

In vitro fermented nuts exhibit chemopreventive effects in HT29 colon cancer cells

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Abstract

It is proven that nuts contain essential macro- and micronutrients, e.g. fatty acids, vitamins and dietary fibre (DF). Fermentation of DF by the gut microflora results in the formation of SCFA which are recognised for their chemopreventive potential, especially by influencing cell growth. However, little is known about cellular response to complex fermentation samples of nuts. Therefore, we prepared and analysed (pH, SCFA, bile acids, tocopherol, antioxidant capacity) fermentation supernatant (fs) fractions of nuts (almonds, macadamias, hazelnuts, pistachios, walnuts) after *in vitro* fermentation and determined their effects on growth of HT29 cells as well as their genotoxic/anti-genotoxic potential. The fermented nut samples contained 2- to 3-fold higher amounts of SCFA than the faeces control, but considerable reduced levels of bile acids. While most of the investigated native nuts comprised relatively high amounts of tocopherol (α -tocopherol in almonds and hazelnuts and γ - and δ -tocopherol in pistachios and walnuts), rather low concentrations were found in the fs. All nut extracts and nut fs showed a strong antioxidant potential. Furthermore, all fs, except the fs pistachio, reduced growth of HT29 cells significantly. DNA damage induced by H₂O₂ was significantly reduced by the fs of walnuts after 15 min co-incubation of HT29 cells. In conclusion, this is the first study which presents the chemopreventive effects (reduction of tumour-promoting desoxycholic acid, rise in chemopreventive SCFA, protection against oxidative stress) of different nuts after *in vitro* digestion and fermentation, and shows the potential importance of nuts in the prevention of colon cancer.

Key words: Nuts: *In vitro* fermentation: Dietary fibre: Human colon cells: Cell growth: Antioxidant capacity

Traditionally, nuts have been considered to be unhealthy because of their high content of fat^(1,2). However, recent findings attribute a wide range of health benefits resulting from nut consumption. Nuts are an important source of essential macro- and micronutrients, e.g. fatty acids, vitamins and minerals. In addition, some species provide 10% of the recommended daily intake of thiamine, niacin, P and Zn under allowance of the daily intake of 40 g nuts a day, according to the US Department of Agriculture⁽¹⁾. Furthermore, nuts contain polyphenols and carotenoids which also have health-improving effects^(3,4). Evidence exists that an increased consumption of nuts can protect from CVD⁽⁵⁾ and Hu *et al.* demonstrated that death from heart disease as well as non-lethal heart attacks can be reduced. These effects are probably a cause of the lipid profile of nuts, because they contain mainly MUFA and PUFA which can reduce the levels of

LDL-cholesterol⁽⁶⁾. Different studies demonstrated that the protection against CVD is at least partly caused by the modulation of serum lipids through unsaturated fatty acids. The consumption of only four walnuts a day over 3 weeks (in addition to the regular diet), e.g. significantly increased blood levels of α -linoleic acid and its metabolite eicosapentaenoic acid^(7–9). In addition, Torabian *et al.*⁽¹⁰⁾ demonstrated that including walnuts as part of a habitual diet (free-living situation) also favourably altered the plasma lipid profile. Importantly, no additional weight gain was observed in human studies with nuts^(11–13). Nuts are also rich in different tocopherol forms and can deliver more than 20% of the recommended daily allowance⁽¹⁴⁾. It is notable that different nuts contain divergent tocopherol profiles. For example, hazelnuts and almonds are the best source for α -tocopherol (15 mg/100 g and 16 mg/100 g), pistachios and walnuts contain mainly γ -tocopherol

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); BA, bile acids; DAPI, 4',6-diamidino-2-phenylindol; DCA, desoxycholic acid; DF, dietary fibre; DPPH, 1,1-diphenyl-2-picrylhydrazyl; fs, fermentation supernatants; TEAC, trolox equivalent antioxidant capacity.

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(22 mg/100 g and 21 mg/100 g)^(15,16). Nuts are also an excellent source of health-promoting dietary fibre (DF). They can deliver between 5 and 12% of the recommended daily intake (30 g) of DF^(4,17,18). This is highly interesting because of the beneficial effects of DF on the digestive tract, especially the large intestine. DF absorb water and thus increase stool bulk and prevent obstipation⁽¹⁹⁾. The beneficial effects also include the reduction of symptoms caused by chronic inflammatory bowel disease and chemoprevention of colorectal cancer⁽²⁰⁾. Reports of Davis & Iwahashi⁽²¹⁾ suggest that nuts can be considered to be a protective factor of the colon. Whole-almond fractions reduced incidences of histological alterations of the colon mucosa of rats. Results of the European Prospective Investigation into Cancer and Nutrition study with 36 994 subjects in ten different countries demonstrated an inverse association between consumption of nuts and seeds and the risk of colorectal cancer in women⁽²²⁾. DF do not only increase stool bulk, they can also increase the amounts of SCFA, like butyrate, propionate and acetate in the lumen of the colon⁽²³⁾. Butyrate and, to a minor extent, propionate have been shown to inhibit the growth of colon tumour cells, by blocking proliferation and initiating apoptosis^(24,25). In contrast, butyrate and other metabolites of DF fermentation act as growth factors in non-transformed colon cells and can increase the toxicological defence systems^(26,27). Selected DF, termed prebiotic fibre, increases the amounts of SCFA in the colon and stimulates the growth of beneficial lactic acid-producing bacteria^(28–30). Especially bifidobacteria can improve colonic health by lowering the pH-value in the colonic lumen, thereby inhibiting the growth of pathogenic bacteria^(31,32). Furthermore, prebiotics are able to reduce the genotoxic potential of faecal water and thus reduce the exposure of the colon epithelium with potential carcinogens^(33–35). Up till now, the effects of nuts on colonic health have not been well analysed and little is known about the metabolites which are formed during their fermentation. Considering that colorectal cancer is one of the most frequent and deadly cancer forms worldwide⁽³⁶⁾, studying these effects may increase the knowledge on the health benefits of nuts, next to their role in the prevention of CVD. Therefore, we generated fermentation supernatants (fs) of five different nut species (almonds, macadamias, hazelnuts, pistachios and walnuts) by using an *in vitro* fermentation system simulating the human digestion^(25,37,38). The first goal of the present work was to characterise and to compare the fermentation products of different nuts regarding concentrations of SCFA (acetate, propionate and butyrate), tocopherols, antioxidant capacity and bile acids (BA). HT29 colon adenocarcinoma cells were treated with these fs and the effects on cell growth, cell cycle modulation as well as the genotoxic/anti-genotoxic potential of the fs were studied.

Material and methods

In vitro fermentation of nuts

The five different nut varieties used in this study were delivered from Blue Diamond Growers (almonds), Max Kiene

GmbH (macadamias), Turkish Hazelnut Promotion Group (hazelnuts), Paramount Farms (pistachios) and Mariani Nut Company (walnuts). The nuts were digested using an *in vitro* simulation of the human gastrointestinal passage according to a previously described protocol^(38–40). In brief, 2 g of each nut variety were ground and suspended in anaerobic potassium phosphate buffer (0.1 M, pH 7.0). A positive control containing 2 g Synergy1[®] (inulin enriched with oligofructose; ORAFIT) and a negative control (blank: fermentation buffer only) were included. Pre-digestion was simulated by incubation with α -amylase (Sigma A-0521; 17.36 μ l from a 500 U/500 μ l stock solution in 20 mM-NaH₂PO₄ buffer) 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, the samples were incubated with an intestinal extract of pancreatin and oxgall (Sigma P-1750, Fluka-Sigma 70 168, 2.6 mg and 5.0 mg, respectively in 5 ml of 11 mM-bicarbonate buffer; pH 6.5) in a dialysis membrane (molecular weight cut-off: 1000 Da) under a semi-anaerobic condition at 37°C for 6 h. Semi-anaerobic conditions were achieved by removing a part of the air in the fermentation bottles via a cannula at 0.5 bar for 1 min and subsequently injecting a fermentation gas mixture (86% N₂, 10% CO₂ and 4% H₂) at 0.8 bar for 1 min for seven cycles repeated (15 min) according to Stein *et al.*⁽⁴¹⁾.

For the *in vitro* fermentation, the suspensions were mixed to equal parts with human faecal slurries (approximately 25 ml pooled suspension from three human donors) and fermented under anaerobic conditions for 24 h at 37°C and a starting pH-value of 6.5. Anaerobic conditions were achieved by gas exchange as described previously for 30 min (fourteen cycles repeated). The fermentation process was stopped by placing the suspensions on ice and the pH was measured.

Preparation of nut fermentation supernatants

fs were obtained by centrifugation of the faeces suspension for 30 min at 4200 g. The supernatant fractions were centrifuged again for 15 min at 4200 g. The fs from three different fermentations were pooled and centrifuged for 15 min at 16 000 g. All centrifugation steps were performed at 4°C. Afterwards, the fs were sterilised by filtration (pore size 0.22 μ m) to obtain the final fs for further investigations. Before the sterilised fs were used for the incubation of respective colon cells, they were diluted with cell-culture medium to reach a final concentration of 2.5, 5, 10 or 20% (v/v).

Cell line and culture conditions

The human colon adenocarcinoma cell line HT29 (American Type Culture Collection no. HTB-38) was used for cell culture experiments in this study. The origin, properties and cell culture conditions of this cell line have been described previously⁽³⁹⁾. In brief, the cells were grown in Dulbecco's modified Eagle's medium (Gibson BRL) supplemented with 10% fetal calf serum. For cell culture experiments, HT29 cells were seeded in ninety-six-well plates (determination of cell growth) or in



six-well plates (determination of genotoxic- and anti-genotoxic effects), respectively. The cells were grown for 24 h before incubation with the fs (2.5–20%). After incubation, the cells were used for different assays, as described next. Cells of passages 10–21 were used for the experiments.

Analysis of characteristic constituents of the fermentation supernatant

The fs were analysed considering SCFA, BA, tocopherol content, and also antioxidative capacity. Additionally, the antioxidant potential and tocopherol concentration of raw material (ground nuts) were determined.

To analyse the concentrations of SCFA by GC, the fs were mixed with an internal standard (iso-caproic acid in concentrated formic acid). In brief, 1 μ l of the mixture was injected and SCFA were separated on a Zebron™ FFAP Capillary GC Column 15 m (Phenomenex, Inc.). Each measurement followed a defined temperature protocol (105°C 1.5 min \rightarrow 35°C/min \rightarrow 170°C 2 min) as described elsewhere⁽⁴²⁾.

The BA concentrations of the fs were investigated by tandem MS according to a previous published protocol^(25,43). Briefly, the fs were diluted (1:10 in fermentation buffer) and solid phase extraction was carried out using 101 sorbent cartridges (Separtis) and cholic acid – d_4 as an internal standard. The tested BA were cholic acid, lithocholic acid, desoxycholic acid (DCA), dihydroxycholic acid, ursodesoxycholic acid, chenodesoxycholic acid, sodium taurochenodesoxycholic acid and sodium glycochenodesoxycholic acid. BA were eluted and separated on an RP-C8 column (100 \times 4 mm, 5 μ m; MZ-Analysentechnik); and 10 mM-ammonium acetate buffer (pH 5.0) was used as the mobile phase with eluant A (formic acid, 0.012%) and eluant B (acetonitrile). The eluants were linearly changed from 70% A and 30% B to 30% A and 70% B within 38 min, held for 10 min and finally changed to their original ratio. The determination of BA was performed with a tandem mass spectrometer API 4000™ (Applied Biosystems/MDS SCIEX), equipped with an ESI ionisation source operating in the negative mode at –4.5 kV at 450°C. BA were quantified by external calibration in the multiple reaction monitoring (MRM) mode using the Analyst 1.4 software (Applied Biosystems/MDS SCIEX).

Tocopherol concentrations of the ground nuts and fs were measured by HPLC according to an established protocol⁽⁴⁴⁾. Tocopherols were extracted with *n*-hexane, ethanol and tert-butylmethylether and separated on a Knauer Europher 100 DIOL column (250 \times 4.0 mm, 7 μ m) with a mixture of *n*-hexane–tert-butylmethylether (98 + 2, v/v) as the mobile phase. Tocopherol isomers were determined by fluorescence (λ_{ex} 292 nm, λ_{em} 330 nm) and quantified via an external standard curve.

Analysis of the antioxidant potential

To analyse the antioxidant capacity of the raw material (ground nuts) and the fs, it was necessary to use different extraction methods. Considering that the antioxidant substances could be lipo- or hydrophilic, we used the trolox equivalent antioxidant capacity (TEAC)- and the 1,1-diphenyl-2-picrylhydrazyl

(DPPH)-assay. The TEAC-test for foodstuffs with lipophilic antioxidants was used for the ground nuts after a slightly modified protocol⁽⁴⁵⁾ of Miller *et al.*⁽⁴⁶⁾. The ground nuts were extracted in 100 μ l hexane. ABTS solution (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) in phosphate buffer) was then added. After centrifugation (30 s), the extinction of the ABTS-nut extract was measured after 2 min with the V-530 spectrophotometer (Jasco). To determine the antioxidant potential considering lipophilic and hydrophilic antioxidants, the DPPH-test modified according to Liua *et al.*⁽⁴⁷⁾ was used. Therefore, extraction of the ground nuts was done with ethanol and before measuring the extinction at 540 nm with the V-530 spectrophotometer (Jasco) DPPH solution was added. The fs as more hydrophilic mixtures were analysed with a TEAC-test for hydrophilic antioxidants after a protocol described previously^(45,48) with an extinction of 743 nm.

Determination of cell growth

The 4',6-diamidino-2-phenylindol (DAPI) assay was used to examine the time- and dose-dependent effects of fs. HT29 cells were seeded in ninety-six-well plates and grown for 24 h. After an additional 24, 48 or 72 h of incubation with the fs (5–20%), the relative cell number was quantified using a protocol described previously⁽⁴⁹⁾. After 30 min of cell incubation with the DNA intercalating fluorescent dye DAPI, the DNA content as a reflection of the remaining number of cells was detected by fluorimetric analysis (excitation 360 nm; emission 465 nm) in a microplate reader (Spectra Fluor Plus Tecan). The results were calculated on the basis of the medium control which was set to 100%.

Cell cycle analysis

To analyse the influence of nut fs on the cell cycle, HT29 cells were seeded in six-well plates and grown for 24 h. Afterwards, the cells were incubated with fs (5%) for 24, 48 and 72 h, subsequently trypsinised and dissolved in PBS. After measuring the cell viabilities and cell number with a CASY-cell counter (CASY® model TT; Roche Innovatis AG CASY® Technology), they were stained with NIM-DAPI (0.6% Nondinet P40 and 10 μ g/l DAPI dissolved in PBS). Then, 10 min later, the intercalated DAPI was quantified by flow cytometry, whereas counted cells were allocated to the phases of the cell cycle using cytometry analysis software (Cell Lab Quanta TM SC_MPL 1.0; Beckman Coulter)⁽²⁵⁾.

Determination of genotoxic and anti-genotoxic effects

To determine the genotoxic/anti-genotoxic potential of the nut fs, the single-cell micro gel electrophoresis (comet assay) was used as has been described elsewhere^(50,51). To identify the genotoxic effects of the nut fs, HT29 cells were seeded in six-well plates and 24 h later they were incubated with different concentrations (2.5 and 5%) of the nut fs or controls for an additional 1 or 24 h. Subsequently, the cells were washed, trypsinised and dissolved in PBS. H₂O₂ (75 μ M, 5 min at 4°C) and PBS were used as positive and negative

controls, respectively. To analyse the anti-genotoxic effects, HT29 cells were grown for 24 h, incubated for 15 min (short term, co-incubation with H₂O₂) and 24 h (long term) with nut fs and challenged with 75 μM-H₂O₂ for 15 min at 37°C as has been described elsewhere⁽⁵²⁾. Viabilities and cell numbers were determined with a CASY-cell counter (CASY® model TT; Roche Innovatis AG CASY® Technology) and a defined cell number of 0.4 × 10⁶ cells were mixed with 75 μl 0.7% low-melting agarose (Biozym) dissolved in PBS and distributed onto microscopic slides coated with 0.5% normal-melting agarose (Biozym). Further steps were carried out as described elsewhere^(38,52). DNA was stained with the fluorescent dye SYBR® Green (Sigma Aldrich Chemie GmbH) and detected with a fluorescence microscope (ZEISS Axiostar plus; Carl Zeiss Jena GmbH) using an image analysis system (Comet Assay IV, Perceptive Instruments). The degree of DNA damage correlates with the fluorescence in the tail (% tail intensity). For each concentration, means of sixty cells were the basis for calculating the effect in one experiment.

Statistical evaluation

Means and standard deviations were calculated from at least three independent experiments. Statistical differences were analysed by one- or two-way ANOVA, including Bonferroni post-test using GraphPad Prism® version 5 for Windows (GraphPad software). 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, the comparison of two groups was done using Student's *t* test.

Results

Analysis of pH

The *in vitro* fermentation of all nuts resulted in a significantly decreased pH value (pH 6.12 (SD 0.09)) compared with the fs blank (pH 6.53 (SD 0.05)). The positive control Synergy1® showed the strongest effect (pH 4.58 (SD 0.26)). The lowered

pH is a first indication of the generation of acidic metabolites by *in vitro* fermentation (Table 1).

Analysis of characteristic constituents of the fermentation supernatant

Analysis of SCFA. Table 1 demonstrates that the total amounts of SCFA in the nut fs were explicitly higher (67.83–85.93 mM) than in the blank control (32.15 mM). More importantly, the molar ratio of acetate–propionate–butyrate shifted from 56.94:21.31:21.75 in the blank to an average of 44.50:20.86:34.64 in the nut samples. Thereby, the ratio of acetate was decreased in favour of the highly bioactive SCFA butyrate. No major differences between the nut varieties were found. Fermentation of Synergy1® resulted in the highest amount of SCFA (112.00 mM).

Analysis of bile acids. Only three of eight investigated BA were detectable in noteworthy quantities. Hence, concentrations of the primary BA cholic acid as well as the secondary BA DCA and lithocholic acid are proven in the nut fs (Table 1). The fermentation of all nut varieties lowered especially the DCA concentrations (8.20–88.65 mM) in comparison to fs blank (125.05 μM). Almond-, walnut- and pistachios fs showed comparable DCA concentrations. The lowest concentrations of DCA were detectable in the fs of hazelnuts (16.48 μM) and macadamias (8.20 μM). These very low concentrations of DCA were even lower than the amount found in Synergy1® fs. A further notable finding was that cholic acid was only detectable in the fs of Synergy1® but hardly in that of nuts.

Analysis of tocopherol concentrations. To compare the tocopherol concentrations in nuts before and after *in vitro* fermentation, ground nuts and the fs were analysed. Table 2 shows that α-tocopherol was the main form found in almonds and hazelnuts. The total amounts of tocopherol in these nut varieties were 16.5 mg/100 g and 25.2 mg/100 g. In contrast, the amounts of tocopherol in pistachios (9.8 mg/100 g) and walnuts (9.3 mg/100 g) were considerably lower. In macadamias, only α-tocotrienol was found at a concentration of 1.4 mg/100 g. On the other hand, only in the fs of hazelnuts and almonds, respectively, rather low amounts of α-tocopherol, α-tocotrienol and β-tocotrienol were detectable (data not shown).

Table 1. Comparison of pH, SCFA concentrations, ratio of main SCFA (acetate–propionate–butyrate) as well as the concentration of bile acids (BA) in fermentation supernatant (fs) after *in vitro* fermentation of different nuts, blank (fermentation buffer only as negative control) and Synergy1® (as positive control)

(Mean values and standard deviations)

fs	pH		Total SCFA (mmol/l)	Main SCFA (mmol/l)†			Ratio of main SCFA %	BA (μM)†		
	Mean	SD		Acetate	Propionate	Butyrate		CA	DCA	LCA
Blank	6.53	0.05	32.15	16.26	6.08	6.21	56.94:21.31:21.75	1.59	125.08	7.71
Synergy1®	4.58**	0.26	112.00	70.69	8.79	28.71	65.34:8.13:26.53	14.07	23.82	7.67
Hazelnuts	6.10**	0.02	67.83	27.42	12.35	20.51	45.48:20.49:34.03	n.d.	16.48	5.61
Macadamias	6.14*	0.14	71.47	28.58	13.10	22.65	44.43:20.37:35.21	n.d.	8.20	5.49
Almonds	6.09**	0.05	85.93	35.93	16.54	24.41	46.74:21.51:31.76	n.d.	68.27	6.27
Pistachios	6.03**	0.07	70.83	27.87	12.78	23.87	43.20:19.81:37.00	0.48	81.01	7.39
Walnuts	6.26*	0.04	72.19	27.40	14.22	22.62	42.65:22.14:35.21	0.38	88.65	7.49

CA, cholic acid; DCA, desoxycholic acid; LCA, lithocholic acid; n.d., not detectable.

Mean values were significantly different from those of the fs blank: * *P* < 0.05, ** *P* < 0.01 (*t* test)

† Three different fermentations were pooled, only one determination in triplicate could be conducted. A statistical analysis was therefore not possible.

Table 2. Overview of the analysed vitamin E forms of different nut varieties

Nuts	Concentration (mg/100 g)†							
	Total tocopherol	α-Tocopherol	α-Tocotrienol	β-Tocopherol	γ-Tocopherol	γ-Tocotrienol	δ-Tocopherol	δ-Tocotrienol
Hazelnuts	25.2	17.97	n.d.	1.23	5.06	n.d.	0.79	0.03
Macadamias	1.1	n.d.	1.41	n.d.	n.d.	n.d.	n.d.	n.d.
Almonds	16.5	14.31	n.d.	0.02	2.70	n.d.	n.d.	n.d.
Pistachios	9.8	n.d.	n.d.	n.d.	7.87	0.30	1.59	n.d.
Walnuts	9.3	0.26	n.d.	n.d.	7.60	n.d.	1.67	n.d.

n.d., not detectable.

† Three different fermentations were pooled, only one determination in triplicate could be conducted. A statistical analysis was therefore not possible.

Analysis of the antioxidant potential

Using the TEAC and DPPH assays, it was possible to demonstrate (Fig. 1) that different natural nuts have a significant higher antioxidant capacity as the fibre source Synergy1[®], which had no effects regarding either the hydrophilic or the lipophilic assay. While hazelnuts, almonds, pistachios and walnuts showed similar effects, macadamias represented a nearly 50% lower antioxidant potential.

The antioxidant capacity of fermented nuts was measured with the TEAC assay. Apart from macadamias, all nut fs at least tended to show a higher antioxidant capacity than the blank control (Fig. 2). It is noteworthy that walnut fs had the strongest effect with an about 2.5-fold higher antioxidant capacity than the blank control. In comparison to the blank control, fermentation of Synergy1[®] had no additional effect.

Determination of cell growth

The fermented nut samples were used to analyse their effect on cell growth of HT29 adenocarcinoma cells. Treatment of cells with the fs affected the cell number in a time- and dose-dependent manner. No effects were observed after an incubation of 24 h (data not shown). However, the cell number was significantly reduced after the incubation of HT29 cells with 20% of the fs of hazelnuts and almonds for 48 h compared to the medium control (Fig. 3). A significant decrease in cell number was also seen for the fs Synergy1[®]. After 72 h incubation, apart from pistachios all fs of nuts and Synergy1[®] were able to decrease the cell number in comparison to the medium control (Fig. 4), but without an additional effect to the blank. Significant results were already seen for

5% of the almond fs. Surprisingly, low concentrations of pistachio fs resulted in a slight increase in cell growth.

Cell cycle analysis

Cell cycle distribution was determined to analyse the anti-proliferative effects of the fermented nuts on HT29 cells. However, none of the fs (5%) were able to modulate the cell cycle progression after short- (24 h) or longer incubation times (48, 72 h) (data not shown).

Determination of genotoxic and anti-genotoxic effects

The treatment of HT29 cells with fs of different nut samples (2.5–10%) for 1 and 24 h (data not shown) did not induce DNA damage as reflected by tail intensities comparable to the negative control. Therefore, fermented nuts are considered to be not genotoxic in HT29 cells.

To analyse whether treatment with fermented nuts caused an improved protection against oxidative stress, HT29 cells were incubated for 15 min as well as 24 h with the test substances and challenged with H₂O₂ to induce DNA damage. No cytotoxic impact of either substance was detected at the applied concentrations and times (data not shown). Treatment of HT29 cells for 24 h did not result in reduced levels of DNA damage (data not shown). Co-incubation with the fs of nuts tended to reduce the levels of H₂O₂-induced DNA damage after 15 min (Fig. 5) in comparison to the positive control. In particular, the treatment with the walnut fs resulted in a significantly reduced level of DNA damage compared to the positive control and the fs of blank and Synergy1[®].

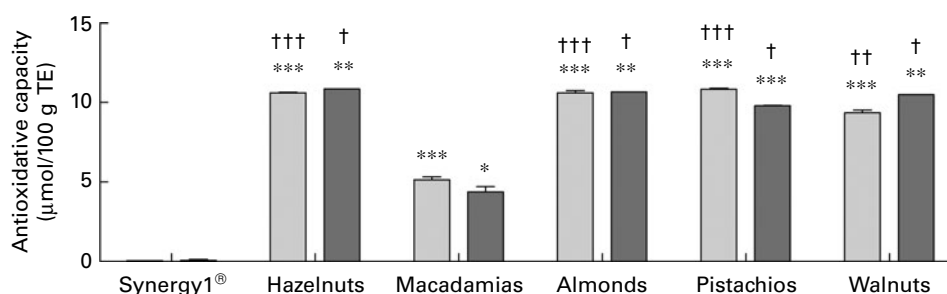


Fig. 1. Antioxidant potential of natural nuts analysed with □ trolox equivalent antioxidant capacity- and ■ 1,1-diphenyl-2-picrylhydrazyl-assay. Values are means, with standard deviations represented by vertical bars (*n* 3). Student's *t* test was used to calculate differences from Synergy1[®] (**P*<0.05, ***P*<0.01, ****P*<0.001) and from macadamia (†*P*<0.05, ††*P*<0.01, †††*P*<0.001). TE, tocopherol equivalent.

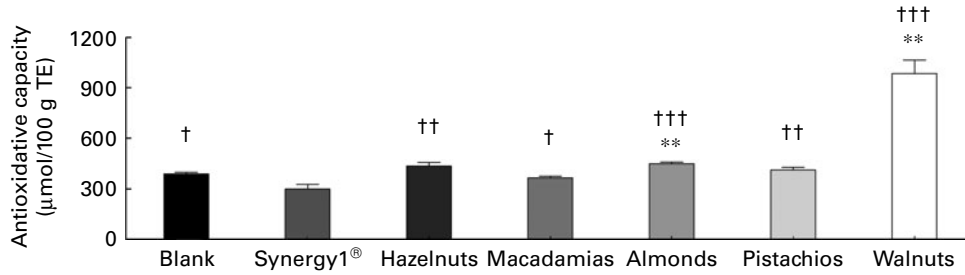


Fig. 2. Antioxidant capacity of nut fermentation supernatant determined with hydrophilic trolox equivalent antioxidant capacity-assay. Values are means, with standard deviations represented by vertical bars (*n* 3). Student's *t* test was used to calculate the differences from blank (***P*<0.01) and from Synergy1® (†*P*<0.05, ††*P*<0.01, †††*P*<0.001). TE, tocopherol equivalent.

Discussion

Numerous health-promoting effects were attributed to the consumption of nuts⁽⁵³⁾. These effects resulted from special ingredients, e.g. unsaturated fatty acids which are responsible for lower cholesterol levels and protection against CVD. However, nuts also contain antioxidant compounds (e.g. tocopherols, polyphenols) and health-promoting DF⁽⁴⁾. The consumption of DF might be associated with a reduced risk for developing colorectal cancer, one of the most common types of cancer in industrialised countries⁽³⁶⁾. Despite this, a recent national survey showed that in Germany only 2 g of nuts were consumed per day^(2,54). Our knowledge about the positive effects of nuts results almost from human intervention trials and is based on chemical analysis of nut ingredients. So far there are no experimental studies available that explain the mechanism of action in the human gut. Hence, the aim of this project was to investigate the potential health-promoting effects of fermented nuts (hazelnuts, macadamias, almonds, pistachios, walnuts) using the human colon carcinoma cell line HT29.

In vitro fermentation of nuts resulted in an increased level of chemopreventive SCFA. Various studies showed the main faecal SCFA acetate, propionate and butyrate in a ratio of 60:20:20⁽⁵⁵⁾. The ratio of fs blank of 56:94:21:31:21:75 was almost identical and confirms the comparability to physiological *in vivo* conditions. Fermentation of DF (Synergy1® and nuts) resulted in an increased level of SCFA and shifted the ratio to a higher level of butyrate. This is in line with former studies^(25,56) investigating the effect of different fibre sources on SCFA production. It is notable that all nut varieties showed a comparable influence on the concentration of

SCFA (67.83–85.93 mmol/l) and the butyrate content (20.51–24.41 mmol/l). The increased SCFA level resulted in a significantly reduced pH value of the fs. This might affect the gut microflora by a growth benefit of health-promoting lactobacilli and bifidobacteria^(28–30,32). BA were metabolised during the gut fermentation (7- α -dehydroxylase activity of bacteria) and could be a risk factor for developing colon cancer, because high amounts of secondary BA are associated with tumour-promoting effects⁽⁵⁷⁾. The presented results show that fermentation of nuts lowered the concentration of the secondary BA DCA compared to fs blank control. Thereby, hazelnuts and macadamias were more effective than the fs of the pure fibre source Synergy1®. One reason for this protective effect might be the lower pH value in these fs which reduces the activity of converting enzymes and increases bacteria strains which are able to bind BA, as has been discussed elsewhere⁽²⁵⁾. Furthermore, DF are able to bind BA directly⁽⁵⁸⁾.

Another mechanism by which nut ingredients (e.g. tocopherols and secondary plant products like polyphenols and flavonoids) could exhibit chemopreventive effects is the prevention of oxidative DNA damage⁽⁵⁹⁾. The present study confirmed that all the nut varieties used are an important source of vitamin E, with substantial differences between the nuts. High tocopherol concentrations were found in almonds and hazelnuts (total tocopherol 16.5/25.2 mg per 100 g). Total tocopherol concentrations of pistachios and walnuts were considerably lower (approximately 9 mg/100 g). Our results are in line with literature data which also concur that macadamias contain only low amounts of vitamin E and only in the form of α -tocotrienol⁽⁴⁾.

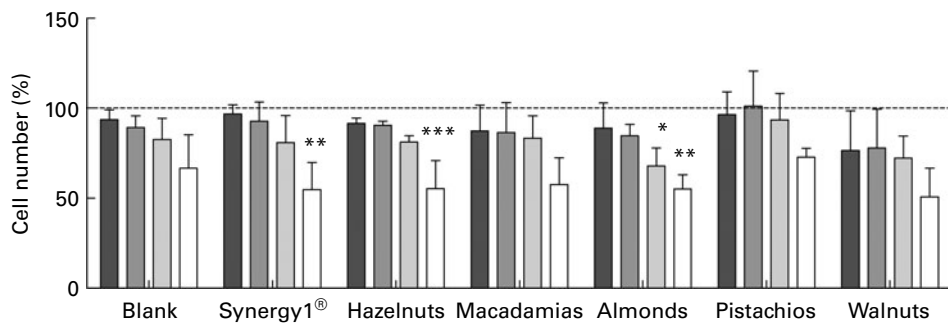


Fig. 3. Effects of nut fermentation supernatant (fs) (2.5 ■, 5 ■, 10 □, 20% □) on growth of HT29 cells after 48 h incubation obtained by 4',6-diamidino-2-phenylindol-assay on the basis of the medium control which was set 100% (dashed line). Values are means, with standard deviations represented by vertical bars (*n* 3). Statistical variance was analysed with one-way ANOVA/Bonferroni post-test, all fs *v.* medium control (**P*<0.05, ***P*<0.01, ****P*<0.001).

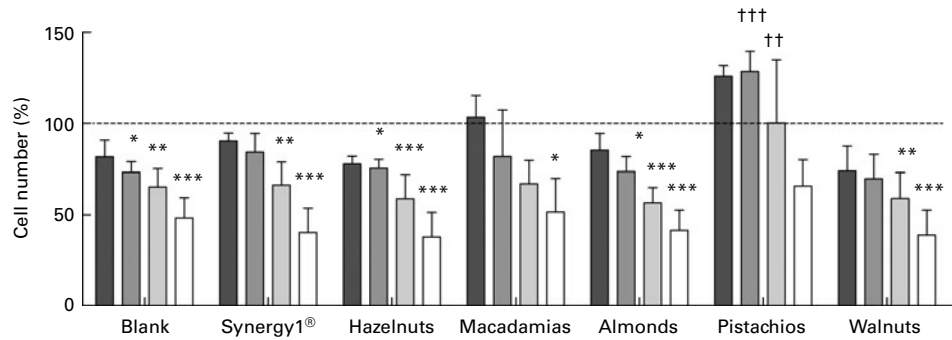


Fig. 4. Effects of nut fermentation supernatant (fs) (2.5 ■, 5 ■, 10 □, 20% □) on growth of HT29 cells after 72 h incubation obtained by 4',6-diamidino-2-phenylindol-assay on the basis of the medium control which was set 100% (dashed line). Values are means, with standard deviations represented by vertical bars (*n* 3). Statistical variance was analysed with one-way ANOVA/Bonferroni post-test, all fs v. medium control (**P*<0.05, ***P*<0.01, ****P*<0.001) and two-way ANOVA/Bonferroni post-test, all fs v. blank (††*P*<0.01, †††*P*<0.001).

Alasalvar *et al.*⁽¹⁵⁾ reported about the high antioxidant capacity of hazelnuts, which is due to their high content of phytochemicals. We were able to confirm this statement and disclosed high antioxidant capacities of almost all nut varieties investigated in our present study. Furthermore, for the first time we demonstrated that fs of nuts had an antioxidant capacity, much higher than ground nuts. Particularly, walnut fs showed considerably higher effects than the other fs, maybe as a result of the high content of the bioactive ellagic acid⁽³⁾. The antioxidant activity of the Synergy1[®] fs could be caused by the added faeces suspension. This reflects that not only the ingredients of nuts but also so far unidentified metabolites of the faeces inoculum have antioxidant effects and act synergistically with nut phytochemicals. Which and how much phytochemicals are really bioavailable after digestion is currently not clarified. Furthermore, since information about the complete phytochemical profile of nuts is lacking, further research is necessary^(60,61).

Effects on the cell number of transformed cells such as HT29 are thought to be a useful marker for secondary chemoprevention⁽²⁵⁾. The present study showed that the number of HT29 cells was efficiently diminished by all fs in a time- and dose-dependent manner. Surprisingly, pistachio fs showed a

slight increase of cells, but only after 72 h of incubation. Further studies are necessary to disclose the ingredients responsible for this effect. The nut fs decreased cell growth similar to Synergy1[®] and both were more effective compared to the medium control. This is due to the content of SCFA in fs. SCFA, especially butyrate, are biologically active and able to diminish the growth of cancer cells as shown recently by Borowicki *et al.*⁽²⁵⁾. Beside butyrate, also propionate demonstrated important anti-proliferative properties^(24,26,34). Additionally, it has been discussed that also the secondary BA DCA could reduce the cell number⁽³⁸⁾, which could be the reason for the growth inhibitory potential of the control fs blank (DCA 125 μM) despite the low amount of SCFA of only 32 mmol/l and the missing significant difference of the nut fs. Thus, the growth inhibiting effect of the fs is probably not only due to the butyrate content. It may be the result of the additional activities of different partly unidentified fermentation metabolites, ellagic acid⁽⁶²⁾ and/or synergistic effects with other SCFA like propionate. To get more insights into the mechanism which could be responsible for the effects on cell growth, the impact of fs on the cell cycle was also investigated. However, cell cycle modulation effects could not be detected. While butyrate in concentrations over 5 mM

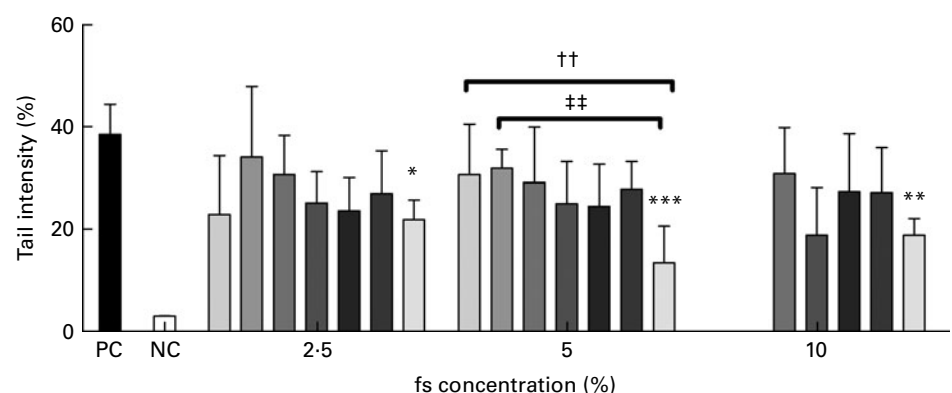


Fig. 5. Anti-genotoxic effects of nut fermentation supernatant (fs) (positive control (PC) 75 μM-hydrogen peroxide ■; negative control (NC) □, medium incubated cells treated with PBS; blank □; Synergy1[®] ■; hazelnuts ■; macadamias ■; almonds ■; pistachios ■; walnuts □) in concentrations of 2.5–10% after 15 min incubation of HT29 cells with nut fs and co-incubation with hydrogen peroxide analysed with comet assay. Values are means, with standard deviations represented by vertical bars (*n* 3). Statistical variance was analysed with one-way ANOVA/Bonferroni post-test, all fs v. positive control (**P*<0.05, ***P*<0.01, ****P*<0.001) and two-way ANOVA/Bonferroni post-test, all fs v. blank (††*P*<0.01) and two-way ANOVA/Bonferroni post-test, all fs v. Synergy1[®] (‡‡*P*<0.01).

has been shown to inhibit cell proliferation by arresting cells in the G0/G1 phase of the cell cycle, lower concentrations did not modulate the cell cycle progression^(25,63). The effective dose of butyrate in 5% fs was too low (only between 1 and 1.2 mM) to stimulate cell cycling modulation effects. In contrast, complex fs of wheat aleurone containing more than 5 mM-butyrate were able to arrest the cell cycle⁽⁶⁴⁾.

In addition to the analysis of growth inhibition, the genotoxic and anti-genotoxic potential of fermented nuts, as a relevant marker for primary chemoprevention, were investigated for the first time. All fs of nuts and also the blank fs tended to prevent oxidative damage induced by H₂O₂. The fermented nuts showed a higher anti-genotoxic potential compared to Synergy1[®], but only the fs of walnuts indicated a significant reduction of the formation of oxidative DNA damage caused by H₂O₂. One reason for the anti-genotoxic effects of the fs walnut could be their high content of phenolic compounds⁽⁶⁵⁾ and the resulting prominent antioxidant potential. Walnut phenolics are reported to display strong antioxidant and free-radical-scavenging capacities⁽⁶⁶⁾. This could be also shown by the present study. Anti-genotoxicity can be the result of direct antioxidant activities^(40,67) and/or modification of DNA repair mechanisms and/or modulation of mRNA expression and activity of biotransformation and detoxification enzymes⁽⁴⁰⁾. Given that a long-term incubation (24 h) did not show any protective effect, the measured reduction of DNA damage by nuts might be the result of direct quenching of reactive hydroxyl radicals and this could prevent the formation of mutated cells.

In conclusion, this is the first study which presents the chemopreventive effects (reduction of tumour-promoting DCA, rise in chemopreventive SCFA, protection against oxidative stress) of different nuts after *in vitro* digestion and fermentation, and shows the potential importance of nuts in the prevention of colon cancer.

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