JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Interactions of a Lignin-Rich Fraction from Brewer's Spent Grain with Gut Microbiota in Vitro

Piritta Niemi,*[,†] Anna-Marja Aura,[†] Johanna Maukonen,[†] Annika I. Smeds,[‡] Ismo Mattila,[†] Klaus Niemelä,[†] Tarja Tamminen,[†] Craig B. Faulds,[†,§] Johanna Buchert,[†] and Kaisa Poutanen[†]

[†]Bio and Process Technology, VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT Espoo, Finland

[‡]Laboratory of Wood and Paper Chemistry, Process Chemistry Centre, Åbo Akademi University, Porthansgatan 3, FI-20500 Turku, Finland

ABSTRACT: Lignin is a constituent of plant cell walls and thus is classified as part of dietary fiber. However, little is known about the role of lignin in gastrointestinal fermentation. In this work, a lignin-rich fraction was prepared from brewer's spent grain and subjected to an in vitro colon model to study its potential bioconversions and interactions with fecal microbiota. No suppression of microbial conversion by the fraction was observed in the colon model, as measured as short-chain fatty acid production. Furthermore, no inhibition on the growth was observed when the fraction was incubated with strains of lactobacilli and bifidobacteria. In fact, the lignin-rich fraction enabled bifidobacteria to survive longer than with glucose. Several transiently appearing phenolic compounds, very likely originating from lignin, were observed during the fermentation. This would indicate that the gut microbiota was able to partially degrade lignin and metabolize the released compounds.

KEYWORDS: *brewer's spent grain, dietary fiber, lignin, lignan, in vitro fermentation, colon microbiota, lactobacilli, bifidobacteria*

INTRODUCTION

Cereal dietary fiber (DF) is commonly known to provide several benefits for human health, including reduced risk of obesity, cardiovascular disease, and type 2 diabetes.[1] DF consists mainly of the carbohydrates resistant to human digestive enzymes, such as arabinoxylan and β-glucan, but other noncarbohydrate compounds, for example, lignin, are also included in DF. According to the European Union (EU) definition, lignin is included as a component of DF when it remains closely associated with the original plant polysaccharides.[2] Lignin is a polymeric and phenolic compound acting as the glue between the cellulose−hemicellulose matrix in plant cell walls. It is formed from three monomers: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Figure 1), which are linked together in a branched network structure by radical-induced condensation reactions. Depending on the plant origin, lignins vary in monomer ratios and in the types of linkages between the monomers. The most typical linkage between the monolignol units is the β-arylether (β-O-4) linkage (Figure 1D). Other types of linkages include phenylcoumaran (β-5), biphenyl (5-5′), diarylpropane (β-1), α-arylether (α-O-4), diarylether (4-O-5), and pinoresinol (β-β) linkages.[3] Wood lignin has been widely studied, but very little is known about cereal lignin. However, the presence of lignin in cereal grains has been demonstrated.[4] Cereal lignin differs from wood lignin in the monomer composition and by having ferulate cross-links to carbohydrates.

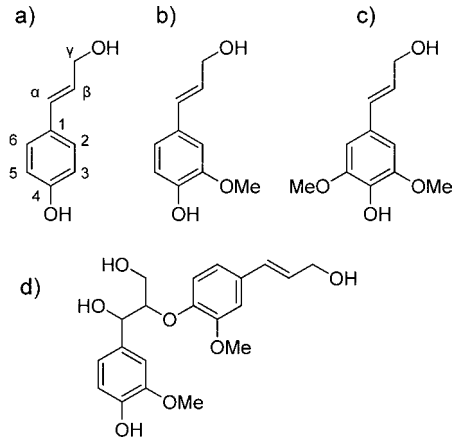

Figure 1. Lignin monomers: (a) *p*-coumaryl alcohol; (b) coniferyl alcohol; (c) sinapyl alcohol; (d) β-O-4 linked dilignol structure.

Lignin is generally considered to be an inert compound in the human gastrointestinal tract and resistant to the metabolic activities of gut microbiota.[5] In in vitro studies, fiber fractions high in lignin have been shown to be less extensively fermented by gut microbiota than low lignin fibers.[6] As lignin in cereals is covalently linked to carbohydrates,[4] it causes steric hindrance for carbohydrate-degrading enzymes and could hinder the fermentation of lignin-rich DF. Lignin can also adsorb enzymes, such as cellulases, and thus inhibit their action.[7] Nevertheless, some wood-feeding insects including termites have the ability to digest lignin.[8] There is also some evidence of lignin degradation in animals such as goats, cows, and dogs.[9,10] A few studies demonstrating lignin digestion in humans have been reported,[11,12] which contradicts the common conception of the inertness of lignin. Biodegradation of lignin by white rot fungi is better known,[13] and there is also evidence of lignin degradation by soil bacteria.[14] However, the interactions between lignin and

Received: April 19, 2013
Revised: June 13, 2013
Accepted: June 15, 2013
Published: June 15, 2013

human colon microbiota have hardly been studied. Although lignin has been consumed in the human diet since ancient times, its possible degradation in the human gastrointestinal tract and its effects on the gastrointestinal microbiota have not been adequately elucidated.

Because lignin is a polymer, it is not absorbed but remains in the gut lumen and could interact with dietary components or have effects on gut microbiota or its conversion activities as has been demonstrated for isolated apple and grape tannins.[15,16] Isolated condensed tannins inhibit DF fermentation, which can be monitored as a formation of short-chain fatty acids (SCFA).[15] Tannins have been shown to bind proteins and thus inhibit enzymes,[17] which may explain the suppression of SCFA formation.

The aim of this study was to investigate if a lignin-rich fraction from brewer's spent grain (BSG) is degraded in an in vitro human colon metabolic model with concurrent release and conversion of low molecular weight lignin-like components. The fraction was extracted from BSG by enzymatic methods as described previously[18] and contained a low amount of carbohydrates in relation to lignin, which in all likelihood makes the lignin more accessible for the microbiota than as part of an intact cell wall. The second aim was to investigate if the lignin-rich fraction is inhibitory to carbohydrate degradation in the in vitro colon model and if the fraction has an effect on the growth of lactobacilli and bifidobacteria.

MATERIALS AND METHODS

Material. The lignin-rich fraction was prepared from BSG as described previously.[18] In brief, BSG was first treated with carbohydrases and then with an alkaline protease. The hydrolysate of the proteolytic treatment was acidified to produce a precipitate, which was called protease-alkaline extracted fraction (P-AE fraction). The composition of the fraction was 4.0% carbohydrates, 24% lignin, 27% protein, 40% lipids, and 4.5% ash.[18] For the studies with lactobacilli and bifidobacteria, lipids were removed by extracting the material with heptane for 5 h in a Soxhlet apparatus.

Chemicals. In the targeted analysis *trans*-2-hydroxycinnamic acid (Aldrich, St. Louis, MO, USA) was used as the internal standard. In addition, the following compounds were quantitated using the same compounds for external standards: 3-hydroxybenzoic acid, 3-(4′-hydroxyphenyl)propionic acid, 3-(3′,4′-dihydroxyphenyl)propionic acid (Aldrich, Steinheim, Germany); 4-hydroxybenzoic acid, 2-(3′-hydroxyphenyl)acetic acid, 2-(3′,4′-dihydroxyphenyl)acetic (Sigma, St. Louis, MO, USA); 3-phenylpropionic acid, 3,4-dihydroxybenzoic acid (Fluka, Buchs, Switzerland); 3-(3′-hydroxyphenyl)propionic acid (Alfa Aesar, Karlsruhe, Germany); 4-methylcatechol (Aldrich, Steinheim, Germany); vanillic acid (Fluka); *p*-coumaric acid (Sigma); gallic acid (Extrasynthèse, Genay, France); and ferulic acid (Sigma-Aldrich, St. Louis, MO, USA). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (Sigma) and methoxyamine (Thermo Scientific, Bellefonte, PA, USA) were used as the derivatization reagents. The standards used in SCFA analysis were acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and heptanoic acid (internal standard) (Fluka Analytical, Buchs, Switzerland). Deuterated lignans (matairesinol-d_6, secoisolariciresinol-d_6, enterolactone-d_6, and dimethylated pinoresinol-d_6) used as internal standards in the lignan analysis were prepared at the Laboratory of Organic Chemistry at Åbo Akademi University as described previously.[19,20]

Fermentation of the P-AE Fraction in the Colon Model. In vitro fermentation was performed as described previously,[21] with minor modifications. Human feces were collected from five healthy volunteers, who had not received antibiotics within the previous 3 months. The donors of the feces were not given any limitations regarding their diets prior to the feces collection. Freshly passed feces were immediately placed in an anaerobic chamber, pooled, and homogenized (2 min) with the culture medium using a Waring blender (18000 rev/min). The slurry was diluted to 20.8% (w/v) by adding the culture medium, filtered through a 1 mm sieve, placed on ice, and used immediately as an inoculum in the experiment. Two hundred milligrams of the P-AE fraction was weighed into bottles (50 mL) and suspended with 2 mL of the culture medium 1 day before incubation to reduce the lag in fermentation rate due to the hydration of the fraction. Prehydrated P-AE fractions were inoculated with 8 mL of fecal suspension, and a 16.7% (w/v) final concentration of fresh fecal matter was obtained. Bottles were tightly closed and incubated in a water bath at 37 °C under agitation (250 rev/min) for 0, 2, 4, 6, 8, and 24 h. Fecal controls were similarly produced except that they did not contain any P-AE fraction. Fermentations were carried out in triplicate. After the incubation, the fermentation samples were rapidly cooled using an ice water bath, and the pH was measured. They were then rapidly frozen using liquid nitrogen and stored at −20 °C for further analyses. Averages and standard deviations were calculated from triplicate measurements at each time point.

Extraction of Fermentation Samples. Extraction of SCFA to diethyl ether was carried out as described previously.[22] For phenolic compound, lignan, and metabolome analyses 1 mL of the fermented sample was thawed. One milliliter of 2% NaCl solution was added to salt out the lipids contained within the P-AE fraction. Fifty microliters of 6 M HCl was added to lower the pH to nearly 1. For the analysis of phenolic compounds and the metabolome 15 μL of internal standard (123 mg/L in MeOH) was added to the fermented samples, which were then extracted twice with 3 mL of ethyl acetate. The organic phases were collected, combined, and evaporated under nitrogen. For lignan extraction, 20 μL of an ethyl acetate solution containing the internal standards matairesinol-d_6 (430 ng), secoisolariciresinol-d_6 (524 ng), enterolactone-d_6 (316 ng), and dimethylated pinoresinol-d_6 (756 ng) was added to 1 mL of the fermented samples, which were then extracted twice with methanol (3 mL), and the liquid phases were combined. The methanol−water mixture was evaporated under nitrogen stream. The dried samples were stored under a nitrogen atmosphere at −20 °C until analyzed.

SCFA Analysis. Diethyl ether extracts (2 μL, splitless injection) were analyzed with gas chromatography with a flame ionization detector (GC/FID) (Agilent 6890 Series, Palo Alto, CA, USA). Analytes were separated on a DP-FFAP capillary column (30 m × 0.32 mm) with a phase thickness of 0.25 μm (Agilent). Helium was used as the carrier gas at 2.7 mL/min. Both the injector and FID were kept at 250 °C. The temperature program started at 50 °C with 3 min of holding time, then increased at 25 °C/min to 100 °C, and finally increased at 10 °C/min to the final temperature of 240 °C, which was maintained for 10 min. Compounds were quantitated with corresponding standards.

Metabolomics. The analysis was performed as described previously[15] using a two-dimensional gas chromatograph coupled with a time-of-flight mass detector (GC×GC-TOFMS). Sample derivatization was done automatically by Gerstel MPS autosampler and Maestro software. The data processing of GC×GC-TOFMS responses has been described earlier.[15] Briefly, the peaks were identified by ChromaTOF software, which matches deconvoluted spectra against the NIST05 mass spectral library. The compounds in different data sets were aligned and normalized using an in-house developed software, Guineu,[23] for further analyses. Alignment of the data was performed on the basis of retention indices, second-dimension retention times, and spectra.

The GOLM Metabolome Database (GMD)[24] and the Guineau program[23] were utilized for second-stage identification of those compounds that lacked spectral matches from the NIST05 or in-house collected libraries. GMD allows searching of the database on the basis of submitted GC-MS spectra, retention indices, and mass intensity ratios. In addition, the database allowed a functional group prediction, which helped characterize the unknown metabolites without available reference mass spectra in the GMD.

The visualization was performed by calculating 2-based logarithmic fold-changes of the relative peak areas from GC×GC-TOFMS analysis against the fecal control. The profile of the individual metabolite was visualized as color intensities (red as overexpression and blue as

underexpression) and the time point specific significances (*t* test *p*-values) as asterisks against the corresponding control. The nontargeted metabolite profiling was semiquantitative. The names of the overexpressed metabolites were verified by comparing the mass spectra with those found in GMD, and the unkowns were named according to the group specifications and displayed in the final heat maps. Clustering of the metabolites was performed according to the similarity of the time profiles.

Lignans. To the dry fermentation methanol extracts was added 1.5 mL of 10 mM sodium acetate buffer (pH 5.0), and the solutions were sonicated for 1−2 min or until the material was completely dissolved. The solutions were then centrifuged for 15 min, and the supernatant was carefully removed and then centrifuged again for 15 min. The clear supernatant was solid-phase extracted using Oasis HLB 30 mg cartridges (Waters Corp., Milford, MA, USA) according to a previously described method.[20] After evaporation of the solvent to dryness, 200 μL of methanol/0.1% acetic acid in Milli-Q water (20:80, v/v) was added, the solution was sonicated for 1−2 min, and 10−20 μL was injected into the HPLC-MS/MS. The HPLC-MS/MS method and conditions were the same as described previously.[25] Quantitation was carried out using standard solutions containing the internal standards and six concentration levels of the analyzed lignans, as described previously.[25] The standard solutions were solid-phase extracted and redissolved as the real samples.

Studies with Lactobacilli and Bifidobacteria. To evaluate possible microbiological impurities within the P-AE fraction after removal of lipids, 2% heptane extracted P-AE fraction was anaerobically incubated for 3 days at 37 °C in de Man−Rogosa−Sharpe (MRS) medium (Oxoid, Basingstoke, UK), Reinforced Clostridial Medium (RCM) (Difco, Franklin Lakes, NJ, USA), and *Bifidobacterium* medium (DSMZ medium 58; www.dsmz.de). After incubation, the optical density (OD) and pH of each medium were measured. The media containing the fraction were serially diluted in prereduced peptone saline containing 0.5 g of L-cysteine/HCl (pH 6.3; Merck, Darmstadt, Germany) and plated on corresponding solid culture medium (i.e., MRS medium to MRS agar) in an anaerobic workstation.

Four lactobacilli strains (*Lactobacillus rhamnosus* VTT E-97800, *L. rhamnosus* VTT E-97948, *Lactobacillus paracasei* VTT E-97949, and *Lactobacillus salivarius* VTT E-981006) and three bifidobacteria strains (*Bifidobacterium adolescentis* VTT E-981074, *Bifidobacterium breve* VTT E-981075, and *Bifidobacterium longum* VTT E-96664) were used to evaluate the potential of the lignin-rich fraction as a bacterial growth substrate. The following culture media were utilized (incubation at 37 °C): (1) unmodified MRS medium as a positive control medium for lactobacilli; (2) MRS medium without carbohydrates and the fraction as a negative control medium for lactobacilli; (3) MRS medium without carbohydrates as a basal medium with 0.5, 1.0, or 2.0% (w/w) addition of the fraction as test media for lactobacilli; (4) unmodified MRS medium as a basal medium with 0.5, 1.0, or 2.0% (w/w) addition of the fraction as extra media for lactobacilli; (5) unmodified *Bifidobacterium* medium as a positive control medium for bifidobacteria; (6) *Bifidobacterium* medium without carbohydrates and the fraction as a negative control medium for bifidobacteria; (7) *Bifidobacterium* medium without carbohydrates as a basal medium with 0.5, 1.0, or 2.0% (w/w) addition of the fraction as test media for bifidobacteria. Bacterial growth was monitored using OD and pH. In addition, the bacterial suspensions containing 1% of the P-AE fraction were serially diluted as described above and plated on MRS agar (lactobacilli) or RCM agar (bifidobacteria). The plates were incubated in Anoxomat WS8000 anaerobic jars (Mart Microbiology, Lichtenvoorde, The Netherlands) containing 10:5:85 $H_2/CO_2/N_2$ for 3 days (lactobacilli and bifidobacteria) and 7 days (bifidobacteria) at 37 °C.

Statistical Analyses. For statistical analyses of the quantitated phenolic compounds and SCFA, the responses were measured in triplicates, and two-way ANOVA with repeated measures using a Bonferroni adjustment was used to test significance ($p < 0.05$) between the fraction and the control. The statistics were performed with using MatLab Version R2008b. Two-way ANOVA was also performed for nontargeted GC×GC-TOFMS data as described previously.[15] Significantly different response levels between lignin fraction and the fecal control within a time point are indicated with asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

RESULTS

Interactions of the Lignin-Rich Fraction with Fecal Microbiota. *SCFA Formation.* The conversion of carbohydrates to SCFA was measured to determine if the lignin-rich P-AE fraction suppressed the fermentation of remaining carbohydrates from the fraction and fecal suspension, expressed as SCFA formation (Figure 2). No suppression of SCFA formation was observed in the presence of the fraction. In the beginning of the fermentation, acetic acid, which was the most abundant SCFA, was formed more rapidly from the P-AE fraction than from the fecal suspension, but toward the end of the fermentation the formation rate decreased, and at the end the concentration of acetic acid in the fecal control reached the same level as in the P-AE fraction. Propionic and butyric acids were constantly produced in higher concentrations in the P-AE fraction than in the fecal control. All data points were found to be significantly different from the fecal control ($p < 0.05$) with the *t* test, except for the 24 h point for acetic acid.

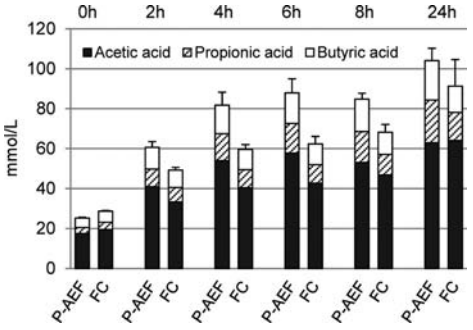

Figure 2. Concentrations of linear short-chain fatty acids formed during in vitro fermentation. FC, fecal control; P-AEF, protease-alkaline extracted fraction. The error bar is the combined standard deviation of the three fatty acids.

Effects of the Lignin-Rich Fraction on Lactobacilli and Bifidobacteria. No bacterial growth was observed after incubation of 2% (delipidated) P-AE fraction in MRS, RCM, or *Bifidobacterium* medium. The effect of the fraction on growth medium pH was first evaluated to assess the possibility of using pH as a measurement of growth. All of the concentrations of the fraction decreased the pH of the medium, independent of bacteria, during the 2 day incubation period. The pH in MRS medium with 0.5% fraction inoculated with lactobacilli was 6.1−6.2, depending on the lactobacilli strain used, whereas the pH in MRS medium with 0.5% fraction and no bacterial inoculum was also 6.1. In addition, the P-AE fraction concentration affected the pH; the higher the concentration of the fraction, the lower the pH. With 0.5% concentration of the P-AE fraction, the pH of MRS medium was 6.1, whereas with 2% concentration the pH was 5.5. Therefore, pH was not further used for growth measurements.

The effect of the P-AE fraction in the OD values was also evaluated. The addition of the fraction increased the OD, independently of bacteria. The ODs of 0.5, 1, and 2% PA-E fraction in MRS medium without the addition of other carbohydrates were 0.110, 0.140, and 0.180, respectively. The ODs of the same medium with the fraction inoculated with *L. salivarius* did not differ from the OD of the P-AE fraction,

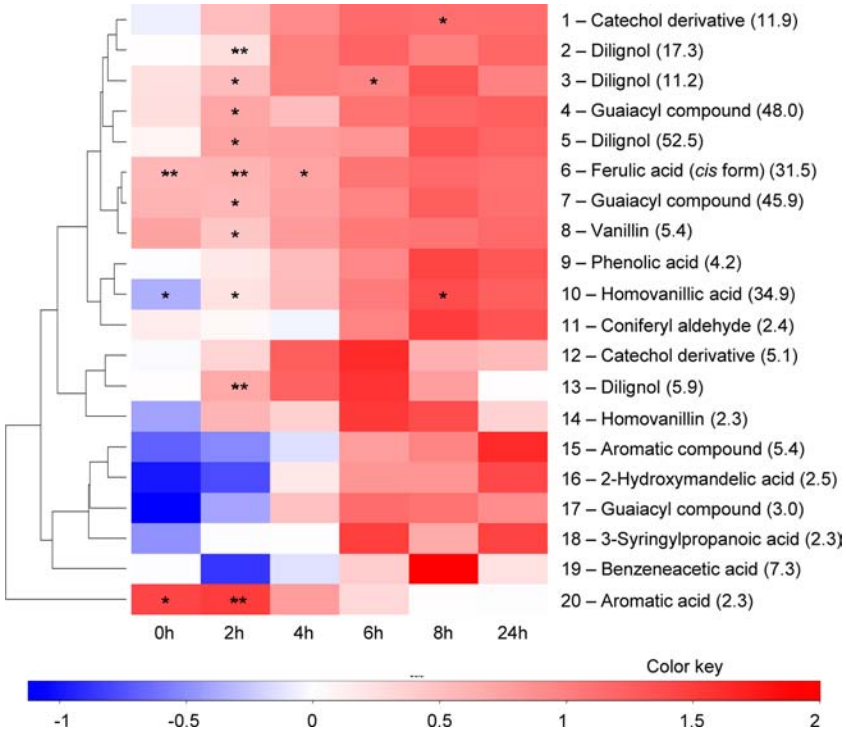

Figure 3. Heat map of the 20 aromatic metabolites from the metabolome. The number after the name in parentheses is the FC of the compound. The color key is the 2-log value of the FC. A blue color indicates an underexpression and red color represents an overexpression of the metabolite compared to the fecal control.

suggesting that *L. salivarius* did not grow on the P-AE fraction as the only substrate. Better growth was observed with the other lactobacilli, and the average ODs of the other lactobacilli with 0.5, 1, and 2% addition of the fraction were 0.220, 0.270, and 0.340, respectively. When basal MRS (including carbohydrates) enriched with the P-AE fraction was used for the incubation of lactobacilli, the OD was significantly higher (1.250−1.500) in the lactobacilli-inoculated media than in the medium without lactobacilli (OD = 0.180−0.300).

The culture-based studies indicated that the growth of *L. rhamnosus* strains in medium supplemented with 1% P-AE fraction (8.0−8.1 log units) was similar to the growth in the glucose-supplemented medium (8.2−8.3 log units). The colony counts of *L. paracasei* were slightly lower in 1% P-AE fraction supplemented medium (7.8 log units) than in the glucose-supplemented medium (8.2 log units). The colony counts of *L. salivarius* were 1.5 log units lower in the medium supplemented with 1% P-AE fraction than in the glucose-supplemented medium. The bifidobacterial colony counts after 3 days of incubation in the medium supplemented with 1% P-AE fraction were similar to the ones in the glucose-supplemented medium (7.5−8.2 log units, depending on the strain). However, after 7 days of incubation, the colony counts of *B. adolescentis*, *B. breve*, and *B. longum* in the medium supplemented with 1% P-AE fraction were 6.3, 7.1, and 6.5 log units, respectively, whereas the colony counts in the glucose-supplemented medium were below 2 log units after 7 days.

Metabolite Formation in the in Vitro Colon Model. *Metabolomics.* The formation of metabolites from the P-AE fraction in the in vitro colon model fermentation was followed over 24 h, and approximately 1800 metabolites were discovered. The metabolites were filtered according to the difference in responses between the fraction and the fecal control, that is, the fold-change (FC). FC was calculated as average from all six time points. Metabolites with FC < 2 were excluded. There were 699 metabolites with FC > 2, and from those the compounds with structural similarity to lignin-derived components were selected. With these criteria, the total number of compounds was narrowed down to 26. On the basis of the mass spectra, as many as 20 of them turned out to be aromatic in nature; they are presented in the heat map with their FC values (Figure 3). Several of the identified metabolites showed low response in the beginning of the fermentation. However, toward the end of the fermentation the response of most compounds was intensified, displayed as a deeper red color in the heat map.

Several monomeric compounds could be fully identified using a commercial library and extensive collection of literature data.[26,27] These included several typical lignin-type compounds with guaiacyl (4-hydroxy-3-methoxyphenyl) structure (compounds **8**, **10**, **11**, and **14**) and one with syringyl (4-hydroxy-3,5-dimethoxyphenyl) structure (compound **18**). In addition, the mass spectra of several other compounds (**4**, **7**, and **17**) showed certain features characteristic of guaiacyl derivatives. Two of them (**17** and **7**) are clearly homologous compounds, with molar masses of 414 and 428 (as TMS derivatives). Despite that and the presence of intense *m*/*z* 209 ions, their final structures are still unknown. Compound **4** represents a 4-hydroxy-3-methoxybenzyl alcohol derivative (as shown by the base peak at *m*/*z* 297), but the molar mass could not be reliably determined. In addition to the above lignin-type monomeric compounds, several other aromatic monomers (**1**, **9**, **12**, **15**, **16**, **19**, and **20**) were also found. Some of them could be characterized only as catechol derivatives. Compound **15** represents 3,4-dihydroxycinnamyl alcohol with high probability.

Several late-eluting (retention indices >2700) aromatic compounds, dilignols or lignans, were also found. Without more detailed discussions, it can be said that in most cases their

dx.doi.org/10.1021/jf401738x | J. Agric. Food Chem. 2013, 61, 6754−6762

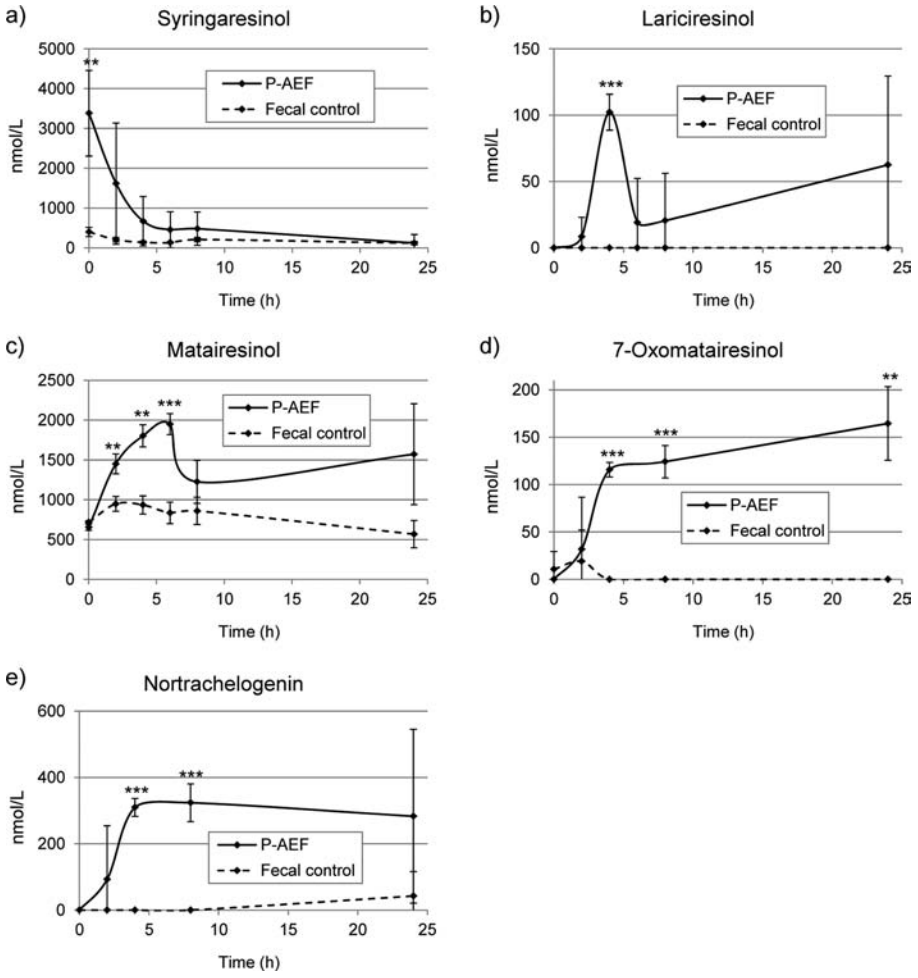

Figure 4. Concentrations of lignans during in vitro fermentation: (a) syringaresinol; (b) lariciresinol; (c) matairesinol; (d) 7-oxomatairesinol; (e) nortrachelogenin. P-AEF, protease-alkaline extracted fraction.

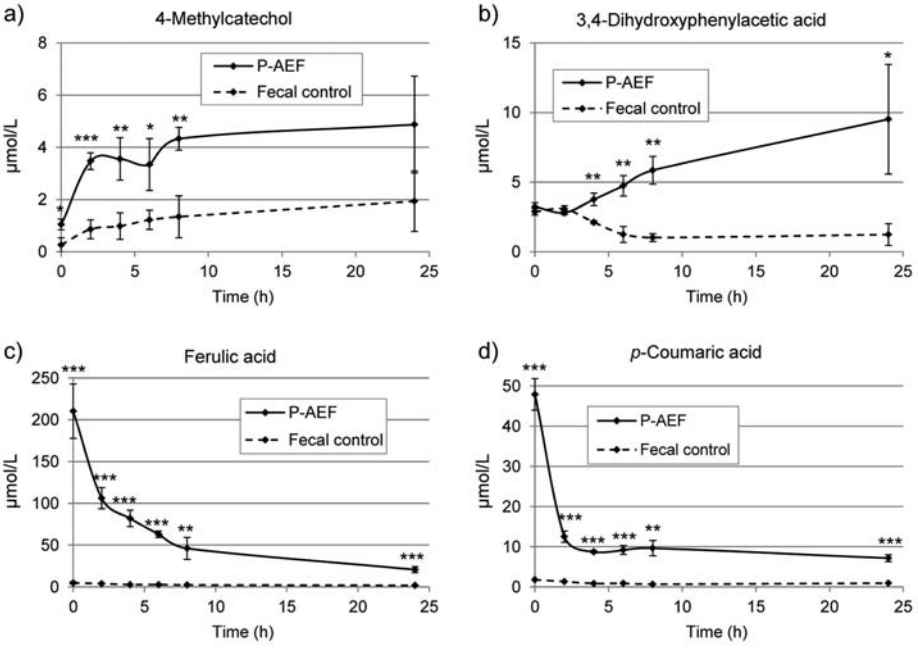

Figure 5. Concentrations of phenolic compounds during the in vitro fermentation: (a) 4-methylcatechol; (b) 3,4-dihydroxyphenylacetic acid; (c) ferulic acid; (d) *p*-coumaric acid. P-AEF, protease-alkaline extracted fraction.

dx.doi.org/10.1021/jf401738x | *J. Agric. Food Chem.* 2013, 61, 6754−6762

mass spectra exhibited intense m/z 179, 209, 267, or 297 ions, indicating the presence of guaiacyl or catechol structures. Their molar masses (as the TMS derivatives) could not always be reliably determined, but several peaks in the m/z range from 500 to 650 were usually found.

Lignans. The concentrations of several lignans, analyzed by HPLC-MS/MS, increased in the course of the fermentation of the lignin-rich fraction (Figure 4). Nortrachelogenin was formed in quantities many times higher than in the fecal control. Matairesinol and its derivative 7-oxomatairesinol also showed significant increase compared to the control. Lariciresinol was above the control, but there were large variations between the replicates. However, as lariciresinol was always below the detection limit in the control, it was considered a metabolite and not originating from the fecal matrix. Enterolactone and its derivative 7-hydroxyenterolactone levels were below the control, and enterodiol formation was somewhat above the control, but there were large variations between the replicates (data not shown). Cyclolariciresinol, pinoresinol, syringaresinol, and α-conidendrin seemed to be present already in the P-AE fraction, because their concentrations were significantly higher in the beginning of the fermentation compared to fecal control, so they were not considered metabolites, and secoisolariciresinol seemed to originate from the fecal background (data not shown except for syringaresinol).

Phenolic Compounds. A number of different phenolic compounds were identified and quantitated, from which 4-methylcatechol and 3,4-dihydroxyphenylacetic acid differed significantly from control (Figure 5). The concentrations of both ferulic acid and *p*-coumaric acid declined rapidly during the fermentation. Other detected phenolic acids did not differ significantly between the P-AE fraction and the fecal control and thus were not considered metabolites from the P-AE fraction.

DISCUSSION

BSG is the most abundant side-stream generated by the brewing industry. It contains the hulls and outer layers of the barley kernels, and it is thus rich in cellulose, arabinoxylan, lignin, and protein. A lignin-rich fraction was prepared from BSG by enzymatic methods as described previously,[18] by precipitation of the liquid phase after an alkaline protease treatment carried out on carbohydrase-pretreated BSG. The fraction, named the protease-alkaline extracted fraction, was considered lignin-rich compared to commonly consumed foods, even though its lignin content was no higher than 24%. In addition, the insoluble residue after the protease treatment contained more lignin (34%)[18] than the P-AE fraction; nevertheless, the P-AE fraction was used for the in vitro studies, because the lignin in it was considered more accessible for the microbiota and enzymes as it was not trapped inside a carbohydrate network. It can be assumed that lignin in the P-AE fraction is not as much cross-linked to carbohydrates as lignin in the insoluble residue, which contains the most recalcitrant carbohydrates. For the experiments with lactobacilli and bifidobacteria, lipids were removed from the fraction to avoid giving the bacteria an alternative carbon source, which increased the lignin content of the fraction to 41%.

The carbohydrates that are resistant to human digestive enzymes are fermented by colon microbiota to SCFA, which are major metabolites in the colon. It is possible to use the SCFA formation as an indicator in determining whether or not the fermented substrate suppresses the conversion activity of the microbiota, as shown earlier.[15] As seen in Figure 2, the concentrations of linear SCFA were higher in the P-AE fraction than in the fecal control, suggesting that the lignin in the P-AE fraction did not suppress microbial metabolism. The small increase in SCFA production was most likely due to the residual carbohydrates present in the fraction. The production of SCFA in the colon is desirable, as, for example, butyric acid is the major energy source for colon epithelial cells and may help to protect against colon cancer.[28] Propionic acid is also associated with beneficial health effects, such as lower lipogenesis and serum cholesterol.[29] Therefore, a diet high in slowly fermentable DF is necessary for the health and well-being of humans and especially of the gut. It is also worth pointing out that according to these results, even the very high ratio of lignin to carbohydrates (6:1) did not prevent microbes from fermenting the carbohydrates present in the P-AE fraction to SCFA.

The lignin-rich fraction as a growth substrate did not inhibit the growth of lactic acid bacteria and bifidobacteria, which are regarded as beneficial bacteria in the human colon. Moreover, it seemed that some component of the fraction enabled a longer growth of bifidobacteria, because after 7 days of incubation, there was still growth in bifidobacterial cultures with 1% lignin preparation as the sole carbohydrate source (10^7−10^8 cfu/mL), whereas with glucose the numbers of bifidobacteria were below 10^2 cfu/mL. A similar protective effect on bifidobacteria has been previously observed with oat fiber preparations.[30] The carbohydrate content of solvent-extracted P-AE fraction was only 6.7%, but there was also 45% of proteinaceous material. There is not much information in the literature regarding proteolytic abilities of bifidobacteria. Most of the studies are related to dairy products and report only limited proteolytic activity of bifidobacteria toward dairy proteins.[31] The effect of cereal proteins on bifidobacterial growth is not well studied, and therefore it is difficult to estimate if bifidobacteria could survive on the peptides extracted from BSG. Nevertheless, some component in the P-AE fraction kept the bifidobacterial cells alive longer than an equal amount of glucose.

The in vitro colon model used in this investigation is a metabolic model designating biochemical changes in introduced components due to their metabolism by the fecal microbiota, which is pooled and collected from several donors and used as an inoculum. This model has been used in biochemical conversions of carbohydrates, phenolic components, and pharmaceuticals detected by targeted GC-MS and nontargeted GC×GC-TOFMS methods as described in previous studies.[15,32] The batch model is not suitable for studies concerning changes in microbial composition, because the incubation time and batch design are not sufficient, but changes in microbial metabolites depend only on the components in the studied substrates and are not due to changes of the microbiota. Therefore, the model is well suited for the detection of the release of low molecular weight aromatic compounds from lignin.

The major phenolic compounds in BSG are *p*-coumaric acid, ferulic acid and its dimers,[33] and lignin. As the possible degradation of lignin was studied, low molecular weight phenolic compounds were the most searched metabolites. Lignin is generally considered to be an inert material in the colon. However, in this study several metabolites, which very likely originated from lignin, were identified (Figure 3). For some of the metabolites the exact structure could not be

determined, and therefore they were indicated with a group-specific name only. The possibility of some of the metabolites originating from phenolic precursors other than lignin, such as ferulic acid, cannot be excluded. However, the metabolism of ferulic acid in the gut is well-known; the main metabolite from monomeric ferulic acid is 3-(3′,4′-dihydroxyphenyl)propionic, and in addition 3′,4′-dihydroxyphenylacetic acid is formed from 8-O-4-linked diferulate with homovanillic and dihydroferulic acids as intermediates.[34] Approximately two-thirds of the ferulic acid in BSG occurs in monomeric form and one-third as different types of dimers.[33]

Different types of guaiacyl compounds and dilignols with FC as high as around 50 were detected, but, on the other hand, many of the possible lignin-derived compounds also had lower FC, such as vanillin (5.4), coniferyl aldehyde (2.4), and 3-syringylpropanoic acid (2.3) (Figure 3). The latter is especially interesting, as it contains two methoxyl groups and thus cannot be formed from ferulic acid, which has only one methoxyl group. The release of these low molecular weight phenolic compounds would indicate that partial degradation of lignin could have occurred due to microbial action. As the metabolite analysis is semiquantitative, it is difficult to estimate the amount of the released phenolic metabolites, but it was assumed to be very limited as indicated by the low FCs. The late appearance of many of the lignin-related metabolites also suggests that the release of these compounds occurs slowly. This would seem reasonable and in line with the idea that lignin is a difficult substrate to be degraded by the colon microbiota.[5,6] However, these results should be considered as preliminary and indicative, because to have solid proof of lignin degradation by gut microbiota, isotope-labeled lignin should be used as the substrate.

Several lignans have been previously identified in the extracted P-AE fraction.[18] Lignans are low molecular weight phenolic compounds found in plants, and they are commonly known to have several health-promoting properties, such as antitumor and antioxidant activities.[35] The structure of lignans consists of two β-β-linked phenylpropan units with various hydroxyl and methoxyl substitution patterns in the aromatic rings and other structural modifications. Structures of some lignans, for example, pinoresinol and lariciresinol, exist in lignin,[36] and therefore it is possible that lignans or lignan-like compounds could be released from lignin as a result of microbiological activity, as shown by Begum et al.[36] In this study several lignans were released or formed from the P-AE fraction at significantly higher concentrations than from the fecal control. Syringaresinol is the most abundant lignan in the P-AE fraction,[18] and according to Heinonen et al.[37] it is converted to several other metabolites, and only to a low extent to enterodiol and enterolactone. In this study syringaresinol was shown to disappear during the incubation. Nortrachelogenin is the second most abundant lignan in the P-AE fraction,[18] but it seemed to be incorporated into the matrix and released by microbial activity over time. 7-Oxomatairesinol was not detected in the fraction, but was probably converted from 7-hydroxymatairesinol. It is possible that a part of the lignans could have been released from lignin structure and a part would have been converted from other lignans, as the gut microbiota is capable of releasing and transforming lignans.

Potential health effects on digestion have been suggested to arise from lignin. Lignin has been shown to be a precursor of the mammalian lignans enterodiol and enterolactone,[36] higher concentrations of which have been associated with a lower risk of cancers and cardiovascular disease.[38,39] Lignin-enriched DF can adsorb carcinogenic compounds in the colon,[40,41] and the adsorption of the carcinogens by lignin may prevent their absorption from the gut into circulation and thus reduce the risk of cancer.[41] Insoluble fiber is known to deliver antioxidative, phenolic compounds into the gastrointestinal tract,[42] and due to its phenolic structure, lignin also possesses such antioxidative and radical scavenging activity in the lumen.[43]

In conclusion, a lignin-rich fraction originating from BSG did not suppress the conversion activity of gut microbiota in an in vitro colon metabolic model. Neither did it inhibit the growth of beneficial gut bacteria, lactobacilli and bifidobacteria. Moreover, some component in the P-AE fraction enabled the growth of bifidobacteria for a longer time than glucose. Several low molecular weight, lignin-like phenolic metabolites were formed during the fermentation, which suggests partial degradation of lignin in the colon. Most likely, the release of these metabolites occurs only slowly and to a limited extent. More research is needed to confirm the origin of these compounds as other analogous compounds were also present in the P-AE fraction.

AUTHOR INFORMATION

Corresponding Author

*(P.N.) Phone: +358 207 227 558. Fax: +358 207 227 071. E-mail: piritta.niemi@vtt.fi.

Present Address

[§]Laboratoire de Biotechnologie des Champignons Filamenteux, INRA-Université Aix Marseille, 163 Avenue de Luminy, 13288 Marseille, France.

Funding

Financial support from the Academy of Finland is gratefully acknowledged.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We acknowledge Maria Saarela and Hanna-Leena Alakomi for their valuable input in the planning of the lactobacilli and bifidobacteria experiments. We thank Kaarina Viljanen for supervising the SCFA analysis. We also thank Niina Torttila, Marja-Liisa Jalovaara, Siv Matomaa, Annika Majanen, Airi Hyrkäs and Liisa Änäkäinen for technical assistance.

REFERENCES

(1) Smith, C. E.; Tucker, K. L. Health benefits of cereal fibre: a review of clinical trials. *Nutr. Res. Rev.* **2011**, *24*, 118−131.

(2) Commission Directive 2008/100/EC. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:285:0009:0012:EN:PDF (accessed Feb 20,2013).

(3) Vanholme, R.; Demedts, B.; Morreel, K.; Ralph, J.; Boerjan, W. Lignin biosynthesis and structure. *Plant Physiol.* **2010**, *153*, 895−905.

(4) Bunzel, M.; Ralph, J.; Lu, F.; Hatfield, R. D.; Steinhart, H. Lignins and ferulate−coniferyl alcohol cross-coupling products in cereal grains. *J. Agric. Food Chem.* **2004**, *52*, 6496−6502.

(5) Holloway, W. D.; Tasman-Jones, C.; Lee, S. P. Digestion of certain fractions of dietary fiber in humans. *Am. J. Clin. Nutr.* **1978**, *31*, 927−930.

(6) Garleb, K. A.; Fahey, G. C.; Lewis, S. M.; Kerley, M. S.; Montgomery, L. Chemical composition and digestibility of fiber fractions of certain by-product feedstuffs fed to ruminants. *J. Anim. Sci.* **1988**, *66*, 2650−2662.

(7) Palonen, H.; Tjerneld, F.; Zacchi, G.; Tenkanen, M. Adsorption of *Trichoderma reesei* CBH I and EG II and their catalytic domains on steam pretreated softwood and isolated lignin. *J. Biotechnol.* **2004**, *107*, 65−72.
(8) Geib, S. M.; Filley, T. R.; Hatcher, P. G.; Hoover, K.; Carlson, J. E.; Jimenez-Gasco, M. d. M.; Nakagawa-Izumi, A.; Sleighter, R. L.; Tien, M. Lignin degradation in wood-feeding insects. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 12932−12937.
(9) Csonka, F. A.; Phillips, M.; Jones, D. B. Studies on lignin metabolism. *J. Biol. Chem.* **1929**, *85*, 65−75.
(10) Silanikove, N.; Brosh, A. Lignocellulose degradation and subsequent metabolism of lignin fermentation products by the desert black Bedouin goat fed on wheat straw as a single-component diet. *Br. J. Nutr.* **1989**, *62*, 509−520.
(11) Williams, R. D.; Olmsted, W. H.; Hamann, C. H.; Fiorito, J. A.; Duckles, D. The effect of cellulose, hemicellulose and lignin on the weight of the stool: a contribution to the study of laxation in man. *J. Nutr.* **1936**, *11*, 433−449.
(12) Kelsay, J. L.; Goering, H. K.; Behall, K. M.; Prather, E. S. Effect of fiber from fruits and vegetables on metabolic responses of human subjects: fiber intakes, fecal excretions, and apparent digestibilities. *Am. J. Clin. Nutr.* **1981**, *34*, 1849−1852.
(13) Leonowicz, A.; Matuszewska, A.; Luterek, J.; Ziegenhagen, D.; Wojtaś-Wasilewska, M.; Cho, N.; Hofrichter, M.; Rogalski, J. Biodegradation of lignin by white rot fungi. *Fungal Genet. Biol.* **1999**, *27*, 175−185.
(14) Bugg, T. D.; Ahmad, M.; Hardiman, E. M.; Singh, R. The emerging role for bacteria in lignin degradation and bio-product formation. *Curr. Opin. Biotechnol.* **2011**, *22*, 394−400.
(15) Aura, A.-M.; Mattila, I.; Hyötyläinen, T.; Gopalacharyulu, P.; Cheynier, V.; Souquet, J.; Bes, M.; Bourvellec, C.; Guyot, S.; Orešič, M. Characterization of microbial metabolism of Syrah grape products in an *in vitro* colon model using targeted and non-targeted analytical approaches. *Eur. J. Nutr.* **2012**, 1−14.
(16) Bazzocco, S.; Mattila, I.; Guyot, S.; Renard, C.; Aura, A.-M. Factors affecting the conversion of apple polyphenols to phenolic acids and fruit matrix to short-chain fatty acids by human faecal microbiota *in vitro*. *Eur. J. Nutr.* **2008**, *47*, 442−452.
(17) Scalbert, A. Antimicrobial properties of tannins. *Phytochemistry* **1991**, *30*, 3875−3883.
(18) Niemi, P.; Tamminen, T.; Smeds, A.; Viljanen, K.; Ohra-aho, T.; Holopainen-Mantila, U.; Faulds, C. B.; Poutanen, K.; Buchert, J. Characterization of lipids and lignans in brewer's spent grain and its enzymatically extracted fraction. *J. Agric. Food Chem.* **2012**, *60*, 9910−9917.
(19) Smeds, A. I.; Eklund, P. C.; Sjöholm, R. E.; Willför, S. M.; Nishibe, S.; Deyama, T.; Holmbom, B. R. Quantification of a broad spectrum of lignans in cereals, oilseeds, and nuts. *J. Agric. Food Chem.* **2007**, *55*, 1337−1346.
(20) Smeds, A. I.; Hakala, K.; Hurmerinta, T. T.; Kortela, L.; Saarinen, N. M.; Mäkelä, S. I. Determination of plant and enterolignans in human serum by high-performance liquid chromatography with tandem mass spectrometric detection. *J. Pharm. Biomed. Anal.* **2006**, *41*, 898−905.
(21) Barry, J.-L.; Hoebler, C.; MacFarlane, G. T.; MacFarlane, S.; Mathers, J. C.; Reed, K. A.; Mortensen, P. B.; Nordgaard, I.; Rowland, I. R.; Rumney, C. J. Estimation of the fermentability of dietary fibre *in vitro*: a European interlaboratory study. *Br. J. Nutr.* **1995**, *74*, 303−322.
(22) Schooley, D. L.; Kubiak, F. M.; Evans, J. V. Capillary gas chromatographic analysis of volatile and non-volatile organic acids from biological samples as the t-butyldimethylsilyl derivatives. *J. Chromatogr. Sci.* **1985**, *23*, 385−390.
(23) Castillo, S.; Mattila, I.; Miettinen, J.; Orešič, M.; Hyötyläinen, T. Data analysis tool for comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry. *Anal. Chem.* **2011**, *83*, 3058−3067.
(24) Golm metabolome database; http://gmd.mpimp-golm.mpg.de/search.aspx (accessed May 30, 2012).
(25) Smeds, A. I.; Eklund, P. C.; Willför, S. M. Content, composition, and stereochemical characterisation of lignans in berries and seeds. *Food Chem.* **2012**, *134*, 1991−1998.
(26) Niemelä, K.; Sjöström, E. Simultaneous identification of aromatic and aliphatic low molecular weight compounds from alkaline pulping liquor by capillary gas-liquid chromatography−mass spectrometry. *Holzforschung* **1986**, *40*, 361−368.
(27) Niemelä, K. Low-molecular-weight organic compounds in birch kraft black liquor. *Ann. Acad. Sci. Fenn., Ser. A II, Chem.* **1990**, *229*, 1−142.
(28) Comalada, M.; Bailón, E.; de Haro, O.; Lara-Villoslada, F.; Xaus, J.; Zarzuelo, A.; Gálvez, J. The effects of short-chain fatty acids on colon epithelial proliferation and survival depend on the cellular phenotype. *J. Cancer Res. Clin. Oncol.* **2006**, *132*, 487−497.
(29) Hosseini, E.; Grootaert, C.; Verstraete, W.; Van de Wiele, T. Propionate as a health-promoting microbial metabolite in the human gut. *Nutr. Rev.* **2011**, *69*, 245−258.
(30) Kontula, P.; Jaskari, J.; Nollet, L.; De Smet, I.; von Wright, A.; Poutanen, K.; Mattila-Sandholm, T. The colonization of a simulator of the human intestinal microbial ecosystem by a probiotic strain fed on a fermented oat bran product: effects on the gastrointestinal microbiota. *Appl. Microbiol. Biotechnol.* **1998**, *50*, 246−252.
(31) Janer, C.; Arigoni, F.; Lee, B. H.; Peláez, C.; Requena, T. Enzymatic ability of *Bifidobacterium animalis* subsp. *lactis* to hydrolyze milk proteins: Identification and characterization of endopeptidase O. *Appl. Environ. Microbiol.* **2005**, *71*, 8460−8465.
(32) Aura, A.-M.; Oikarinen, S.; Mutanen, M.; Heinonen, S.-M.; Adlercreutz, H. C. T.; Virtanen, H.; Poutanen, K. S. Suitability of a batch *in vitro* fermentation model using human faecal microbiota for prediction of conversion of flaxseed lignans to enterolactone with reference to an *in vivo* rat model. *Eur. J. Nutr.* **2006**, *45*, 45−51.
(33) Hernanz, D.; Nuñez, V.; Sancho, A. I.; Faulds, C. B.; Williamson, G.; Bartolomé, B.; Gómez-Cordovés, C. Hydroxycinnamic acids and ferulic acid dehydrodimers in barley and processed barley. *J. Agric. Food Chem.* **2001**, *49*, 4884−4888.
(34) Braune, A.; Bunzel, M.; Yonekura, R.; Blaut, M. Conversion of dehydrodiferulic acids by human intestinal microbiota. *J. Agric. Food Chem.* **2009**, *57*, 3356−3362.
(35) Adlercreutz, H. Lignans and human health. *Crit. Rev. Clin. Lab. Sci.* **2007**, *44*, 483−525.
(36) Begum, A. N.; Nicolle, C.; Mila, I.; Lapierre, C.; Nagano, K.; Fukushima, K.; Heinonen, S.; Adlercreutz, H.; Rémésy, C.; Scalbert, A. Dietary lignins are precursors of mammalian lignans in rats. *J. Nutr.* **2004**, *134*, 120−127.
(37) Heinonen, S.; Nurmi, T.; Liukkonen, K.; Poutanen, K.; Wähälä, K.; Deyama, T.; Nishibe, S.; Adlercreutz, H. In vitro metabolism of plant lignans: new precursors of mammalian lignans enterolactone and enterodiol. *J. Agric. Food Chem.* **2001**, *49*, 3178−3186.
(38) Pietinen, P.; Stumpf, K.; Männistö, S.; Kataja, V.; Uusitupa, M.; Adlercreutz, H. Serum enterolactone and risk of breast cancer: a case-control study in eastern Finland. *Cancer Epidemiol. Biomarkers Prev.* **2001**, *10*, 339−344.
(39) Vanharanta, M.; Voutilainen, S.; Lakka, T. A.; van der Lee, M.; Adlercreutz, H.; Salonen, J. T. Risk of acute coronary events according to serum concentrations of enterolactone: a prospective population-based case-control study. *Lancet* **1999**, *354*, 2112−2115.
(40) Funk, C.; Weber, P.; Thilker, J.; Grabber, J. H.; Steinhart, H.; Bunzel, M. Influence of lignification and feruloylation of maize cell walls on the adsorption of heterocyclic aromatic amines. *J. Agric. Food Chem.* **2006**, *54*, 1860−1867.
(41) Funk, C.; Braune, A.; Grabber, J. H.; Steinhart, H.; Bunzel, M. Model studies of lignified fiber fermentation by human fecal microbiota and its impact on heterocyclic aromatic amine adsorption. *Mutat. Res.* **2007**, *624*, 41−48.
(42) Vitaglione, P.; Napolitano, A.; Fogliano, V. Cereal dietary fibre: a natural functional ingredient to deliver phenolic compounds into the gut. *Trends Food Sci. Technol.* **2008**, *19*, 451−463.

(43) Dizhbite, T.; Telysheva, G.; Jurkjane, V.; Viesturs, U. Characterization of the radical scavenging activity of lignins − natural antioxidants. *Bioresour. Technol.* **2004**, *95*, 309−317.