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# Effects of synbiotic fermentation products on primary chemoprevention in human colon cells☆

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**Abstract**

The consumption of synbiotics, a mixture of probiotics and indigestible food constituents such as dietary fiber, has been reported to reduce colon cancer risk. We investigated the effects of fermented wheat aleurone enriched with the probiotics *Lactobacillus rhamnosus* GG/*Bifidobacterium animalis* supsp. *lactis* on the gene expression and functional end points related to cellular defence in HT29 and primary human colon cells. Aleurone was digested and fermented *in vitro* with/without probiotics. The resulting fermentation supernatants (fs) were analyzed for concentrations of deoxycholic acid and ammonia. The cells were treated with the fs, and effects on gene expression of catalase, *GSTP1* and *SULT2B1*, enzyme activity of catalase and glutathione *S*-transferase as well as $H_2O_2$-induced DNA damage were examined. Fermentation of aleurone reduced deoxycholic acid concentration by 84%, while the probiotics enhanced this effect. Ammonia was increased by fs aleurone, whereas a reduction occurred by the addition of *L. rhamnosus* GG/*B. animalis* supsp. *lactis* 12. *GSTP1* expression tended to result in an increase by the fs aleurone in both cell types, whereas the probiotics could not additionally increase the effect. Catalase was not modulated by fs aleurone enriched with probiotics. Only in HT29 cells, expression of *SULT2B1* was enhanced by fs aleurone. Enzyme activity of catalase and glutathione *S*-transferase was induced (2–3.6 fold, 72 h) in HT29 cells only. Addition of probiotics had no influence on this effect. In HT29 cells, a reduced $H_2O_2$-induced DNA damage by the fs aleurone after 48 h, enhanced by the addition of probiotics, was detected. The observed effects could improve detoxification of xenobiotics and therefore may lower colon cancer risk.


*Keywords:* Wheat aleurone; Catalase; Glutathione *S*-transferases; Human colon cells; Probiotics; Sulfotransferases

## 1. Introduction

Colorectal cancer is one of the most common causes of death from cancer worldwide. Approximately one million people are diagnosed with colon cancer every year, over the last 25 years [1]. It is suggested that diet and other lifestyle factors are substantially involved in cancer development [2,29].

The intestinal microflora acts as an important determinant for general health of the human body, for example, by influencing the immune system and the aetiology of colon cancer. Therefore, a beneficial modulation of the composition of the gut microbiota represents an interesting approach to improve health. This modulation can be realized either by ingestion of probiotics, living microbial feed supplements, which improve the intestinal microbial balance; or prebiotics, which are selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits upon host well-being and health [3-5]. Meanwhile, there is some evidence indicating an inverse association of probiotic intake and the incidence of colon cancer [6]. Even though the mechanisms by which probiotics may inhibit colon cancer are not fully characterized, some potential mechanisms were disclosed, for example, alteration of the metabolic activities of the intestinal microflora, changing physicochemical conditions in the colon, binding of potential carcinogens, short-chain fatty acid (SCFA) production and production of antitumorigenic or antimutagenic compounds [7,8].

Dietary fiber represents a substrate for fermentation by the human gut flora and has been shown to convey a prebiotic effect, which has been linked to colon cancer prevention [3]. Consequently, the fermentation results in a selective stimulation of growth and/or activity of the gut microflora, particularly the beneficial *Bifidobacteria* and *Lactobacilli* [9,10]. However, fermentation of dietary fiber also results in the formation of SCFA, namely, acetate, propionate and butyrate. Of these, butyrate, in particular, has received much attention

*Abbreviations:* CAT, catalase; DCA, deoxycholic acid; GST, glutathione *S*-transferases; fs, fermentation supernatant; SCFA, short-chain fatty acids; SULT, sulfotransferases.

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as a potential chemopreventive agent [11,12]. While acting as energy source for nontransformed cells, butyrate possibly reduces survival of tumor cells by inducing apoptosis and differentiation as well as inhibiting proliferation. These mechanisms may play an important role in the reduction and/or inhibition of promotion and progression of cancer development [13,14].

Many prebiotics belong to the group of nondigestible oligosaccharides, which resist digestion and absorption in the human small intestine and are fermented in the colon [4]. Fructo-oligosaccharides and inulin are the best-studied prebiotic oligosaccharides. The main complex nondigestible polysaccharide of cereal grains is the dietary fiber arabinoxylan, which is an important part of the aleurone layer of whole grains, for which a prebiotic potential was suggested [4,15]. Rafter et al. demonstrated by a human intervention trial that synbiotic products, a combination of pro- and prebiotics, are more effective than probiotics or prebiotics alone in terms of cancer prevention [16].

Next to influencing cell survival of cancer cells, another mechanism by which prebiotics and dietary fiber may be protective against colon cancer is by preventing the formation of ultimate carcinogens or reactive oxygen species (ROS) [12].

Experimental evidence supports an important role for ROS in carcinogenesis. ROS are highly reactive oxidants, which are formed during numerous physiological processes, for example, aerobic respiration, cell metabolism, detoxification of foreign compounds and immune response [17]. Oxidative stress, the imbalance between the formation and degradation of ROS, can cause damage of lipids, proteins and/or DNA, thereby contributing to numerous degenerative diseases, including the initiation and promotion/progression of cancer [18-21].

Mammalian cells express different types of protective stress–response enzyme systems against oxidative stress, for example, catalase (CAT) or superoxide dismutase. Hydrogen peroxide ($H_2O_2$), a highly reactive ROS, is rapidly detoxified by CAT, the key defence enzyme against oxidative stress. A high level of CAT expression and/or activity is therefore associated with a reduction of genetic damage and consequently a lower risk for cancer [20,22-25].

Glutathione *S*-transferases (GST) and sulfotransferases (SULTs) are phase II enzymes that can detoxify a number of carcinogens by conjugation of diverse electrophiles with glutathione or a sulfate group, respectively. It has been proposed that an enhancement of GST and SULT activity might result in more efficient elimination of carcinogens and therefore reduces the susceptibility to cancer [26,27].

In this study, the colon adenocarcinoma cell line HT29 was used as a well-established and stable cell model for human colon cells, which has been used in many other studies [28-31]. Primary human colon cells were used to investigate the effects on primary chemoprevention.

Up to now, the effects of complex fermentation supernatants (fs) containing probiotics on primary chemoprevention have rarely been the subject of *in vitro* studies. Thus, the first aim of the study was to find out whether expression of genes involved in primary cancer prevention can be changed by complex fs of wheat aleurone generated with an *in vitro* batch model [31]. Another aim was to determine whether a combined administration of aleurone and *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium animalis* supsp. *lactis* (Bb12) during fermentation would increase the potency of chemoprotective effects of the fs. Furthermore, the content of potential tumor-promoting metabolites [deoxycholic acid (DCA) and ammonia] in the fs was determined. To examine the functional consequences of a modulated messenger RNA (mRNA) expression of the selected genes involved in detoxification, the enzyme activities of CAT and GST were analyzed in both colon cell models. As mentioned above, GST and CAT are connected with inactivation of cancer risk factors, for example, $H_2O_2$ [20,24]. Hence, it was of interest if the complex fs exhibit an increased chemoprotective potential toward $H_2O_2$.

## 2. Methods and materials

### 2.1. Dietary fiber source and probiotics

Wheat aleurone from Kampffmeyer Food Innovation GmbH (Hamburg, Germany) was used as a source of dietary fiber. Preparation of the aleurone fraction (we used an aleurone standard preparation [ASP-2] with high purity [70]) was done by Bühler AG (Uzwil, Switzerland) according to Bohm et al. [32]. To maintain stability of the aleurone, aliquots were prepared and stored in air- and light-proof flasks at 4°C. To analyze synergistic effects, we used a mixture of two probiotic strains — LGG and Bb12. LGG from Valio (Helsinki, Finland) and Bb12 from Christian Hansen (Hørsholm, Denmark) were both available as lyophilized highly viable powders (>log10 colony-forming unit/g product).

### 2.2. In vitro digestion and fermentation of wheat aleurone

This experiment was conducted by using wheat aleurone with (aleurone+) or without addition of the probiotic strains LGG/Bb12 (each $3\times10^9$ colony-forming unit) (aleurone−) as substrates. The test substances were digested and fermented *in vitro* in two repetitions in a batch–culture system according to the described procedure of Borowicki et al. [31]. Samples without the dietary fiber source but with (blank+) or without (blank−), the probiotic strains were used as controls. The obtained fs were stored at −80°C. Before using in the cell culture experiments, the samples were thawed quickly, aliquoted in 2-ml tubes, centrifuged (16,000×g, 4°C) and sterilized by filtration (pore size, 0.22 μm).

### 2.3. Analytics of potential tumor-promoting metabolites in fs

The concentrations of DCA and ammonia in the fs were determined by HPLC-MS/MS (high-performance liquid chromatography tandem mass spectrometry) and colorimetrically by the Berthelot reaction, respectively, as described elsewhere [31].

### 2.4. Preparation of primary human colon cells

Primary human colon cells were isolated from tissue specimens obtained during surgery of colorectal tumors, diverticulitis and colon polyps. The tissues were taken from the very edges of the resected colon segments. The surgeon and the pathologist confirmed that they did not show any micro- or macroscopic signs of malignant or inflammatory pathology. The university ethics committee approved the study, and the patients gave their informed consent (approval number: 1601-08/05). Mean age (±S.D.) of the six donors (three male, three female) of colon tissue, whose cells were used in the form of epithelial stripes for RNA isolation and cytosol extraction after incubation with the test substances, was 65 (S.D., 11) years. The tissue was prepared by separating the colon epithelium from the underlying layers of the tissue according to a described procedure of Schäferhenrich et al. [33]. The obtained epithelial stripes were used for incubation and subsequent analysis of gene expression and enzyme activities. To use noncytotoxic concentrations of the test substances in investigations on gene expression and enzyme activity, cell number and viability of the primary human colon cells had to be determined after incubation with the test substances. For this, single cells were isolated from the epithelial stripes of three male and three female patients [mean (S.D.) age, 79 (4) years] using proteinase K (Sigma, Steinheim, Germany) and collagenase C (Roche, Penzberg, Germany) as described before [33].

### 2.5. Cell culture of HT29 cells

The human colon adenocarcinoma cell line HT29 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), and culture conditions used are described in Glei et al. [28]. Passages 9–18 were used for the experiments. In regular intervals (~once a month), a mycoplasma test (MycoAlert Detection Kit) was performed, and contamination with mycoplasma was excluded.

### 2.6. Determination of cell number and viability of primary colon cells

Single cells ($2.8\times10^6$ cells/ml), isolated from epithelial stripes, were incubated for 1–12 h with 0–20% fs aleurone− and fs aleurone+ and the corresponding fs blank (feces control) diluted in minimal essential medium with Earle's salts enriched with 20% fetal calf serum, 2 mM glutamine, 1% penicillin/streptomycin, 100 μg/ml gentamicin, 2.5 μg/ml Fungizone, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite [34] in a shaking thermomixer at 37°C. The trypan blue exclusion test was routinely used according to Strober [35] to determine cell number and cell viability after incubating the cells to exclude cytotoxic effects of fs for the following experiments.

### 2.7. Treatment of primary colon cells to analyze gene expression and enzyme activity

Effects of fermented aleurone with and without LGG/Bb12 on gene expression and enzyme activity were studied after incubating epithelial tissue stripes with 10% (subtoxic concentrations) of the fs. This was done because cells incubated as epithelial stripes displayed an improved survival after 10 h of incubation in comparison with

single cells in suspension. Therefore, primary colon tissue pieces were placed in Petri dishes (35 $mm^2$), and after allowing the tissue pieces to settle for 15 min, they were treated with the fs dissolved in minimal essential medium. After 10 h, epithelial stripes were washed and immediately submerged in RNA*later* RNA stabilization reagent (Qiagen, Hilden, Germany), or they were ground in liquid nitrogen for experiments on enzyme activity. Storage of the samples took place at −80°C until further use for total RNA isolation and cytosol extraction.

### 2.8. Treatment of HT29 cells to analyze gene expression, enzymatic activity and antigenotoxicity

HT29 cells were grown for 24 h in $T_{25}$-cell culture flasks ($3\times10^6$ cells per flask) to analyze mRNA expression by real-time quantitative polymerase chain reaction (real time qPCR) or in 6-well plates ($1.5\times10^6$ cells/well) to analyze enzyme activity and antigenotoxic capacity. Then they were treated with 10% fs aleurone± for 24 h for analysis of gene expression, for 24–72 h for analysis of enzyme activity or for 24–48 h for analysis of the antigenotoxic potential of the fs against $H_2O_2$-induced DNA damage, respectively. After harvesting the cells with trypsin and a washing step, the pellets were used immediately to extract cytosols or to determine the antigenotoxicity. Onto analysis of mRNA expression, the cell pellets were stored in RLT buffer (for lysis of cells before RNA isolation; Qiagen) at −80°C until further use for total RNA isolation. Cell number and viability were determined with a CASY cell counter (CASY model TT; Roche Innovatis AG CASY Technology, Bielefeld, Germany).

### 2.9. Isolation of RNA

To isolate total RNA, epithelial stripes stored in RNA*later* solution were thawed, transferred into RLT buffer (for lysis of cells before RNA isolation) and were subsequently homogenized using the Polytron PT-DA 2107/2EC homogenizer (Fisher Scientific GmbH, Nidderau, Germany). HT29 cells stored in RLT buffer were thawed and completely lysed using Qiashredder minispin columns. Afterward, total RNA was isolated using the RNAeasy plus Mini Kit according to the manufacturer's protocol (Qiagen), dissolved in RNase-free water and stored at −80°C until further use. Yield and purity of RNA were quantified with a NanoDrop ND-1000 Spectrophotometer (Peqlab, Erlangen, Germany), and RNA stability (RNA integrity number) was measured using a Bioanalyzer (Agilent Technologies Deutschland GmbH, Böblingen, Germany).

### 2.10. Gene expression analysis of CAT, GST and SULT2B1 using real-time qPCR

To analyze mRNA expression, 1 μg (primary colon cells) or 2.5 μg (HT29 cells) of total RNA (RNA integrity numbers 7–10) was converted into first-strand complement DNA using SuperScript II as described in the manufacturer's protocol (Invitrogen GmbH, Karlsruhe, Germany). For real-time qPCR analysis, we used the system of iCycler iQ1 (Bio-Rad GmbH, München, Germany). Real-time qPCR was carried out using specific primer pairs and qPCR Eva Green Master according to the manufacturer's protocol (Jena Bioscience, Jena, Germany). All reactions were performed in duplicate. The PCR reaction profile included an initial denaturation of 2 min at 95°C, followed by 40 cycles of denaturation (15 s at 95°C), annealing and extension (30 s at 60°C). Cumulative fluorescence was measured at the end of the extension of each cycle. Product-specific amplification was confirmed by melting curve analysis, which was conducted with temperature gradient from 60°C to 95°C at 0.10°C/s to differentiate nonspecific primer dimer and specific amplicon. Gene-specific primer sequences for quantification of *CAT* (forward 5′-TGGACAAG-TACAATGCTGAG-3′ and reverse 5′-TTACACGGATGAACGCTAAG-3′), *GAPDH* (forward 5′-ACCCACTCCTCCACCTTTGAC-3′ and reverse 5′-TCCACCACCCTGTTGCTGTAG-3′), *GSTP1* (forward 5′-CTGCGCATGCTGCTGGCAGATC-3′ and reverse 5′-TTGGACTGGTA-CAGGGTGAGGTC-3) and *SULT2B1* (forward, 5′-ACGACGACATCTTTATCATCAC-3′ and reverse, 5′-CATGTAGATCACCTTGGCCT-3′) were used in both cell types, whereas only *CAT* and *GSTP1* were used for primary human colon cells because of the small size of the available samples. All primers were designed using the freely available PerlPrimer software version 1.1.17 (http://perlprimer.sourceforge.net).

The iCycler iQ1 optical version 3.0a software was utilized for determining the relative threshold cycle numbers ($C_t$).

The relative quantification of the target-mRNA expression was calculated with the comparative $\Delta\Delta C_t$ ($\Delta CT=\Delta C_{t\ control}-\Delta C_{t\ reference}$) method. For normalization, $\Delta C_t$ values were calculated by subtracting the average of the $C_t$ value in the control for the reference gene (*GAPDH*) from the average of the $C_t$ value for the target gene and subtracting the average of the $C_t$ value in the treated sample of the reference gene from the target gene. Then, the difference between the $\Delta C_t$ values of control and treatment ($\Delta\Delta C_t$) was calculated. The fold change was ascertained according to the efficiency method ($E=2$; fold change$=E^{difference}$) [36,37].

Changes of expression were determined as the fold change to the medium control, which was set to 1.

### 2.11. Preparation of cytosols, measurement of enzyme activities and cytosolic protein

To analyze the enzyme activity of CAT and GST, HT29 cells were lysed by incubation in a cold lysis buffer containing 1 mM EDTA, 0.1% Triton X-100, 50 mM potassium phosphate buffer (pH 6.5) and 1 mM pefabloc (Roth, Karlsruhe, Germany) for 10 min on ice. Epithelial stripes were thawed and homogenized before cytosol extraction in the presence of the lysis buffer using the Polytron PT-DA 2107/2EC homogenizer (Fisher Scientific GmbH). Cells were subsequently incubated for 10 min on ice. After centrifugation (10,000×*g*, 10 min, 4°C), the supernatants for both primary colon as well as HT29 cells were aliquoted and subsequently used for enzyme activity measurements. CAT activity was calculated according to Aebi [38] using $H_2O_2$ as substrate by photometrically measuring its decrease at 240 nm and 22°C. Total GST activity was determined photometrically as described in Habig et al. [39] using 1 chloro-2,4-dinitrobenzene as substrate. The increase of *S*-2,4-dinitrophenylglutathion, the product of conjugated 1 chloro-2,4-dinitrobenzene, was photometrically measured at 340 nm and 30°C. Results for HT29 cells were calculated on the basis of $1\times10^6$ cells and expressed as fold change to the medium control, which was set to 1. Results for primary colon cells were calculated on the basis of 1 μg protein and expressed as fold change to the medium control, which was set 1. Total protein content was measured using the method of Bradford [40] with bovine serum albumin as standard protein.

### 2.12. Detection of antigenotoxicity

DNA damage was measured with the single-cell microgel electrophoresis (Comet assay) after challenging the fs blank± as well as the fs aleurone± preincubated HT29 cells with 75 μM $H_2O_2$ for 15 min at 37°C according to the described procedure of Glei et al. [41]. Comet images, revealing the amount of damaged DNA (intensity of fluorescence in the comet tail; % tail intensity = TI) [42], were quantified using the image analysis system of Perceptive Instruments (Halstead, UK). For each concentration, means of 60 cells were the basis for calculating effects in one experiment.

### 2.13. Statistical evaluation

Means and standard deviations (S.D.) were calculated from at least three independent experiments (HT29 cells) and six donors (colon epithelial stripes), respectively. Differences were calculated by one- or two-way analysis of variance (ANOVA), including Bonferroni posttest with selected pairs, by Student's *t* test or by Friedman test, including Dunn's posttest with selected pairs using GraphPad Prism Version 5.02 for Windows (GraphPad Software, San Diego, CA, USA). The statistical analyses used depended on the respective experimental design and are specified in the legends to the figures and tables.

## 3. Results

### 3.1. Potential tumor-promoting metabolites in fs

The concentrations of the secondary bile acid DCA and ammonia were determined in the fs blank± and fs aleurone± (Table 1). Fermentation of aleurone− resulted in a considerable decrease in the amount of the secondary bile acid DCA by 84% compared with the fs blank−. The probiotics alone (fs blank+) also decreased the DCA concentration by 76% in comparison with the fs blank−. Importantly, the combination of both aleurone and LGG/Bb12 (fs aleurone+) showed the strongest decreasing effect with an almost completely reduction (of about 99.5%).

The level of ammonia was higher in the fs aleurone− than in the fs blank−. The probiotics alone (fs blank+) decreased the level of ammonia about 20% from 16.33 mM in the fs blank− to 12.96 mM. The addition of the probiotics to aleurone (fs aleurone+) also resulted in a reduction of ammonia in the fs in comparison with fs aleurone−.

### 3.2. Cell number and viability of primary colon cells

Cell number and viability of primary colon cells were determined by the trypan blue exclusion test after treatment of single cells with 0–20% of fs blank± and fs aleurone± to examine cytotoxicity. Only after 12 h, the viability of the untreated primary colon cells decreased significantly by 47% (S.D., 6%), and the recovery (remaining cells after incubation) of the cells was significantly reduced to 11% (S.D., 4%). However, an incubation for up to 12 h with all selected concentrations (0, 2%.5%, 5%, 10% and 20%) of our fs caused no impairment of the cell viabilities and the recovered number of cells in comparison with the control (data not shown). Thus, related to those two parameters, no dose effects were seen within each test substance. Therefore, a

concentration of 10% fs (analog to HT29 cells) was selected for subsequent experiments, because effects on mRNA expression and enzyme activities were supposed to be determined using a non-cytotoxic concentration. Accordingly, the cell viabilities of 10% fs [blank−: 59% (S.D., 4%); blank+: 63% (S.D., 10%); aleurone−: 61% (S.D., 11%); aleurone+: 62% (S.D., 9%)] did not differ significantly from the medium control [53% (S.D., 6%)] after the longest incubation time.

### 3.3. Gene expression analysis of CAT, GSTP1 and SULT2B1 by real-time qPCR

The influence of the fs blank± and fs aleurone± (10%) on mRNA expression of the selected genes *CAT*, *GSTP1* and *SULT2B1* was determined by real-time qPCR. The effects after 24 (HT29) and 10 h (primary cells) are summarized in Table 2 and Fig. 1, respectively. In HT29 cells, the expression level of *CAT* was increased by trend only by the probiotics alone with a fold change of over 1.5 in comparison with the medium control (Table 2). The expression of *GSTP1* was increased by the fs aleurone−, whereas the up-regulation (two-fold) was significantly different to the fs blank−. An addition of probiotics did not further enhance this effect. Incubation with the fs aleurone− resulted in an increased mRNA expression of *SULT2B1*. The probiotics alone had the same impact. However, treatment of the cells with the fs blank− revealed an identical effect. As opposed to the results in HT29 cells, in primary human colon cells, only the mRNA expression of the *GSTP1* gene was modulated (Fig. 1). The expression was comparably up-regulated by 10% of the fs aleurone−, the probiotics alone (fs blank+) and the combination of both (fs aleurone+). But a significant difference to the fs blank was not detectable. For *CAT*, no altered expression levels were measured after the used incubation time.

### 3.4. Quantification of total GST activity

Total GST activity, a marker of primary chemoprevention, was measured to analyze a possible consequence of the modulated gene expression after incubation with 5% and 10% (HT29 cells) and 10% (primary cells) of fs blank± and aleurone±. The experiments revealed that GST activity was significantly modulated in a time-dependent manner in HT29 cells only in response to 10% of the test substances. Since the strongest effects were seen after 72 h, only results after 72 h are illustrated in Table 3 (left column). The fs aleurone− and the fs aleurone+ induced GST activity, whereas the addition of LGG/Bb12 to the fermentation did not further increase the effect of the fs aleurone−. However, cells incubated with the probiotics alone showed a significantly higher enzyme activity compared with the medium control and to the fs blank−, respectively. In primary human colon cells, GST activity was not induced by any of the test substances (data not shown).

Table 1
Concentrations of the potential tumor-promoting substances DCA and ammonia in the fs

| fs | DCA (μM)[a] | | Ammonia (mM)[a] | |
|---|---|---|---|---|
| | Mean | S.D. | Mean | S.D. |
| Blank− | 30.70 | 4.30 | 16.33 | 1.42 |
| Blank+ | 7.47 | 0.82 | 12.96 | 0.41 |
| Aleurone− | 5.02 | 0.38 | 22.42 | 5.34 |
| Aleurone+ | 1.81 | 0.42 | 20.58 | 1.06 |

−, without LGG and Bb12; +, with LGG and Bb12.
[a] Because fs from two fermentations were pooled, only one determination in triplicate (shown are means and S.D.) could be conducted. A statistical analysis was therefore not possible.

### 3.5. Analysis of CAT activity

As a further marker of primary chemoprevention, the CAT activity was determined. Therefore, both HT29 and primary cells were incubated with 5% and 10% (HT29 cells) and 10% (primary cells) of all fs for 24–72 and 10 h, respectively. This analysis revealed that the CAT activity was time dependently induced in HT29 cells only in response to the incubation with 10% fs aleurone−, which was significantly different to the fs blank− (Table 3, right column). The fs aleurone+ (10%) also enhanced the enzyme activity of CAT significantly, whereas the added probiotics did not enforce the effect induced by the fs aleurone−. Since the effects were more prominent after 72 h, the enzyme activities are illustrated only after 72 h. Enzyme activity was not affected by the probiotics alone.

In contrast, the CAT activity was not affected in primary human colon cells by the incubation for 10 h with 10% of the test substances (data not shown).

### 3.6. Antigenotoxicity

To analyze whether treatment with fermented aleurone± caused an increased protection against $H_2O_2$-induced DNA damage, HT29 cells were preincubated for 24 and 48 h with the test compounds and were afterward challenged with $H_2O_2$. The treatment with the fs blank+ and fs aleurone± for 48 h (Fig. 2) but not for 24 h (data not shown) resulted in significantly reduced levels of DNA damage induced by $H_2O_2$. Interestingly, the impact of the reducing effect of fs aleurone− was significantly enhanced by the addition of the probiotics (fs aleurone+). Noteworthy, both fermented aleurone− and aleurone+ showed a difference to their respective fs blank ($P<.05$ fs aleurone−, $P\leq.10$ fs aleurone+). Neither fs blank± nor the fs aleurone± induced DNA damage on their own (data not shown). No cytotoxic impact of either substance was detectable at the applied concentration and time.

## 4. Discussion

An inverse association between dietary fiber intake and risk of colon cancer was suggested by large human trials [43,44]. The mechanisms, which may be involved in this protection, are dilution of carcinogens and procarcinogens contained in the feces, reduction of transit time, production of chemopreventive SCFA and reduction of tumor-promoting substances, for example, DCA and ammonia [45].

Table 2
Gene expression analysis in HT29 and primary human colon cells after treatment with 10% of the fs of the feces control (blank±) and of aleurone± for 24 (HT29 cells) and 10 h (primary human colon cells), respectively, using real-time qPCR[a]

| | HT29 cells (fold change) | | | | | | Primary human colon cells (fold change) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CAT | | GSTP1 | | SULT2B1 | | CAT | | GSTP1 | |
| | Mean | S.D. | Mean | S.D. | Mean | S.D. | Mean | S.D. | Mean | S.D. |
| fs blank− | 1.3 | 0.2 | 0.9 | 0.2 | 3.0* | 0.8 | 1.1 | 0.3 | 1.4 | 0.3 |
| fs blank+ | 1.8(*) | 0.4 | 1.2 | 0.4 | 3.0* | 1.2 | 1.3 | 0.6 | 1.6* | 0.4 |
| fs aleurone− | 2.1 | 0.7 | 2.0(*),** | 0.5 | 2.8* | 1.0 | 1.2 | 0.5 | 1.7* | 0.6 |
| fs aleurone+ | 1.9 | 1.0 | 2.5(*),(**) | 0.9 | 2.2 | 1.3 | 1.2 | 0.3 | 1.8* | 0.5 |

−, without LGG and Bb12; +, with LGG and Bb12.
[a] Results are normalized based on *GAPDH*, calculated to the medium control and expressed as fold change. Values are means and S.D. of fold change (HT29: $n=3$; primary human colon cells: $n=6$). In HT29 cells, Student's $t$ test was used to calculate the differences from the medium control, which was set to 1 (*$P<.05$) and to the respective fs blank (**$P<.05$). Friedman test with Dunn's posttest was used to calculate the differences from the medium control (*$P<.05$) in primary human colon cells. Parentheses denote that the effect varies only by trend ($P\leq.10$).

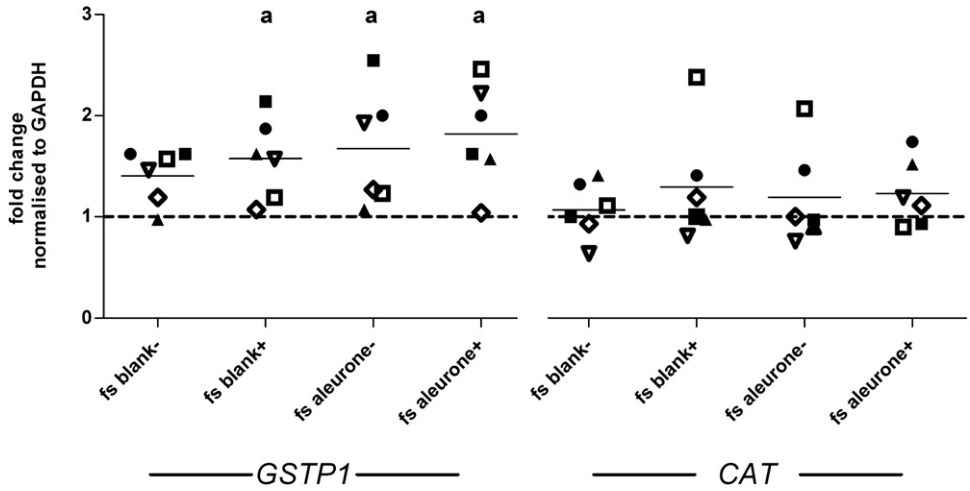


Fig. 1. Gene expression analysis of *CAT* and *GSTP1* using real-time qPCR in primary human colon cells after treatment with 10% of the fs of the feces control (blank±) and of aleurone± for 10 h. Results are normalized based on GAPDH, calculated to the medium control, which was set to 1 (dotted line) and expressed as fold change. Shown are means of six donors and the variability of response of each donor. Friedman test with Dunn's posttest was used to calculate the differences from the medium control ([a]$P<.05$). Same symbols represent the response in one donor.

Especially, the secondary bile acid DCA, produced by a bacterial dehydroxylation of primary bile acids, showed tumor-promoting properties by acting directly on the mucosa or by promoting the effects of carcinogenic substances present in the colon [46]. Previous studies from Borowicki et al. [31] showed a decreased concentration of DCA after fermentation of wheat aleurone as a consequence of SCFA production and the resulting decrease in pH. The present study confirms that the fermentation of wheat aleurone considerably reduces the DCA concentration by about 84% compared with the fs blank−. Interestingly, this effect was increased by the addition of probiotics (LGG and Bb12). In addition, probiotics were also able to decrease the amount of DCA by remarkably 76% on their own. This reduction of DCA may be caused by an increased level of SCFA resulting in a lower colonic pH and reduced activities of bacterial enzymes important for DCA formation, for example, 7α-dehydroxylase [3,47]. Furthermore, an altered microflora may modulate the activities of additional enzymes involved in the formation of carcinogens, for example, β-glucuronidase and/or nitroreductase [47]. In addition, an enhanced binding of bile acids by probiotics and/or the increase of acidophilic bacteria may possibly support bile acid reduction [48]. Moreover, direct binding of bile acids by dietary fiber, for example, by the arabinoxylans contained in the aleurone, could also lead to reduced concentrations in the gut lumen [49]. Thus, we could show that the addition of probiotics (LGG/Bb12) to the aleurone contributes to the protective effects of aleurone, which may be a first indication of synergistic effects. The observed decline in bile acids in the presence of dietary fiber and/or probiotics can be considered to be favorable in terms of primary colon cancer prevention.

Table 3
Enzyme activities of GSTs and CAT in HT29 cells after treatment with 10% of the fs of the feces control (blank±) and of aleurone± for 72 h[a]

| | HT29 cells | | | | | |
|---|---|---|---|---|---|---|
| | Relative GST activity (fold change) | | | Relative CAT activity (fold change) | | |
| | Mean | S.D. | | Mean | S.D. | |
| fs blank− | 1.4 | 0.2 | | 1.6 | 0.3 | |
| fs blank+ | 2.1 | 0.5 | *, ** | 2.4 | 1.0 | |
| fs aleurone− | 3.6 | 0.9 | *, *** | 3.6 | 1.4 | *, *** |
| fs aleurone+ | 3.2 | 0.5 | *, *** | 3.0 | 0.4 | * |

−, without LGG and Bb12; +, with LGG and Bb12.

[a] Results are calculated to the medium control and expressed as fold change. Values are means and S.D. of fold change ($n=3$). Statistical analysis was performed using one-way ANOVA with Bonferroni posttest to calculate the differences to the medium control (*$P<.05$) and two-way ANOVA with Bonferroni posttest to calculate the differences to the corresponding fs blank (***$P<.05$) and to the corresponding sample without LGG and Bb12 (**$P<.05$).

In this study, the ammonia concentration was higher in the fs with aleurone than in the blanks, reflecting the higher protein content in the respective samples. This finding is in line with earlier results of Borowicki et al. [31]. However, the content of ammonia was reduced by the addition of the LGG and Bb12, which were used alone or in combination with aleurone. Undigested dietary protein is fermented by colonic microbes leading to the formation of cytotoxic agents like ammonia. Ammonia may promote carcinogenesis by stimulating cell proliferation, which favors the growth of already transformed cells [50]. Several studies demonstrated that the presence of fermentable carbohydrates decreases the fecal ammonia content [51-53]. This is due to the stimulation of the rapid growth of colonic bacteria by undigested carbohydrates. Bacteria can act as “nitrogen sinks” using the protein and the resulting metabolites for their own metabolism and growth [50]. The reduction of ammonia by probiotics was also shown in investigations of Sakata et al. [54], at which probiotics decreased the ammonia concentration about 56% after a one-batch fermentation. This is an indication of decreased protein degradation or increased bacterial metabolism and/or growth of colonic bacteria in the mixed bacteria culture of the fecal inoculum.

Furthermore, the effects of the different fs on mRNA expression of selected genes (*CAT*, *GSTP1* and *SULT2B1*), involved in detoxification of xenobiotics, were measured. An induction of these genes is important for primary cancer prevention by preventing the formation of ultimate carcinogens or ROS as well as by antioxidative effects [12].

SULTs represent key components of the detoxification system. They are important for different physiological processes, for example, metabolizing numerous drugs and small endogenous compounds such as bile acids. Bile acids are known substrates of the hydroxysteroid SULTs (SULT2) [55]. Therefore, an increased expression could improve colon cancer prevention. Fs aleurone− showed a 2.8-fold increase on mRNA expression of *SULT2B1* in HT29 cells, whereas the addition of the probiotics to the aleurone (fs aleurone +) did not result in an increased *SULT2B1* gene expression. This effect was, however, also observed for the fs blank±. The higher concentrations of secondary bile acids, for example, DCA (30.70 μm),

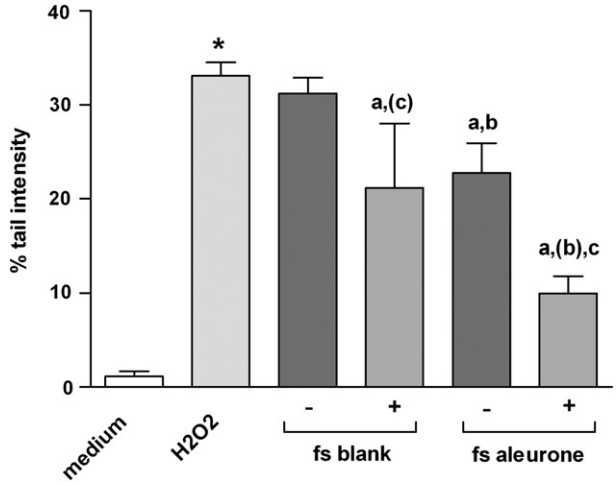


Fig. 2. Modulation of $H_2O_2$-induced DNA damage by pretreatment with 10% of fs of the feces control (blank±; −, without LGG and Bb12; +, with LGG and Bb12) and of aleurone± for 48 h in HT29 cells. Values are means and standard deviations ($n$=3). Statistical analysis was performed using Student's $t$ test to calculate the differences to the $H_2O_2$-treated cells ($^aP$<.05), to the corresponding fs blank ($^bP$<.05) and to the corresponding sample without LGG and Bb12 ($^cP$<.05). Also, Student's $t$ test was used to determine the difference of the $H_2O_2$-treated cells to the medium control (*$P$<.05). Parentheses denote that the effect varies only by trend ($P$≤.10).

in the fs blank− could possibly explain the high induction of *SULT2B1* expression by the fs blank−. However, it may be suggested that the same impact on mRNA expression by fs blank+ and fs aleurone− was due to the fermentation metabolite butyrate. Since to date, no data are available whether complex fs affect *SULT2B1* mRNA expression *in vitro* and *in vivo*, more studies are necessary to elucidate the underlying mechanisms of the regulation by complex fermentation samples. We were not able to examine mRNA expression of *SULT2B1* in primary cells because of the small size of the colon surgery samples.

Our study further demonstrates a significant induction of the *GSTP1* expression by 10% fs aleurone− and fs aleurone+ after 24 h in a comparable manner (2–2.5-fold) in HT29 cells, whereas the respective feces controls (fs blank±) had no effect. Other studies of our group also showed an induction of *GSTP1* mRNA expression after incubation with 10% of fermented aleurone after 24 h [56]. A study of Treptow-van Lishaut et al. [57] demonstrated an enhanced *GSTP1* mRNA expression in the colon of rats after feeding a diet rich in resistant starch. Pool-Zobel et al. [58] showed an induction of several GST after butyrate treatment of human colon primary cells, premalignant human LT97 adenoma and malignant human HT29 cancer cells. In addition, Ebert et al. [30] were able to show a significant induction of *GSTP1* mRNA expression (~1.5-fold) by 4 mM butyrate. Possibly, the comparable effects by both fs aleurone− and fs aleurone+ may be a result of the similar butyrate concentrations (fs aleurone−: 2.4 mM, fs aleurone+: 3.0 mM [59]). The ability of butyrate to inhibit histone deacetylases is a possible mechanism of inducing gene expression. This results in histone hyperacetylation and relaxation of DNA making regulatory elements of the DNA more accessible for transcription factors, resulting in an increase of transcriptional activity [12]. However, the concentration of butyrate was up to 1.6-fold lower in the fs than the used butyrate concentration (4 mM) in the study of Ebert et al. [30], and thus, synergistic effects of butyrate with other so far unknown fermentation metabolites may increase the efficacy of the fs.

As a consequence of the increased mRNA expression, the enzyme activity of total GST was increased about threefold in a comparable manner by fs aleurone− and aleurone+ after 72 h in HT29 cells. Both fs significantly differed to their respective feces control (fs blank±). Even though the probiotics alone (fs blank+) enhanced enzyme activity on a lower level, no additional effect was seen for fs aleurone+ compared with fs aleurone−. The comparable effects on enzyme activity by fs aleurone± may be explained by the similar butyrate concentrations (fs aleurone−: 2·4 mM, fs aleurone+: 3·0 mM [59]).

Since other studies showed an increased enzyme activity of total GST after treatment with 4 mM butyrate in HT29 cells [12], our suggestion supported that butyrate could be responsible for the observed effects of the fs. Overall, these results (mRNA expression and enzyme activity) demonstrate a possible enhancement of glutathione detoxification by fermented aleurone as an important mechanism of primary chemoprevention.

In this context, on one hand, an induction of GST is favorable since it results in an enhanced detoxification of risk factors in primary cells. On the other hand, in tumor cells, the induction could increase resistance to chemotherapeutic agents. However, it may be speculated that the high concentration of butyrate (10–20 mM) *in vivo* could impair tumor cell growth before GST induction can occur [58]. Nevertheless, the HT29 cell line was often applied to investigate cancer preventive effects of food constituents as a well-established cell model for human colon cells [28-31,60].

To improve our knowledge about the effects in the target cells of carcinogenesis, we analyzed whether the fermentation samples may also protect healthy primary colon cells from carcinogens by increasing gene expression of enzymes involved in detoxification and antioxidative defence. Actually, we were able to show an induction of the *GSTP1* mRNA expression (>1.5-fold) by 10% fs aleurone− and fs aleurone+ in primary colon cells, whereas the addition of probiotics could not enhance the effect. This may be a result of similar butyrate concentrations in the respective fs. In spite of a lower butyrate concentration (1.4 mM [59]), the blank containing the probiotics had comparable effects as the fs, probably pointing to yet unidentified factors in the fecal matrix, possibly the antioxidant and antimutagenic phenol acids [61,62]. Our findings further showed that the modulation of *GSTP1* expression occurred with a high variability between the different donors, which points to differences in cellular susceptibility to xenobiotics. In primary human colon cells, the altered *GSTP1* expression was not associated with an increase of total GST activity. Possibly a 10-h incubation is too short to detect changes in protein expression levels. Therefore, an increase of the survival time of human primary cells in culture, a major limitation of this interesting model of healthy colon cells, may be necessary to analyze the effects on enzyme activities in detail. Efforts to improve the models of healthy colon cells are therefore important to analyze the effects of nutritional factors on markers of chemoprevention and a major focus of research of our and other groups.

In HT29 cells, the expression of *CAT* was increased (≥1.5-fold) by trend for the fs containing only the probiotics (fs blank+). Interestingly, the potential prebiotic (fs aleurone−, 2.1-fold) and synbiotic (fs aleurone+, 1.9-fold) indicated the same effect on *CAT* compared with the fs blank+ (Student's $t$ test: $P$=.13 for fs aleurone−, $P$=.27 for fs aleurone+). Measurements of enzyme activity of CAT as a possible consequence of mRNA expression resulted in an enhanced activity (threefold) by both fs aleurone− and fs aleurone+ in HT29 cells, whereas the addition of probiotics did not increase this effect. In general, the increased enzyme activity may be considered to be important because CAT potentially plays a key role in cancer prevention by reducing oxidative stress, in particular, due to $H_2O_2$ [22]. In primary colon cells, the mRNA expression and enzyme activity of CAT were not modulated, which could be a result of the shorter incubation time (10 h) or a reduced susceptibility to the treatment with the fs in comparison with HT29 cells. Furthermore, it cannot be excluded that primary colon cells

could already be at a higher level of gene expression that could not increase any more. In particular, it has to be kept in mind that the primary cells are isolated from human colon surgeries, and therefore, cells from different donors may react differently. This is supported by our findings showing that the *CAT* mRNA was expressed with an interindividual variability in human primary colon cells, which points to large differences in cellular susceptibility to xenobiotics between the donors. However, Sauer et al. showed an induction of CAT activity by butyrate treatment (10 mM) in primary human colon cells after 2 h. In this study, it was further demonstrated that 15% of the butyrate was taken up from the cell culture medium by primary cells after 4 h, whereas after 12 h, only 5% of butyrate were still found in the cells [23]. Fitch and Fleming [63] reported that in the colon, the absorbed butyrate is partly secreted out of the cells and reaches the blood stream. Thus, the increase of butyrate concentration in the culture medium may be due to the enhanced secretion of butyrate out of the cells. Hence, it could be speculated that after an incubation of 10 h, most of the butyrate of the fermented samples is probably secreted by the primary cells, and thus, no effects were seen at this time.

This study further reports a reduction of DNA damage induced by $H_2O_2$ by the fs aleurone− in the preincubated HT29 cells after 48 h. Interestingly, the addition of the probiotics (fs aleurone+) increased this antigenotoxic effect of the fs. The more efficient reduction of the DNA damage by the addition of LGG/Bb12 is in line with results from earlier studies. *In vivo* rat studies, for example, have shown that fecal water derived from prebiotic- or synbiotic-treated animals exerts antigenotoxic effects in human cell lines [64]. In a human intervention trial using a synbiotic (oligofructose-enriched inulin and LGG/Bb12), a significantly decreased level of DNA damage in the colonic mucosa in polyp patients was also measured at the end of the intervention period [16]. In addition, *in vivo* rat studies by Rowland et al. [65] showed a most potent inhibitory effect on azoxymethane-induced aberrant crypt foci in rats treated with the combination of the probiotic *Bifidobacterium longum* and prebiotic inulin compared with the single compounds.

Moreover, butyrate can affect intracellular antioxidative enzymes, which could also be responsible for the reduction of DNA damage [66]. In the present study, the reduced DNA damage could be possible caused by the increased enzyme activity of both GST and CAT, which are involved in the cellular protection from the products of oxidative stress [22,67]. Thereby, the CAT is the most prominent enzyme for $H_2O_2$ decomposition. Moreover, it could be assumed that the antigenotoxicity possibly arises from direct antioxidative activities, for example, by ferulic acid [68], or modification of DNA repair mechanisms [11,19]. Thus, especially for butyrate, it has been shown that it increases chromatin accessibility to DNA repair enzymes in HT29 cells and could therefore decreases DNA damage [19]. An explanation for the additional effect of the probiotics in reducing DNA damage could possibly be the scavenging of carcinogenic intermediates, for example, $H_2O_2$ by exocellular polysaccharides, which was demonstrated for yogurt starter culture bacteria [69]. Furthermore, the additional reduction of DNA damage by the addition of the probiotics (fs aleurone+) may be also the consequences of an enhanced release of ferulic acid from the arabinoxylane, which is highly enriched in wheat aleurone [70] and is well recognized for its free radical scavenging property [68,71].

In conclusion, our investigations highlighted mechanisms that support an enhanced potential of detoxification of cancer risk factors by the complex dietary fiber source wheat aleurone that may influence initiation and progression of cancer. More importantly, we showed evidence that protection can be increased not only in HT29 cells but also in normal colon epithelial cells from human surgeries, which emphasizes a possible role of wheat aleurone in primary cancer prevention.

Hence, the modulation of markers of primary chemoprevention indicates that wheat aleurone may possibly have a cancer preventive potential, which could be partially favored by the addition of the probiotics LGG/Bb12. Nevertheless, further studies using isolated human primary cells as well as animal studies and/or human intervention trials are required to define a role in primary cancer prevention by fermentation products of wheat aleurone and probiotics under *in vivo* conditions.

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