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ORIGINAL CONTRIBUTION

Chemopreventive effects of in vitro digested and fermented bread in human colon cells

Wiebke Schlörmann · Beate Hiller · Franziska Jahns · Romy Zöger · Isabell Hennemeier · Anne Wilhelm · Meinolf G. Lindhauer · Michael Glei

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Abstract
Purpose Bread as a staple food product represents an important source for dietary fibre consumption. Effects of wheat bread, wholemeal wheat bread and wholemeal rye bread on mechanisms which could have impact on chemoprevention were analysed in colon cells after in vitro fermentation.
Methods Effects of fermented bread samples on gene expression, glutathione S-transferase activity and glutathione content, differentiation, growth and apoptosis were investigated using the human colon adenoma cell line LT97. Additionally, apoptosis was studied in normal and tumour colon tissue by determination of caspase activities.
Results The expression of 76 genes (biotransformation, differentiation, apoptosis) was significantly upregulated (1.5-fold) in LT97 cells. The fermented bread samples were able to significantly increase glutathione S-transferase activity (1.8-fold) and glutathione content (1.4-fold) of the cells. Alkaline phosphatase activity as a marker of differentiation was also significantly enhanced (1.7-fold). The fermented bread samples significantly inhibited LT97 cell growth and increased the level of apoptotic cells (1.8-fold). Only marginal effects on apoptosis in tumour compared to normal tissue were observed.
Conclusions This is the first study which presents chemopreventive effects of different breads after in vitro fermentation. In spite of differences in composition, the results were comparable between the bread types. Nevertheless, they indicate a potential involvement of this staple food product regarding the prevention of colon cancer.

Keywords Apoptosis · Bread · Colon cancer · Dietary fibre · In vitro fermentation

Introduction

Among cancer diseases, colorectal carcinomas are the second and third most frequently causes of death in developed countries for men and women, respectively [1]. Colon cancer also ranks among the most diet-related cancer forms [2]. Dietary fibre (DF) could prevent the development of colon cancer. Even though results from intervention studies are controversial, there are some experimental studies on animals showing that DF could reduce the risk of colon cancer [3]. Furthermore, the most comprehensive 'EPIC'-study has revealed that consumption of DF is inversely associated to the risk for the development of colon cancer [4]. But until now, the mechanisms underlying these cancer preventing effects are not fully understood. It has been shown, that DF reduces passage times by means of water binding and faecal bulking effects leading to a dilution of harmful components [5]. The fermentation of DF by intestinal microorganisms in the colon leads further to the

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W. Schlörmann (✉) · F. Jahns · R. Zöger · I. Hennemeier · A. Wilhelm · M. Glei
Department of Nutritional Toxicology, Institute of Nutrition, Friedrich-Schiller-University Jena, Dornburger Strasse 24, 07743 Jena, Germany
e-mail: wiebke.schloermann@uni-jena.de

B. Hiller · M. G. Lindhauer
Department of Safety and Quality of Cereals, Max Rubner-Institute, Schuetzenberg 12, 32756 Detmold, Germany

production of short chain fatty acids (SCFA). SCFA reduce the intestinal pH resulting in a growth benefit of acidophilic microorganisms with colon health-promoting effects (e.g. bifidobacteria) [6] and a reduced conversion of primary into colon cancer promoting secondary bile acids as well as a lower solubility of secondary bile acids [7, 8]. The SCFA butyrate is utilised as an energy source in non-transformed cells [9], whereas in tumour cells, butyrate reduces survival by inducing apoptosis and inhibiting proliferation [10]. Butyrate also plays a role in the induction of glutathione S-transferases (GSTs), which are involved in detoxification of many carcinogens [11]. Especially whole grain cereal products, such as bread, are rich in DF and could contribute to the protection against the development of colon cancer [12]. General health-promoting effects of whole grain bread were shown by Jacobs et al. [13] who demonstrated that hazard rate ratios for total mortality (and mortality from different diseases like coronary heart disease, cardiovascular disease and cancer) are inverse to the amount of consumed whole grain bread among whole grain bread eaters in comparison to white bread eaters. Furthermore, the studies of Dewettinck et al. [14] reported that whole grain bread has a better nutritional value (e.g. higher content of ingredients such as vitamins, minerals, antioxidants and phytochemicals) and could therefore have more influence on men's health than white bread. A human dietary intervention study revealed that compared to white bread, the intake of whole grain rye bread caused a higher faecal output, stool frequency and higher SCFA production as well as reduced faecal bacterial enzyme activities and secondary bile acid concentrations [15, 16]. Rye bread intervention studies with animals showed higher concentrations of faecal butyrate and lactate as well as an increased stool wet weight than wheat products [17–19].

But until now, investigations examining the influence of different bread types on parameters of primary and secondary chemopreventive effects in colon cells are still missing. Primary prevention describes the inhibition of initiation, the first step of tumourigenesis by e.g. reduction of toxification and induction of detoxification, whereas the promotion of initiated cells to preneoplastic cells or the tumour progression is inhibited by secondary prevention e.g. reduction of cell growth or enhancement of differentiation and apoptosis [20]. Therefore, the object of this study was to analyse the chemopreventive properties of different bread samples after an in vitro simulated digestion and fermentation in a batch fermentation model of the human intestine. The fermentation products were tested in LT97 colon adenoma cells and also partly in matched normal and tumour colon tissue derived from human individuals.

Materials and methods

Production of bread samples

Wheat flour bread (WB), wholemeal wheat bread (WMWB) and wholemeal rye bread (WMRB) were produced according to the German guidelines for the production of bread and small baked goods [21] as described by Hiller et al. [22]. Bread samples were air dried and ground to a particle size of $\leq$500 µm.

In vitro fermentation of bread samples

Digestion and in vitro fermentation of bread samples were performed in eight replications according to a procedure described by Stein et al. [23]. Briefly, 0.5 g fermentable substance of bread samples were reconstituted with 10 mL anaerobic potassium phosphate buffer (0.1 M, pH 7.0), incubated with α-amylase (Sigma A-0521; 17.36 U/sample) for 5 min at 37 °C (simulation of the mouth) and for 2 h with pepsin (Sigma P-7012) at 37 °C (1.11 mg in 0.94 mL 20 mM HCl; pH 2.0; simulation of the stomach). In order to simulate the small intestine, bread samples were incubated with an intestinal extract of pancreatin and ox-gall (Sigma P-1750, Fluka-Sigma 70168, 2.6 and 3.0 mg, respectively in 5 mL of 11 mM bicarbonate buffer; pH 6.5) in a dialysis membrane (molecular weight cut-off: 1,000 da) under semi-anaerobic conditions (37 °C, 6 h). Subsequently, in vitro fermentation was performed using a faeces inoculum mixture of three healthy donors in an anaerobic atmosphere (37 °C, 24 h). Synergy1®, a mixture of inulin enriched with oligofructose, was used as positive control, whereas a blank fermentation sample of the faecal inoculum without bread supplementation served as negative control.

Preparation of fermentation supernatants (fs) and chemical analysis

Fs were obtained by three centrifugation steps of the faeces suspension (30 min, 4,200×*g*; 15 min, 4,200×*g*; 15 min, 10,300×*g*; 4 °C). The fs from 8 different fermentations were pooled to have enough volume for the chemical analysis [22] and the in vitro experiments. Sterile filtration was performed using a Millipore filter with a pore size of 0.22 µm for sterilisation prior to usage. Hiller et al. [22] have been provided an extensive chemical characterisation of the fs used in this study.

Cell culture

For cell culture experiments, the human colon adenoma cell line LT97 was used. LT97 cells were established from

a colon adenoma representing an early stage of development of colon tumours [24]. Culture conditions for LT97 cells are described by Klenow et al. [25]. Primary epithelial strips as well as epithelial cells were isolated from paired normal and/or tumour colon tissue obtained after surgery of patients suffering from colon carcinomas (3 male, 1 female, 56 to 85 years) as described by Schäferhenrich et al. [26]. This study was approved by the Ethics Committee of the Friedrich-Schiller-University Jena (No. 1601-08/05). Written informed consent was obtained from all subjects.

RNA extraction

RNA isolation was performed after incubation of LT97 cells with 2.5% of the fs using the RNeasy Plus Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). RNA quality was analysed using the Agilent RNA 6000 Nano Kit and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only RNA samples with a RIN (RNA Integrity Number) >9 were used for further experiments.

Microarray analysis

For microarray analysis, a customised cDNA microarray system and the PIQOR antisense Microarray kit (Miltenyi Biotech, Bergisch Gladbach, Germany) were used as described by Stein et al. [23]. Fluorescence signals for Cy3 and Cy5 were detected using the GeneSpotter® Microarray scanner and the MicroDiscovery GeneSpotter® 2.6.0 software (MicroDiscovery GmbH, Berlin, Germany). Fluorescence signals of the samples were normalised to the mean of 6 housekeeping genes (α-actin 2, cyclophilin A, glycerinaldehyde-3-phosphatdehydrogenase, hypoxanthine guanine phosphoribosyl transferase, α-tubulin, β-tubulin) and a fold change was generated to the fermentation blank. Significantly regulated genes were calculated from three independent experiments.

Analysis of glutathione S-transferase (GST) activity, glutathione (GSH) content and alkaline phosphatase (AP) activity in LT97 cells

Cells were grown in 6-well plates to a confluence of about 30% and subsequently incubated with 2.5 and 5% fs or 4 mM butyrate as positive control for 24, 48 and 72 h. Cells were washed with PBS and harvested with trypsin/versene.

GST activity

For quantification of GST activity, cytosols were prepared. Therefore, cells were re-suspended in lysis buffer (50 mM Na/K-phosphate buffer, 1 mM Na-EDTA, 0.1% Triton-X, 1 mM Pefabloc; pH 7), incubated on ice for 10 min and finally centrifuged at 10,000×*g* for 10 min. The GST activity was determined using 1-chloro-2,4-dinitrobenzene as a substrate at 340 nm according to Habig et al. [27].

GSH content

LT97 cells were re-suspended in 5% metaphosphoric acid, homogenised with ultrasound (2 min, six cycles, 40% power, on ice, Sonoplus, Bandelin Electronics, Berlin, Germany) and centrifuged (13,000×*g*, 10 min, 4 °C). The GSH content was analysed in duplicate with the Glutathione Assay Kit from Calbiochem (Merck, Darmstadt, Germany) according to the manufacturer's instructions with minor variations. GSH quantification was performed in 96-well plates using a GSH dilution series (0–100 µM) based on a 25 mM stock solution to generate a standard curve. The amount of GSH was determined spectrophotometrically at 400 nm (SpectraFluor Plus, Tecan GmbH, Crailsheim, Germany) and normalised to the cell number of 1×10^6.

AP activity

Cytosol extraction was performed according to Ebert et al. [28]. Briefly, cells were homogenised with ultrasound (1 min, six cycles, 40% power, on ice, Sonoplus, Bandelin Electronics, Berlin, Germany) in homogenisation buffer (250 mM sucrose, 20 mM Tris/HCl, 1 mM dithiothreitol, 1 mM Pefabloc) and centrifuged (13,000×*g*, 60 min, 4 °C). The supernatant was used for quantification of AP activity in duplicate as described by Beyer-Sehlmeyer et al. [29] with slight modifications. Therefore, p-nitrophenylphosphate (5 mM) was used as substrate and 0–800 µM p-nitrophenol was used to generate a standard curve. The amount of p-nitrophenol was determined spectrophotometrically at 400 nm (SpectraFluor Plus, Tecan GmbH, Crailsheim, Germany).

Enzyme activities were calculated on the basis of the protein content determined by the method of Bradford with bovine serum albumin as standard protein [30].

Effects on GST and AP enzyme activities as well as GSH content were determined in three independent experiments relative to a medium control, which was set to 1 (fold change).

Analysis of cell growth after incubation of LT97 cells with fs

Growth and survival of LT97 adenoma cells were determined in 96-well plates using the DAPI-assay as described by Klenow et al. [25]. After seeding (48 h), the cells were

incubated with 2.5–20% fs for 24, 48 and 72 h. Fixing and permeabilising of cells was achieved by incubation with methanol for 5 min and DNA was stained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; Sigma-Aldrich, Deisenhofen, Germany) for 30 min at 37 °C. The DNA content was detected fluorimetrically at Ex/Em 360/450 nm (SpectraFluor Plus, Tecan Germany GmbH, Crailsheim, Germany) as a reflection of the relative cell number. Mean values (six determinations per experiment) were recorded for final evaluation. The results obtained for the fs-incubated cells were calculated in comparison to a medium control which was set to 100%. To calculate the sub-toxic concentrations for further analysis, the effective mean doses (EC_{25}) of the fs that inhibited growth by 25% were determined with GraphPad Prism® (GraphPad Software, Inc. San Diego, CA, USA) nonlinear regression/one phase exponential decay of three independent experiments and expressed as percentages. Additionally, the influence of synthetic mixtures as well as single substances containing SCFA, bile acids and phenolic acids (according to contents found in fs, see Table S1) was analysed.

Detection of apoptosis in LT97 cells after incubation with fs

Apoptotic LT97 cells were detected in an annexin V binding buffer using the annexin V-FITC/7-AAD (fluorescein isothiocyanate/7-aminoactinomycin D) kit (Beckman Coulter, Krefeld, Germany) in 4 independent experiments. Therefore, cells were seeded into 6-well plates, grown to a confluence of about 70% and incubated with 2.5–5% fs for 12 and 24 h. Due to the experimental design, only the fs WB and WMRB were used. Butyrate (4 mM) served as positive and medium as negative control. After harvesting, the cell number was determined with a CASY-cell counter (CASY model TT, Innovatis AG CASY Technology, Reutlingen, Germany) and set to 1×10^6 cells in 200 µl of 1 × binding buffer. After staining with 7-AAD and annexin V- FITC, samples were measured and the level of apoptotic cells was quantified by flow cytometry (Beckman Coulter, Krefeld, Germany) according to the manufacturer's instructions. Furthermore, the level of caspase-3, -8 and -9 activity was determined according to Borowicki et al. [31] in LT97 cells treated with 2.5 and 5% fs of WB, WMWB and WMRB for 24, 48 and 72 h in three independent experiments. Results for caspase activity are presented as fold changes relative to the medium control, which was set to 1.

Detection of apoptosis in normal and tumour colon tissue after incubation with fs

To analyse the apoptotic potential of fs, epithelial strips from normal colon tissue, prepared according to Schäferhenrich et al. [26], as well as pieces of colorectal tumour tissue (obtained from 4 different donors) were treated with 5% fs in medium defined by Rogler et al. [32] for 1, 6 and 12 h, which represents the longest duration of in vitro culture yielding sufficient viable cells [11]. Cytosols were extracted using a caspase assay lysis buffer by homogenisation with a Polytron homogeniser (Kinematica AG, Littau, Lucerne, Switzerland). Caspase activities were determined in duplicate according to the method described by Borowicki et al. [31] and the results were calculated based on the protein content, which was measured using the method of Bradford with bovine serum albumin as standard protein [30].

Statistical analysis

LT97 cells: means and standard deviations (SD) were calculated from at least three independent experiments. Differences were identified by one- or two-way ANOVA, including Bonferroni post-test with selected pairs, using GraphPad Prism® 5.01 (GraphPad Software, Inc. San Diego, CA, USA). Comparisons of two groups were performed with Student's t test.

Primary colon tissue: medians were calculated from 4 independent experiments (representing 4 different colon tissue donors) carried out in duplicate. Statistical analysis was done by Friedman test including Dunn's post-test to define differences between three or more groups. The Wilcoxon matched pairs test was used to compare two groups.

Results

Impact of fs on gene expression in LT97 cells

Analysis of gene expression patterns of about 300 genes involved in colon cancer development using microarray technology revealed that 76 genes were significantly upregulated (fold change >1.5) in LT97 cells upon 24 h treatment with fs from different bread samples. None of the genes showed a significant downregulation. From the 76 upregulated genes, 23 genes were regulated by the fs WB, 55 by WMWB and only 1 by WMRB. *GSTT1* (glutathione S-transferase theta 1), *ERCC4* (excision repair cross-complementing rodent repair deficiency) and *WNT2B* (wingless-type MMTV integration site family member 2B) were affected by both WB and WMWB. Generally, the upregulated genes included genes from biotransformation phase I, II and III, stress and signal transduction, DNA repair, apoptosis, cell cycle and others (Table 1, Table S2). To verify the microarray data, qPCR was performed for 6 genes, including *GSTT1* and *WNT2B* influenced by WB

Table 1 Major changes in gene expression in LT97 cells upon treatment with fermentation supernatants WB and WMWB (2.5%) for 24 h

Gene class	WB			WMWB		
	Gene	FC	*p* value	Gene	FC	*p* value
Biotransformation phase I	CYP2C8 (Cytochrome P450, family 2, subfamily C, polypeptide 8)	3.72	0.0041	CYP7A1 Cytochrome P450, family 7, subfamily A, polypeptide 1	9.95	0.0205
Biotransformation phase II	GSTT1# (Glutathione S-transferase theta 1)	5.79	0.0339	GSTT1# (Glutathione S-transferase theta 1)	2.49	0.0216
	CHST1 (Carbohydrate sulfotransferase 1)	4.49	0.0034	SULT2A1 (Sulfotransferase family. cytosolic. 2A. member 1)	16.78	0.0368
	SULT2B1 (Sulfotransferase family.cytosolic. 2B. member 1)	4.30	0.0317	UGT2B7 (UDP glycosyltransferase 2 family. polypeptide B7)	5.52	0.0111
Biotransformation phase II-gene regulation	c-JUN (MAPK9) (c-Jun N-terminal kinase 2)	3.89	0.0082	PIK3CA (Phosphoinositide-3-kinase. catalytic. alpha polypeptide)	4.91	0.0111
Stress- and signal transduction	SOD2 (Superoxide dismutase 2. mitochondrial)	17.09	0.0004	RAD9 (RAD9 homologue A; S. pombe)	5.97	0.0067
	GPX1 (Glutathione peroxidase 1)	6.69	0.0001	RAD50 (RAD50 homologue; S. cerevisiae)	5.37	0.0363
	ERCC4# (Excision repair cross-complementing rodent repair deficiency)	2.37	0.0351	ERCC4# (Excision repair cross-complementing rodent repair deficiency)	4.46	0.0048
	TNFSF10 (TRAIL) (Tumour necrosis factor (ligand) superfamily. member 10)	4.39	0.0413	TNFRSF6 (Fas; TNF receptor superfamily. member 6)	5.84	0.0189
	WNT2B# (Wingless-type MMTV integration site family. member 2B)	4.21	0.0008	WNT2B# (Wingless-type MMTV integration site family. member 2B)	4.03	0.0005

FC fold change; *WB* wheat bread; *WMWB* wholemeal wheat bread. Significant differences between bread fermentation supernatants and blank ($n = 3$) were obtained by Student's *t* Test. # genes regulated by WB and WMWB

and WMWB. *SOD2*, *GPX1*, *SULT2B1* and *UGT2B7* were also chosen because of their relative high fold changes (see Table 1, Table S2). The qPCR results only confirmed the microarray data concerning the genes *SOD2* and *SULT2B1*, which were also significantly regulated by WMRB (data not shown).

Impact of fs on GST activity, GSH content and AP activity in LT97 cells

While GST activity in LT97 cells was not significantly affected after incubation with fs for 24 and 72 h in comparison to the blank control (Fig. 1a), a significant increase in GST activity could be detected for WB and WMRB (5%) as well as the positive control butyrate (4 mM) after 48 h. The fs were not able to increase the GSH content in LT97 cells after 24 h incubation (Fig. 1b). In contrast, the content was significantly higher after 48 h incubation with 5% fs WB and WMWB and after 72 h incubation with 5% fs WB and WMRB. This effect was almost comparable to the butyrate control. Similar results were found regarding AP activity which was increased significantly after 48 h incubation with 2.5% fs WB, WMRB and inulin compared to the blank control (Fig. 1c). Significant differences between the distinct types of bread were not detectable.

Impact of fs on cell growth of LT97 cells

Cell growth analysis revealed that all fs of the different bread samples were able to modulate the adenoma cell growth in a time and dose-dependent manner (Fig. 2). Treatment of cells for 24 h with fs already resulted in reduced cell numbers, whereas an incubation time of 48 or 72 h exhibited the strongest effects. At these time points, the fs were able to inhibit cell growth significantly even at low concentrations (2.5 and 5%, respectively) compared to the blank control. The growth inhibitory effects are also reflected by the calculated EC_{25} values which ranged e.g. from 0.84% (WB) to 2.90% (blank) for the 48 h treatment (Table 2). Additionally, synthetic mixtures reconstituted from SCFA, bile acids and phenolic acids according to their concentrations in the fs (Table S1) were analysed. Here, the 72 h treatment had the strongest effect, shown exemplarily in Fig. S1 for WB, WMRB, inulin and blank control. From 10 combinatory mixtures or single substances only those containing butyrate (mixtures 1, 2, 3, 5, 10) were able to reduce the cell number significantly. Other

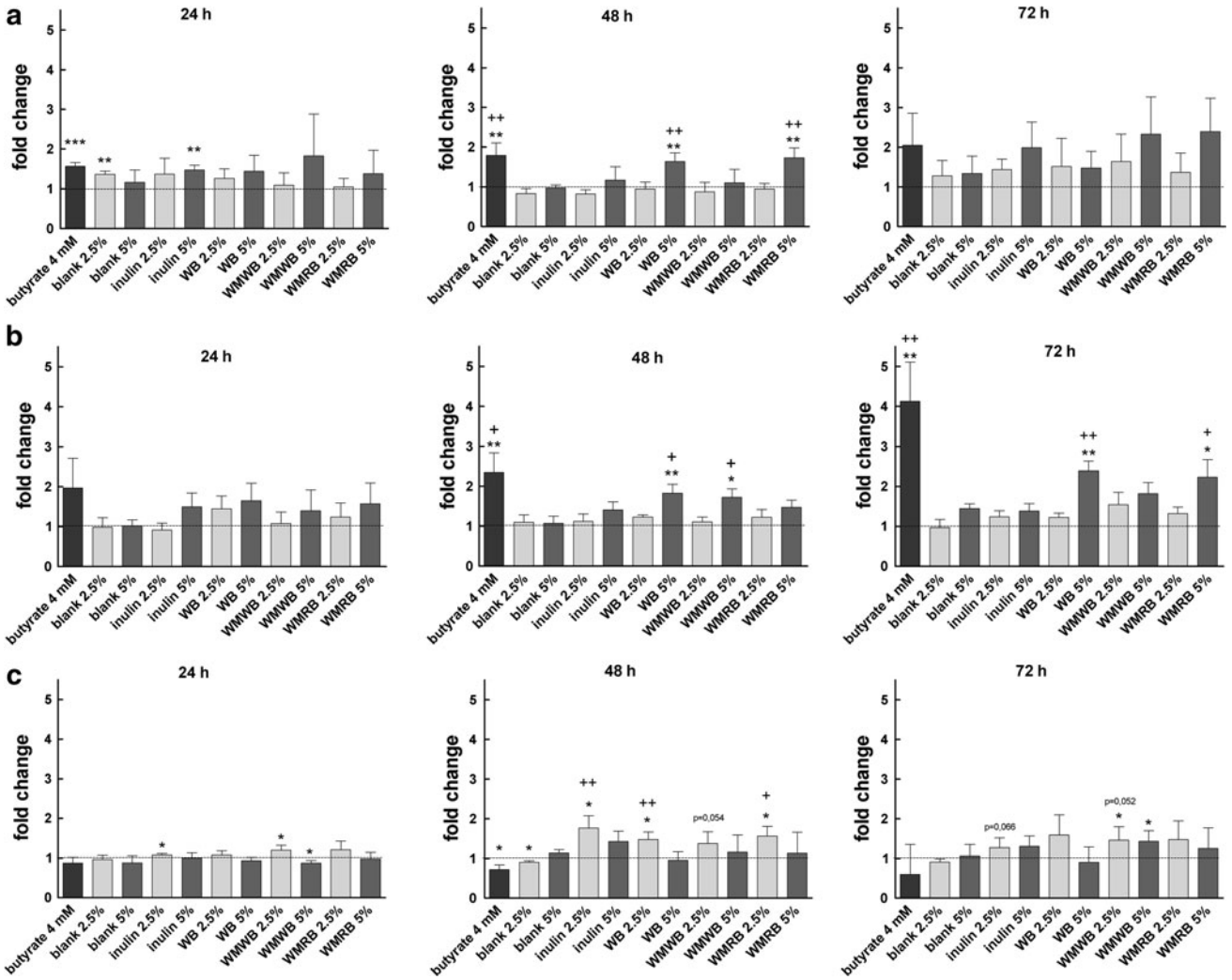

Fig. 1 GST activity (**a**), GSH content (**b**) and AP activity (**c**) in LT97 adenoma cells after incubation with fermentation supernatants for 24–72 h in concentrations of 2.5 and 5%. Results (fold changes) were obtained on the basis of a medium control which was set to 1 (*dashed line*). Significant differences between bread fermentation supernatants and medium control $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$ and significant differences to the blank control $+ p \leq 0.05$; $++ p \leq 0.01$ were obtained by Student's t test (mean + SD, $n = 3$). *WB* wheat bread; *WMWB* wholemeal wheat bread; *WMRB* wholemeal rye bread

components had little (acetate or propionate) or no effect (bile acids and phenolic acids). Significant differences between various bread fs were not detectable.

Impact of fs on apoptosis in LT97 cells

Induction of early apoptosis in LT97 cells after 12 and 24 h treatment with fs WB and WMRB was analysed using flow cytometry. Fig. 3a shows that fs from both bread samples (5%, WB: 1.5-fold, WMRB: 1.7-fold) as well as the positive control butyrate (4 mM, 1.8-fold) induced apoptosis significantly in the adenoma cells after 12 h incubation compared to the blank and medium control, respectively. Treatment of LT97 cells with the fs for 24 h increased the early apoptosis rate only by trend. The apoptotic potential of selected fs was also examined using a caspase assay. These results showed a significant increase in caspase-3 activity in LT97 cells after 24 h treatment with fs WB, WMWB and WMRB (Fig. 3b) except 2.5% WMRB. A 24 h exposure was chosen as starting time point since previous investigations could not detect any caspase activity at earlier incubation times in LT97 cells (data not shown). Treatment of cells for 48 h resulted in lower but partly significant effects. Butyrate (4 mM) was also able to increase caspase-3 activity significantly. Caspases-8 and -9 were not modulated by the fs (data not shown). The different bread samples did not display any significant differences.

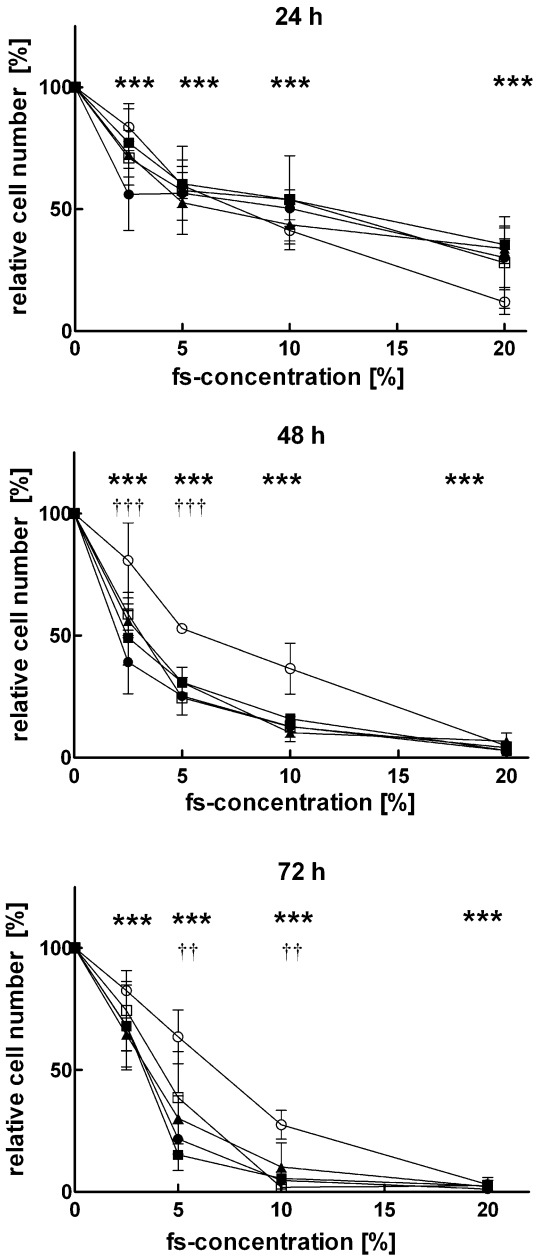

Fig. 2 Growth inhibiting potential of fermentation supernatants after incubation of LT97 adenoma cells for 24–72 h in concentrations of 2.5–20%. Blank (*empty circle*); inulin (*empty square*); WB (wheat bread) (*filled circle*); WMWB (wholemeal wheat bread) (*filled triangle*); WMRB (wholemeal rye bread) (*filled square*). Significant differences between bread fermentation supernatants and medium control (0%) *** $p \leq 0.001$ (mean + SD, $n = 3$) were obtained by one-way-Anova/Bonferroni post-test and significant differences to the blank control †† $p \leq 0.01$; ††† $p \leq 0.001$ (mean + SD, $n = 3$) were determined by two-way-Anova/Bonferroni post-test

Impact of fs on apoptosis in normal and tumour colon tissue

Cytotoxic effects on primary colon cells could be excluded by testing the influence of fs from different bread samples in isolated primary cells (2.5 and 5% after 1, 6 and 12 h; data not shown). Therefore, a concentration of 5% was used for treatment of primary colon tissue strips followed by detection of apoptosis using the caspase assay. None of the caspases (3, 8, 9) were affected by fs in normal tissue, neither after 1 and 6 nor 12 h (caspase-3, 1 and 12 h, Fig. S2). The tumour tissue seemed to be more susceptible to a treatment with fs showing an increased caspase-3 activity after 12 h incubation with the fs WMRB by trend ($p = 0.125$) (Fig. S3). Caspase-8 activity also tended to increase by treatment with WMRB after 12 h ($p = 0.125$), whereas caspase-9 activity was not at all affected by treatment with bread fs (data not shown). These results are preliminary since no significant effects could be observed due to the limited number of patients.

Discussion

Studies on DF which have long been discussed to have preventive effects on colon cancer are inconsistent. Some investigations were unable to show an association between the intake of DF and a reduced risk of colon cancer [33, 34], whereas others found significant inverse correlations [4, 35]. But the mechanisms underlying the chemopreventive effects of DF are still not completely understood. Bread as a staple food product is one of the most important DF sources, especially in Germany. Nevertheless, there are only a few studies which analysed the health-promoting effects of bread [13–15, 19, 36] and investigations on the influence of different bread types on chemopreventive effects in colon cells are still missing. Hence, the aim of this study was to gain insight into chemopreventive properties of different types of bread (WB, WMWB, WMRB). The highest fibre contents could be found in WMRB, followed by WMWB, whereas the lowermost content was detectable in WB. WMRB also had the highest content in soluble fibre, followed by WB and WMWB [22].

Microarray analysis of LT97 cells disclosed an impact of fs (predominantly WB and WMWB) on the expression of genes involved in DNA repair (e.g. *ERCC4*), differentiation (e.g. *TGF*) or apoptosis (e.g. *CASP10*) as well as biotransformation phase I (*CYP*, cytochrome pP450 family) and II (e.g. *GST*). WMRB only induced the expression of one gene (*CYP4F2*), compared to WB and WMWB which regulated 23 and 55 genes, respectively. But qPCR also showed an induction of *SOD2* and *SULT2B* for WMRB. In general, microarray data has to be verified by qPCR, because such discrepancies are not excludable [37]. Nevertheless, the obtained information provides an overview of potential regulated genes. Our data showed that WB fs increased the expression of e.g. *SOD2* and *GPX* and might enhance the antioxidant capacity of the colon cells

Table 2 EC_{25}-values of fermentation supernatants from different bread types and controls

Obtained on the basis of growth inhibition analysis in LT97 cells ($n = 3$)

WB wheat bread; *WMWB* wholemeal wheat bread; *WMRB* wholemeal rye bread

	EC_{25}-values (%)		
	24 h incubation	48 h incubation	72 h incubation
WB	1.76 ± 1.40	0.84 ± 0.39	1.41 ± 0.60
WMWB	2.26 ± 0.86	1.22 ± 0.32	1.46 ± 0.40
WMRB	3.02 ± 1.26	1.06 ± 0.27	1.27 ± 0.34
Negative control (blank)	3.33 ± 1.01	2.90 ± 1.15	3.07 ± 0.36
Positive control (inulin)	4.47 ± 4.71	1.20 ± 0.21	1.87 ± 0.99

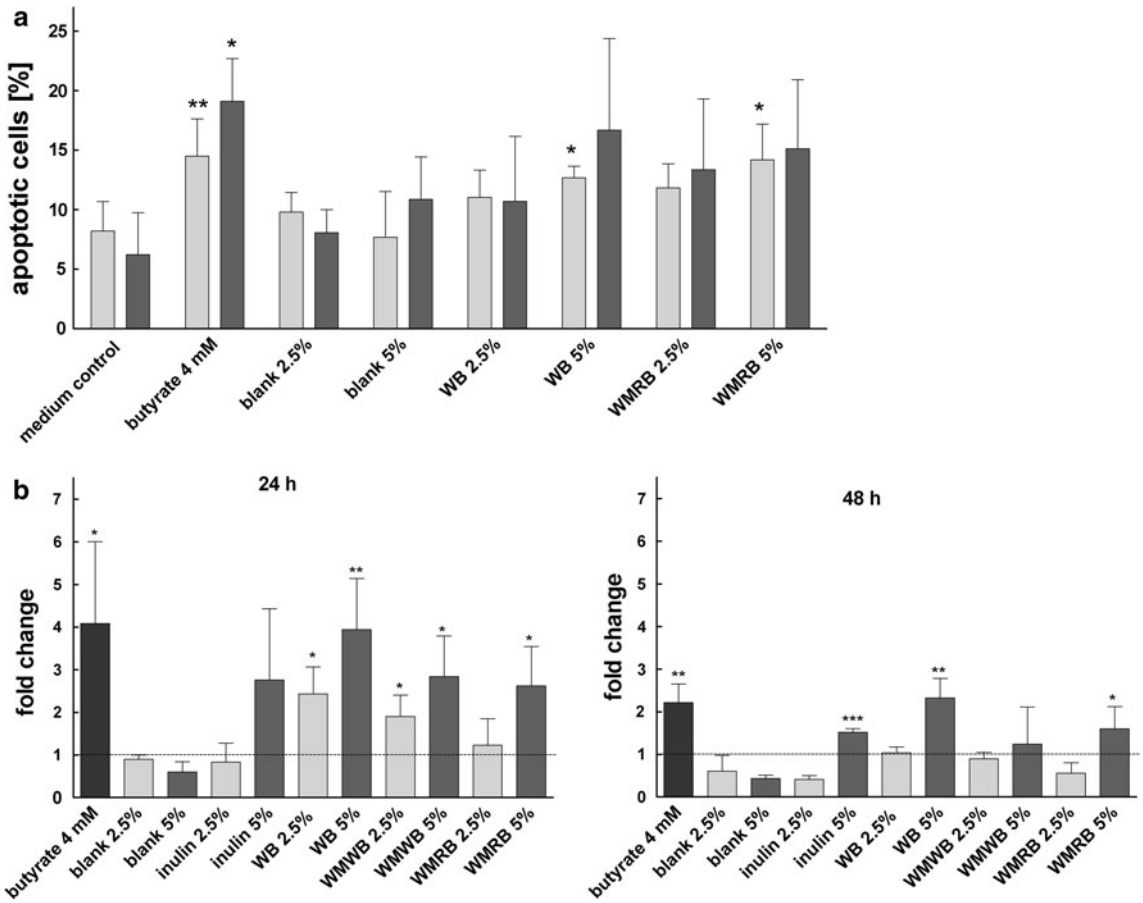

Fig. 3 **a** Early apoptosis in LT97 adenoma cells after incubation for 12 (*light grey shaded bar*) and 24 h (*dark grey shaded bar*) with fermentation supernatants in concentrations of 2.5 and 5%. Significant differences between bread fermentation supernatants and blank as well as butyrate and medium control $*p \leq 0.05$; $**p \leq 0.01$ (mean + SD, $n = 4$) were obtained by Student's t test. **b** Induction of caspase-3 activity in LT97 adenoma cells after 24 and 48 h incubation with fermentation supernatants in concentrations of 2.5 and 5%. Results (fold changes) were obtained on the basis of a medium control which was set 1 (*dashed line*). Significant differences between bread fermentation supernatants and blank $*p \leq 0.5$,$**p \leq 0.01$, $***p \leq 0.001$ (mean + SD, $n = 3$) were obtained by Student's t test. *WB* wheat bread; *WMWB* wholemeal wheat bread; *WMRB* wholemeal rye bread

which could lead to reduced oxidative DNA damage as for example shown by Glei et al. [38] in HT29 cells after incubation with fs of wheat aleuron arabinoxylans. The fs WB also induced *WNT2B* and *WNT1*, whereas WMWB increased the expression of *WNT2B* and *WNT5B* as well as the frizzled receptors *FZD5* and *FZD2* involved in the WNT signalling pathway which is activated in colon carcinoma [39]. Borowicki et al. [31] already described an induction of *WNT2B* expression after incubation of LT97 and HT29 cells with fs from wheat aleuron and butyrate (4 mM). Bordorano et al. [39] could also show that butyrate (5 mM) leads to a hyperinduction of the WNT

pathway in colon cell lines from different transformation levels with a linear induction of apoptosis. The pro-apoptotic effects of butyrate could therefore be directly correlated to the induction of the WNT pathway. All three bread fs induced biotransformation phase I-associated genes (*CYP3A4*, *CYP2C8*, *CYP4F2*), whereas phase II-associated genes were affected predominantly by WMWB and WB. The WB fs induced expression of *GSTT1*, *SULT2B1* and *SULT4A*. Additionally, WMWB increased the expression of acetyl transferase (*CHAT*), methyl transferase (*HNMT*) and UGT (*UGT2B7*). An increased expression of phase I-associated together with phase II-associated genes could be explained by a combined gene regulation of phase I- and II enzymes mediated by the aryl-hydrocarbon receptor and xenobiotic response element as assumed by Helsby et al. [40] who analysed the antimutagenic effects of a wheat bran diet in rats. A higher expression of phase II enzymes could enhance the detoxification of carcinogens in the colon. Stein et al. [23] disclosed that an incubation of primary colon cells and HT29 adenocarcinoma cells with fs of wheat aleuron resulted in an increased mRNA expression of *SULT2B* and former studies of Pool-Zobel et al. [11] demonstrated that the fermentation product butyrate has an elevating impact on the expression of phase I (CYP p45*0* family) and II (acetyltransferases, sulfotransferases, GSTs) genes. Here, we also analysed the influence of complex bread fs on the activity of GSTs as relevant detoxification enzymes as well as the GSH content. WB, WMRB (5%) and the positive control butyrate (4 mM) induced GST activity significantly in LT97 adenoma cells. Butyrate as key fermentation product of the bread samples [2.5% fs: 0.5 mM (WB)—0.7 mM (WMRB) butyrate and 5% fs: 1.1 mM (WB)—1.4 mM (WMRB) butyrate] could at least be partially responsible for this induction. But, fs represent complex mixtures of substances which could contribute to the activation or inhibition of enzymes as shown for the blank which also increased GST activity significantly, although the butyrate concentration was much lower than in the bread fs (Table S1).

The increased GST activity partially matches with the GSH content found in LT97 cells upon treatment with bread fs. The elevated GSH levels after treatment with fs WB and WMWB (48 and 72 h) are consistent with other studies which showed increased GSH contents upon butyrate treatment [41, 42]. Low GSH levels might be accompanied by a greater risk for cancer development, possibly due to DNA damage induced by genotoxic substances [43, 44]. In contrast, an increased GST activity and GSH content could protect the cells from reactive substances like 4-hydroxynonenal or benzo(a)pyrene [20, 45]. This might be associated with a reduced risk of cell initiation (mutation) [46].

The loss of differentiation is one hallmark of degenerated cells [47]. Several studies have demonstrated that butyrate is a potent differentiating agent inducing the expression and/or activity of the differentiation marker AP in colon cells [48, 49]. Borowicki et al. [31] could show that in vitro fermented aleurone was able to increase AP activity in HT29 adenocarcinoma cells. In the present study, a treatment with complex fs of WB, WMRB and inulin also significantly increased AP activity in LT97 adenoma cells (48 h). Incubation with WMWB tended to result in an increase after 48 and 72 h. The missing modulation of AP activity upon butyrate treatment (4 mM), which was also shown by Borowicki et al. [31], could be due to a higher sensitivity of LT97 towards butyrate [31, 50, 51], resulting in an enhanced apoptosis rate in comparison to HT29 cells. Therefore, butyrate would fail to stimulate differentiation. This was also reported by Orchel et al. [49] for butyrate concentrations higher than 5 mM in HT29 cells. Additionally, the present study demonstrated that butyrate (4 mM) as well as 5% [1.1 mM (WB)—1.4 mM (WMRB) butyrate] of the bread fs induced early apoptosis in LT97 cells. Compared to 2.5% fs [0.5 mM (WB)—0.7 mM (WMRB) butyrate], these concentrations might already be too high to induce differentiation. Differences between fermented bread samples could not be detected (regarding GST, AP activity and GSH content). Since the obtained effects are likely to be induced by butyrate, the lack of significant differences between the various bread fs could be due to the only marginal differences of butyrate concentrations in the fermented bread samples.

Investigations on the cell number after incubation with different concentrations of fs showed a time and dose-dependent inhibition of adenoma cell growth. Such growth inhibitory effects of fs from sources rich in fibre were also reported by others [38, 51, 52]. The growth inhibiting effects of the blank control might be a result of metabolites from the faeces inoculum (e.g. butyrate). In contrast, the bread fs exhibited increased growth inhibitory effects after 48 and 72 h. Studies with synthetic mixtures showed that only samples containing butyrate displayed significant growth inhibitory effects. EC_{25} values of complex fs were lower than those of the synthetic mixtures with butyrate or butyrate alone (data not shown), indicating that SCFA, bile or phenolic acids might not be the only effective components. Aglycones emerged from glycosylated polyphenols, for example, showed also growth inhibitory effects in HT29 cells [53, 54].

Induction of apoptosis is a relevant mechanism of chemoprevention. We were able to show that fs WB and WMRB induced early apoptosis in LT97 cells after 12 h treatment. In addition, elevated levels of caspase-3 activity, a marker of advanced apoptosis, were triggered by bread fs

and butyrate (24 and 48 h). These results are in line with those of Borowicki et al. [31], who observed a significant increase in early apoptotic cells and caspase-3 activity after incubating LT97 adenoma cells with fermented aleurone. Induction of apoptosis in preneoplastic adenoma cells could reduce the number of cells with inherent risk for transformation to carcinoma cells [20]. The microarray data also revealed that genes involved in apoptosis were upregulated upon treatment with bread fs probably due to butyrate. The mechanisms of butyrate mediated pro-apoptotic effects are not fully elucidated, but extrinsic as well as intrinsic pathways of programmed cell death might be involved [55, 56]. Therefore, the mechanisms and compounds responsible for the disclosed effects of bread fs have to be investigated in greater detail.

An additional aim of this study was to analyse the influence of fermented bread samples on cells from different stages of carcinogenesis. It is assumed that on the one hand butyrate has trophic effects in non-transformed cells, and on the other hand, it reduces survival of tumour cells [10]. To increase our knowledge about the so-called butyrate paradox [57], primary epithelial colon tissue and tumour tissue from the same patients were treated with fs and analysed regarding apoptotic effects. A treatment of tumour tissue strips with WMRB tended slightly towards a higher caspase-3 activity. But, due to the low number of patients no significant effects of the treatment on activities of caspases-3, -8 and -9 were detectable. The lack of differences between normal and tumour tissue could also be due to the fact that morphologically appearing normal tissue from cancer patients could already be altered in gene expression and metabolic activity [58]. Therefore, it is not excludable that normal tissue already shows and altered response upon treatment with bread fs. Nevertheless, an induction of apoptosis in this tissue could suppress the formation of degenerated cells at an early stage of colon cancer development. To gain more insights into the effects of butyrate in normal and tumour colon tissue, more patients have to be tested, prospectively.

It is noteworthy, that the selected bread fs did not show any significant differences regarding the observed chemopreventive outcomes, although the DF content differed [22] (WB: 2.74 ± 0.09, 2.86 ± 0.08; WMWB: 2.47 ± 0.11, 12.10 ± 0.14; WMRB: 4.97 ± 0.14, 12.67 ± 0.24; soluble, insoluble fibre content in g/100 g dry matter, respectively). Hence, we could not confirm that bread with a higher content of DF (WMRB) also exhibits stronger effects. One reason could be the limitation of the in vitro digestion and fermentation process which is only an approximation to the in vivo situation. Metabolites of the complex matrices might not have been completely removed during dialysis and could therefore contribute to the fermentation and the obtained results. However, this is also possible under in vivo conditions. Carbohydrates may only be incompletely digested and absorbed in the small intestine and thus become available to fermentation in the colon [59, 60]. Besides, bread is a complex food and other components than DF could influence and contribute to the potential protective effects (e.g. polyphenolic compounds) [61]. Assuming that butyrate may primarily be responsible for the disclosed effects, as also shown by Borowicki et al. [52], the small differences in the butyrate content between the fermented bread samples could explain the marginal variations in the observed effects. The high butyrate content of WB could probably be due to the higher content of starch compared to WMRB which contributes to the production of butyrate [22, 60]. Our results are in contrast to intervention studies which could show higher faecal butyrate excretion upon administration of WMRB in comparison to WB [15]. The differences between our in vitro and former in vivo results could be due to the relative static in vitro fermentation conditions, e.g. fermentation of different bread samples with standardised fermentation times of 24 h for batch fermentation [62]. Although a 24 h simulation of the large intestine reflects the mean normal in vivo condition [63], Aura et al. [62] also discussed that for incubation times longer than 24 h, the application of a semi-continuous model would be a better choice to reflect the conditions in the colon. Using a dialysis step is reasonable to remove digestion products but the choice of the cut-off is critical. It cannot be excluded that a cut-off of 1 kDa removed e.g. small non-digestible oligosaccharides which could have contributed to the fermentation. But a smaller cut-off might have left to many undesired metabolites which, if not previously removed, would have masked the results from DF fermentation. Apart from butyrate as the key fermentation product also other compounds or mechanisms could contribute to the chemopreventive effects of bread. Hiller et al. [22] showed that fermentation of WB exhibited the highest stimulation of SCFA and lactic acid production as well as reduction of detrimental bacterial enzyme activities. In contrast, the strongest retardation of bacterial bile acid degradation and stimulation of phenolic acid and metabolite release was induced by WMRB. Furthermore, other positive in vivo effects of bread rich in DF (e.g. WMRB) should not be disregarded. These include the reduction of passage times by means of water binding and faecal bulking effects as well as prebiotic properties.

In summary, the present study shows that different types of bread exhibit chemopreventive potential post-digestion and in vitro fermentation which is mediated by distinct mechanisms. These include the upregulation of genes from DNA repair, biotransformation, differentiation and apoptosis, increase in GST activity, GSH content and

differentiation, growth inhibition and induction of apoptosis in LT97 cells.

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