Fermentation Supernatants of Wheat (*Triticum aestivum* L.) Aleurone Beneficially Modulate Cancer Progression in Human Colon Cells

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Wheat aleurone contains high amounts of dietary fibers that are fermented by the microflora, resulting in the formation of short-chain fatty acids (SCFA), which are recognized for their chemopreventive potential. This study investigated the effects of fermented aleurone on growth, apoptosis, differentiation, and expression of several genes using two different human colon cell lines (LT97 and HT29). In LT97 cells, the fermentation supernatant (fs) aleurone reduced significantly the cell growth (EC_{50} after 48 h = 7.6−8.3%), whereas the level of apoptotic cells was significantly increased (2.1−2.3-fold). Differentiation was enhanced in HT29 cells (1.8-fold) more than in LT97 cells (1.6-fold). Cell growth and apoptosis-related genes, namely *WNT2B* and *p21*, were induced by the fs (LT97, 1.7−3.3-fold; HT29, 7.9−22.2-fold). In conclusion, fermented wheat aleurone is able to act as a secondary chemopreventive agent by modulating parameters of cell growth and survival, whereas cells of an early transformation stage are more sensitive.

KEYWORDS: Aleurone; apoptosis; colon cancer; differentiation; fermentation; *Triticum aestivum* L.

INTRODUCTION

Some epidemiological studies suggest that dietary fiber and complex carbohydrates in the diet may help to reduce colon cancer risks, but scientific evidence is still lacking (*1*, *2*). Whereas recent large trials document a reduction in the risk of colorectal cancer development after dietary fiber intake (*3*), other well-conducted studies do not support this inverse association (*4*). Dietary fiber might improve colonic health by acting as a substrate for luminal health-promoting bacteria species that increase fermentation products, mainly short-chain fatty acids (SCFA), particularly butyrate, propionate, and acetate. Butyrate is considered to be the major energy source for normal, non-transformed colon cells, thereby enhancing survival of these cells. In addition, butyrate has been suggested to play a central role in reducing cancer risk by promoting differentiation, inducing apoptosis, and inhibiting growth of neoplastic cells in vitro (*5*).

Physiologically, butyrate affects human colonocytes in conjunction with other fermentation products, which are formed simultaneously by microbial degradation of dietary fiber. Detailed evidence is limited on how fermentation products from complex dietary fiber sources such as grains interact with human intestinal cells and how generated fermentation supernatants (fs) affect cells in comparison to analogous SCFA concentrations (*6*, *7*). Wheat grains are particularly high in dietary fiber and other physiologically beneficial substances, which are mainly concentrated in the bran fraction, more precisely, in the aleurone layer, which forms the outer part of the endosperm (*8*). Schatzkin et al. have shown in a large prospective study that particularly wheat-grain products were associated with a modest reduced risk of colorectal cancer development (*9*). Some epidemiological and experimental studies have indicated that wheat bran might be one of the most effective parts of the whole grain in protecting against colorectal cancer (*10*).

We have previously shown that fermented wheat aleurone was able to induce apoptosis and to block the cell cycle in highly transformed HT29 colon adenocarcinoma cells (*11*). In terms of chemoprevention, it is useful to affect also cells of earlier stages in carcinogenesis such as preneoplastic adenoma cells, because microadenomas are very frequent in the human population (*12*). Therefore, an adenoma cell line (LT97) representing an early premalignant genotype (*13*) was used for the presented study to analyze which effects the same samples would have on a cell line of a different stage of transformation. It was of special interest to find out if fermented aleurone and analogous SCFA concentrations would inhibit the growth and induce apoptosis in human LT97 adenoma cells similar to HT29 adenocarcinoma cells or if they would react in different manners.

Synergy1 (oligofructose-enriched inulin) was used as a reference because growth-inhibiting and apoptosis-inducing effects of fs were indicated previously (*14*). In addition to growth inhibition and induction of apoptosis, the promotion of differentiation is another relevant marker of secondary chemoprevention. Previously, it has been shown that butyrate induces differentiation by enhancing the enzyme activity of alkaline phosphatase (AP) in colon carcinoma cells (*15*). Hence, the AP activity was examined after treatment with the fermented aleurone in LT97 adenoma and HT29 adenocarcinoma cells.

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2002   *J. Agric. Food Chem.,* Vol. 58, No. 3, 2010

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Table 1. Concentration and Ratio of SCFA in the Fermentation Supernatant (fs) after in Vitro Fermentation of Aleurone Published in Borowicki et al. (*11*) and of Synergy1

	SCFA (mM)			
	acetate	propionate	butyrate	ratio of SCFA (%)
fs blank	10.75	4.56	4.56	54:23:23
fs EU[a] aleurone	21.01	7.12	20.32	43:15:42
fs U.S.[b] aleurone	25.41	9.06	24.23	43:16:41
fs Synergy1	31.78	7.09	25.19	50:11:39

[a] EU, European. [b] U.S., American.

To obtain more detailed information about the chemopreventive potential of the fermented aleurone as well as Synergy1, the mRNA expression of selected genes involved in pathways of cell cycle arrest (*p21*) and apoptosis (*DR5*) was investigated in both cell lines. Additionally, another gene (*WNT2B*) was chosen, which is associated with processes involved in proliferation and differentiation (*16*). However, in butyrate-treated cells, a hyperactivation of the WNT signaling pathway seems to be a required event to achieve high levels of apoptosis (*16*).

Overall, the aim of our study was to enhance our understanding of the chemoprotective properties of wheat aleurone and its specific role in secondary cancer prevention.

MATERIALS AND METHODS

Dietary Fiber Sources. Wheat aleurone of two varieties [European (EU) and American (U.S.)] was used as dietary fiber source (obtained from Kampffmeyer Food Innovation GmbH, Hamburg, Germany). Preparation of the aleurone fraction (ASP-2 preparation with high purity) was done by Bühler AG, Uzwil, Switzerland, according to the method of Bohm et al. (*17*). Synergy1, a commercially available mixture of inulin enriched with oligofructose, was obtained from ORAFTI, Tienen, Belgium, and used as a reference. To guarantee the stability of the samples, aliquots were prepared and stored in air- and light-proof flasks at 4 °C.

Fermentation of Dietary Fiber Sources and Preparation of the Synthetic Mixtures. Wheat aleurone (EU and U.S.) and Synergy1 were digested and fermented in vitro in six repetitions according to a described procedure of Borowicki et al. (*11*). A sample without dietary fiber was used (blank − feces control) as a control. The synthetic mixtures containing identical concentrations and ratios of SCFA as the corresponding fs (**Table 1**) were prepared according to the method of Borowicki et al. (*11*).

Cell Lines and Culture Conditions. The cell lines (HT29 and LT97 human colon cells) and the culture conditions used are described in Glei et al. (*7*) and Klenow et al. (*18*). Passages 14−31 of HT29 cells and passages 6−38 of LT97 cells were used for the experiments in this study. In regular intervals a mycoplasma test (MycoAlert Mycoplasma Detection Kit, Lonza, Switzerland) was performed, and contamination with mycoplasma was excluded.

For cell culture experiments, LT97 cells were incubated with the fs (2.5−20%) for 1−4 days after seeding in 96-well plates (determination of cell growth), 6-well plates (determination of apoptosis and enzyme activities), or T$_{25}$ cell culture flasks (experiments on mRNA expression) after reaching a confluence of 50−60%. HT29 cells were seeded in 6-well plates (1.5×10^6 cells/well) to analyze AP activity or in T$_{25}$ flasks (3×10^6 cells/T$_{25}$ flask) to quantify mRNA expression and were grown for 1 day before exposure to the fs (10%). After incubation, LT97 and HT29 cells were trypsinized and suspended in PBS. Viabilities and cell numbers were determined with a CASY-cell counter (CASY model TT, Innovatis AG CASY Technology, Reutlingen, Germany).

Determination of Cell Growth. After 24, 48, or 72 h of incubation with the fs or the synthetic mixtures, the amount of DNA was quantified as described previously (*18*) as a relative measure of cell number. The results were calculated on the basis of the medium control, which was set to 100%. The effective mean doses (EC$_{50}$) of the fs and the synthetic mixtures that inhibited growth by 50% were determined and expressed as percentages.

Detection of Apoptosis. To detect the level of apoptosis in LT97 cells, 1×10^6 cells were stained with annexin V-FITC as well as 7-aminoactinomycin D (7-AAD) in annexin V binding buffer using the annexin V-FITC/7-AAD kit (Beckman Coulter, Krefeld, Germany), and the level of apoptotic cells was quantified by flow cytometry as described previously (*11*). Furthermore, the enzyme activity of caspase-9, caspase-8, and caspase-3 was determined to obtain more detailed information on the induction of apoptosis according to ref *11*. For interpretation of all results, fold changes were calculated on the basis of the medium control, which was set to 1.0.

Determination of Alkaline Phosphatase Activity. Cell lysates of LT97 and HT29 cells were prepared as described before (*19*). AP activity was measured as described by Beyer-Sehlmeyer et al. (*6*) with slight modifications. Briefly, AP activity was measured at 37 °C using *p*-nitrophenylphosphate (5 mM) as substrate. *p*-Nitrophenol (0−800 μM) was used to generate a standard curve. The amount of *p*-nitrophenol liberated was determined spectrophotometrically at 400 nm (SpectraFluor Plus, Tecan GmbH, Crailsheim, Germany). One unit is defined as the activity that hydrolyzes 1 μmol of subtrate per minute at 37 °C. Results are expressed relative to the cellular protein content, which was determined spectrophotometrically according to Bradford's method (*20*).

RNA Extraction and mRNA Expression Analysis. Total RNA from LT97 and HT29 cells was isolated using the RNeasy Plus Mini Kit according to the manufacturer's manual (Qiagen, Hilden, Germany), dissolved in 50 μL of RNase-free water, and stored at −20 °C until further use. RNA was quantified by measuring the absorbance at 260 nm, and the purity of the samples was verified by measuring the ratios of absorbance at 260:280 or 260:230, respectively, using a NanoDrop ND-1000 photometer (NanoDrop Technologies, Wilmington, DE). RNA was checked for integrity using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA) on the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). RNA quality was expressed as an RNA integrity number (RIN). Only RNA samples with a RIN > 8 were used for further experiments.

The expression of selected target genes was analyzed with 2.5 μg of total RNA converted into first-strand cDNA using SuperScript II (Invitrogen, Karlsruhe, Germany). Real-time quantitative PCR was carried out using specific primer pairs and qPCR GreenMaster (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 50 cycles of denaturation (15 s at 95 °C), annealing, and extension (30 s at 56−60 °C, depending on the primer pairs). Cumulative fluorescence was measured at the end of the extension phase of each cycle. Product specific amplification was confirmed by melting curve analysis. The following gene-specific primer sequences were used for the quantification: *GAPDH* (5′-ACCCACTCCTCCACCTTTGAC-3′ and 5′-CACTGTCTTGTA-CCCTTG-3′); *DR5* (5′-CCACCTGGACACCATATCTC-3′ and 5′-TA-CAATCACCGACCTTGACC-3′); *p21* (5′-CACTGTCTTGTACCCTT-GTG-3′ and 5′-CTTCCTCTTGGAGAAGATCAG-3′); and *WNT2B* (5′-TCATGCAGAAGTAGCCGA-3′ and 5′-ACACCGTAGTGGATG-TTGTC-3′). All primers were designed using the freely available PerlPrimer v1.1.17 (http://perlprimer.sourceforge.net) software. The mRNA expression of the target genes was calculated on the basis of the house-keeping gene *GAPDH* according to the method of Pfaffl et al. (*21*). For interpretation of all results, fold changes were calculated on the basis of the medium control, which was set to 1.0.

Statistical Evaluation. Means and standard deviations (SD) were calculated from at least three independent experiments. Differences were calculated by one- or two-way ANOVA, including a Bonferroni post test with selected pairs, using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA). The one-way ANOVA was done to define differences within one group if more than two concentrations were used. The two-way ANOVA was used to define differences between two groups if more than two concentrations were used. Otherwise, comparisons of two groups were done with Student's *t* test. The statistical analyses used depended on the respective experimental design and are specified in the legends to the figures and tables.

RESULTS

Modulation of Cell Growth by the fs and the Corresponding SCFA in LT97 Cells. The effects of fs of different varieties of aleurone (EU and U.S.) on cell growth of LT97 cells were

Table 2. EC_{50} of the Fermentation Supernatant (fs) and the Corresponding Synthetic Mixtures of SCFA (Acetate, Propionate, and Butyrate), Propionate + Butyrate, and Butyrate Alone in LT97 Cells

	$EC_{50}{}^a$ (%)															
	fs				SCFA				propionate + butyrate				butyrate			
	48 h		72 h		48 h		72 h		48 h		72 h		48 h		72 h	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
blank	15.8	4.3	15.5	2.9	×^{b}	×	×	×	×	×	×	×	×	×	×	×
EU^c aleurone	8.3	1.7	7.9	0.5	13.7	1.2	13.0	2.0	×	×	17.0	4.3	14.8	2.4	13.7	2.4
U.S.^{d} aleurone	7.6	1.6	7.4	0.7	10.7	1.8	11.3	1.3	18.3	4.7	14.9	2.6	12.2	2.6	12.2	1.5

^{a} EC_{50} (%) is defined as the effective concentration at which cell number decreased to 50% of viable cells. ^{b} × indicates that EC_{50} was not achieved. ^{c} EU, European. ^{d} U.S., American.
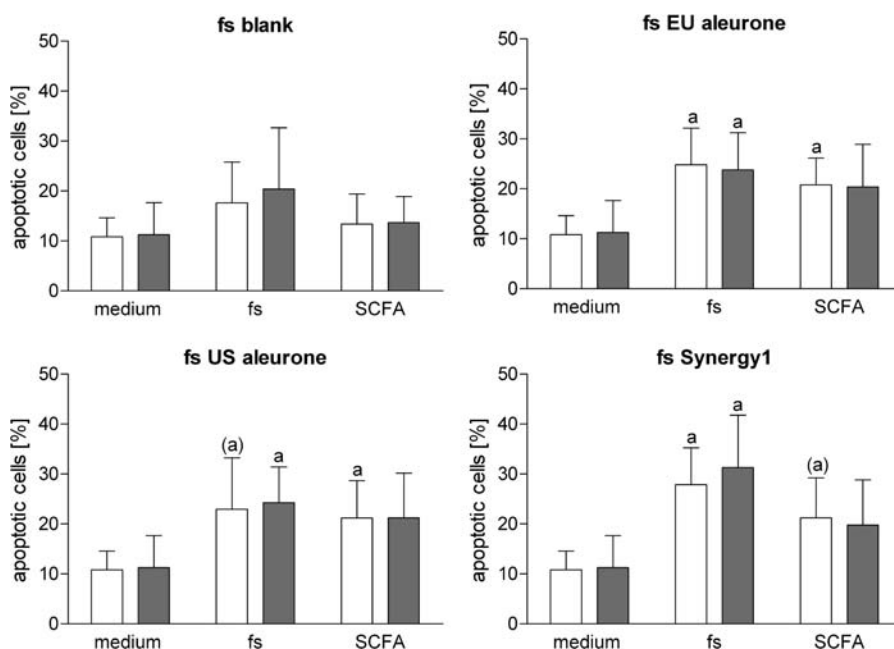

Figure 1. Effects of fermentation supernatant (fs, 5%) and its corresponding SCFA mixtures (acetate, propionate, and butyrate) on early apoptosis in LT97 cells after treatment with fs blank, fs EU aleurone, fs U.S. aleurone, and fs Synergy1 for 24 h (white bars) and 48 h (gray bars). Apoptosis was assessed by binding of annexin V and exclusion of 7-AAD. Values are means with their standard deviations depicted by vertical bars ($n = 4$). Student's t test was used to calculate the difference from the respective medium control (a, $P < 0.05$). Parentheses denote that effects vary by trend only ($P < 0.07$).

analyzed using the DAPI assay. Treatment of LT97 cells with the fs aleurone (EU and U.S.) affected the cell number in a time- and dose-dependent manner, even after 24 h on a significant level. However, after 24 h, the calculated EC_{50} was not detectable for any fs, whereas after 48 h, EC_{50} ranged from 7.6 to 15.8% (**Table 2**). After 48 and 72 h of treatment, the fermented aleurone reduced cell growth more effectively than the fs blank independent of the variety. In addition, the growth of cells was efficiently retarded in LT97 cells by the SCFA mixtures as well as mixtures of butyrate and propionate or butyrate alone in concentrations corresponding to both fs aleurone. Nevertheless, all synthetic mixtures were less effective than the fs, but the general profiles of response were similar. The higher concentrations of butyrate present in fs U.S. aleurone (24 mM) compared to fs EU aleurone (20 mM) did not result in a measurable additional influence on cell growth.

The concentration of 5% fs was selected for subsequent experiments in LT97 cells, because effects on apoptosis, differentiation, and mRNA expression should be determined using a subtoxic concentration.

Induction of Apoptosis by the fs of Both Aleurone Varieties in LT97 Cells. The fermentation supernatants of both aleurone varieties and Synergy1 were used to analyze the effect on apoptosis as a relevant marker of secondary chemoprevention.

The basal levels of early apoptotic cells detected in LT97 cells were $10.81 \pm 3.79\%$ after 24 h and $11.26 \pm 6.40\%$ after 48 h, respectively. The fs (5%) of different aleurone varieties (EU and U.S.) and of Synergy1 induced a significant increase in apoptosis (2.1−2.8-fold) in LT97 cells time-independently (**Figure 1**). The fs blank was not able to induce apoptosis, but also did not differ significantly from the fs aleurone. Particularly after 24 h, the synthetic mixtures of SCFA enhanced levels of apoptotic cells comparable to the fs. Furthermore, there were no differences between the fs aleurone and the fs Synergy1.

To confirm these results and to get more detailed information about the underlying molecular mechanisms, the influence of the fs on caspase-3, -8, and -9 activity was measured. The fermented aleurone of both varieties (5%) as well as Synergy1 (5%) significantly induced the caspase-3 activity compared to the medium control, whereas the fs blank could not modulate the enzyme activity (**Figure 2**). Generally, the caspase-3 activity was more inducible after 24 h than after 48 h. The initiator caspases-8 and -9 were not modulated by any fs (data not shown).

Induction of Alkaline Phosphatase by the fs of Both Aleurone Varieties in LT97 and HT29 Cells. Tumor cells are typified by a loss of responsiveness to some or all of the factors regulating cell growth, differentiation, and apoptosis, resulting in more
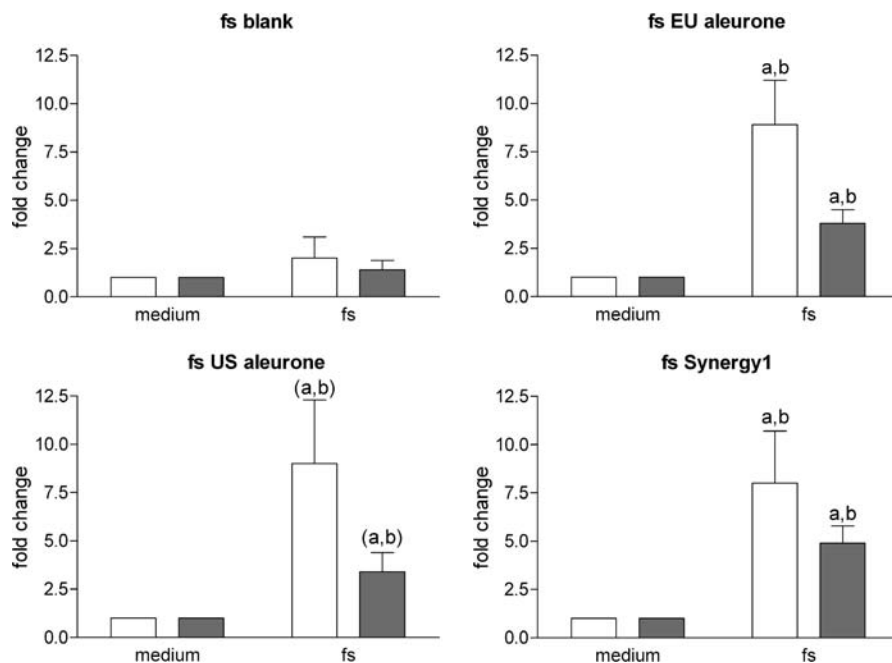
Figure 2. Effects of fermentation supernatants (fs, 5%) on caspase-3 activation in LT97 cells after treatment with fs blank, fs EU aleurone, fs U.S. aleurone, and fs Synergy1 for 24 h (white bars) and 48 h (gray bars) as indicator for apoptotic effects. The caspase-3 activity was assessed by measuring the amount of liberated AMC from the respective substrate. The specificity of the assay was confirmed by inhibition of enzymatic activity with the specific caspase-3 inhibitor Ac-DEVD-CHO. Fold changes of results of three separate experiments were calculated on the basis of the respective medium control, which was set to 1.0. Values are means with their standard deviations depicted by vertical bars ($n = 3$). Student's *t* test was used to calculate the difference from the respective medium control (a, $P < 0.05$) and from blank (b, $P < 0.05$). Parentheses denote that effects vary by trend only ($P = 0.05$).
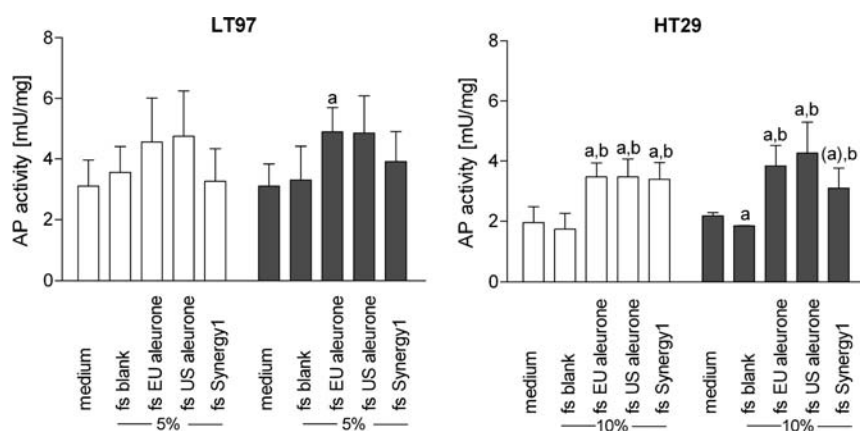

Figure 3. Effects of fermentation supernatants (fs, 5 and 10%) on alkaline phosphatase activity in LT97 and HT29 cells after treatment with fs blank, fs EU aleurone, fs U.S. aleurone, and fs Synergy1 for 48 h (white bars) and 72 h (gray bars) as marker of differentiation. The enzyme activity was assessed by measuring the amount of liberated *p*-nitrophenol. Values are means with their standard deviations depicted by vertical bars ($n = 3$). Student's *t* test was used to calculate the difference from the respective medium control (a, $P < 0.05$) and from blank (b, $P < 0.05$). Parentheses denote that effects vary by trend only ($P = 0.08$).

undifferentiated cell types. Thus, the fermatation supernatants of both aleurone varieties and Synergy1 were used to analyze the effect on differentiation by measuring the modulation of the AP activity in the cell lysates. Because no modulation of AP activity was seen after 24 h in both cell lines, only effects after 48 and 72 h are illustrated in **Figure 3**. In LT97 cells, the AP activity was significantly induced only by 5% fs EU aleurone (1.6-fold after 72 h) even though the effect was not significantly different from the fs blank. Interestingly, butyrate (4 mM) did not enhance the AP activity (data not shown). In HT29 cells, the fermentation supernatants (10%) of both aleurone varieties (EU and U.S.) significantly induced the AP activity 1.8-fold after 48 and 72 h and were significantly more effective than the fs blank, which did not

modulate the AP activity after 48 h and even reduced activity after 72 h. Synergy1 significantly enhanced the enzyme activity only after 48 h (1.8-fold) but did not differ from the fs aleurone of both varieties. Butyrate (4 mM) induced the AP activity already after 24 h (1.5-fold) as well as after 48 h (2.8-fold) and 72 h (2.0-fold), respectively (data not shown).

Effect of the fs of Both Aleurone Varieties on mRNA Expression of Selected Target Genes in LT97 and HT29 Cells. To obtain more detailed information about a chemopreventive potential of the fermented aleurone as well as Synergy1, the mRNA expression of selected genes involved in pathways of cell cycle arrest (*p21*), apoptosis (*DR5*), or both events (*WNT2B*) was investigated in LT97 adenoma and HT29 adenocarcinoma cells (**Table 3**).

Table 3. Modulated mRNA Expression of *p21*, *DR5*, and *WNT2B* in LT97 and HT29 Cells Treated with Fermentation Supernatant (fs) (5 or 10%) or Butyrate (4 mM) for 24 h[a]

	LT97 (5% fs)			HT29 (10% fs)		
	mean	SD		mean	SD	
p21						
butyrate, 4 mM	4.02	1.97		13.95	2.67	a
fs blank	1.11	0.12		10.38	3.86	a
fs EU[b] aleurone	3.34	1.34	(a), b	19.25	4.85	a, (b)
fs U.S.[c] aleurone	3.26	0.87	a, b	22.24	5.15	a, b
fs Synergy1	2.32	0.69	(a), b	20.45	1.91	a, b
DR5						
butyrate, 4 mM	3.09	1.35		1.26	0.31	
fs blank	0.81	0.13		0.86	0.29	
fs EU aleurone	1.20	0.59		1.11	0.19	
fs U.S. aleurone	1.80	0.41	(a), b	1.31	0.07	a, (b)
fs Synergy1	1.03	0.52		1.38	0.24	(b)
WNT2B						
butyrate, 4 mM	2.17	0.37	a	9.54	0.85	a
fs blank	0.85	0.10		3.36	2.02	
fs EU aleurone	1.80	0.34	(a), b	7.90	1.35	a, b
fs U.S. aleurone	1.72	0.44	(a), b	8.34	2.58	a, (b)
fs Synergy1	1.62	0.00	a, b	9.20	0.64	a, b

[a] Results are presented as fold changes calculated on the basis of the medium control, which was set to 1.0. Student's *t* test was used to calculate the difference from the respective medium control (a, $P < 0.05$), from blank (b, $P < 0.05$), and from Synergy1 (c, $P < 0.05$). Parentheses denote that effects vary by trend only ($P \leq 0.1$). [b] EU, European. [c] U.S., American.

(*a*) *Effect on mRNA Expression of p21*. In LT97 cells *p21* was induced by all fermentation supernatants of dietary fiber sources, but only for the fs U.S. aleurone on a significant level. There were no differences seen either between both aleurone varieties or between the fs aleurone and Synergy1. The fs blank did not modulate the mRNA expression. In HT29 cells *p21* was significantly induced by all fermentation supernatants compared to the medium control, whereas the fs blank induced *p21* on a lower level than the fs aleurone (EU and U.S.) and the fs Synergy1. Again, fermented aleurone was comparably effective as Synergy1. In LT97 cells, butyrate (4 mM) did not modulate *p21* on a significant level. In HT29 cells, in contrast, butyrate significantly induced mRNA expression of *p21*. In general, *p21* was less regulated in LT97 adenoma cells compared to HT29 adenocarcinoma cells, possibly due to the higher concentrations of the used fs (5% in LT97 versus 10% in HT29).

(*b*) *Effect on mRNA Expression of DR5*. In LT97 cells only the fs U.S. aleurone induced *DR5* expression on mRNA level compared to the medium control and was significantly different from the fs blank. In contrast, the fs EU aleurone and Synergy1 did not modulate mRNA expression of *DR5*. In HT29 cells, again up-regulation of *DR5* was detected only by treatment with fs U.S. aleurone compared to the medium control. The fs U.S. aleurone and the fs Synergy1 were more effective than the fs blank. Butyrate (4 mM) had no effect on *DR5* mRNA expression in both cell lines used.

(*c*) *Effect on mRNA Expression of WNT2B*. The mRNA expression level of *WNT2B* was induced by all fermentation supernatants except from the fs blank compared to the medium control in LT97 as well as HT29 cells. The fermented aleurone was comparably effective as Synergy1. In addition, butyrate (4 mM) significantly induced mRNA expression in both cell lines. *WNT2B* was less regulated in LT97 cells compared to HT29 cells.

DISCUSSION

Chemoprevention of colorectal cancer is a major concern for improving public health, because this is the third and the second most common type of cancer in developed countries in men and women, respectively (*22*). The consumption of dietary fiber from fruits and grains might be associated with a reduced risk of colorectal cancer development, whereby high but also lower amounts may exert beneficial effects (*1*, *4*). Nevertheless, it is apparent that more detailed studies are needed on how different types of foods and dietary fibers contribute to gut health and how they may act on a molecular basis.

Hence, the present study was performed to elucidate in depth which chemopreventive effects can ensue from fermented wheat aleurone in human colon cancer cells of different transformation stages. The wheat aleurone used in this study, in which beneficial substances such as arabinoxylans and polyphenols are concentrated, was previously characterized. It was shown that an in vitro fermentation of aleurone by the colonic microflora obtained from fecal slurry resulted in a marked increase of SCFA concentrations in comparison to the feces control (*11*). Because the fermentation of Synergy1, a pure dietary fiber source made of oligofructose-enriched inulin, generated similar SCFA concentrations, the fiber source of aleurone should be as highly fermentable as the prebiotics inulin or oligofructose.

LT97 adenoma cells are highly relevant target cells for studying aspects of chemoprevention in an earlier stage of carcinogenesis (*5*). In the present study we found a significant time- and dose-dependent growth inhibition in LT97 cells by the fermented aleurone, which may be attributed to the increase in SCFA, in particular butyrate, after fermentation of this dietary fiber source. Thus, the synthetic mixtures of the SCFA and butyrate alone followed a similar pattern of response in mediating growth inhibition, reflecting the potential of SCFA in secondary chemoprevention. However, the fermentation samples were more effective in inhibiting the growth of LT97 cells than the corresponding SCFA mixtures. Furthermore, the feces control also reduced the growth of LT97 cells. The additional activity of the fermentation samples and of the feces control reflects the growth-inhibitory properties of other so far unidentified metabolites, probably from the fecal inoculum. After 48 h, the growth-inhibiting properties of the fs and the synthetic mixtures were 1.6-fold more efficient in LT97 adenoma than in HT29 adenocarcinoma cells (*11*). After 72 h, the impacts were similar in both cell lines. These findings are almost in line with previous experiments in which several fermentation supernatants of various fiber sources were analyzed for their effects on cell growth and survival (*6*, *14*). It was demonstrated that the difference in growth inhibition may be potentially caused by varying butyrate uptake rates in adenoma and carcinoma cell lines (*23*) resulting from a down-regulation of butyrate transporters in human colon cancer tissues compared to normal cells (*24*). Hence, primarily after 48 h the uptake of butyrate might be delayed, and this delay may be accompanied by a reduced sensitivity against butyrate exposure in HT29 cells compared to LT97 cells. Furthermore, dietary fiber is physiologically consumed by the intake of cereals, fruits, and vegetables several times per day. The human colon epithelium is therefore continuously exposed by metabolites of the bacterial degradation of fiber. In the case of wheat fiber it was reported that when humans were given one serving including wheat bran, the transit was up to 41 h (*25*). Hence, the in vitro growth-inhibiting properties of wheat aleurone might have important consequences for chemoprevention when translated to the in vivo situation, because it indicates that fermentation products of wheat aleurone could efficiently suppress the growth of cells of earlier cancer stages.

In addition to growth inhibition, several in vitro studies indicated that butyrate induces apoptosis and promotes differentiation in various colon cancer cells (*24*) and thus could be important for secondary chemoprevention of colorectal cancer. In contrast, effects of fs or synthetic SCFA mixtures have not been well investigated. In the present study, we observed a significant increase in apoptotic cells and caspase-3 activity after incubating the LT97 adenoma cells with the fermented aleurone. The synthetic SCFA mixtures mimicking the concentration in the fs enhanced the level of apoptotic cells in a similar response compared to the fs. These findings are opposite to effects on apoptosis in HT29 cells in which SCFA did not induce apoptosis (*11*). This may have important implications for possible chemopreventive activities in earlier stages of the cancer process, because it is of high benefit to induce apoptosis in adenoma cells and thereby reduce the formation of more degenerated carcinoma cells.

Agents that act by modulating the differentiation of neoplastic and carcinoma cells, thereby slowing or even halting their growth, exhibit a chemopreventive activity (*26*). HT29 cells synthesize the intestinal form of AP at extremely low levels, and the expression can be enhanced by differentiating agents (*27*). Several studies have shown that butyrate induces the AP activity in HT29 adenocarcinoma cells accompanied by a more differentiated phenotype (*15, 24*). In the present study, butyrate (4 mM) induced AP activity in adenocarcinoma cells after only 24 h, which is in line with other findings (*6, 15*). The fermentation supernatants of aleurone and Synergy1 were of equal potency in enhancing AP activity, which is probably due to the amount of butyrate (2–2.5 mM). Interestingly, in LT97 adenoma cells treatment with butyrate or the fs had nearly no influence on AP activity. Previously it has been suggested that butyrate stimulates colonic cancer cells to progress to a more differentiated phenotype followed by an apoptotic death (*26*). Orchel et al. demonstrated that higher doses of butyrate (> 5 mM) induced apoptosis in the cells and failed to stimulate colonocyte differentiation (*15*). Because LT97 cells are more sensitive to a treatment with fs, particularly SCFA, this could be a reason for no effect on the enzyme activity level. Because LT97 cells represent an earlier stage of carcinogenesis, they are more differentiated than HT29 cells. Therefore, basal AP activity may be higher in LT97 cells, and the cells may be more resistant to induction of AP activity (*28*).

Furthermore, we have chosen three selected target genes, which are involved in pathways of cell cycle arrest (*p21*) and apoptosis (*DR5*) or are associated with both processes (*WNT2B*), to obtain more detailed information about the protective capability of the fermented aleurone as well as Synergy1.

In HT29 cells, the fs aleurone was able to induce p21 on a transcriptional level, and this is associated with a cell cycle arrest as we have shown before (*11*). Because the fermentation supernatants containing just 2–2.5 mM butyrate were much more effective than butyrate (4 mM) alone and even the feces control could enhance *p21* mRNA expression, again additional factors seem to be present in the fermentation supernatants bearing an effect on cells. Interestingly, in LT97 adenoma cells *p21* mRNA expression was less inducible, but still on a remarkable level. Because butyrate (4 mM) failed to modulate mRNA expression, probably most of the LT97 cells were already in apoptosis caused by an enhanced susceptibility against butyrate in comparison to HT29 cells (unpublished data).

Furthermore, butyrate (4 mM) and the fs (containing 1–2.5 mM butyrate) mostly failed to induce DR5 on a transcriptional level in both cell lines, contrary to findings of Kim et al., which have demonstrated that 4 mM butyrate induced a 2.4-fold increase of *DR5* expression in HT29 cells (*29*). Therefore,

experimental conditions such as the time frame (we used 24 h of exposure; no information by Kim et al.) could be a reason for the lack of induction. Medina et al. described that in LIM 1215 colorectal cancer cells the hyperacetylation of DNA–histone complexes appear early after induction with butyrate, resulting in an activation of caspase-3 within 16 h, which is rapidly followed by an apoptotic death (within 24 h) (*30*). Hence, a shortened exposure time to butyrate could be associated with an increased mRNA expression of *DR5*, which is an early step within the signal cascade of apoptosis. Furthermore, induction of the intrinsic pathway could be responsible for the demonstrated apoptotic effects of the fermented aleurone as has been described for butyrate before (*24*).

The WNT signaling pathway is constitutively activated in the majority of colorectal cancers (*16*) by mutation of the *APC* gene, which LT97 and HT29 cells also carry. However, it was reported that a hyperactivation of the WNT signaling pathway in butyrate-treated colon cancer cells is a required event to achieve high levels of apoptosis in these cells (*16*), which is still mentioned as a marker of secondary chemoprevention. This is in line with our findings because we could report that the fs induced *WNT2B* on the transcriptional level accompanying an enhanced level of apoptotic cells in both cell lines. Again, the fs aleurone and the fs Synergy1 were of equal potency in modulating mRNA expression of all three selected genes, which indicates that the complex dietary fiber source is as effective as the well-known pure dietary fiber source consisting of oligofructose-enriched inulin.

In conclusion, this study provides new interesting insights as to how fermentation products of wheat aleurone may support secondary chemoprevention. Hence, the effects of fermented aleurone point to mechanisms involved in inducing cell cycle arrest as well as apoptosis and differentiation, thereby suppressing the progression of carcinogenesis. Because adenoma cells primarily were found to be more susceptible, this may have important implications for chemoprevention when translated to the in vivo situation, because the survival of neoplastic cells could be reduced. Thus, a fiber-rich diet (e.g., with aleurone-enriched products) might be an effective strategy to inhibit growth of colon cancer cells.

ABBREVIATIONS USED

7-AAD, 7-aminoactinomycin D; AP, alkaline phosphatase; APC, adenomtosis polyposis coli; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; DR5, death receptor 5; EU, European; FITC, fluorescein isothiocyanate; fs, fermentation supernatant; RIN, RNA integrity number; SCFA, short-chain fatty acids; SD, standard deviation; U.S., American; WNT2B, wingless-type MMTV integration site family, member 2B.

Note Added after ASAP Publication

The abstract was modified in the version of this paper published ASAP December 2, 2009; the corrected version published ASAP December 4, 2009.

Article

J. Agric. Food Chem., Vol. 58, No. 3, 2010 **2007**

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