Chemopreventive potential of in vitro fermented nuts in LT97 colon adenoma and primary epithelial colon cells

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Due to their beneficial nutritional profile the consumption of nuts contributes to a healthy diet and might reduce colon cancer risk. To get closer insights into potential mechanisms, the chemopreventive potential of different in vitro fermented nut varieties regarding the modulation of genes involved in detoxification (CAT, SOD2, GSTP1, GPx1) and cell cycle (p21, cyclin D2) as well as proliferation and apoptosis was examined in LT97 colon adenoma and primary epithelial colon cells. Fermentation supernatants (FS) of nuts significantly induced mRNA expression of CAT (up to 4.0-fold), SOD2 (up to 2.5-fold), and GSTP1 (up to 2.3-fold), while GPx1 expression was significantly reduced by all nut FS (0.8 fold on average). Levels of p21 mRNA were significantly enhanced (up to 2.6-fold), whereas all nut FS significantly decreased cyclin D2 expression (0.4-fold on average). In primary epithelial cells, expression of CAT (up to 3.5-fold), GSTP1 (up to 3.0-fold), and GPx1 (up to 3.9-fold) was increased, whereas p21 and cyclin D2 levels were not influenced. Nut FS significantly inhibited growth of LT97 cells and increased levels of early apoptotic cells (8.4% on average) and caspase 3 activity (4.6-fold on average), whereas caspase 3 activity was not modulated in primary colon cells. The differential modulation of genes involved in detoxification and cell cycle together with an inhibition of proliferation and induction of apoptosis in adenoma cells might contribute to chemopreventive effects of nuts regarding colon cancer.

KEYWORDS
apoptosis, cell cycle, colon cancer, dietary fiber, nuts

1 | INTRODUCTION

Nuts exhibit a unique nutrient profile comprised of several compounds which can exert health promoting properties such as polyunsaturated fatty acids (PUFA), essential micronutrients, high-quality proteins, different phytochemicals, or dietary fiber.1,2 Therefore, it is recommended to consume, for example, 25 g3 or 30 g4 nuts on a daily basis as part of a healthy diet. Several studies indicate the potential of a regular nut intake on health promoting effects supporting an inverse association between nut intake and colon cancer risk in both men and women.13 Also a recent follow-up of the Nurses’ Health Study showed that nut consumption was inversely, though not statistically significant, associated with a lower risk for colon cancer.16 Results from a recent study also indicate an inverse association between nut intake and colon cancer risk in both men and women.17

Colon cancer is one of leading causes of all cancer deaths worldwide representing the second and third most common form of cancer in women and men, respectively.18 The risk for developing sporadic colon cancer, which comprises about 75% of all colon cancer cases, is mainly influenced by lifestyle factors such as high consumption of red meat, a high fat and low fiber diet, alcohol and tobacco consumption, obesity and a lack in physical activity.19,20 In contrast, the intake of dietary fiber may protect against the formation of colon cancer,21 as shown in the EPIC study which revealed an inverse relationship between dietary fiber consumption and the risk for colon cancer development.22 The consumption of nuts can contribute up to 10% of the recommended dietary fiber intake of 30 g per day.1,2,23,24
Metabolites which are formed during fiber fermentation in the colon by the intestinal microbiota contribute to the protective effects of dietary fiber. Especially, the short chain fatty acid butyrate exerts colon health promoting properties and chemopreventive effects. Butyrate serves as an energy source for normal epithelial colon cells, whereas it inhibits the proliferation of colon adenoma or cancer cells.\textsuperscript{25,26} The fermentation of nuts also leads to the formation of metabolites which could exert chemopreventive effects regarding colon cancer development such as butyrate.\textsuperscript{27}

The aim of the present study was to examine the chemopreventive potential of nuts after in vitro fermentation in colon adenoma cells in comparison to primary epithelial colon cells. Therefore, we analyzed the effects of nut fermentation products on enzymes involved in detoxification and proliferation processes as well as cell growth and apoptosis in order to address primary (elimination of ROS) and secondary chemopreventive effects (inhibition of proliferation, induction of apoptosis) of nuts in these different cell types, respectively.\textsuperscript{28}

2 | METHODS

2.1 | Characteristics of nuts

Five different nut varieties were used in the present study. Macadamia nuts (South Africa), pistachios (California), and walnuts (California) were obtained from the Southern African Subtropical Growers’ Association, Paramount Farms and the California Walnut Association, respectively. Hazelnuts (Turkey) and almonds (California) were derived from Viba Sweets (Floh-Seligenthal, Germany).

2.2 | In vitro digestion and fermentation of nuts

The different nut varieties were subjected to an in vitro simulated digestion and fermentation as described previously\textsuperscript{29,30} with slight modifications.\textsuperscript{27} In brief, 2 g of ground nuts were reconstituted with anaerobic potassium phosphate buffer (0.1 M, pH 7.0) and incubated with α-amylase (17.4 U/sample from a 1000 U/mL stock solution in 20 mM NaH\textsubscript{2}PO\textsubscript{4}) and NaCl (0.85%) for 5 min and additionally with pepsin (1.11 mg in 0.94 mL 20 mM HCl, pH 2.0) for 2 h at 37°C. Subsequently, nut samples were incubated with an intestinal extract (2.6 mg pancreatin and 5.0 mg oxgall in 5 mL of 11 mM bicarbonate buffer; pH 6.5) in a dialysis membrane (molecular weight cut off: 500-1000 Dalton) under semi-anaerobic conditions (6 h, 37°C). A feces inoculum mixture of at least three healthy donors was used for in vitro fermentation in an anaerobic atmosphere (37°C, 24 h). An oligofructose enriched with inulin (Synergy1\textsuperscript{®}, Beneo, Mannheim, Germany) served as positive control. A blank fermentation sample without nuts, representing the pure feces sample of the donors, was used as negative control.

2.3 | Preparation of fermentation supernatants and pellets

Subsequently to the in vitro fermentation of the nut samples, fermentation supernatants (FS), and fermentation pellets (FP) were obtained. Therefore, the fermented nut samples were subjected to two centrifugation steps (30 min, 4200g and 15 min, 4200g at 4°C, respectively). FS and FP from two separate fermentations were pooled. Final FS were obtained by centrifugation (15 min, 10300g, 4°C) and subsequent sterile filtration (pore size 0.22 μm). Aliquots of FS and FP were stored at −80°C until further use.

2.4 | Cell culture

For cell culture experiments the human colon adenoma cell line LT97 (a kind gift from Professor B. Marian, Institute for Cancer Research, University of Vienna, Austria) was used. The cell line was established from a colon adenoma. Therefore, LT97 cells represent an early stage of colon tumor development.\textsuperscript{31} A detailed description of the origin, properties, and culture conditions of the cell line is provided by Klenow et al.\textsuperscript{32} Recently, an authentication of LT97 cells was performed by STR (short tandem repeat) profiling (Leibnitz-Institute DSMZ, German Collection of Microorganisms and Cell Cultures, March 2015). Cells from passages 3 to 26 were used for cell culture experiments.

Primary epithelial strips as well as epithelial cells were isolated from normal colon tissue obtained from intestinal resections after surgery of patients suffering from colon carcinomas (5 male, 50-74 years) at least 10 cm away from the tumor site as described by Schäferhenrich et al.\textsuperscript{33} The study was approved by the Ethics Committee of the Friedrich Schiller University Jena (No. 1601-08/05) and written informed consent was obtained from all subjects.

2.5 | Isolation of total RNA

Total RNA was isolated after incubation of LT97 cells with nut FS (2.5% and 5%) as well as butyrate (4 mM), which served as positive control, for 24 and 48 h using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA from epithelial strips was isolated after incubation with nut FS (5%) in medium defined by Rogler et al.\textsuperscript{24} for 12 h representing the so far most certain duration of in vitro culture still yielding sufficient viable cells.\textsuperscript{35,36} The strips were homogenized in RLT Plus buffer (Qiagen, Germany) with the Polytron homogenizer 2100 (Kinetmatica, Littau/Lucerne, Switzerland), and total RNA was isolated as described above. RNA was eluted in 50 μL RNase free water and stored at −80°C until further use. RNA concentrations were measured at 260 nm absorbance with a NanoDropND-1000 photometer (NanoDrop Technologies, Wilmington, DE). Furthermore, RNA integrity was controlled using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

2.6 | cDNA synthesis

Synthesis of complimentary DNA was performed using the SCRIPT Reverse Transcriptase Kit (Jena Bioscience, Jena, Germany). Reverse transcription of 1.5 μg total RNA was conducted in a 20 μL reaction mix for 50 min at 42°C. The reaction was stopped at 72°C for 15 min and remaining RNA was removed by RNaseH treatment for 20 min at 37°C. The cDNA was diluted 1:50 with RNase free water for further experiments and stored at −80°C, respectively.
2.7 | mRNA expression

Expression levels of CAT, SOD2, GSTP1, GPx1, p21, and cyclin D2 mRNA were measured using qPCR. In previous experiments, efficiency of qPCR (95-110%) as well as the optimal annealing temperatures of the specific primers were determined. The qPCR experiments were performed in duplicate in a 25 μL reaction mix using the GoTaq® qPCR Master Mix (Promega, Mannheim, Germany) and the iCycler iQ Real time PCR Detection System (Bio-Rad Laboratory, Munich, Germany). RNase-free water and 10 pmol of gene-specific primers: CAT forward 5'-TGGGACAATGAAATGCTGAG-3' and reverse 5'-TTACAC GGAATGCTAG-3', SOD2 forward 5'-GGCCCTGAACCTCAGAT-3' and reverse 5'-GAC TACCCGAGAAGTC-3', GSTP1 forward 5'-TGACTGGCTGATCCTGGACTAC-3' and reverse 5'-GTGGCTGCTGGCA AGAC-3', GPx1 forward 5'-GACTACCCAGATGAACGA-3' and reverse 5'-ACGTACTGGAGGAATCTCAG-3', p21 forward 5'-CACTGTTGTACCTGCTCT-3' and reverse 5'-CTTCCTTGGAGGAA TCT-3', cyclin D2 forward 5'-CCACCGACTT TAAGTTGGGC-3' and reverse 5'-CTTGGAGACATCCAAGCTG-3', β-actin forward 5'-AGAGCCCTGCCTT GCGAT-3' and reverse 5'-CCACGATGGAGGGAAAGAC-3', GAPDH forward 5'-ACA CACTTCCTCCTCCCTGAC-3' and reverse 5'-TCCACCA CACCCGTTGCTGATC-3'. The qPCR profile included an initial denaturation step of 2 min at 95°C followed by 40 cycles of denaturation (30 s at 94°C), annealing (30 s at 60°C), and extension (30 s at 72°C) and a melting curve analysis. The expression of mRNA levels was normalized to the geometric mean of the two reference genes β-actin and GAPDH based on the equation of Pfaffl et al.37

2.8 | Determination of cell growth

The time- and dose-dependent effects of nut FS on the growth of LT97 adenoma cells were analyzed via DAPI (4',6-diamidino-2-phenylindol) assay as described previously.29 Briefly, LT97 cells were seeded into 96-well plates and were grown to a confluence of 20-30% prior to incubation with different concentrations of nut FS (2.5, 5, 10, and 20%) for 24, 48, and 72 h. After fixing and permeabilization of the cells with methanol for 5 min, DNA was stained with DAPI (Sigma-Aldrich, Munich, Germany; 30 min, 37°C) and the DNA content as a reflection of the cell number was detected fluorimetrically at Ex/Em 380/465 nm (SpectraFluor Plus, Tecan Germany GmbH, Crailsheim, Germany). Results were calculated in comparison to a medium control which was set to 100%. Sub-toxic concentrations of the FS (EC25/EC50: 2.5 and 5% FS, respectively) were determined via nonlinear regression/one phase exponential decay from three independent experiments (GraphPad Prism®, GraphPad Software, San Diego, CA) for additional experiments.

2.9 | Detection of apoptosis

LT97 cells were grown to a confluence of about 70% in 6-well plates and incubated with nut FS (2.5 and 5%) for 12 and 24 h as well as butyrate (4 mM) as positive control. Early apoptotic cells were quantified by flow cytometry (Cell Lab Quanta™ SC MPL 1.0, Beckman Coulter, Krefeld, Germany) using the annexin V-FITC/7-AAD (fluorescein isothiocyanate/7-aminoactinomycin D) kit (Beckman Coulter, Krefeld, Germany) in 200 μL of annexin V binding buffer according to the manufacturer's instructions. In addition, the level of caspase-3, -8, and -9 activity was determined as described by Borowicki et al.38 in LT97 cells treated with nut FS (2.5 and 5%) for 24 and 48 h as well as butyrate (4 mM). In brief, 2.4 × 10⁶ cells were lysed in caspase assay lysis buffer (250 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 25 mM 3-[(3-cholamidopropyl)-dimethylamino]-2-hydroxy-1-propanesulfonate (CHAPS), 25 mM threo-1,4-dimercapto-2,3-butanediol (DTT), 1 mg/mL leupeptin, 1 mg/mL pepstatin A, 100 mg/mL pefabloc SC, 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM sodium orthovanadate) for 20 min at 4°C. Cell lysates were centrifuged (16,000g, 15 min, 4°C) and one half of the lysates was treated with 50 nM caspase inhibitors (caspase 3: Ac-DEVD-CHO; caspase 9: Ac-LEHD-CHO; caspase 8: Ac-IETD-CHO, Enzo Life Science, Lörrach, Germany) for 10 min. Subsequently, all lysates were treated with 25 mM caspase substrate (caspase 3: Ac-DEVD-AMC; caspase 9: Ac-LEHD-AMC; caspase 8: Ac-IETD-AMC, Enzo Life Science) for 2 h at 37°C. Modulation of caspase activity was detected fluorimetrically with Ex/Em 380/465 nm (SpectraFluor Plus, Tecan Germany GmbH, Crailsheim, Germany). Furthermore, caspase activities were measured in primary epithelial strips treated with nut FS (5%) and staurosporine as positive control (10 μM) for 12 h in medium defined by Rogler et al.34 as described above. Therefore, cytosols from primary epithelial strips were obtained by homogenization in caspase assay lysis buffer with a Polytron homogenizer (Kinematica) and caspase activities were calculated on the basis of the protein content determined by the method of Bradford.39 Results for caspase activity of both cell types are presented as fold changes relative to the medium control, which was set to 1.

2.10 | Cell viability

Cytotoxic effects of nut FS in LT97 cells were excluded by measuring the cell number and viability using the ViCell cell counter (Beckman Coulter, Krefeld, Germany) in each experiment. To exclude cytotoxic effects in primary epithelial cells, cell viability of isolated primary epithelial colon cells was determined after incubation with 5% nut FS for 1, 6 and 12 h with the trypan blue exclusion test.

2.11 | Statistical analysis

Means and standard deviations of at least three independent experiments were calculated and statistical differences were analyzed by one-way or two-way ANOVA including Bonferroni post-test using GraphPad Prism® version 5 for Windows (GraphPad Software, San Diego, CA) or Student’s t-test for comparison of two groups. Results from primary epithelial strips were calculated as means from five independent experiments (representing five different colon tissue donors). 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.
3 | RESULTS

3.1 | Effect of nut FS on mRNA expression of selected target genes in LT97 cells

The mRNA expression of CAT was significantly enhanced almost in a dose dependent manner in LT97 cells after incubation with FS from different nut varieties and butyrate (4 mM) as positive control compared to the medium control (Fig. 1a). CAT mRNA expression increased between 1.7- and 2.9-fold in cells incubated with 2.5% FS from hazelnuts and pistachios, respectively, and between 2.6- and 4.1-fold in cells incubated with 5% FS from blank and almonds, respectively. Butyrate increased CAT mRNA levels by 3.3-fold. Incubation of LT97 cells with FS from almonds, macadamia and pistachios (5%) led to a significant increase in CAT expression compared to the respective blank control (1.3-1.6-fold).

In general, the relative increase in mRNA expression of SOD2 was lower compared to CAT, but was significant after incubation with 5% FS of the different nut varieties compared to the medium control (Fig. 1b). SOD2 mRNA expression ranged from 1.0- to 1.7-fold upon incubation with 2.5% FS from hazelnuts and pistachios, respectively, and from 1.4- to 2.5-fold after incubation with 5% FS from pistachios and almonds, respectively. Butyrate enhanced SOD2 mRNA levels by 2.0-fold. Incubation with the FS from almonds (5%) significantly increased SOD2 mRNA levels compared to the respective blank control (1.5-fold).

Levels of GSTP1 mRNA were significantly higher than the medium and blank control after incubation with 5% FS of hazelnuts, almonds, macadamia and walnuts (~2.0-fold, Fig. 1c). GPx1 mRNA expression was significantly reduced upon incubation with butyrate as well as the FS from hazelnuts and walnuts compared to the medium control (~0.7-fold, respectively). In contrast, 2.5% FS from the blank control significantly increased GPx1 mRNA levels (1.3-fold). Incubation with 2.5% FS from all nut varieties led to a significant down-regulation of GPx1 mRNA levels compared to the respective blank control (0.5-0.7-fold, Fig. 1d).

The expression of the cell cycle regulatory gene p21 was significantly increased by 5% almond FS (2.6-fold) compared to the medium control, whereas it was only slightly enhanced by butyrate or other nut FS (5%, Fig. 1e). In contrast, mRNA levels of cyclin D2 were significantly down-regulated by nut FS (0.3- to 0.5-fold on average) and butyrate (0.3-fold) in comparison to the medium control as well as to the blank control (0.9-fold) (Fig. 1f).

3.2 | Effect of nut FS on mRNA expression of selected target genes in primary epithelial colon cells

The incubation of primary epithelial strips with 5% of nut FS resulted in higher levels of CAT, GSTP1, and GPx1 mRNA (Fig. 2a, c, and d) in comparison to the medium control, whereas the expression of SOD2, p21, and cyclin D2 was not affected by incubation with nut FS or butyrate (Fig. 2b, e, and f). The highest induction of CAT, GSTP1, and GPx1 mRNA expression was observed after incubation with FS from almonds (3.0-, 2.6-, and 3.0-fold, respectively) and macadamia (3.5-, 3.0-, and 3.9-fold, respectively). But, only butyrate significantly increased CAT (3.6-fold) and GSTP1 mRNA expression (4.0-fold) compared to the medium control. The FS from almonds and macadamia enhanced mRNA levels of GSTP1 compared to the blank control by trend (P = 0.0625). An insignificant increase could also be observed for CAT (P = 0.125) and GPx1 (P = 0.0625) expression after incubation with FS from macadamia.

3.3 | Modulation of LT97 cell growth by nut FS

Incubation with FS of nuts as well as controls significantly decreased the growth of LT97 colon adenoma cells in a time- and dose-dependent manner (Fig. 3). With an average of 66% and 29% after incubation with 2.5% and 20% FS, respectively, cell number was already significantly reduced after 24 h (Fig. 3a) by all fermented nut samples and controls compared to the medium control which was set to 100% (dashed line). The incubation of LT97 cells for 48 with the FS of Synergy1® and the different nut varieties (except hazelnuts) led to a significant lower cell growth of 43 and 31% on average compared to the blank control with 63 and 43% at concentrations of 2.5 and 5%, respectively (Fig. 3b). Strongest growth inhibitory effects could be observed after incubation of LT97 cells with FS of nuts and controls after 72 h (Fig. 3c). On average, cell number ranged from 46 to 1.6% after incubation with 2.5 and 20% FS from nuts and controls, respectively. At this time point, FS of almonds (2.5 and 5%), walnuts and pistachios (2.5%) significantly reduced growth of LT97 cells compared to the respective blank control.

3.4 | Induction of apoptosis in LT97 cells by nut FS

Detection of apoptosis by flow cytometry revealed a significant increase of early apoptotic cells after incubation of LT97 cells with FS from hazelnuts (5% FS: 8.2%), almonds (2.5% FS: 6.1%), and pistachios (2.5% FS: 7.0%) compared to the medium control (1.8%) after 12 h (Fig. 4a). Incubation with FS from hazelnuts and pistachios also led to a significant induction of apoptosis in comparison to the FS from the blank control (2.5% FS: 3.0%, 5% FS: 2.6%). Induction of apoptosis in LT97 cells was more pronounced and almost dose-dependent after incubation with nut FS after 24 h (Fig. 4b). FS from almonds (2.5% FS: 5.5%), walnuts (2.5% FS: 5.6%), Synergy1® (5% FS: 5.9%), and butyrate (5.3%) significantly enhanced levels of early apoptotic cells compared to the medium control (2.1%). All nut FS (5% FS: 8.3% on average) significantly induced early apoptosis in comparison to the medium and the respective blank control (3.2%). The examination of caspase activity also confirmed the apoptotic potential of nut FS as shown in Fig. 5. Caspase 3 activity was significantly induced by all nut FS (2.5% FS: 4.1-fold, 5% FS: 5.1-fold on average) as well as butyrate (7.3-fold) and the FS of Synergy1® (2.5% FS: 3.0-fold, 5% FS: 5.1-fold) in LT97 cells after incubation for 24 h compared to the medium control (=1) and the respective blank control (2.5% FS: 1.1-fold, 5% FS: 1.0-fold). The activity of caspases 8 and 9 were not induced in LT97 cells upon incubation with nut FS (data not shown).
3.5 Detection of apoptosis in primary epithelial colon tissue

The activity of caspase 3 in primary epithelial colon tissue was not induced by nut FS or controls as shown in Fig. 6. The incubation of primary epithelial strips with staurosporine slightly enhanced relative caspase 3 activity in comparison to the medium control ($P = 0.0625$) and additionally, caspase 3 activity was significantly higher than after incubation with butyrate and FS from Synergy1®, almonds, and macadamia. Activities of caspase 8 and 9 were not affected upon incubation with nut FS (data not shown).

4 DISCUSSION

Reactive oxygen species (ROS) are involved in the progression and transition of preneoplastic into malign cells by inducing further DNA...
Protection from excessive oxidative stress by induction of antioxidant and detoxifying enzymes as part of the defense mechanisms of colon cells is an important chemopreventive step in order to inhibit the initiation of cells into preneoplastic lesions, a process termed primary chemoprevention. An induction of antioxidant and phase II enzymes by nutritional compounds or metabolites might therefore protect from colon cancer development. The results from the present study show for the first time that fermentation products obtained from different nut varieties induce mRNA expression of enzymes involved in the elimination of ROS and detoxification of carcinogens like CAT, SOD2, and GSTP1 in LT97 colon adenoma cells. The mRNA levels of these enzymes were especially enhanced by FS from hazelnuts, almonds, and macadamia and were comparable or even higher than mRNA levels after incubation with the well-known preventive agent butyrate. In contrast, mRNA levels of GPx1 were significantly reduced. In primary epithelial colon cells, FS...
from macadamia and partly almonds also induced CAT, GSTP1, and GPx1 mRNA expression. This induction was not significant probably due to the limited number of samples and the relative large inter-individual variation between the different donors. In contrast to nut FS, butyrate significantly induced mRNA levels of CAT and GSTP1 as well as GPx1 by trend ($P = 0.0625$) in primary colon cells. An induction of antioxidant or phase II enzymes in colon cells of different transformation grades upon incubation with fermentation products from fiber rich sources was also observed by Stein et al.\textsuperscript{36} Fermented wheat aleurone increased mRNA expression of CAT and GSTP1 in HT29 colon carcinoma and GSTP1 in primary epithelial cells. Former studies indicated that the induction of these genes can be attributed to butyrate as the key fermentation product of dietary fiber.\textsuperscript{28,35,41,42} Though there is no direct link between the daily recommended nut intake and FS used in the present study, we could recently confirm that the fermentation of nuts results in up to 2.8-fold higher concentrations of short chain fatty acids compared to the control and a shift of molar ratios towards butyrate production ranging in physiological observed

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\textbf{FIGURE 3} Growth inhibiting of LT97 colon adenoma cells after incubation with fermented nut samples and controls (blank, Synergy1®) in concentrations of 2.5-20\% for 24 h (a), 48 h (b), and 72 h (c) (mean $\pm$ SD, $n = 3$). Significant differences between blank and fermentation supernatants (FS) of Synergy1® or nuts ($^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^*P \leq 0.05$) and between FS ($^{##}P \leq 0.001$, $^{##}P \leq 0.01$, $^#P \leq 0.05$) and between different concentrations were obtained by two-way Anova/Bonferroni post-test. Significant differences between different concentrations were obtained by one-way Anova/Bonferroni post-test ($^{###}P \leq 0.001$, $^{##}P \leq 0.01$, $^#P \leq 0.05$). All fermentation samples were significantly different compared to the medium control which was set to 100\% (dashed line).
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concentrations. Therefore, butyrate might be mostly responsible for the induction of antioxidant and phase II enzymes by fermented nut samples which contain up to 1.5 mM butyrate while the blank control only contains 0.4 mM butyrate (FS 5%, respectively). Since FS represent complex mixtures, other nut compounds or metabolites might also contribute to the modulation of ROS eliminating enzymes. Next, effects of the feces matrix cannot be excluded since the blank control also increased mRNA levels, although to less extents. Butyrate regulates gene expression via its function as a histone deacetylase inhibitor. In addition, the induction of phase II enzymes by butyrate in normal intestinal epithelial cells is associated with increased levels of NF-E2-related factor 2 (Nrf2). The transcription factor Nrf2 regulates the expression of many antioxidant and phase II enzymes via antioxidant response elements (ARE). Several studies demonstrated that many dietary compounds such as flavonoids and polyphenols activate Nrf2 as reviewed by Su et al. Therefore, butyrate together with other nut compounds or metabolites resulting from the fermentation process might act synergistically in the induction of Nrf2-related downstream target genes involved in the elimination of ROS which could initiate or promote colon carcinogenesis.

The observed differential expression pattern of GPx1 in LT97 and primary colon cells may reflect a differential role for GPx in different stages of colon carcinogenesis. In primary colon cells, an up-regulation of GPx is associated with the protection against H2O2 or...
ROS-mediated DNA or cell damage to inhibit the initiation of the cells. But, an induction of GPx1 could promote survival of already transformed cells by inhibiting ROS inducible apoptotic processes. Loss of the tumor suppressor gene APC, which is deleted in LT97 cells, leads to ROS accumulation which in turn could sensitize the cells towards ROS mediated apoptosis, as discussed by Emmink et al.\textsuperscript{44} They demonstrated that suppression of oxidative stress by GPx2 is associated with the formation of differentiated tumor mass and metastatic capacity in colon cancer. Therefore, a down-regulation of GPx in LT97 cells could be associated with a lower risk for colon cancer promotion.

Promotion of initiated into preneoplastic cells is also inhibited by secondary chemopreventive mechanisms, such as reduced cell growth and increased apoptosis. Therefore, we analyzed the effect of nut FS on the cell cycle relevant genes p21 and cyclin D2. Gene expression of p21 was partly significantly enhanced compared to the medium control, whereas cyclin D2 mRNA levels were significantly decreased in LT97 cells by FS from all nut varieties compared to the medium and blank control. In comparison, mRNA levels of p21 and cyclin D2 were not influenced in primary colon cells. Elevated levels of p21 mRNA in LT97 upon incubation with FS from a dietary fiber rich source could also be demonstrated by Borowicki et al.\textsuperscript{28} The gene modulatory effects in adenoma cells can be attributed to butyrate which shows a similar influence on p21 and cyclin D2 expression as the nut FS. Several studies showed that butyrate modulates expression of these cell cycle relevant genes due to its function as histone deacetylase inhibitor in colon adenoma and carcinoma cells.\textsuperscript{38,45-47} Also other nut compounds could exert chemopreventive effects by modulating cell cycle relevant genes, as it has been shown for phenolic acids or flavonoids such as ellagic acid or genistein, respectively.\textsuperscript{48} Recently, Gonzalez et al.\textsuperscript{49} showed that urolithins as colonic metabolites of ellagitannins and ellagic acid induce p21 mRNA expression. The induction of the CDK inhibitor p21 together with the down-regulation of cyclin D2 by nut FS might exert chemopreventive effects by initiating cell cycle arrest, thereby inhibiting proliferation of colon adenoma or cancer cells.\textsuperscript{25} This is supported by the growth inhibitory effects of nut FS on LT97 adenoma cells shown in the present study. All nut FS, except FS from hazelnuts, were able to significantly reduce proliferation of the cells similar to the FS from Synergy1® (oligofructose enriched with inulin as positive control of fermentation) in comparison to the blank control, especially after 48 h incubation. These results are line with growth inhibition of HT29 colon carcinoma cells upon incubation with FS from different nut varieties shown by Lux et al.\textsuperscript{50} Results from other studies also demonstrated that the fermentation of fiber rich sources results in the formation of metabolites, such as butyrate.\textsuperscript{25,26,29,51} with anti-proliferative effects on colon adenoma or cancer cells.\textsuperscript{29,38,51} Beside butyrate, propionate which was also formed during fermentation of nuts\textsuperscript{57} might contribute to the growth inhibitory effects.\textsuperscript{52} Furthermore, a recent study demonstrated that also deoxycholic acid (DCA) inhibits the growth of HCT116 colon cancer cells.\textsuperscript{53} These results might explain the growth inhibitory potential of the blank control which contained significantly higher levels of DCA than the nut FS.\textsuperscript{27} In a recent study, we have shown that the fermentation of walnuts resulted in the formation of CLA (c9,t11 conjugated linoleic acid) which is also able to reduce the growth of colon adenoma or carcinoma cells.\textsuperscript{27,54} Also phenolic acids and flavonoids present in nuts or urolithins as colonic metabolites could contribute to the growth inhibitory effects.\textsuperscript{48,55}

The anti-proliferative effects of nut FS could be the result of apoptotic processes. The present study demonstrates that all nut FS significantly enhanced the percentage of early apoptotic cells as well as caspase 3 activity as a marker of advanced apoptosis in LT97 cells after 24 h treatment. No information could be drawn whether the intrinsic or extrinsic pathway is involved in induction of apoptosis by nut fs since caspase 8 and 9 were not modulated in the present study. Similar results were also obtained by Borowicki et al using wheat aleurone,\textsuperscript{51} whereas results from another study indicate, that caspase 9 is involved in butyrate mediated induction of apoptosis in HT29 cells.\textsuperscript{56} Results from other studies confirm the induction of apoptosis in HT29 and LT97 cells by complex FS from dietary fiber rich sources, which could be mainly mediated by butyrate.\textsuperscript{29,51,57} Important to note, no modulation of caspase 3 activity by nut FS or butyrate was observed in primary epithelial colon strips. These results indicate the differential role of the fermentation product butyrate as it serves as energy source in normal epithelial cells while it induces apoptosis in transformed colon cells.\textsuperscript{28} The pro-apoptotic effects of butyrate are at least partly mediated by the inhibition of histone deacetylases. But, the exact mechanism of action is not uncovered and several pathways may be involved. Studies indicate that butyrate mediated induction of apoptosis is related to an activation of death receptor (DR) 4 and 5 or TGF-β.\textsuperscript{58,59} Studies also indicate that the JNK MAP kinase pathway as well as mitochondrial pathways are involved in butyrate mediated induction of apoptosis in colon cancer cells.\textsuperscript{56,60} It has been also shown that butyrate leads to a hyperactivation of the WNT pathway in colon cancer cells, which is directly associated with the induction of apoptosis.\textsuperscript{61} This association was also found for LT97 colon adenoma cells.\textsuperscript{52} Furthermore, other studies indicate that WNT signaling is involved in the induction of apoptosis in LT97 cells by FS from dietary fiber rich sources containing butyrate.\textsuperscript{29,38} Again, in addition to butyrate, other nut components or metabolites which are formed during the fermentation process may be involved in the pro-apoptotic effects, such as phenolic acids, flavonoids, lignans, phytosterols,\textsuperscript{48,63,64} tocotrienols,\textsuperscript{65} CLA resulting from the fermentation process of LA (linoleic acid) in walnuts\textsuperscript{27,54} or urolithins.\textsuperscript{49}

In summary, the results from the present study demonstrate that nuts or metabolites formed during fermentation, respectively, exert chemopreventive effects via several mechanisms including the induction of antioxidant, phase II and cell cycle relevant genes, reduction of proliferation as well as induction of apoptosis in colon adenoma cells. In conclusion, the chemopreventive potential of nuts might be associated with a lower risk for colon cancer development and may indicate that frequent nut consumption contributes to a healthy diet.

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Nothing to disclose.

POTENTIAL CONFLICTS OF INTEREST

Nothing to disclose.

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