which is silylated. In the parental 8-*O*-4-DFA two methoxy groups are available to be theoretically demethylated. We hypothesize that due to sterical hindrance, demethylation did not preferentially occur at the *C*-4-coupled ferulate monomer but occurred at the methoxy group of the *C*-8-coupled ferulate monomer, yielding caffeic acid-8-*O*-4-ferulic acid. However, this question needs to be addressed in more detail in the future, for example, by semipreparative isolation and NMR characterization of this compound.

On the basis of the metabolites detected, the following pathways of 8-*O*-4-DFA conversion by human fecal microbiota are proposed (**Figure 2**). The ether bond of 8-*O*-4-DFA was cleaved, resulting in the formation of ferulic acid and likely 3-(4-hydroxy-3-methoxyphenyl)pyruvic acid. Both metabolites were further converted by decarboxylation, reduction, O-demethylation, and dehydroxylation, respectively. From 3-(4-hydroxy-3-methoxyphenyl)pyruvic acid was formed 3,4-dihydroxyphenylacetic acid via homovanillic acid. Ferulic acid was converted mainly to dihydroferulic acid but also to caffeic acid. From the latter two compounds was formed 3-(3,4-dihydroxyphenyl)propionic acid, which was further dehydroxylated to 3-(3-hydroxyphenyl)propionic acid. The incomplete conversion of homovanillic acid and dihydroferulic acid to 3,4-dihydroxyphenylacetic acid and 3-(3,4-dihydroxyphenyl)propionic acid, respectively, by some of the individual fecal slurries indicates that the O-demethylating activity of bacteria was limited. However, besides the monomeric phenolic acids, also the dimeric 8-*O*-4-DFA was a substrate for microbial O-demethylation, resulting in the formation of an 8-*O*-4-coupled caffeic acid/ferulic acid dimer. As observed for demethylation, dehydroxylation of 3-(3,4-dihydroxyphenyl)propionic acid was catalyzed by only four of the fecal suspensions.

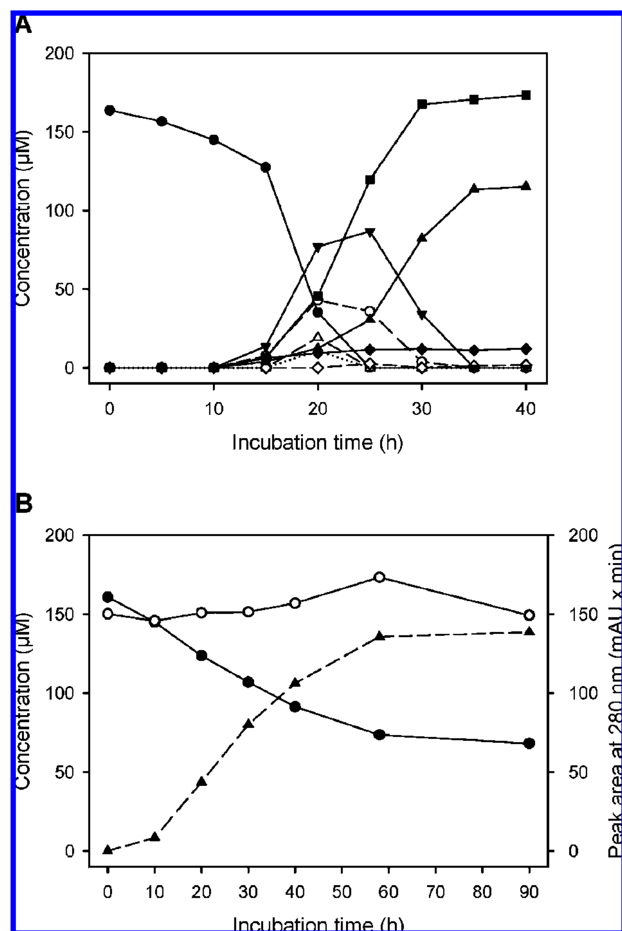


**Figure 1.** (**A**) Time course of degradation of 8-*O*-4-dehydrodiferulic acid (8-*O*-4-DFA) by a human fecal suspension (FS 5). From 8-*O*-4-DFA (●) were formed 3-(4-hydroxy-3-methoxyphenyl)pyruvic acid (···), ferulic acid (△), homovanillic acid (▼), dihydroferulic acid (○), caffeic acid (◇), 3-(3,4-dihydroxyphenyl)propionic acid (■), 3-(3-hydroxyphenyl)propionic acid (◆), and 3,4-dihydroxyphenylacetic acid (▲). For the 8-*O*-4-DFA control without fecal inoculum see panel **B**. (**B**) Time course of partial conversion of 8-*O*-4-DFA by a human fecal suspension (FS 6). From 8-*O*-4-DFA (●) was formed the proposed demethylated metabolite, caffeic acid-8-*O*-4-ferulic acid (▲) (*y* axis on the right). 8-*O*-4-DFA without fecal inoculum (○) served as a control.

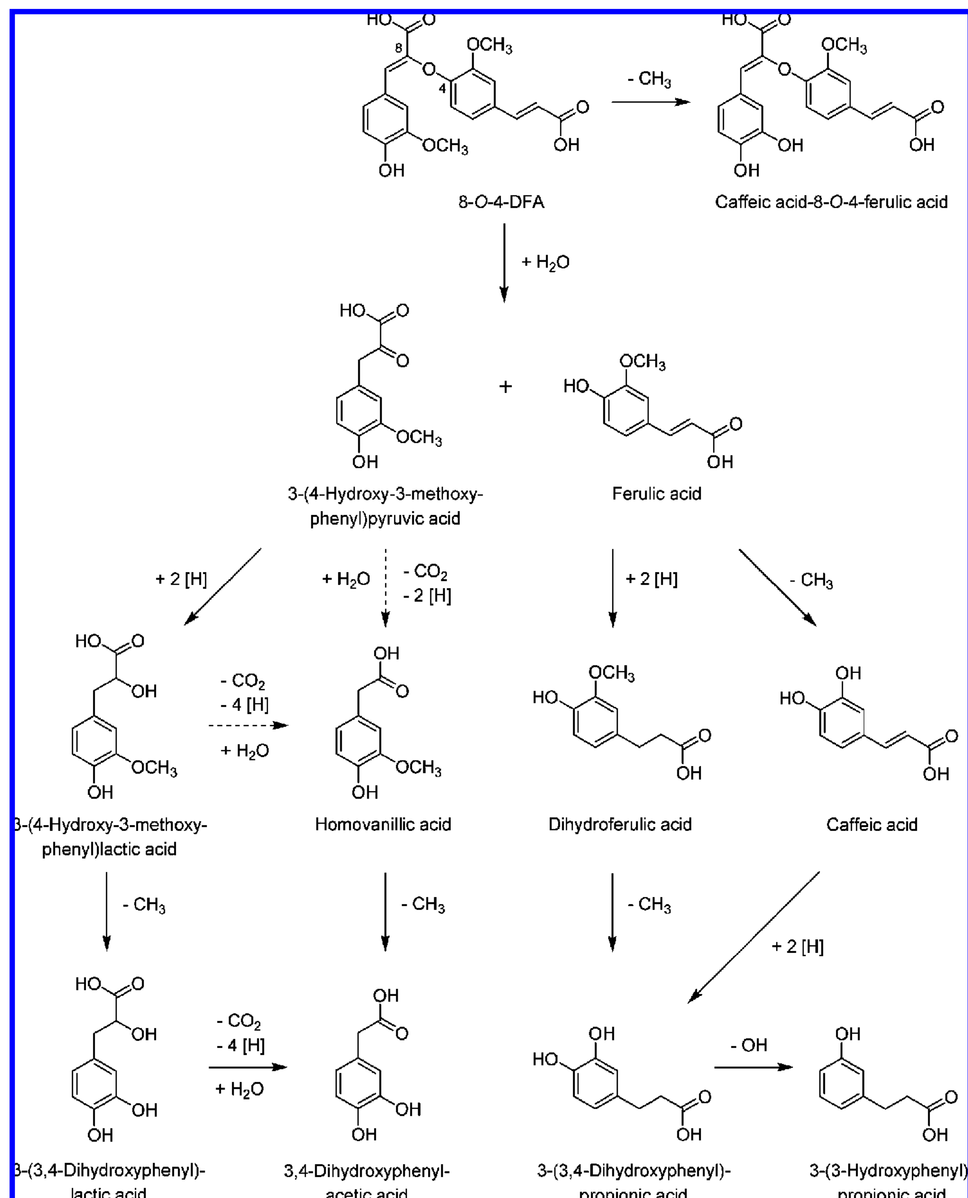


**Figure 2.** Proposed pathways of 8-*O*-4-dehydrodiferulic acid (8-*O*-4-DFA) conversion by human intestinal microbiota. Pathways indicated by dashed arrows could not be verified in this study.

The ability of human gut bacteria to convert 3-(4-hydroxy-3-methoxyphenyl)pyruvic acid, the proposed initial product of 8-*O*-4-DFA cleavage besides ferulic acid, was tested. 3-(4-Hydroxy-3-methoxyphenyl)pyruvic acid was incubated with fecal suspensions of two donors (FS 2, FS 3; **Table 1**), which in previous experiments showed a complete conversion of 8-*O*-4-DFA. 3-(4-Hydroxy-3-methoxyphenyl)pyruvic acid (190 $\mu$M) was completely converted within 5 h of incubation (data not shown). The end product was identified as 3,4-dihydroxyphenylacetic acid. Two intermediates, M1 ($\lambda_{max}$ 227, 280 nm) and M2 [$\lambda_{max}$ 229(S), 281 nm], were formed consecutively. Both metabolites could not be identified by comparison to standard reference compounds. Therefore, the compounds were isolated from supernatants of incubation experiments and subjected to GC-MS analysis. On the basis of the mass spectra of the silylated metabolites, M1 and M2 were tentatively identified as 3-(4-hydroxy-3-methoxyphenyl)lactic acid and 3-(3,4-dihydroxyphenyl)lactic acid, respectively. Metabolite M1 showed a molecular ion with *m*/*z* 428 (6% of the base peak) and characteristic fragments of *m*/*z* 338 (M − TMSOH) and 209 (base peak). A similar fragmentation pattern was described for 3-(4-hydroxyphenyl)lactic acid in the literature. However, due to the lack of an aromatic methoxy group the molecular ion is shifted to *m*/*z* 398, and the characteristic fragments are shifted to *m*/*z* 308 and 179 (base peak) (*24*). Metabolite M2 had a molecular ion with *m*/*z* 486 (8% of the base peak), and characteristic fragments were noted at *m*/*z* 396 (M − TMSOH) and 267 (base peak). From this mass spectrum it can be deduced that the aromatic methoxy group was demethylated, creating a new hydroxyl group to be silylated during the derivatization process. Following their assignment as metabolites of the conversion of 3-(4-hydroxy-3-methoxyphenyl)pyruvic acid (**Figure 2**), 3-(4-hydroxy-3-methoxyphenyl)lactic acid and 3-(3,4-dihydroxyphenyl)lactic acid were also detected in several samples taken during initial 8-*O*-4-DFA transformation by some of the fecal slurries. However, homovanillic acid was not observed in the course of the conversion of 3-(4-hydroxy-3-methoxyphenyl)pyruvic acid. Homovanillic acid had been identified as an intermediate of 8-*O*-4-DFA transformation and proposed to be a metabolite resulting from 3-(4-hydroxy-3-methoxyphenyl)pyruvic acid (**Figure 2**).

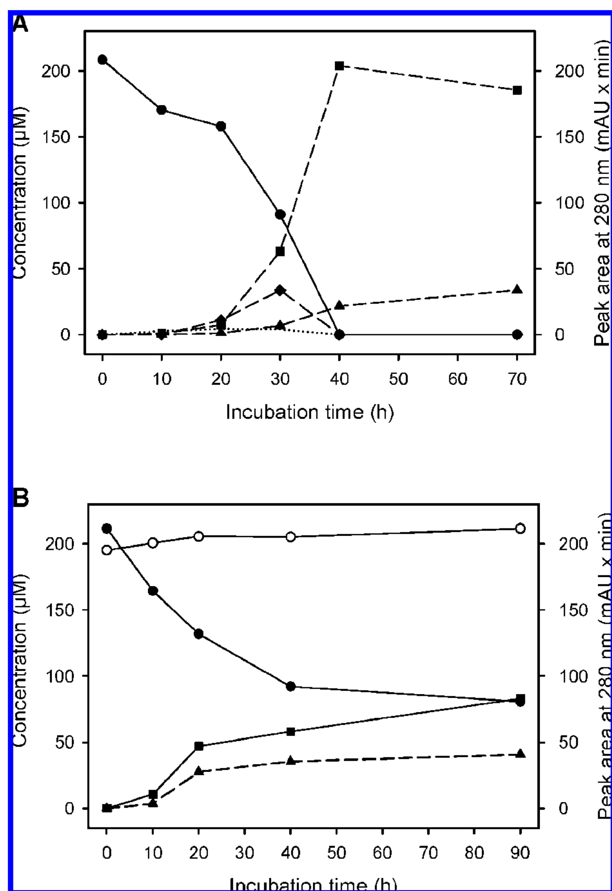


**Figure 3.** (**A**) Time course of conversion of 5-5-dehydrodiferulic acid (5-5-DFA) by a human fecal suspension (FS 5). From 5-5-DFA (●) were formed ferulic acid-5-5-dihydroferulic acid (···), ferulic acid-5-5-caffeic acid (◆), caffeic acid-5-5-caffeic acid (■), and caffeic acid-5-5-dihydrocaffeic acid (▲). Broken lines refer to the *y* axis on the right. For the 5-5-DFA control without fecal inoculum see panel **B**. (**B**) Time course of partial conversion of 5-5-DFA by a human fecal suspension (FS 4). From 5-5-DFA (●) were formed ferulic acid-5-5-dihydroferulic acid (▲) (*y* axis on the right) and dihydroferulic acid-5-5-dihydroferulic acid (■). 5-5-DFA without fecal inoculum (○) served as a control.

**Conversion of 5-5-DFA by Human Intestinal Microbiota.** The fecal suspensions used for conversion experiments with 8-*O*-4-DFA were also tested for their ability to transform 5-5-DFA (**Table 1**). The compound was stable during the total incubation period of 164 h in medium without an inoculum. Only one of seven different fecal suspensions catalyzed the complete conversion of 5-5-DFA [recovery at 0 h (±SD), 210 ± 10 $\mu$M] within 40 h of incubation (**Figure 3A**). In the course of 5-5-DFA conversion by this fecal suspension (FS 5), four main metabolites were detected (**Figure 3A**). Following isolation, these metabolites were characterized by GC-MS analysis as ferulic acid-5-5-dihydroferulic acid (molecular ion and base peak *m*/*z* 676), ferulic acid-5-5-caffeic acid (molecular ion and base peak *m*/*z* 732), caffeic acid-5-5-caffeic acid (molecular ion and base peak *m*/*z* 790), and caffeic acid-5-5-dihydrocaffeic acid (molecular ion and base peak *m*/*z* 792). Whereas ferulic acid-5-5-dihydroferulic acid and ferulic acid-5-5-caffeic acid were formed transiently, caffeic acid-5-5-caffeic acid and caffeic acid-5-5-dihydrocaffeic acid represented the end products of microbial 5-5-DFA conversion. After 70 h of incubation, no major changes in the concentration of the two end products were observed.

In the course of incubation for 164 h with the other six fecal suspensions, 10−60% of the initially available 5-5-DFA was transformed. The conversion of 5-5-DFA essentially occurred within 40 h of incubation. Two metabolites were formed from 5-5-DFA at various concentrations depending on the fecal suspension. One of these compounds, ferulic acid-5-5-dihydroferulic acid, had already been observed as an intermediate of 5-5-DFA conversion by the fecal suspension FS 5. The second metabolite was identified as dihydroferulic acid-5-5-dihydroferulic acid by comparison to a standard reference compound. The transformation of 5-5-DFA by the fecal suspension forming the highest amounts of both metabolites (FS 4) is shown in **Figure 3B**. On the basis of the metabolites detected following incubation with the individual human fecal slurries, **Figure 4** summarizes the pathways of microbial 5-5-DFA conversion. The ferulate dimer was not cleaved by human intestinal microbiota. Only the side-chain double bonds were reduced, and methoxy groups were partially demethylated.

## DISCUSSION

The fermentation of dietary fiber by gut bacteria is proposed to have beneficial effects on human health (*25*). The fiber degradability may be affected by cross-linking polysaccharide chains by ferulate dimerization. In vitro analyses of carbohydrate fermentation products show that cross-linking via ferulate derivatives reduces the rate of arabinoxylan degradation (*8*). On the other hand, low to moderate levels of diferulates (0.8−2.6 mg/g) do not alter the release of carbohydrates or the overall degradation of nonlignified cell walls by human gut microbiota (*26*). The ester bonds between the polysaccharides and the (di)ferulates may be cleaved by both intestinal and microbial enzymes (*19, 20, 27*). Microbial feruloyl esterases have been characterized from several species, including the gut-relevant *Lactobacillus acidophilus* (*28*). In contrast, data on both the cleavage of fiber-bound diferulates and the conversion of the free dimers as released by esterases in the gut are still limited. In the present study, the ability of the human intestinal microbiota to convert two regioisomeric DFA, 8-*O*-4-DFA and 5-5-DFA, was elucidated. These dimers are among the dominating diferulates of cereal grains. For example, in insoluble dietary fiber from maize (total DFA content = 12.6 mg/g) 8-*O*-4-DFA and 5-5-DFA contribute 21 and 25% to the total diferulates (*2*).

The 8-*O*-4-coupled DFA was completely transformed by human fecal suspensions within 15−40 h. This time period corresponds to the average colonic transit time in healthy humans of 36 h (*29*). The observed metabolites indicate that the first step of 8-*O*-4-DFA conversion was the cleavage of the ether bond (**Figure 2**). Ether linkages in both biological and xenobiotic compounds show a high degree of resistance against enzymatic degradation. Although only a few ether-cleaving enzymes have been described to date, these enzymes exhibit a variety of mechanisms (*30*). We assume that 8-*O*-4-DFA was cleaved analogously to the hydrolysis of vinyl ethers catalyzed by the isochorismate pyruvate hydrolase (isochorismatase, EC 3.3.2.1), which is found in several bacteria including *Escherichia coli* (*31*). According to the reaction mechanism described for the isochorismatase (*30, 32*), the cleavage of 8-*O*-4-DFA may take place by hydration of the double bond to yield the hemiacetal, cleavage via $\beta$-elimination releasing ferulic acid and 3-(4-hydroxy-3-methoxyphenyl)pyruvic acid, the latter formed by tautomerization. The transformation of ferulic acid by intestinal microbiota has been described in the past (*21, 33*). As reported previously, hydrogenation of the side chain proceeded more quickly than O-demethylation and dehydroxylation. On the basis of kinetic data, homovanillic acid and 3,4-dihydroxyphenylacetic acid were most likely formed from 3-(4-

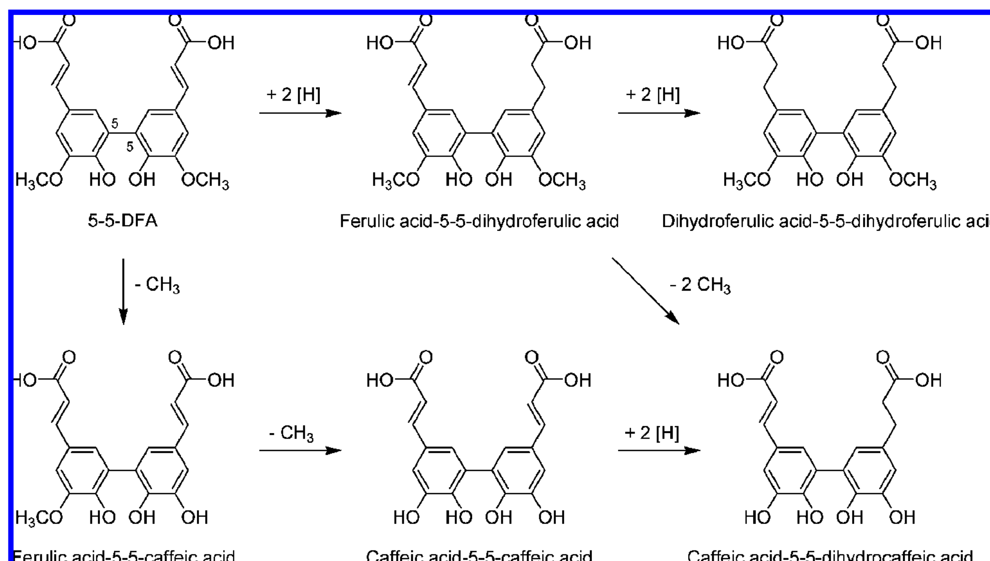


**Figure 4.** Proposed pathways of 5-5-dehydrodiferulic acid (5-5-DFA) conversion by human intestinal microbiota.

hydroxy-3-methoxyphenyl)pyruvic acid. However, when incubated with fecal suspensions, 3-(4-hydroxy-3-methoxyphenyl)pyruvic acid was converted to 3,4-dihydroxyphenylacetic acid via 3-(4-hydroxy-3-methoxyphenyl)lactic acid and 3-(3,4-dihydroxyphenyl)lactic acid (**Figure 2**), but we were not able to detect homovanillic acid. Therefore, we cannot exclude a concurrent mechanism in which homovanillic acid is formed from the parental 8-*O*-4-DFA or from one of the primary cleavage products described here.

In contrast to 8-*O*-4-DFA, the 5-5-coupled DFA was not cleaved into monomers by any of the human fecal suspensions tested. Only the side chains of 5-5-DFA were finally hydrogenated, yielding dihydroferulic acid-5-5-dihydroferulic acid (**Figure 4**). Demethylation of the methoxy groups was less frequently observed. However, methoxy-substituted phenolic acids formed in the course of 8-*O*-4-DFA conversion were readily demethylated by the same microbiota. The conversion of 5-5-DFA and other synthesized diphenolic model compounds by rumen and human intestinal microbiota was tested in a previous study (*21*). Following incubation of the differently coupled diphenolic compounds with rumen or fecal slurries in buffer, some of the investigated compounds including the 5-5-DFA were mostly recovered intact. The use of a culture medium containing glucose, vitamins, and trace elements may have led to a more efficient transformation of 5-5-DFA as observed in our study.

The results obtained in this study indicate that human intestinal microbiota may structure-dependently transform DFA, which are released from fiber carbohydrates by esterases into the gut lumen. This in turn may affect the absorption of DFA, which has been demonstrated for several of these dimers to occur in the rat intestine (*19*). However, no absorption of unaltered DFA was observed in humans after high-bran cereal consumption, which indicates that the bulk of free or still ester-linked dimers remains in the intestinal content and is available for metabolization by gut bacteria (*34*). For both the DFA and the phenolic acids, which were formed from 8-*O*-4-DFA, such as ferulic acid, caffeic acid, and 3,4-dihydroxyphenylacetic acid, antioxidant and chemoprotective effects have been reported (*14−16, 35, 36*). Because the phenolic acids are presumably better absorbed, for example, by the monocarboxylic acid transporter (*37*), the microbial conversion of DFA might result in bioactivation of these compounds. Besides cleavage of the free 8-*O*-4-DFA, intestinal microbiota could also contribute to the depolymerization of dietary fiber in the gut by cleaving the existing 8-*O*-4-DFA cross-links prior to ester hydrolysis, thereby increasing the fiber fermentability.

## ABBREVIATIONS USED

BSTFA, bis(trimethylsilyl)trifluoroacetamide; DFA, dehydrodiferulic acid(s); 5-5-DFA, 5-5-dehydrodiferulic acid; 8-*O*-4-DFA, 8-*O*-4-dehydrodiferulic acid; FS, fecal suspension; TMS, trimethylsilyl.

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