

Release of Small Phenolic Compounds from Brewer's Spent Grain and Its Lignin Fractions by Human Intestinal Microbiota in Vitro

Anna-Marja Aura,^{*,†} Piritta Niemi,[†] Ismo Mattila,[†] Klaus Niemelä,[†] Annika Smeds,[‡] Tarja Tamminen,[†] Craig Faulds,^{†,§} Johanna Buchert,[†] and Kaisa Poutanen^{†,#}

[†]TTT Technical Research Centre of Finland, P.O. Box 1000, Tietotie 2, Espoo, Finland

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

[#]University of Eastern Finland, Kuopio, Finland

S Supporting Information

ABSTRACT: Brewer's spent grain (BSG), the major side-stream from brewing, is rich in protein, lignin, and nonstarch polysaccharides. Lignin is a polyphenolic macromolecule considered resilient toward breakdown and utilization by colon microbiota, although some indications of release of small phenolic components from lignin in animals have been shown. The aim of this study was to investigate if the human intestinal microbiota can release lignans and small phenolic compounds from whole BSG, a lignin-enriched insoluble fraction from BSG and a deferuloylated fraction, in a metabolic in vitro colon model. The formation of short-chain fatty acid (SCFA) was also investigated. More lignin-related monomers and dilignols were detected from the lignin-enriched fraction than from BSG or deferuloylated BSG. SCFA formation was not suppressed by any of the fractions. It was shown that small lignin-like compounds were released from these samples in the in vitro colon model, originating most likely from lignin.

KEYWORDS: *brewer's spent grain, intestinal microbiota, in vitro colon model, lignin, lignan*

■ INTRODUCTION

Brewer's spent grain (BSG) is the major side-stream from the brewing of beer. It is composed of the husks and outer layers of malted barley grains together with the residual endosperm remaining after mashing. As such, it is rich in protein and dietary fiber (DF), including arabinoxylan, cellulose, and lignin.¹ So far, the utilization of BSG has been limited to ruminant feed with low commercial value. However, BSG could be a source of nutritional ingredients or platform chemicals, if cost-efficient fractionation techniques can be developed.

As a component of feed, lignin is considered to be poorly digested by rumen microbiota,² and thus it most likely remains in the gut lumen, where it could interact with other dietary components or affect conversion activities of gut microbiota. The breakdown of lignin has been demonstrated in the rumen of goats,³ and lignin was shown to be a precursor of the mammalian lignans enterodiols and enterolactone in rats,⁴ suggesting that nonruminants could also be able to degrade lignin. However, the ability of human gut microbiota to degrade lignin has not yet been proven. The in vitro metabolic colon model used in this study is a model designating biochemical changes in introduced components due to fecal microbial enzymes. This model combined with gas chromatographic analysis coupled with mass detection (GC-MS or GC×GC-TOFMS) and metabolite profiling has been used to investigate biochemical conversions of plant foods and their components, carbohydrates, and phenolic compounds.^{5–8}

Lignin is a polyphenolic macromolecule acting as the glue between the cellulose–hemicellulose matrix in plant cell walls. Lignin is formed from three monomers: *p*-coumaryl alcohol,

coniferyl alcohol, and sinapyl alcohol, which are linked together in a branched network structure by radical-induced condensation reactions. The ratio of these alcohols is dependent on the plant species. Lignin units are derived from methoxylated hydroxycinnamic acids, such as ferulic and sinapinic acids, via the phenylpropanoid pathway, and similar substitutions on the phenol ring with guaiacyl (4-hydroxy-3-methoxyphenyl) or syringyl (4-hydroxy-3,5-dimethoxyphenyl) alcohols, respectively.⁹ This creates challenges for the identification of monomers that are uniquely lignin-derived, and therefore the removal of ester-linked ferulates might exclude the impact of the formation of methoxylated degradation products from hydroxycinnamic acids.

Lignin could also act as a suppressive component in the colon model. Dose-dependent suppression of short-chain fatty acid (SCFA) formation by tannins, a polymer formed from the flavanols (–)-epicatechins and (+)-catechins, isolated from apples and grapes, has been previously demonstrated in the in vitro colon model, showing lower SCFA concentrations than in fecal control.^{7,8} This was possibly caused by inhibition of cell wall degrading enzymes present in the fecal inoculum through the phenolics binding to these enzymes.¹⁰

The hypothesis of the present study is that gut human microbiota is able to release low molecular weight phenolic compounds, which can potentially be considered as DF

Received: June 3, 2013

Revised: September 4, 2013

Accepted: September 12, 2013

Published: September 12, 2013

phytochemicals, from lignin. Therefore, lignin-rich fractions were prepared from BSG by enzymatic treatments hydrolyzing carbohydrates and proteins and by an alkaline treatment removing ester-linked ferulates. The obtained fractions were studied using the in vitro metabolic colon model to investigate the microbial release of lignin-derived monomeric and dimeric compounds. The formation of SCFA was also studied to exclude the possibility of suppression of microbial conversion activities. The suppression of SCFA formation was also studied, even though lignin-rich fractions contained a low amount of accessible carbohydrates and therefore were not eminent precursors for SCFA formation.

MATERIALS AND METHODS

Preparation of BSG Fractions. BSG was obtained from Sinebryhoff brewery (Kerava, Finland) directly after the mashing process. BSG was composed of 42% carbohydrates, mainly arabinoxylan and cellulose, 19% lignin, 23% protein, 11% lipids, and 4.7% ash.¹¹ Although most of the glucan in BSG was cellulose, residual amounts of starch (1.3%) and mixed-link β -glucan (0.36%) were also present. A portion of BSG was dried and milled with a Hosokawa Alpine mill with a 0.3 mm sieve (Hosokawa Alpine AG, Augsburg, Germany). The dried and milled BSG was used as one of the samples in the in vitro fermentation. To remove ester-linked ferulates, BSG was incubated in 2 M NaOH at room temperature for 2 h with continuous stirring. After the incubation, the solids were separated by centrifugation, washed thoroughly with distilled water, and neutralized with HCl. Finally, these solids were lyophilized, and the dried material denoted deferuloylated sample (DEFE). Nondried BSG was milled with a Masuko Supermasscolloider MKZA10-15J (Masuko Sangyo Co. Ltd., Kawaguchi-city, Japan) and sequentially digested first with carbohydrases and then with proteases as described previously (Figure 1).¹¹ The residue after the proteolytic treatment was further digested

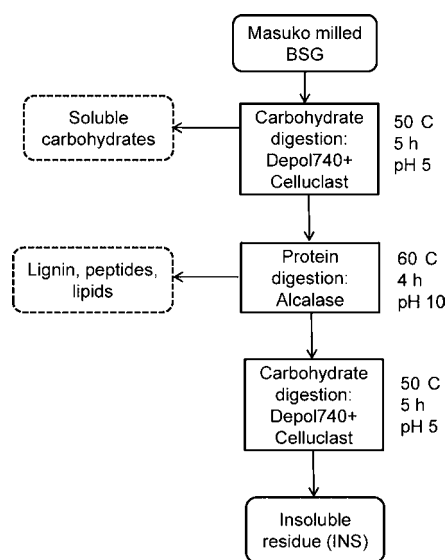


Figure 1. Sequential enzymatic hydrolyses of brewer's spent grain (BSG).

with Celluclast 1.5L (Novozymes, Bagsvaerd, Denmark) (50 FPU/g material) and Depol740L (Biocatalysts Ltd., Cefn Coed, Wales, UK) (5000 nkat of xylanase activity/g material) (5 h, 50 °C, pH 5.0) to remove residual carbohydrates. After the hydrolysis, the solids were separated by centrifugation, washed with distilled water, and lyophilized. The sample was denoted insoluble residue (INS). The composition of BSG and the modified fractions is described in Table 1. The compositions of BSG and its fractions were determined as

Table 1. Compositions of the Fermented Fractions as Mass Percent of Dry Matter

component	initial BSG	insoluble residue (INS)	deferuloylated BSG (DEFE)
carbohydrates	42.2	39.2	53.4
arabinoxylan	22.2	25.7	20.3
glucan	17.1	11.2	30.9
lignin	19.4	40.3	21.9
protein	22.8	6.6	7.6
lipids	11.0	3.1	2.9
ash	4.7	8.7	6.0

described previously.¹¹ Klason lignin was determined gravimetrically and acid-soluble lignin as UV absorbance of the hydrolysate.¹¹

Microscopy Imaging. Light microscopy was carried out as described previously.¹² In brief, samples were embedded in a hydroxyethyl methacrylate matrix from which 2 μ m thick sections were cut. Prior to imaging with the microscope, the sections were treated with chemical dyes Calcofluor and Acid Fuchsin to enable visualization of different components of BSG. Acid Fuchsin stains protein red, and Calcofluor stains β -glucan bright blue. The autofluorescence of lignin and other phenolics is seen as yellow and green. The excitation and emission wavelengths used were 330–385 and >420 nm, respectively.

In Vitro Colon Model. Fermentation of BSG and the modified fractions in the in vitro colon model was performed according to the method of Barry et al.¹³ with the following modifications: 200 mg (on dry weight basis) of BSG or its fractions was weighed into bottles (50 mL) and hydrated with 2 mL of medium 1 day before inoculation. Medium was an 0.11 M carbonate–0.02 M phosphate buffer (pH 6.5) including 0.5 g/L L-cysteine with the addition of 5 mL of mineral solution.¹³ Human feces were collected from five healthy volunteers who had not received antibiotics for at least 6 months and had given written consent. The collection of fecal samples was performed with the approval of and according to the guidelines given by the Ethical Committee of VTT Technical Research Centre of Finland. Freshly passed feces were immediately taken in an anaerobic chamber or closed in a container with an oxygen-consuming pillow (Anaerocult Mini; Merck, Darmstadt, Germany) and a strip testing the anaerobiosis (Anaerotest; Merck). Fecal suspension was prepared under strictly anaerobic conditions. Equal amounts of fecal material from all donors were pooled and diluted to a 20.8% (w/v) suspension, 8 mL of which was dosed to the fermentation bottles to obtain a 16.7% (w/v) final fecal concentration described previously.^{5,14} The fermentation experiments were performed in triplicate, and a time course of 0, 2, 4, 6, 8, and 24 h was followed using the same inoculum for all of the substrates. Incubation was performed at 37 °C in tightly closed bottles and in magnetic stirring (250 rpm). Fecal background was incubated without addition of the supplements.

Extraction of Fermentation Samples. Extraction of SCFA to diethyl ether was carried out as described previously.¹⁵ For phenolic compound, lignan, and metabolome analyses, 1 mL of the fermented sample was thawed. One milliliter of 2% NaCl solution was added to break the emulsion and to ensure the separation of the solvent phase. Fifty microliters of 6 M HCl was added to lower the pH to near 1. For the analysis of phenolic compounds and the metabolome, *trans*-2-hydroxycinnamic acid (Aldrich, St. Louis, MO, USA) was used as the internal standard, and 15 μ L (123 mg/L in MeOH) was added to the fermented samples. The samples were subsequently extracted twice with 3 mL of ethyl acetate. The organic phases were collected, combined, and evaporated under nitrogen. For lignan extraction, 20 μ L 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) (all prepared at the Laboratory of Organic Chemistry at Åbo Akademi University) was added to 1 mL of the fermented sample. The samples were 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 ($2\ \mu\text{L}$, splitless injection) were analyzed with gas chromatography with flame ionization detector (GC-FID) (Agilent 6890 series, Palo Alto, CA, USA). Analytes were separated on a DP-FFAP capillary column ($30\ \text{m} \times 0.32\ \text{mm}$) with a phase thickness $0.25\ \mu\text{m}$ (Agilent). Helium was used as the carrier gas ($2.7\ \text{mL}/\text{min}$). Both the injector and FID were kept at 250°C . The temperature program started at 50°C with a 3 min holding time, then increased at $25^{\circ}\text{C}/\text{min}$ to 100°C , finally increasing at $10^{\circ}\text{C}/\text{min}$ to the final temperature of 240°C , which was held for 10 min. Compounds were quantitated with corresponding standards. SCFA formation was expressed as a sum of acetic, propionic, and butyric acids. The individual SCFA concentrations were calculated from the averages of three replicates at each time point (0–24 h). The initial SCFA formation rate was calculated as follows: The averages of the sum of acetic, propionic, and butyric acid concentrations (mM) were named SCFA (total). SCFA(total, 0 h) was reduced from the SCFA (total, 2 h), and this reduction was divided by 2 h, the time interval.

Metabolomics. The analysis was performed using a two-dimensional gas chromatograph equipped with a time-of-flight mass spectrometer (GC \times GC-TOFMS) as previously described.⁸ The phenolic acids were quantitated with authentic standards. The following compounds were used as standards: Benzoic acid, 3-hydroxybenzoic acid, 3-(4'-hydroxyphenyl)propanoic acid, and 3-(3',4'-dihydroxyphenyl)propanoic acid were products from Aldrich (Steinheim, Germany). 4-Hydroxybenzoic acid, 2-(3'-hydroxyphenyl)acetic acid, and 2-(3',4'-dihydroxyphenyl)acetic acid were purchased from Sigma (St. Louis, MO, USA). 3-Phenylpropionic acid and 3,4-dihydroxybenzoic acid were from Fluka (Buchs, Switzerland), and 3-(3'-hydroxyphenyl)propanoic acid was purchased from Alfa Aesar (Karlsruhe, Germany). 4-Methylcatechol (Aldrich), vanillic acid (4-hydroxy-3-methoxybenzoic acid, Fluka), *p*-coumaric acid (Sigma), and gallic acid (3,4,5-trihydroxybenzoic acid were from Extrasynthèse (Genay, France), and ferulic acid was from Sigma-Aldrich (St. Louis, MO, USA). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and methoxyamine 2% hydrochloride in pyridine (MOX) from Pierce (Rockford, IL, USA) were used as the derivatization reagents.

The data processing of GC \times GC-TOFMS responses has been described earlier.⁸ Briefly, the peaks were identified by ChromaTOF software, which matches deconvoluted spectra against a NIST05 mass spectral library. The compounds in different data sets were aligned and normalized using an in-house-developed software, Guineu,¹⁶ for further analyses. Alignment of the data was performed on the basis of retention indices, second-dimension retention times, and spectra.

GOLM Metabolome Database (GMD),¹⁷ the Guineu program,¹⁶ and relevant literature were utilized for second-stage identification of the compounds, which lacked sufficient spectral matches from the NIST05 or in-house-collected libraries. GMD allows searching of the database based on 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.

Aligned data were filtered using the Guineu program by calculating the fold changes (FC) using the Fold test function describing how many times the test group exceeded the responses of the control group. Aromatic compounds, which showed FC responses >2 , were included in the list of further identified compounds and displayed in the heat map. Heat maps created with Guineu showed the 2-based log FC values as red (overexpressed) or blue (underexpressed) color intensities for each time point and each metabolite. Asterisks showed the time point specific significances (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) for each metabolite, and clustering was performed according to the similarity of the profiles.

Lignan Analysis. To the dried 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 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.¹⁸ 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.¹⁹ Quantitation was carried out using standard solutions containing the internal standards and six concentration levels of the analyzed lignans, as described previously.¹⁹ The standard solutions were solid-phase extracted and redissolved as the real samples.

RESULTS

Potential degradation of lignin from BSG, INS, and the deferuloylated (DEFE) fraction after incubation in an in vitro metabolic colon model was studied by investigating the metabolite profiles corresponding to the release and conversion of lignans and the formation of phenolic acids. In addition, the formation of SCFAs was studied in the in vitro colon model to estimate residual carbohydrate fermentation and potential suppression of the fermentation by high lignin content.

Composition of BSG and Its Fractions. The compositions of BSG, INS, and DEFE are summarized in Table 1. Carbohydrate content was lowest for INS (39.2%) followed by BSG (42.2%), and DEFE showed the highest content of carbohydrates (53.4%) due to reduction in lignin, protein, and lipid levels by the alkaline treatment. Residual starch (1.3%) and β -glucan (0.36%) were present in BSG, but mostly the glucan fraction consisted of cellulose.¹¹ Similarly, in the INS and DEFE fractions, predominantly the glucan was cellulose, but trace amounts of starch and β -glucan may have survived the enzymatic and alkaline treatments. Lignin content was highest, as expected, in INS (40.3%), whereas the lignin contents were similar in DEFE (21.9%) and BSG (19.4%) (Table 1). Lipid content was highest in initial BSG (11.0%) and decreased due to the fractionation steps (3.1 and 2.9% for INS and DEFE, respectively). Protein content in BSG was 22.9%, and it was markedly decreased by the fractionation (6.6 and 7.6% for INS and DEFE, respectively). According to Figure 2A, protein in BSG was mostly encapsulated within the aleurone cells. In the INS fraction, protein was hardly visible due to the preceding proteolysis (Figure 2B). The ash content was between 4.7 and 8.7% in the samples.

Metabolite Profiling. After incubation with fecal microbiota, a wide range of metabolites (FC > 2) were detected for all samples: 694 for BSG, 480 for INS, and 572 for DEFE. From these metabolites, the preliminarily identified (NIST05) small aromatic ones were selected, and structural relevance was confirmed during the final identification (GMD, retention indices and mass spectra) for the heat maps (Figure 3). The heat maps visualize the profiles of overexpressed aromatic metabolites formed from BSG, INS, and DEFE during incubation with human fecal microbiota and analyzed by GC \times GC-TOFMS using nontargeted metabolite profiling against the fecal control (no sample added). The number of identified aromatic metabolites with statistical significance was remarkably higher in the lignin-enriched INS fraction (Figure 3B) than in the BSG or DEFE fractions (Figure 3A,C).

BSG was a source of eight aromatic metabolites, of which compounds 1–3 (4-methylcatechol, a dilignol, and ferulic acid) showed significant ($p < 0.05$) differences from the fecal control at several time points (Figure 3A). The structure of dilignol 2 (Figure 3A) could not be determined, although an intense m/z 180 ion suggests an enterolactone-type compound.²⁰ It is

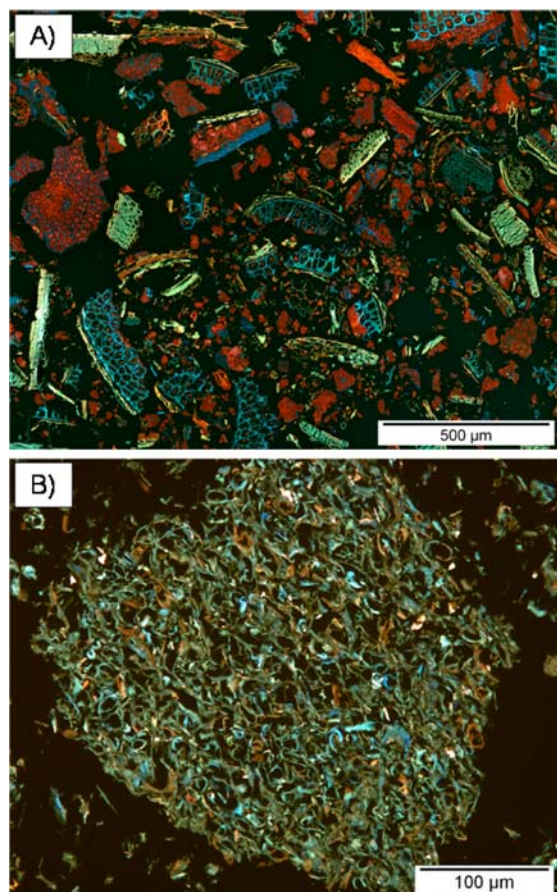


Figure 2. Optical microscopy images of (A) coarse-milled brewer's spent grain (BSG) and (B) insoluble residue (INS). The blue dye indicates cell walls of aleurone and endosperm cells, and the red dye indicates protein. Cell walls containing phenolic compounds are seen as light green due to their autofluorescence.

noteworthy that the other dilignol (compound 6) appears to have the molar mass of 618, suggesting a tetrakis (TMS) derivative of dihydroxylated enterolactone (or a related compound). The base peak at m/z 179 in its mass spectrum indicates a (mono)hydroxyphenyl compound, but no further structure determination was possible. There was also a guaiacyl compound (number 4) present, as indicated by the base peak at m/z 297, characteristic of trimethylsilylated compounds with 4-hydroxy-3-methoxybenzyl alcohol structures. Unfortunately, full identification was not possible.

Many of the metabolites originating from the INS fraction (compounds 8, 13–18, and 20) were recognized as dilignols according to their mass spectra and high retention indices (typically >2700) (Figure 3B), whereas compounds 1–3, 5–7, 10, and 26 were methoxylated aromatic compounds including ferulic acid. Compound 9 (4-methylcatechol), phenolic acids (19, 21, and 22) and compound 25 (4-hydroxymethylcatechol) possessed catechol or other dihydroxyphenyl structures. All or most of these structures can be derived from lignin. It is noteworthy that the aromatic metabolites 2, 5, 6, and 10 in the INS fraction can also be derived from ferulic acid, in addition to lignin. Compound 26 was the only syringyl-type aromatic compound, apparently derived only from lignin. Its final identification was based on published data.²¹

As a whole, eight different dilignols were recognized (Figure 3B). Their full identifications were not possible, but certain

structural features can be distinguished on the basis of a few characteristic ions. Thus, for example, the dilignols 8 and 13 represented compounds with catechol structures (indicated by intense m/z 267 and 179 ions), and compound 15 had a guaiacyl (4-hydroxy-3-methoxyphenyl) unit (intense m/z 209 ion). Three dilignol compounds (16–18) had very intense (up to 100%) m/z 179 ion peaks, suggesting (mono)hydroxyphenyl structures in their molecules. Thus, in most cases, clear structural links to lignin macromolecule are apparent.

The four dilignols detected from the DEFE fraction (Figure 3C) were released at later time points. Of them, compounds 1, 7, and 9 represent (mono)hydroxyphenyl-type dilignols (intense m/z 179 ion) and compound 2 represents a guaiacyl compound (intense m/z 209 ion). Two of them (compounds 1 and 2) showed high statistical significance ($p < 0.01$). It is worth noting that some catechol compounds were released from the DEFE fraction as well.

Fold changes between the metabolites originating from the substrates versus from the fecal control were 2.1–7.0 for BSG, 2.0–13.0 for INS, and 2.0–11.8 for DEFE. It seems that the release of lignin-derived metabolites may occur by human microbiota, but slowly and probably only to a small degree, because the FCs were only between 2 and 13. The highest FCs were observed for dilignols, vanillin, methoxybenzenediol, and a guaiacyl compound (Figure 3). FC of the guaiacylpropanoic acid (compound 5, Figure 3B) was high at both 0 and 24 h but showed lower FC in the middle of the incubation.

Quantitative Analysis of Phenolic Metabolites. Targeted quantitative analysis of the phenolic metabolites from BSG and the INS and DEFE fractions is shown in Figure 4. The four metabolites are shown here because of their structural relevance to lignin (4-methylcatechol) or their connection to feruloylation of lignin and ferulate metabolism in the colon²² (ferulic acid, 3-hydroxyphenylpropanoic acid, and 3',4'-dihydroxyphenylacetic acid). Of the metabolites, 4-methylcatechol was formed in significantly higher concentrations from INS and BSG than from the fecal control, whereas the lowest concentrations of the fractions were from the DEFE fraction, which was slightly above the fecal control (Figure 4A). As could be expected, ferulic acid was released in notably higher concentrations from BSG than from DEFE during the incubation with fecal microbiota (Figure 4B).

3-Hydroxyphenylpropanoic acid was present in surprisingly high concentrations already at the beginning of the incubation, also in the fecal control, after which a declining profile was apparent. After the decline, a small increase was observed at 8 h for fecal control and the INS fraction. 3,4-Dihydroxyphenylacetic acid concentration increased significantly for BSG and INS, but this compound was completely absent from DEFE, because the concentrations were mainly below the fecal control. In addition, 3,4-dihydroxyphenylpropanoic acid, 4-hydroxyphenylpropanoic acid, 3,4-dihydroxybenzoic acid, 3-hydroxybenzoic acid, vanillic acid, caffeic acid, and 4-coumaric acid were detected in the fermented samples. However, they were not considered true metabolites because they did not differ significantly from the fecal control, meaning that they most probably originated from the feces, or their concentrations did not significantly change during the incubation.

Quantitative Analysis of Lignans. Lignan concentrations during the incubation in the model were low but differed significantly from the fecal control at certain time points. Syringaresinol showed significantly different concentrations at 0 h for BSG and its fractions (INS > BSG > fecal control >

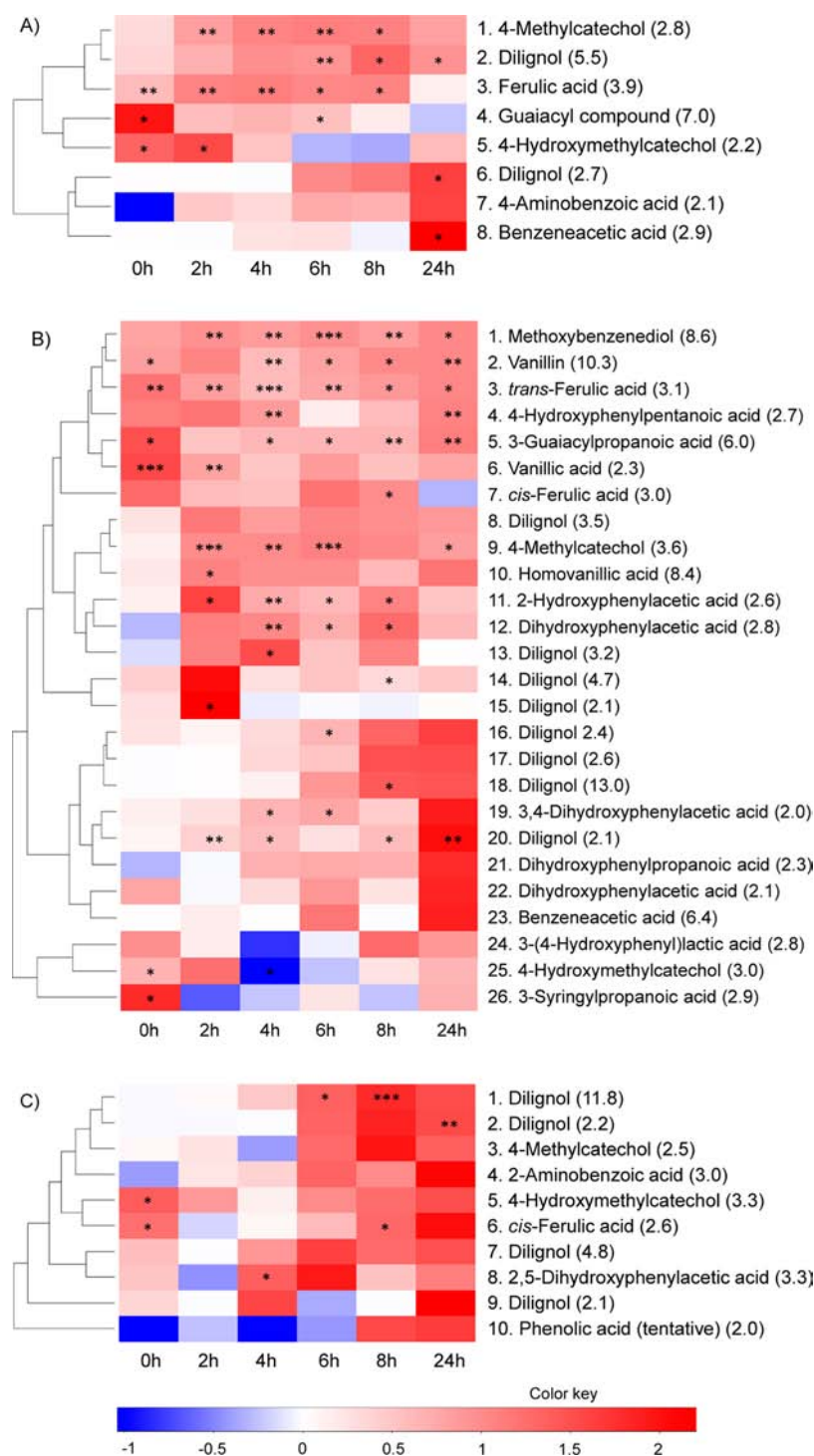


Figure 3. Heat maps of the aromatic metabolites formed in the metabolic *in vitro* colon model for (A) initial brewer's spent grain (BSG), (B) insoluble residue (INS), and (C) deferuloylated BSG (DEFE). The number after the name in parentheses is the fold change (FC) of the metabolite compared to fecal control (microbiota without substrate). The color key is the 2-log value of the FC. Blue color indicates an underexpression and red color represents an overexpression of the metabolite compared to the fecal control. Significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) are expressed as asterisks.

DEFE) (Figure 5A). α -Conidindrin concentration from INS was highest at 0 h and differed from the fecal control, and by 8 h DEFE and BSG incubations reached the same level, but fecal control remained at the baseline. Due to high standard deviations of the INS fraction, no significant differences were observed between samples after 0 h (Figure 5B). Matairesinol concentrations from INS and BSG were significantly higher at 2

h compared to the fecal control, whereas DEFE incubation showed intermediary levels (Figure 5C). All lignans were further converted, because their concentrations declined toward the end of the incubation (Figure 5). In addition, secoisolariciresinol, cyclolariciresinol, enterodiol, enterolactone, 7-hydroxyenterolactone, and 7-oxoenterolactone were detected in the fermented samples. However, their concentrations were

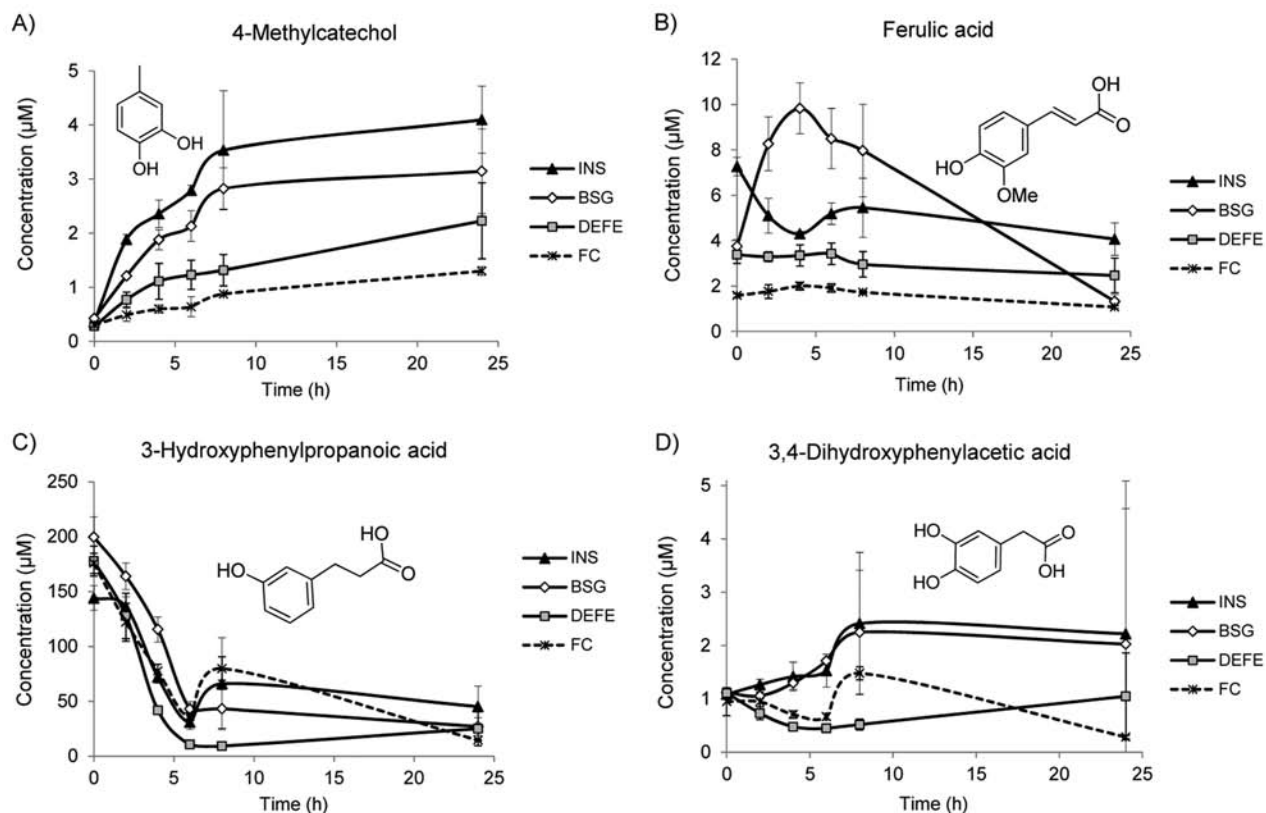


Figure 4. Concentrations of phenolic compounds during the in vitro fermentation of brewer's spent grain and its fractions: (A) 4-methylcatechol; (B) ferulic acid; (C) 3-hydroxyphenylpropanoic acid; (D) 3,4-dihydroxyphenylacetic acid.

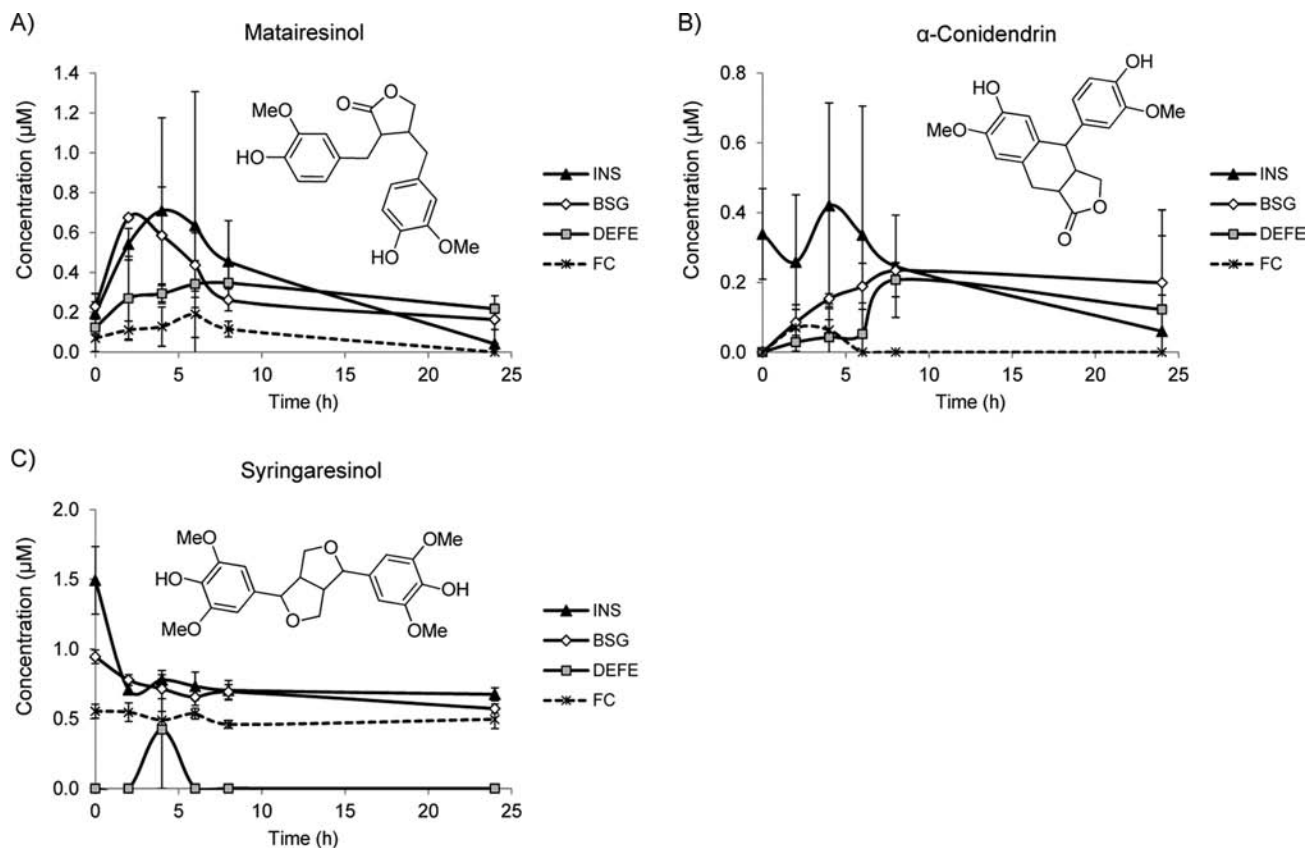


Figure 5. Concentrations of lignans during the in vitro fermentation: (A) matairesinol; (B) α -conidendrin; (C) syringaresinol.

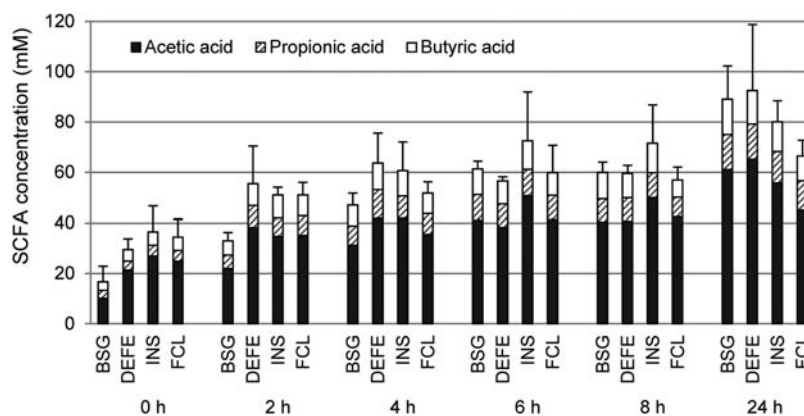


Figure 6. Concentrations of linear short-chain fatty acids formed during in vitro fermentation. The error bar is the combined standard deviation of the three fatty acids.

not significantly different from the fecal control, which means that they most likely originated from the diet of the donors of the feces used in the model and not from the BSG samples.

Formation of SCFA. SCFA formation occurred at a slow rate for all of the substrates (Figure 6). The initial formation rate (0–2 h) was fastest for DEFE (13.0 mM/h) followed by BSG and fecal control (8.2 mM/h). The lowest rate (7.3 mM/h) was observed for INS. There was no indication of suppression of SCFA formation by lignin, because SCFA levels from BSG and its fractions were above those of the fecal control and the carbohydrates originating from the feces and BSG and its fractions were partially fermented. The differences between samples were not significant even in comparison with the fecal control because of high standard deviations between replicates, especially for DEFE and INS fractions.

DISCUSSION

In this study, the emphasis was to study potential release of lignan-like and lignin-derived low molecular weight phenolic compounds from lignin by human fecal microbiota. Release of lignin-derived monomers, phenolic acid conversion products, and formation of SCFAs were analyzed using gas chromatographic techniques combined with identification with authentic standards and mass spectral comparisons with databases.

Conversion of Lignin by Human Intestinal Microbiota.

Release of several low molecular weight aromatic compounds from BSG and the fractions was detected in the in vitro colon model using human fecal microbiota. Even though, on the basis of their structure, many of the metabolites could have derived either from lignin or from ferulic acid, dilignols and 4-methylcatechol were most pronounced in the lignin-rich INS fraction. The formation of 4-methylcatechol could occur from guaiacyl and syringyl residues by demeth(ox)ylation and partial dehydroxylation, which are common conversion activities of fecal microbiota and have been shown for plant lignans^{23,24} and flavonoid metabolites (hydroxylated phenylacetic and propionic acids).^{25,26} The highest concentration of 4-methylcatechol was released from INS, which also had the highest lignin content, suggesting that 4-methylcatechol could originate from lignin.

Identification of Lignin-Derived Metabolites. As a whole, a large number of different phenolic and other aromatic metabolites could be identified or partially characterized. Some structural features (e.g., the presence of catechol or guaiacyl structures) could be linked to the detected dilignols, which

accumulate toward the end of the incubation, and to lignin. Some monomeric compounds can originate either from lignin or from ferulic acid, for example, methoxylated hydroxyphenyl (guaiacyl) compounds. Guaiacylpropanoic acid, homovanillic acid, and vanillic acid can be formed from ferulic acid via reduction of the double bond in the side chain and by shortening of the side chain (α - and β -oxidation), respectively.^{22,27} In this study, the structure of one syringyl-type compound (no. 26 in Figure 3B) was identified and was obviously derived only from lignin. Also, vanillin is more likely to originate from lignin, as also known from its commercial production by oxidation of lignin-containing byproduct of the pulping industry.²⁸ Quantitated 4-methylcatechol showed significantly higher concentrations in INS than in fecal control and is also a structurally promising compound as a released and converted metabolite from lignin. 4-Methylcatechol was formed during incubation with fecal microbiota between 2 and 6 h time points.

As early as in 1929, Csonka et al.²⁹ anticipated that lignin is degraded in the digestive system of cows (ruminant) and dogs (monogastric) on the basis of the loss of methoxyl groups. Later it was suggested that lignin is degraded during human intestinal transit.³⁰ Enterolignan conversion from lignin has also been reported in rats: when deuterated synthetic lignin was fed to rats as part of wheat bran diet, deuterated enterolactone was found in the urine, confirming the release of smaller units from lignin and their conversion to mammalian lignan, enterolactone.⁴ Degradation of lignin from wheat straw in ruminant goats was associated with excretion of aromatic acids (benzoic and hippuric acids);³ however, it is possible that the amount of released compounds may be so small that degradation of lignin will not be apparent. Holloway et al.³¹ studied fiber digestion in ileostomy patients and healthy control subjects of human volunteers and found no indication of lignin degradation in either group measured by mass balances. The methods for lignin analysis are under discussion, and thus the impact of human microbiota in lignin degradation remains to be studied in the future. The release of monomeric products from lignin by human microbiota is relevant as a part of the contribution of colon-derived circulating metabolites and when the methods, for example, metabolomics of the small molecules are available. There is increased interest in dietary fiber related small molecules released and absorbed from the colon that may have potentially health-promoting systemic effects.

In the targeted analysis, none of the known ferulic acid metabolites such as 3-hydroxyphenylpropanoic acid or 3,4-dihydroxyphenylacetic acid²² showed significantly different formation from BSG or its fractions compared with the fecal control. The profiles of the known ferulic acid metabolites in this study were exceptionally different from the previous findings from wheat and rye product, which showed clear formation of 3-hydroxyphenylpropanoic acid during incubation with fecal microbiota in the same model³² and in TNO intestinal models (TIM).³³

Effect of Fractionation on Conversion Products.

Whereas the removal of carbohydrates and protein from BSG in the preparation of the INS fraction increased the lignin content of the insoluble residue, some lignin was solubilized during the proteolytic treatment, which was carried out in mildly alkaline pH.¹¹ The carbohydrate- and protein-degrading enzymes were chosen on the basis of earlier studies,^{11,34} which showed these enzymes to be the most efficient for BSG hydrolysis. The strong alkaline treatment of BSG in the preparation of the DEFE fraction also decreased the lignin content, but, on the other hand, other compounds were simultaneously solubilized by the alkaline conditions and were removed. Therefore, the lignin content in the DEFE fraction was not significantly different from that of the BSG. The alkaline treatment decreased the ferulic acid content in DEFE, as most of the ferulic acid in BSG is ester-bound and can be released with alkali.³⁵ The targeted analysis of colon model metabolites showed low concentrations of ferulic acid for DEFE, as they were only slightly above the fecal control and remained constant during the incubation, indicating successful deferuloylation of the DEFE fraction (Figure 4B). In the colon model, ferulic acid was released mostly from BSG and to a lesser extent from INS, which could be expected on the basis of the enzymatic hydrolysis of BSG with the feruloyl esterase-containing enzyme cocktail and the mild alkalinity in the proteolytic treatment used in the preparation of the INS fraction.¹¹ The ferulic acid and its preliminary microbial metabolite 3-hydroxyphenylpropanoic acid showed clearly that very little conversion occurred from INS or DEFE. Also, 3,4-dihydroxyphenylacetic acid concentration (diferulate metabolite) was low for DEFE. A higher initial SCFA formation rate was seen for DEFE than for BSG or INS, suggesting that the fermentation is retarded if carbohydrate-binding feruloyl bridges are present as in BSG and INS.

Enzymes and altered pH of the hydrolysis solutions in the preparation of the INS fraction had modified the plant matrix structure to make it more susceptible for microbial degradation, shown as promoted initial SCFA formation rate and released lignans and 4-methylcatechol. The strong alkali used in the preparation of the DEFE fraction most probably solubilized the easily released low molecular weight aromatic compounds, such as lignans, which were present in low concentrations in DEFE. It has been shown previously that alkali effectively solubilizes lignans from cereal matrix.³⁶ Also, mild alkali releases lignans, indicating modification of the matrix as shown earlier.¹¹

Formation of SCFA. Garleb et al.² stated that dry matter disappearance in an *in vitro* rumen simulation was higher with low lignin containing fibers than with lignin-rich fibers. It has also been shown in pigs that BSG decreased xylanolytic and cellulolytic enzyme activities in the cecum, and SCFA content in the colon was lower for the group fed BSG than for the control group fed a diet low in fiber.³⁷ These findings could suggest that BSG may interact with intestinal microbiota and

reduce their effectiveness in fermenting available carbohydrates. In contrast, in this study the proportions of fermentable carbohydrates in BSG and its fractions were quite close to each other. Despite the somewhat different initial SCFA formation rates, there were no significant differences between SCFA concentrations between samples and control. In the case of suppression, samples containing lignin should have shown significantly lower concentration of SCFA as compared with fecal control, as shown for apple and grape tannins.^{7,8} However, such an effect was not observed, and it is not likely that enzymatic inhibition occurred to a significant degree. If the lignin were inhibitory, SCFA from fecal inoculum would also have been affected.

The fermentability of BSG and its fractions remained on the same level as in fecal control. In this study the amount of sample was doubled (200 mg) compared with previous experiments (100 mg) studying the carbohydrate fermentation of cereals.^{5,14} The high dose of substrate may cause lower fermentation rate and extent as SCFA formation as indicated previously;¹³ however, in this study carbohydrates were of resilient nature and comprised a maximum 53% of the weight of the substrate, and thus suppression by excessive carbohydrates is unlikely. It is also known from previous studies that BSG carbohydrates are partially resistant toward hydrolytic enzymes.¹¹ Removal of easily fermentable carbohydrates by fractionation leaves the lignin-bound resilient carbohydrates in the fractions, and thus the slow fermentation rate of SCFA formation is most likely caused by the poor accessibility of lignin-bound carbohydrates. In the DEFE fraction, in which the ferulic acid cross-links between carbohydrates and lignin were disrupted, the fermentation rate was increased because carbohydrates were more susceptible to microbial fermentation.

Methods. When the colonic degradation of BSG and the INS and DEFE fractions was studied, the substrates were not digested with alimentary enzymes *in vitro* in the conditions of upper intestine prior to the colon model as in the cases of cereal food samples,⁵ because the BSG and its fractions had been extensively digested in the brewing process and contained only minimally starch (1.3%) and less protein (6.6–22.8%) than was left in digested whole meal rye bread (25%) in a previous study.³⁸ Furthermore, in BSG the protein was shown to be mostly encapsulated in the fiber matrix, so it was considered to be delivered to the colon relatively unchanged (Figure 2A). The fractions contained even less protein, as they were treated with a protease during the processing.

The changes in microbial metabolites in the colon model used in this study depend on the components in the studied substrates, and the microbial composition is not variable during the incubation. The conversions are performed in strictly anaerobic conditions and for precise incubation times in the batch design, which facilitates the detection of metabolite formation as a course of time.

Lignin-Containing Fractions as Dietary Fiber. Lignin is a valuable DF component regardless of its susceptibility to microbial degradation in the colon. Lignin has been shown to adsorb more carcinogenic compounds *in vitro* in the conditions of the small intestine and colon than nonlignified fiber,^{39,40} which could contribute to the cancer-protecting effects of DF. In addition, phenolic compounds within the insoluble fiber are able to quench soluble radicals formed in the gastrointestinal tract,⁴¹ and due to its phenolic structure also lignin possesses such antioxidant and radical scavenging activity in the lumen.^{42,43} In addition, if lignin is a source of small bioavailable

compounds, which may be biologically active and partly responsible for the beneficial health effects of DF complex, it comprises a new group of compounds in addition to enterolignans (enterodiol and enterolactone), urolithins, and phenolic acids,⁴⁴ which are derived from conversions by the intestinal microbiota and are related to plant food and DF intake. Whether the potentially lignin-derived components have health benefits remains to be studied.

In conclusion, small lignin-derived molecules such as dilignols and catechols were released from BSG and its lignin fractions in the metabolic *in vitro* colon model. The significance of the release of these small molecules should be evaluated by quantitation of the formed metabolites in respect to lignin degradation and, finally, by measuring the released components from human urine after consumption of lignin-rich diet. It is possible that the physiological effects of lignin as DF component are mostly due to its binding to carbohydrates, inhibiting their fermentation and increasing the bulking potential of DF. Consequently, nonfermentable fibers absorb and remove toxic components and thus reduce the risk of chronic diseases. Lignin is an underestimated DF component, and its characteristics in the digestive tract deserve more attention.

■ ASSOCIATED CONTENT

● Supporting Information

Concentrations (mean \pm standard deviation) of quantitated phenolic metabolites and lignans and their significances. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*(A.-M.A.) Phone: + 358 20 722 6178. Fax: + 358 20 722 7071. E-mail: anna-marja.aura@vtt.fi.

Present Address

§(C.F.) INRA, UMR 1163 Biotechnologie des Champignons Filamenteux, 163 avenue de Luminy, 13288 Marseille cedex 09, France; Aix-Marseille Université, POLYTECH Marseille, UMR 1163 Biotechnologie des Champignons Filamenteux, 163 avenue de Luminy, 13288 Marseille cedex 09, France.

Funding

Financial support from the Academy of Finland and Graduate School of Biomass Refining (BIOREGS) is gratefully acknowledged.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Niina Torttila, Siv Matomaa, Annika Majanen, Airi Hyrkäs, and Liisa Änäkäinen for technical assistance. We also thank Kaarina Viljanen for supervising the short-chain fatty acid analysis. We gratefully acknowledge Sinebrychoff brewery for providing the BSG.

■ REFERENCES

- (1) Mussatto, S. I.; Dragone, G.; Roberto, I. C. Brewers' spent grain: generation, characteristics and potential applications. *J. Cereal Sci.* **2006**, *43*, 1–14.
- (2) 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.

- (3) 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.

- (4) Begum, A. N.; Nicolle, C.; Mila, I.; Lapierre, C.; Nagano, K.; Fukushima, K.; Heinonen, S.; Adlercreutz, H.; Rémésy, C.; Scalbert, A. Dietary lignans are precursors of mammalian lignans in rats. *J. Nutr.* **2004**, *134*, 120–127.

- (5) 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.

- (6) Aura, A.-M.; Mattila, I.; Seppänen-Laakso, T.; Miettinen, J.; Oksman-Caldentey, K.; Orešič, M. Microbial metabolism of catechin stereoisomers by human faecal microbiota: comparison of targeted analysis and a non-targeted metabolomics method. *Phytochem. Lett.* **2008**, *1*, 18–22.

- (7) 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.

- (8) 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.

- (9) Boerjan, W.; Ralph, J.; Baucher, M. Lignin biosynthesis. *Annu. Rev. Plant Biol.* **2003**, *54*, 519–546.

- (10) Scalbert, A. Antimicrobial properties of tannins. *Phytochemistry* **1991**, *30*, 3875–3883.

- (11) 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.

- (12) Van Craeyveld, V.; Holopainen, U.; Selinheimo, E.; Poutanen, K.; Delcour, J. A.; Courtin, C. M. Extensive dry ball milling of wheat and rye bran leads to *in situ* production of arabinoxylan oligosaccharides through nanoscale fragmentation. *J. Agric. Food Chem.* **2009**, *57*, 8467–8473.

- (13) Barry, J.-L.; Hoebler, C.; MacFarlane, G. T.; MacFarlane, S.; Mathers, J. C.; Reed, K. A.; Mortensen, P. B.; Nordgaard, I.; Rowland, I. R.; Rummen, C. J. Estimation of the fermentability of dietary fibre *in vitro*: a European interlaboratory study. *Br. J. Nutr.* **1995**, *74*, 303–322.

- (14) Karppinen, S.; Liukkonen, K.; Aura, A.-M.; Forssell, P.; Poutanen, K. *In vitro* fermentation of polysaccharides of rye, wheat and oat brans and inulin by human faecal bacteria. *J. Sci. Food Agric.* **2000**, *80*, 1469–1476.

- (15) 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.

- (16) 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.

- (17) Golm metabolome database, <http://gmd.mpimp-golm.mpg.de/search.aspx> (accessed May 30, 2013).

- (18) 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.

- (19) 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.

- (20) Jacobs, E.; Kulling, S. E.; Metzler, M. Novel metabolites of the mammalian lignans enterolactone and enterodiol in human urine. *J. Steroid Biochem. Mol. Biol.* **1999**, *68*, 211–218.
- (21) Niemelä, K. Low-molecular-weight organic compounds in birch kraft black liquor. *Ann. Acad. Sci. Fenn., Ser. A II, Chem.* **1990**, *229*, 1–142.
- (22) Braune, A.; Bunzel, M.; Yonekura, R.; Blaut, M. Conversion of dehydrodiferulic acids by human intestinal microbiota. *J. Agric. Food Chem.* **2009**, *57*, 3356–3362.
- (23) 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.
- (24) Blaut, M.; Clavel, T. Metabolic diversity of the intestinal microbiota: implications for health and disease. *J. Nutr.* **2007**, *137*, 751–755.
- (25) Aura, A.-M.; O'Leary, K. A.; Williamson, G.; Ojala, M.; Bailey, M.; Puupponen-Pimiä, R.; Nuutila, A. M.; Oksman-Caldentey, K.; Poutanen, K. Quercetin derivatives are deconjugated and converted to hydroxyphenylacetic acids but not methylated by human fecal flora in vitro. *J. Agric. Food Chem.* **2002**, *50*, 1725–1730.
- (26) Rechner, A. R.; Smith, M. A.; Kuhnle, G.; Gibson, G. R.; Debnam, E. S.; Srai, S. K. S.; Moore, K. P.; Rice-Evans, C. A. Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. *Free Radical Biol. Med.* **2004**, *36*, 212–225.
- (27) Gonthier, M.-P.; Remesy, C.; Scalbert, A.; Cheynier, V.; Souquet, J.-M.; Poutanen, K.; Aura, A.-M. Microbial metabolism of caffeic acid and its esters chlorogenic and caftaric acids by human faecal microbiota in vitro. *Biomed. Pharmacother.* **2006**, *60*, 536–540.
- (28) Araújo, J. D. P.; Grande, C. A.; Rodrigues, A. E. Vanillin production from lignin oxidation in a batch reactor. *Chem. Eng. Res. Des.* **2010**, *88*, 1024–1032.
- (29) Csonka, F. A.; Phillips, M.; Jones, D. B. Studies on lignin metabolism. *J. Biol. Chem.* **1929**, *85*, 65–75.
- (30) 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.
- (31) 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.
- (32) Nordlund, E.; Aura, A.-M.; Mattila, I.; Kössö, T.; Rouau, X.; Poutanen, K. Formation of phenolic microbial metabolites and short-chain fatty acids from rye, wheat, and oat bran and their fractions in the metabolic in vitro colon model. *J. Agric. Food Chem.* **2012**, *60*, 8134–8145.
- (33) Mateo Anson, N.; van den Berg, R.; Havenaar, R.; Bast, A.; Haenen, G. R. M. M. Bioavailability of ferulic acid is determined by its bioaccessibility. *J. Cereal Sci.* **2009**, *49*, 296–300.
- (34) Niemi, P.; Faulds, C. B.; Sibakov, J.; Holopainen, U.; Poutanen, K.; Buchert, J. Effect of a milling pre-treatment on the enzymatic hydrolysis of carbohydrates in brewer's spent grain. *Bioresour. Technol.* **2012**, *116*, 155–160.
- (35) 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.
- (36) 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 nut. *J. Agric. Food Chem.* **2007**, *55*, 1337–1346.
- (37) Martins, C.; Pinho, M.; Lordelo, M. M.; Cunha, L. F.; Carvalho, J.; Freire, J. P. B. Effect of brewers grain on intestinal microbial activity and mucosa morphology of weaned piglets. *Livest. Sci.* **2010**, *133*, 132–134.
- (38) Aura, A.-M.; Härkönen, H.; Fabritius, M.; Poutanen, K. Development of an *in vitro* enzymic digestion method for removal of starch and protein and assessment of its performance using rye and wheat breads. *J. Cereal Sci.* **1999**, *29*, 139–152.
- (39) 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.
- (40) 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.
- (41) 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.
- (42) Dizhbite, T.; Telysheva, G.; Jurkjane, V.; Viesturs, U. Characterization of the radical scavenging activity of lignins – natural antioxidants. *Bioresour. Technol.* **2004**, *95*, 309–317.
- (43) Lu, F.; Chu, L.; Gau, R. Free radical-scavenging properties of lignin. *Nutr. Cancer* **1998**, *30*, 31–38.
- (44) Aura, A.-M. Microbial metabolism of dietary phenolic compounds in the colon. *Phytochem. Rev.* **2008**, *7*, 407–429.