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# Both Wheat (*Triticum aestivum*) Bran Arabinoxylans and Gut Flora-Mediated Fermentation Products Protect Human Colon Cells from Genotoxic Activities of 4-Hydroxynonenal and Hydrogen Peroxide

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Dietary fibers are fermented by the gut flora to yield short chain fatty acids (SCFAs), which inhibit the growth of tumor cells, induce glutathione S-transferases (GSTs), and protect cells from the genotoxic activity of 4-hydroxynonenal (HNE). Here, we investigated effects of wheat bran-derived arabinoxylans and fermentation products on these parameters of chemoprevention. Newly isolated water extractable (WeAx) and alkali extractable arabinoxylans (AeAx) were fermented under anaerobic conditions with human feces. Resulting fermentation supernatants (FSs) were analyzed for SCFAs and used to treat HT29 colon cancer cells. Cell growth, cytotoxicity, antigenotoxicity against hydrogen peroxide ($H_2O_2$) or HNE, and GST activity were determined. Nonfermented WeAx decreased $H_2O_2$-induced DNA damage by 64%, thus demonstrating chemoprotective properties by this nonfermented wheat bran fiber. The fermentation of WeAx and AeAx resulted in 3-fold increases of SCFA, but all FSs (including the control without arabinoxylans) inhibited the growth of the HT29 cells, reduced the genotoxicity of HNE, and enhanced the activity of GSTs (FS WeAx, 2-fold; FS AeAx, 1.7-fold; and control FS, 1.4-fold), which detoxify HNE. Thus, increases in SCFAs were not reflected by enhanced functional effects. The conclusion is that fermentation mixtures contain modulatory compounds that arise from the feces and might add to the effectiveness of SCFAs.

**KEYWORDS: Chemoprevention; GST; HT29; single cell microgelelectrophoresis (comet assay); triticum aestivum; wheat bran arabinoxylan**

## INTRODUCTION

Dietary fiber and other fermentable carbohydrates are important for maintaining normal bowel function and metabolism of intestinal bacterial flora. Fermentable food ingredients, which enter the large intestine, may change enzyme activities and metabolites of gut bacteria and thus have an effect on colonic health (*1*). Epidemiological studies have reported that a diet rich in fiber is beneficial and may counteract many causes of mortality and morbidity (e.g., ischemic heart disease, cancer) (*2*). Cell wall-rich byproducts from the milling of cereal grains may be of additional value since they also contain many bioactive components such as vitamins, lignans, isoflavones, and phenolic acids. These are probably able to protect the organism by acting as antioxidants or by other mechanisms, e.g., related to inhibition of tumor progression (*3*). Of all food sources of fibers tested in animals and in humans, wheat bran (WB) has been one of the most effective in protecting against colon cancer (*4*). WB inhibited aberrant crypt foci or chemically induced adenomas or adenocarcinomas in animal experiments more consistently than other dietary fibers (*5*−*7*). Host enzymes such as inducible nitric oxide synthase, cyclooxygenase II, and glutathione S-transferase (GST) A1-2 (*8*, *9*) as well as the gut flora enzymes $\beta$-glucuronidase, nitroreductase, and azoreductase (*5*, *10*) were favorably modulated by moderate doses of WB.

An end point of preneoplastic lesions in humans is the recurrence of colorectal adenomas, which, however, was not affected by an increased intake of WB (*11*−*13*). Opposite to these studies, the Australian Polyp Prevention Project did show that the combination of fat reduction and a supplement of WB reduced the incidence of large colorectal adenomas (*14*). The mechanisms of this protective effect have not been elucidated completely, but they are most likely multifactorial. Some mechanisms, such as luminal dilution of potential carcinogens and accelerated transit through the colon, are well-confirmed (*15*). A further mechanism by which this dietary fiber source in general could mediate protection is by acting as a substrate

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for gut flora to yield fermentation products with chemopreventive activity. In particular, butyric acid, one of the major fermentation products in this context, is associated with reduced tumor growth in an animal study (*16*). For WB, in particular, several additional mechanisms have also been discussed, such as effects of phytochemicals and phytates, although there are only few data to support this (*15*).

WB contains different types of dietary fiber. Arabinoxylans are the main nonstarch polysaccharide compounds in WB with 25−30% of dry matter (*17*, *18*). Besides glucuronoarabinoxylans (alkali extractable arabinoxylans, AeAx), extractable from WB by strong alkaline conditions, WB also contains very small amounts of arabinoxylans extractable at neutral pH (water extractable arabinoxylans, WeAx). Common to both arabinoxylan types is a $\beta$-(1→4)-linked D-xylopyranose polymeric backbone chain with α-L-arabinofuranose residues attached as branch points. The chain-forming xylopyranose monomers may be substituted at positions O2 or O3 or O2 and O3 by arabinose residues and may be further linked to other groups such as glucuronic acid residues and ferulic acid (FA) or coumaric acid (CA). In cereals, FA and CA acylate the primary C5-OH of α-L-arabinose moieties of arabinoxylans. In the plant cell wall, FA of adjacent arabinoxylan chains can undergo enzyme-catalyzed dimerization forming isomere dehydrodimers such as 5-8-BendiFA, 8-O-4-diFA, and 5-5-diFA (*19*) while cross-linking different polysaccharide chains. FA is the most abundant hydroxycinnamic acid in cereals followed by *p*-coumaric, synapic, and caffeic acids (*20*).

Only few studies tested the physiological effects of isolated arabinoxylans until now. Investigations by Lu et al. showed that arabinoxylans are a good source for bacterial fermentation in the colon, resulting in acetate formation (*17*). Furthermore, they showed that a supplement of arabinoxylan fiber can significantly improve glycemic control in people with type II diabetes (*21*) and lower postprandial glucose and insulin response in healthy volunteers (*22*). Potential effects related to colonic health have not been reported.

Therefore, in this study, two different fractions of WB fiber—WeAx and AeAx as well as their metabolites produced by fermentation with human gut flora—were investigated for properties related to chemoprotection in the gut lumen. This approach has recently been developed in our laboratory to investigate nonsoluble carbohydrates and dietary fiber under conditions that simulate conditions occurring in the gut lumen in vivo (*23*). Here, it was the aim to characterize these WB fractions and their metabolites for parameters of chemoprevention, namely, inhibition of tumor cell proliferation, modulation of genetic damage induced by putative human colon carcinogens (*24*), and induction of phase II enzyme activities (*25*, *26*). These biological end points may reflect inhibitory activities related to the retardation of initiation and progression of tumor development (*3*).

WeAx and AeAx were different in fine structure details: WeAx contained less arabinose (Xyl/Ara 2.5) and uronic acid (UA, 0.8%) substituents than AeAx (Xyl/Ara 1.2, 2.5% UA) (*18*). Both arabinoxylan fractions did not contain measurable amounts of FA or other hydroxycinnamic acids.

## MATERIALS AND METHODS

**Isolation of the Arabinoxylan Fractions.** Different arabinoxylan fractions were isolated from WB and characterized as described by Hollmann and Lindhauer (*18*). Briefly, WB was purified by boiling in ethanol (70%). This was followed by an extraction with water at ambient temperature. After starch and protein were removed enzymatically from the extract, WeAxs were precipitated with ethanol. For isolating glucuronoarabinoxylans (AeAx), the residual bran was finally extracted with 2% hydrogen peroxide at pH 11 and 40 °C. The residual protein was removed by protease, and AeAx was precipitated with ethanol. Both products were further purified by enzymatic removal of mixed linked (1-3)(1-4)-$\beta$-D-glucans using a combination of lichenase at pH 6.5 and $\beta$-glucosidase at pH 4.0, respectively.

**Fermentation of Arabinoxylans.** All fermentations were conducted in vitro under anaerobic conditions (80% nitrogen, 10% carbon dioxide, and 10% hydrogen at 37 °C), basically according to described procedures (*27*). A mixture of fresh human feces was prepared as a bacterial source. These were used to ferment the reconstituted sample powders. For each inoculum ($n = 4$), fresh feces were collected from three healthy human volunteers, who had given their informed consent. They consumed their normal, nonvegetarian diet without any restrictions. The use of nutritional supplements and antibiotic drugs was forbidden for the last 6 months. The fermentation supernatants (FSs), which were used in each set of experiments, were always derived from the same fermentation procedure.

The study was approved by the Ethical Committee of the Friedrich Schiller University Jena. The samples were immediately weighed and filled in one homogenizing bag. Potassium phosphate buffer (0.1 M, pH 7.0) was added (5:1 v/w), and the mixture was homogenized thoroughly in a Stomacher'400 (Seward, Worthing, United Kingdom). From the fecal homogenate, 40 mL aliquots were filled into 500 mL glass bottles. WB samples were solubilized with anaerobic potassium phosphate buffer to provide 20 g/L fermentable substances (fiber, carbohydrate, and protein). Forty milliliters of each arabinoxylan solution was added to separate bottles to obtain a final fiber content of 10 g/L and a fecal suspension of 10% as recommended by Barry et al. (*27*). As a negative control (blank), potassium phosphate buffer was added to one bottle instead of arabinoxylan solution. Anaerobe conditions in the glass bottles were achieved by removing the available air with an injected cannula (0.5 bar for 1 min). Subsequently, the bottles were filled with the fermentation gas mixture via the cannula (0.8 bar for 1 min). After 30 min (15 cycles repeated), the cannulas were removed and the fermentation suspensions were incubated for 24 h in a shaking water bath at 37 °C. Afterward, the fermentation process was stopped by placing the suspensions on ice. Each sample was transferred to 50 mL tubes and centrifuged (4200*g*, 4 °C) for 15 min. The FSs were divided into aliquots and stored at −80 °C. Samples were sterilized by filtration (pore size, 0.22 $\mu$m) before use in the cell culture experiments.

**Analysis of pH Values, Short Chain Fatty Acids (SCFA), and Bile Acids.** The pH values were determined with a pH meter Hydrus 300 (Fisherbrand, Schwerte, Germany). To determine the SCFAs, the samples were mixed with an isocapronate standard (1:11 v/v), shaken, and centrifuged at 6400*g* for 10 min at 4 °C. Then, the gas chromatographic measurements (GC 17A, Shimadzu, Duisburg, Germany) were performed using a 15 m FFAP column (Phenomenex, Aschaffenburg, Germany) and a specific temperature program (start temperature, 130 °C; increase 35 °C/min; and final temperature, 170 °C) (*28*). Fecal bile acids were analyzed as described previously (*29*). Briefly, aliquots of the FS were hydrolyzed under alkaline conditions with ethanolic NaOH and extracted with cyclohexane. Bile acids were saponified with NaOH, and the samples were then acidified to pH 1 with HCl and extracted with diethyl ether. The extracts were combined in a tube containing internal bile acid standard (hyodeoxycholic acid). The solvent of the combined extracts was evaporated, and the residue was methylated and silylated. After evaporation, the residual content was resolved in decane, shaken, and centrifuged. The clear solution was injected into the gas chromatograph−mass spectrometer (GC17-QP5000, Shimadzu).

**Human Tumor Cell Line HT29.** The human colon carcinoma cell line HT29 was established in 1964 by J. Fogh (Memorial Sloan Kettering Cancer Centre, New York) (*30*) and was obtained from the American Tissue Culture Collection (Rockville, MD). Cells were maintained in stocks in liquid nitrogen, thawed, and grown in tissue culture flasks with DMEM (Dulbecco's modified Eagle medium, Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum, penicillin (50 U/mL), and streptomycin (50 $\mu$g/mL) at 37 °C in a (95%)

humidified incubator (5% $CO_2$). The cultured cells were trypsinized with 1−1.5 mL of trypsin/versene (1:10 v/v) for a maximum of 10 min and subcultivated at a dilution of 1:3 until 1:10 in $T_{75}$ flasks with supplemented DMEM. We used passages 29−54 for the experiments reported in this paper.

**Determination of Cell Growth.** Growth and survival of colon cells were determined in 96 well microtiter plates (MTP). Twenty-four hours after seeding (8000 cells each well), cells were treated with arabinoxylan fractions (0.07−4.5 g/L AeAx, 0.05−3.5 g/L WeAx), or FS (0.01−25%) in culture medium for 24, 48, and 72 h. DNA was isolated by fixing and permeabilizing the cells with methanol for 5 min followed by the addition of 4′,6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich, Deisenhofen, Germany). After 30 min, the DNA content as a reflection of the remaining cells was detected by fluorimetrical analysis with Ex/Em 360/450 nm (SpectraFluor Plus, Tecan Germany GmbH, Crailsheim). Mean values (three determinations per experiment) were recorded for final evaluation.

**Determination of DNA Damage and Chemoprotective Effects with the Single Cell Microgel Electrophoresis Assay.** For short-term incubation, $4 \times 10^6$ cells were seeded in a small cell culture flask (25 $cm^2$) 24 h before starting the experiment. The subconfluently (80−90%) grown cells were trypsinized and dissolved in DMEM at a concentration of $2 \times 10^6$ cells/mL. The cells were incubated with different concentrations of the unfermented arabinoxylan fractions (AeAx and WeAx) or the supernatants of fermented arabinoxylans for 30 min at 37 °C in a Thermomixer (Eppendorf, Hamburg, Germany) at 450 rpm. For long-term incubation, $0.4-0.6 \times 10^6$ cells were seeded in six well plates. After 24 h, the medium was replaced by the incubation mixtures that contained the test substances. The cells were incubated for 24, 48, and 72 h at 37 °C in a humidified incubator (5% $CO_2$/95% air humidity).

The pretreated cells were subsequently incubated with 75 $\mu$M $H_2O_2$ [30% stock solution was diluted in phosphate-buffered saline (PBS)] for 5 min at 4 °C or with 200 $\mu$M 4-hydroxynonenal (HNE) (20 mM stock solution of HNE in 95% ethanol was diluted in RPMI) for 30 min at 37 °C under shaking conditions (450 rpm).

Viabilities were determined with the trypan blue exclusion test, and the remaining cells were mixed with low-melting agarose and distributed onto microscopical slides followed by distribution of one more agarose layer. Further steps were carried out as described elsewhere (*31*−*33*). In short, the slides were lysed for 60 min at 4 °C and subjected to alkaline conditions for 20 min. Electrophoresis was carried out at 1.25 V/cm, 300 mA for 20 min, after which the slides were neutralized and stained with SYBR-Green (Sigma-Aldrich Chemie GmbH, Steinheim, Germany, 10×; 30 $\mu$L per slide). Comet images, revealing more or less damaged DNA (*34*), were quantified using the image analysis system of Perceptive Instruments (Halstead, United Kingdom) evaluating 50 DNA spots per sample and slide. Mean values of tail intensities (the percentage of fluorescence in the comet tail) from three slides per experiment were calculated and were the basis for computing the means of at least three independently reproduced experiments.

**Determination of Cellular Glutathione Peroxidase (cGPx) Activity.** The determination of the cGPX activity was carried out photometrically with the Cellular Glutathione (GSH) Peroxidase Assay Kit from CALBIOCHEM in 96 MTP. After the reaction was started with *tert*-butyl-hydroperoxide, the cGPx oxidized two molecules of GSH to yield the dimer GSSG, which was recycled with added glutathione reductase under NADPH consumption. The oxidation of NADPH to $NADP^+$ was detected by the decrease in absorption at 340 nm, which was proportional to the cGPx activity. For this, cells were treated with different concentrations of the unfermented and fermented arabinoxylans for 30 min and 24 h.

**Determination of GSTP1 and GSTA4-4 Protein and GST Activity.** Cells were incubated with the test substances, after 48 h of incubation time, washed with PBS, and harvested with trypsin/versene. Further steps were essentially performed as described by Ebert et al. (*25*, *35*). Cells were resuspended in cold homogenizing buffer consisting of 250 mM sucrose, 20 mM Tris/HCl, 1 mM dithiothreitol, and 1 mM Pefabloc (Roth, Karlsruhe, Germany; pH 7.4) and homogenized using ultrasound (1 min, six cycles, 40% power, Sonoplus, Bandelin Electronics, Berlin, Germany). Following centrifugation (16000*g*, 60 min, 4 °C), the supernatant was aliquoted and frozen at −80 °C until use. For immunoblotting, cytosols were diluted (1:1.5) with loading buffer [125 mM Tris, 2% sodium dodecyl sulfate (SDS), 10% glycerine, 6 M urea, 324 mM dithiothreitol, and 0.1% bromphenolblue] and incubated for 10 min at 65 °C. For detection of GST isoenzymes, defined amounts of cytosolic protein (63 $\mu$g of protein for GSTA4-4 and 10 $\mu$g of protein for GSTP1) were loaded and subjected to SDS-polyacrylamide gel electrophoresis (stacking gel, 3% w/v acrylamide; separation gel, 12% w/v acrylamide). Afterward, the separated protein was transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany) in a semidry blotting chamber (MTV1, Cti GmbH, Idstein/Taunus, Germany). The immunoblots were subsequently blocked with milk protein (5%) and incubated with the appropriate antibody. We used monoclonal antibodies against human GST class $\alpha$ (generous gift from Prof. E. Gallagher) and $\pi$ (Calbiochem, Schwalbach, Germany) (*36*). As a standard, the housekeeping protein $\beta$-actin (Sigma-Aldrich) was used.

The GST activity in the cytosol was determined spectrophotometrically with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate at 340 nm and 30 °C (*37*). The total protein content was measured using the method by Bradford with bovine serum albumin as a standard protein (*38*).

**Statistical Evaluation.** Data shown in the tables and figures represent mean values ± standard errors of the mean (SEM). Unless otherwise stated, these means were calculated from the means of triplicate replicates obtained in at least three independent experiments. Statistical evaluation was performed with the GraphPad Prism Version 4.0 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). One-way analysis of variance (ANOVA) was used to determine significance of the experimental variables. The significance of individual treatment groups in comparison to the controls was determined with the Bonferroni's multiple comparison posthoc test. The statistical analyses used depend on the respective experimental design and are specified in the legends of the figures and tables.

## RESULTS AND DISCUSSION

Dietary fiber contributes to cancer prevention and especially WB inhibits colon tumors in animal experiments more consistently than other dietary fibers. However, the protective characteristics, which have been proven in numerous studies (*39*−*41*), could be assigned neither to a single compound nor to a certain group of substances so far. Only a few studies analyzed the isolated and chemically defined form of arabinoxylans, which are quantitatively the most important dietary fiber component of WB (*17*) and were therefore the focus of this work.

**Fermentation (SCFA Acids, pH Value, and Bile Acids).** To characterize the FS, we analyzed the fermentation profile (type and amount of the produced SCFA), bile acids, and the pH value. **Table 1** shows that the fermentation of the arabinoxylans resulted in a significant increase of SCFA (FS blank, 27.6 mM; FS AeAx, 92.1 mM; and FS WeAx, 80.5 mM), with a clearly decreased pH value (FS blank, 6.9; FS WeAx, 5.6; and FS AeAx, 5.5). The major products were acetate, followed by propionate and butyrate. The relative molar concentrations, with molar ratios for acetate:propionate:butyrate of 61:23:16 (FS blank), 63:24:13 (FS AeAx), and 65:20:16 (FS WeAx), do not indicate a particular modified SCFA profile. Results of Lu et al. (*17*) indicate that arabinoxylan fiber behaves like a rapidly fermentable, soluble dietary fiber very similar to guar gum. In the rat intestine, predominantly acetate and only very little butyrate were formed as fermentation products after arabinoxylan-rich food intervention. Because of the fast metabolism of the arabinoxylans in the intestine, large quantities of SCFA are formed in proximal intestine sections. The high production of SCFA in vitro is possibly also an indication of protective effects by the diet, since these overall SCFA increases are also a

**Table 1.** Concentration of SCFA (mmol/L) in Fermented Arabinoxylan Fractions[a]

| | FS blank | | FS AeAx | | | | FS WeAx | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | mean | ± SEM | mean | ± SEM | fold change | | mean | ± SEM | fold change | |
| acetate | 14.6 | 1.8 | 54.0 | 1.7 | 3.7 | *** | 48.4 | 5.4 | 3.3 | *** |
| propionate | 5.6 | 0.9 | 20.9 | 3.0 | 3.7 | ** | 14.7 | 1.9 | 2.6 | * |
| *i*-butyrate | 0.9 | 0.2 | 0.8 | 0.1 | 0.8 | | 0.8 | 0.1 | 0.9 | |
| *n*-butyrate | 3.8 | 0.5 | 10.7 | 0.9 | 2.8 | ** | 11.7 | 1.9 | 3.1 | ** |
| *i*-valeriate | 1.2 | 0.2 | 0.7 | 0.1 | 0.6 | | 0.9 | 0.1 | 0.8 | |
| *n*-valeriate | 1.0 | 0.2 | 2.9 | 0.5 | 2.9 | ** | 2.2 | 0.3 | 2.3 | |
| *n*-capronate | 0.4 | 0.1 | 2.0 | 0.1 | 4.5 | *** | 1.7 | 0.2 | 3.9 | *** |
| total | 27.6 | 3.7 | 92.1 | 3.4 | 3.3 | *** | 80.5 | 7.0 | 2.9 | *** |

[a] Fold changes depict changes to the FS blank, and asterisks depict significant differences to the FS blank as the control. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (one-way ANOVA with Bonferroni's multiple comparison posthoc test against control); $n = 4$.

**Table 2.** Influence of Fermented Arabinoxylan Fractions and Blank as Control (0.05–25%) on a Number of HT29 Cells[a]

| | concentration of FSs (v/v) | | | | |
|---|---|---|---|---|---|
| | $EC_{50}$ (%) | 4% (%) | 6% (%) | 10% (%) | 25% (%) |
| FS blank 24 h | ND | | | | –37 (***) |
| FS blank 48 h | 15.1 | | –20 (**) | –33 (***) | –74 (***) |
| FS blank 72 h | 11.1 | –22 (*) | –33 (***) | –44 (***) | –83 (***) |
| FS AeAx 24 h | ND | | –11 (*) | –24 (***) | –45 (***) |
| FS AeAx 48 h | 13.6 | –13 (*) | –21 (***) | –46 (***) | –75 (***) |
| FS AeAx 72 h | 10.6 | –20 (*) | –28 (***) | –53 (***) | –89 (***) |
| FS WeAx 24 h | ND | | –15 (**) | –24 (***) | –51 (***) |
| FS WeAx 48 h | 12.0 | –14 (*) | –27 (***) | –51 (***) | –78 (***) |
| FS WeAx 72 h | 9.0 | –25 (*) | –37 (***) | –59 (***) | –93 (***) |

[a] The table shows $EC_{50}$ values (concentrations of FS in the medium that reduces the number of cells by 50%) and the reduction of cell growth after 24, 48, and 72 h of incubation. Differences between FS blank and FS AeAx or WeAx were not significant. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (one-way ANOVA with Bonferroni's multiple comparison posthoc test against control without FSs); $n = 6$; ND, not detectable.

reflection of enhanced formation of butyrate and propionate, which both have antiproliferative properties (*23*).

The analytical measurements of bile acids revealed concentrations of 1.4 mg/L lithocholic acid (LCA) and 4.3 mg/L desoxycholic acid (DCA) in FS blank, whereas only 0.8 mg/L LCA and 1.0 mg/L DCA were found in FS AeAx, and 0.6 mg/L LCA and 0.8 mg/L DCA were found in FS WeAx. This reduced bile acid formation in the presence of arabinoxylans shows a trend, which is in line with results of Alberts et al. (*42*). These authors reported that the median concentrations of secondary bile acids in stool of participants of the WB fiber colon polyp trial were significantly lower for the high fiber group. Because bile acids are implied as promoting colon cancer growth and progression (*43*) and are known to interfere with antiproliferative properties of butyrate (*44*), this observed decline in bile acids in the presence of fiber may be considered to be favorable in terms of colon cancer prevention.

**Cell Proliferation.** Cellular effects of the FSs on cell proliferation were investigated next. **Table 2** shows that the growth of HT29 cells was efficiently retarded by all FS (including the control) in a time- and concentration-dependent manner. Differences between FS blank and FS AeAx or FS WeAx were not significant. The results show that quite similar dose-response curves were observed for the individual fermentation samples. All samples had significant inhibitory effects after 72 h at concentrations of 4% (FS AeAx, FS WeAx, and FS blank). This is quite remarkable, since they contain different amounts of SCFAs (**Table 1**), which were expected to be the major biologically active and antiproliferative components (*23*). When comparing the effective doses plotted on the basis of butyrate concentrations, a different picture becomes apparent, which shows that the FS blank was the most effective supernatant (data not shown). For example, the calculated $EC_{50}$ value of the effective butyrate concentration after 72 h is only 0.42 mM for the FS blank but 1.11 mM for the FS AeAx and 0.99 mM for the FS WeAx.

The antiproliferative effect of the FS is probably not due to the activity of a single ingredient, like one individual SCFA, because these samples are complex mixtures of many different compounds. The individual compounds have not yet been analyzed in detail, but SCFAs (which were present in known concentrations) are probably involved since they are known to have a high impact on cell growth. Other authors, for example, have demonstrated that 4 mM butyrate for 72 h reduced the growth of HT29 cells by 75% (*45*). A comparable growth reduction could be found in this work in cells treated with the FS WeAx (concentration, 15%) containing butyrate at a concentration of only 1.7 mM. Thus, the antiproliferative effect of the FS in this study is probably not due to their butyrate content. Also, other SCFAs, like propionate, inhibit the growth of HT29 cells. $EC_{50}$ values (after 72 h of incubation) of propionate have been determined to be 2.7 mM (*23*).

Thus, the sum of butyrate and propionate, which amounted to 2.6 and 3.2 mmol in the cell cultures treated with 10% WeAx and AeAx FSs, respectively, could have been responsible for the growth inhibitory properties of these samples but not for the growth inhibitory properties of the controls, which only delivered 0.9 mmol of butyrate and propionate to the cell culture medium. Instead, the control samples probably inhibited growth on account of other ingredients. As mentioned above, they contained 4−5-fold higher amounts of bile acids (sum of DCA, iso-DCA, LCA, and iso-LCA, 19 $\mu$M) than the fermented WeAx and AeAx samples (sum of bile acids was only 3.7 and 4.8 $\mu$M, respectively). Bile acids have been shown to also modulate tumor cell survival, however, by other mechanisms. Thus, selected compounds have been shown to stimulate proliferation of HT29 cells at low concentrations (up to 20 $\mu$M), whereas an approximately 5-fold higher concentration resulted in apoptosis (*46*). Thus, SCFA could be responsible for the growth-modulating effects of the FS AeAx and FS WeAx whereas the secondary bile acids more reflect the activities of the control FS.

**Cytotoxicity and Genotoxicity.** Cytotoxic and genotoxic effects were not detected in HT29 cells after 30 min and 24 h of treatment with unfermented arabinoxylans (data not shown), even though the concentration range used was up to the limit of solubility (7 g/L WeAx and 9 g/L AeAx).

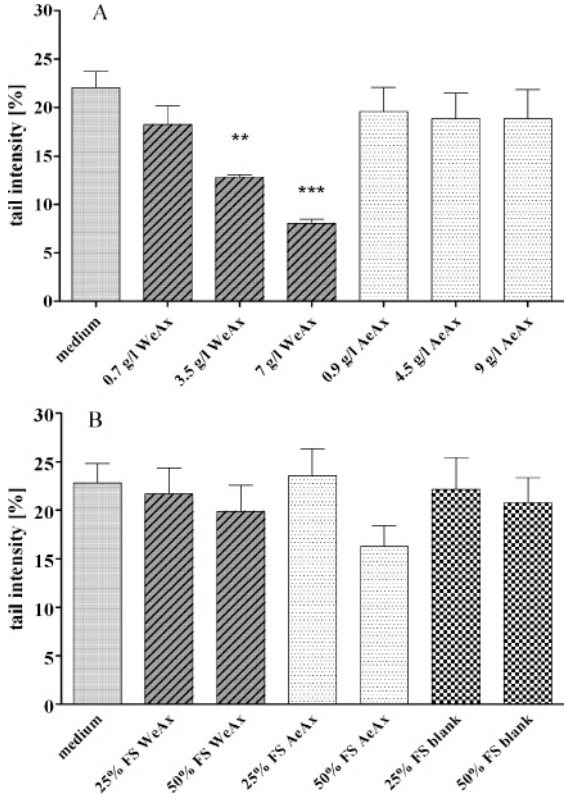


**Figure 1.** $H_2O_2$-induced DNA damage (75 $\mu$M for 5 min on ice) after pretreatment (30 min) of HT29 cells with (**A**) unfermented ($n = 3$) or (**B**) fermented arabinoxylans ($n = 4$). Values are means with standard errors represented by vertical bars. Mean values with asterisks depict significant differences to the medium control (pretreatment with medium and second treatment with $H_2O_2$). $^{**}p < 0.01$ and $^{***}p < 0.001$ (one-way ANOVA with Bonferroni's multiple comparison posthoc test against control).

The FSs were investigated at concentrations of 25 and 50% (for 30 min) and 10% (for 24 h). These concentrations were neither cyto- nor genotoxic to the HT29 cells (data not shown).

**Chemoresistance by Arabinoxylans.** To investigate protection of cells from DNA damage, the HT29 cells were preincubated for 30 min at 37 °C (short-term incubation to assess scavenging potentials) or for 24 h at 37 °C (long-term incubation to assess possible modulation of gene and protein expression) with the different arabinoxylan fractions. Then, the cells were exposed to 75 $\mu$M $H_2O_2$ for 5 min on ice. **Figure 1A** shows that WeAx protected HT29 cells from $H_2O_2$-induced DNA damage (significant reduction of 64% after 30 min of incubation with the highest WeAx concentration), whereas AeAx was without an effect. At first glance, a possible explanation for this apparent antioxidative activity and chemoprotective effect could be due to specific ingredients, namely, hydroxycinnamic acids, which have antioxidative properties (*47*). They are found in WB at concentrations ranging from 99 to 231 $\mu$g/g bran (*48*). Comparable concentrations are also found in arabinoxylan fractions from wheat flour (*49*). Our arabinoxylans AeAx and WeAx contained no measurable contents of FA (data not shown), probably due to the extraction process (*18*). Therefore, other not yet identified bioactive components in WeAx might be responsible for the measured effects. The bacterial activity during the fermentation procedure could be responsible for a reduction of these undefined bioactive compounds. Also, a longer preincubation with arabinoxylan fractions for 24 h failed to prevent $H_2O_2$-induced DNA damage. Neither unfermented nor fermented arabinoxylans were effective (data not shown).

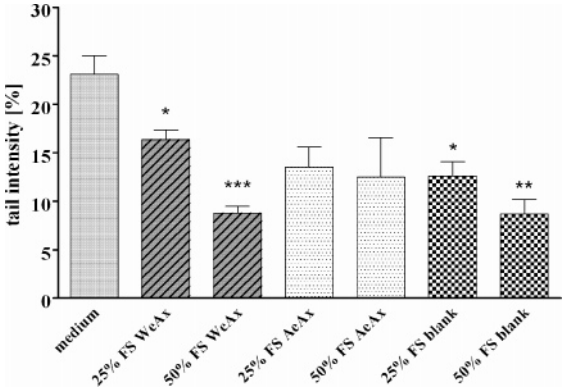


**Figure 2.** HNE-induced DNA damage (150 $\mu$M for 30 min at 37 °C) after preincubation (30 min, 37 °C) of HT29 cells with FSs. Values are means with standard errors represented by vertical bars ($n = 3$). Mean values with asterisks depict significant differences to the medium control (pretreatment with medium and second treatment with HNE). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ (one-way ANOVA with Bonferroni's multiple comparison posthoc test against control).

Oxidative stress causes damage of cellular macromolecules such as DNA, proteins, or lipid bilayers of the cell membranes. The main oxidation products of fatty acids are $\alpha,\beta$-unsaturated aldehydes, like HNE, which can interact with the DNA and damage this macromolecule by adduct formation (*50*). We had previously demonstrated that HNE genotoxicity was efficiently preventable in HT29 cells by their pretreatment with butyrate, an important SCFA product of dietary fermentation (*25*, *51*). We therefore investigated whether unfermented and fermented arabinoxylan fractions may also protect cells from HNE-induced DNA damage. For this, we pretreated the cells for 30 min at 37 °C with the arabinoxylans. The cells were then exposed to HNE for 30 min at 37 °C, and HNE-induced DNA damage was determined. Whereas unfermented samples, in contrast to the results with $H_2O_2$ (maybe due to different chelating affinities between the samples and the genotoxins), had been unable to modulate the genotoxic effect of HNE (data not shown), **Figure 2** shows that the genotoxicity of HNE was clearly reduced after pretreatment with fermented arabinoxylan samples. There was a reduction by 46 and 64% after incubation with FS AeAx (50%) and FS WeAx (50%), respectively. There was, however, also a significant reduction after pretreatment of the cells with the control FS blank, which was prepared without arabinoxylans. Therefore, the protective effect against HNE may result from other ingredients than butyrate, which may have their origin from the fecal samples of the donors. Opposed to the explanation that bile acids and SCFA are probably responsible for growth inhibitory properties of FS blanks and FS from dietary fibers, respectively, this conclusion cannot be made for the parameter of "prevention of HNE genotoxicity". We have, for example, been able to clearly show that HNE-mediated DNA damage is effectively inhibited by pretreating with the SCFA, butyrate (2−4 $\mu$M) (*25*), but have no data available that this could also be mediated by bile acids. Therefore, further investigations are needed to assess the antigenotoxic potential of bile acids in this system to better understand the role of bioactive compounds in control fermentation samples. Previous studies that have also compared the antigenotoxic efficacy of batch fermentation samples did not disclose an effectiveness of the control samples, whereas fermentation samples from wheat and soya did show antigenotoxic potentials (*23*). The activity was, however, less than that observed with a synthetic SCFA mixture composed according to the FS and also less than butyrate, which does not support the presence of HNE modulator compounds in the feces

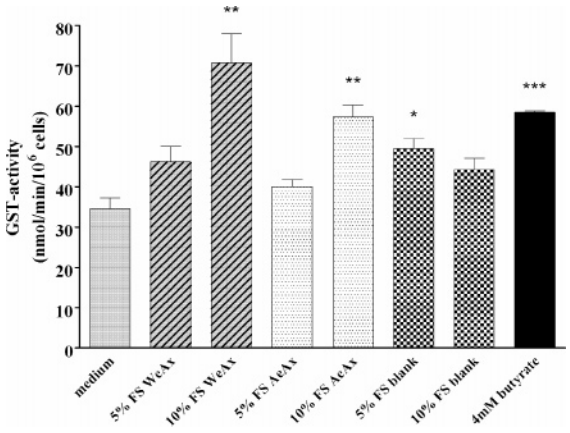


**Figure 3.** GST activity of HT29 cells after incubation with 5 or 10% of fermented arabinoxylan fractions for 48 h. Values are means with standard errors represented by vertical bars ($n = 3$). Mean values with asterisks depict significant differences to the medium control. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (one-way ANOVA with Bonferroni's multiple comparison posthoc test against control and unpaired *t*-test for the butyrate control).

used in those studies. The donors of the feces used in this study were healthy, nonvegetarian volunteers on a regular diet who did not use food supplements or antibiotics, as were all those who supplied the feces also in the first study. Thus, there is no apparent explanation for the observation of why the feces of this study contained or produced as yet unknown antigenotoxic compounds, whereas those of the first study did not.

**Modulation of Enzyme Activities (Glutathione Peroxidase, cGPx, and GST) and Modulation of GST Subunits GSTP1 and GSTA4-4.** It is thought that one mechanism by which FS could decrease the genotoxicity of HNE is by enhancing GSTs, some of which have a high affinity for HNE as substrate (*52*) and thus a good detoxification capacity (*53*). This is why we investigated the effect of the arabinoxylans on enzyme activities and enzyme protein levels.

The unfermented arabinoxylan fractions had no influence on the GST activity (data not shown). Forty-eight hours of incubation with fermented arabinoxylans, however, resulted in a detectable induction of GST activity (**Figure 3**). The GST activity (related to $10^6$ cells) increased significantly for 5% FS blank (factor 1.4), for 10% FS AeAx (factor 1.7), and for 10% FS WeAx (factor 2.0). Ten percent FS blank was only insignificantly less active than the 5% concentration but failed to induce the GST activity ($p = 0.069$) in comparison to the medium control. This might be due to inhibitory compounds of the blank that are active when adding larger amounts to the cell culture medium. The strongest induction of GST activity was observed in the FS WeAx, which had a relatively low butyrate concentration (1.1 mM). The observed increase was significantly higher than the comparable control (FS blank 10%) and insignificantly higher than the butyrate control (4 mM), which only caused a 1.7-fold rise. We have previously shown that butyrate induces GSTs in HT29 cells (*25*, *35*), probably via a butyrate-induced activation of the MAPK cascade, which could be shown by an increased phosphorylation of ERK1/2. Moreover, butyrate markedly induced GSTA4, which has the highest affinity for the substrate HNE (*51*), thus explaining the effectiveness of FS from AeAx and WeAx for this parameter. It is however again unknown which compounds of the FS blank were responsible for enhancing GST activity since it contains too low amounts of butyrate to explain the induction.

The relevance of an increase in GST activity to the in vivo situation must be regarded under two different points of view. In tumor cells, a GST induction would not be favorable for the health of the entire organism, because this increased activity of the GST could improve the resistance against cytostatic drugs. Regarding the chemoprevention of cancer, a GST induction in normal colonocytes or in early stages of carcinogenesis could be a good mechanism for the cell to become chemoresistant against carcinogenic compounds (like HNE) (*25*).

To clarify the reason for the altered GST activity, we examined the influence of the fermented arabinoxylans on the protein quantity of two important GST isoenzymes. For this, we determined cellular concentrations of GSTP1 subunits, the prevailing isoenzyme in the human intestine and in HT29 cells (*25*). We also determined the isoenzyme GSTA4-4, the main enzyme involved in the detoxification of HNE (*51*, *52*). Cells were treated for 48 h with 5 or 10% FS AeAx or FS WeAx (same conditions as during the determination of the GST activity). Neither FS AeAx nor FS WeAx had a significant influence on the expression of the isoenzyme GSTA4-4. Although there were also no significant changes in GSTP1, here, we were able to detect a trend for an increase of the protein content by all test substances [FS blank, factor 1.8 (5%) and 1.6 (10%); FS AeAx, 2.0 (5%) and 2.1 (10%); FS WeAx, 1.4 (5%) and 2.2 (10%); and positive control butyrate, 2.5 (4 mM)] (data not shown). As expected, on the basis of previous studies (*25*, *35*), the butyrate control showed a quantitative increase of the isoenzyme (factor 2.5) but without statistical significance in this particular set of experiments. Previous studies have clearly shown that GST activity and GSTP1 expression are highly correlated and that the majority of the GST activity is reflected by GSTP1 protein expression. It can however not be excluded that the induction of the GST activity is also determined by other groups of isoenzymes. Interestingly, an increase of the enzyme GSTA1-2 could be shown by an intervention trial with WB (*54*), which also conjugates the substrate CDNB used in our method. CDNB, moreover, has a very high affinity for the isoenzyme GSTM2 (*37*). Therefore, the detected rise of the GST activity may be attributed also to a butyrate-induced rise of the GSTM2 and GSTA1-2 protein expression (*35*). For better understanding of how GST activity is influenced by the test substances, further investigations of the fermented arabinoxylans seem necessary and meaningful. It is possible that chemoprotective compounds result from the fermentation of arabinoxylans that have not yet been identified. Furthermore, there could be cross effects between butyrate and other substances (*55*).

In our last set of experiments, we investigated the effect of arabinoxylans on cGPx activity. Here, we found that the activity of this enzyme was not modulated after incubation (30 min and 24 h) with unfermented or fermented arabinoxylan fractions (data not shown).

In conclusion, these results show that arabinoxylans from WB are a good fiber source available for fermentation by human gut bacteria. They demonstrate for the first time chemoprotective properties by WB extracts in human colon cells and indicate that protective effects of FSs may result also from other ingredients, which were not produced during the fermentation of the arabinoxylans but had their origin from the fecal samples of the donors.

## ABBREVIATIONS USED

AeAx, alkali extractable arabinoxylans; CA, coumaric acid; cGPx, cellular glutathione peroxidase; DCA, desoxycholic acid; FA, ferulic acid; FS, fermentation supernatant; GST, glutathione S-transferase; HNE, 4-hydroxynonenal; LCA, lithocholic acid;

SCFA, short chain fatty acid; WB, wheat bran; WeAx, water extractable arabinoxylans.

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