

Chemopreventive Potential of *In Vitro* Fermented Raw and Roasted Hazelnuts in LT97 Colon Adenoma Cells

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Abstract. *Background/Aim:* Due to their unique composition of health-promoting compounds, the consumption of hazelnuts may contribute to the prevention of colon cancer. *Materials and Methods:* Since hazelnuts are often consumed roasted, the impact of different roasting conditions (RC1=140.6°C/25 min, RC2=155.1°C/20 min and RC3=180.4°C/21 min) on chemopreventive effects of *in vitro* fermented hazelnuts was analyzed in LT97 colon adenoma cells. *Results:* FS (2.5%) of raw and roasted hazelnuts reduced H₂O₂-induced DNA damage while 5% FS significantly induced gene expression of SOD2 (3.0-fold) and GSTP1 (2.1-fold). GPx1 mRNA levels were significantly decreased (0.6-fold) by FS (2.5%). The growth of LT97 cells was significantly reduced by hazelnut FS in a time- and dose-dependent manner. Hazelnut FS (5%) increased the numbers of early apoptotic cells (9.6% on average) and caspase-3 activities (6.4-fold on average). *Conclusion:* These results indicate a chemopreventive potential of *in vitro* fermented hazelnuts which is largely unaffected by the roasting process.

Chronic, non-communicable diseases like cardiovascular diseases, cancer or diabetes are the major causes of death worldwide (1). These diseases are heavily influenced by lifestyle factors and nutrition. Especially nuts have the potential to positively influence the risk for these diseases due to their unique composition of health-promoting nutrients such as minerals, vitamins, phytochemicals and unsaturated fatty acids (2, 3). Results from recent studies

indicate that the consumption of nuts is associated with lower risks regarding cardiovascular diseases and diabetes (4-7) or cancer (8-11) as well as total mortality (12). Especially the risk for colon cancer, which is the second and third most frequent cancer worldwide in women and men, respectively (13) is dependent on lifestyle factors and nutrition (14) and can be reduced by nut consumption. Studies support an inverse relationship between nut consumption and the risk for colon cancer in women (9) as well as in men (15). Dietary fibers, which are abundant in nuts, may contribute to the beneficial effect on colon cancer risk. Several studies demonstrated that the consumption of dietary fiber or fiber rich foods is inversely associated with the risk for colon cancer development (16, 17). Especially hazelnuts, one of the most popular nuts consumed worldwide, belong to the nut varieties which are rich in dietary fiber (3, 18). A portion of 30 g nuts per day, which also reflects the daily recommended intake of nuts by the WHO (19) can provide up to 10% of the recommended daily amount of dietary fiber of 30 g (20). Hazelnuts are also a good source for α -tocopherol and phenolic compounds, which also exert health promoting effects as reviewed by Alasalvar and Bolling (2). Only a minor part of hazelnuts is consumed raw, whereas roasted hazelnuts are preferred. Roasting improves sensory properties of the nuts due to the development of the typical flavor and crunchy texture (21, 22). The roasting process can also affect the chemical composition of hazelnuts including health promoting ingredients like tocopherol and phenolic compounds (2, 18). Recently, we demonstrated that different nut varieties including hazelnuts, which were subjected to an *in vitro* digestion and fermentation, exhibit chemopreventive effects in LT97 colon adenoma cells by increasing gene expression of antioxidant and phase II enzymes, inhibition of proliferation and induction of apoptosis (23). Similar results were obtained by Lux *et al.* (24) who investigated the chemopreventive potential of different nuts in HT29 colon

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carcinoma cells. Until now, there is no information if the roasting process has an impact on these chemopreventive effects. Therefore, the aim of the present study was to examine the influence of different roasting conditions on chemopreventive effects of *in vitro* fermented hazelnuts in LT97 colon adenoma cells regarding DNA damage, gene expression of antioxidant and phase II enzymes, proliferation and apoptosis.

Materials and Methods

Roasting of hazelnuts. Hazelnuts were obtained from Viba Sweets (Floh-Seligenthal, Germany, originally derived from Turkey). Roasting of hazelnuts was performed at laboratory scale in charges of 9.5 kg using a FRC-T.1 drum roaster (Probat, Emmerich am Rhein, Germany) as previously described (18). The following roasting conditions (RC) were applied to cover the minimum and maximum range of roasting temperatures and time periods usually used for industrial roasting of hazelnuts (25): RC1=140.6°C/25 min, RC2=155.1°C/20 min and RC3=180.4°C/21 min. Hazelnuts were stored in hermetically sealed bags at 4°C until use.

In vitro digestion and fermentation of hazelnuts. *In vitro* digestion and fermentation of hazelnuts was performed as described previously (23). In brief, 2 g of raw and roasted hazelnuts were ground and reconstituted with anaerobic potassium phosphate buffer (0.1 M, pH 7.0), incubated with α -amylase (17.4 U/sample in 20 mM NaH₂PO₄) and NaCl (0.85%) for 5 min and pepsin (1.11 mg in 0.94 ml 20 mM HCl, pH 2.0) for 2 h at 37°C. Synergy1® (oligofructose-enriched inulin, Beneo, Mannheim, Germany), was used as positive control and a blank fermentation sample without hazelnuts served as negative control. Subsequently, samples were treated with an intestinal extract (26 mg pancreatin, 50 mg oxgall in 5 ml of 11 mM bicarbonate buffer, pH 6.5) and dialyzed (molecular weight cut off: 500-1,000 Da) under semi-anaerobic conditions (6 h, 37°C). A feces inoculum mixture of at least three healthy donors was used to perform *in vitro* fermentation in an anaerobic atmosphere (37°C, 24 h). After stopping fermentation at 4°C, fermentation supernatants (FS) were obtained by centrifugation (30 min, 4200 × g and 15 min, 4,200 × g at 4°C). Final FS were obtained by centrifugation (15 min, 10,300 × g at 4°C) and sterile filtration (pore size 0.22 µm). Aliquots of FS were stored at -80°C until use.

Cell culture. The human colon adenoma cell line LT97 (a kind gift from Professor B. Marian, Institute for Cancer Research, University of Vienna, Austria) was used for cell culture experiments. This cell line was established from a micro-adenoma and represents an early stage of colon tumor development (26). Culture conditions and properties of LT97 cells were already described in detail previously (27). Recently, an authentication of LT97 cells was performed by STR (short tandem repeat) profiling (Leibnitz-Institute DSMZ, German Collection of Microorganisms and Cell Cultures).

Determination of genotoxic and antigenotoxic effects. Potential genotoxic and antigenotoxic effects of hazelnut FS were analyzed using the Comet Assay as described previously (24, 28). LT97 cells were grown to a confluence of about 70%, harvested and washed with PBS. The ViCell cell counter (Beckman Coulter,

Krefeld, Germany) was used to determine cell number and viability. After adjusting the cell number to 0.4×10⁶, LT97 cells were incubated with different concentrations (2.5 and 5%) of FS from raw and roasted hazelnuts and controls (blank, Synergy1®) for 1 h at 37°C. In addition, LT97 cells were challenged with H₂O₂ to analyze antigenotoxic effects. Therefore, 0.4×10⁶ cells were incubated with hazelnut FS for 45 min at 37°C and subsequently co-incubation was carried out with H₂O₂ (75 µM) for additional 15 min. For Comet Assay experiments H₂O₂ (75 µM, 15 min at 37°C) and PBS served as positive and negative controls, respectively. After treatment, LT97 cells were washed with PBS and adjusted to 0.2×10⁶ cells which were mixed with 45 µl 0.7% low-melting agarose (Biozym, Hessisch Oldendorf, Germany) and distributed onto microscopic slides coated with 0.5% normal-melting agarose (Biozym, Hessisch Oldendorf, Germany). Slides were placed in lysis solution (10 mM Tris-HCl, 100 mM Na₂EDTA, 2.5 M NaCl, 10% DMSO, 1% Triton X-100, pH 10) for 60 min at 4°C. Subsequently, slides were placed into a cooled electrophoresis chamber containing alkaline buffer (1 mM Na₂EDTA, 300 mM NaOH, pH 13) for 20 min and then they were electrophoresed for 20 min (20 V, 300 mA, 0.79 V/cm, 4°C). Slides were washed with PBS (3×5 min) for neutralization. After staining DNA with SYBR® Green (Sigma Aldrich, Munich, Germany) DNA damage was detected using a fluorescence microscope (ZEISS Axiostar plus; Carl Zeiss Jena GmbH) and image analysis system (Comet Assay IV, Perceptive Instruments, Suffolk, UK). The tail intensity (% TI) was determined as degree of DNA damage as means of sixty cells.

Isolation of total RNA. LT97 cells were treated with FS from raw and roasted hazelnuts (2.5% and 5%) and controls as well as butyrate (4 mM) as further positive control for 24 h. The RNeasy Plus Mini kit (Qiagen, Hilden, Germany) was used to isolate total RNA according to the manufacturer's instructions. Elution of RNA was performed in 50 µl RNase-free water. The Quality and concentrations of total RNA were measured with a NanoDropND-1000 photometer (NanoDrop Technologies, Wilmington, Delaware, USA). In addition, the RNA integrity number (RIN) was determined using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, California, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's instructions. Only RNA samples with a RIN >9 were used for experiments. RNA was stored at -80°C until use.

cDNA synthesis and mRNA expression. Complimentary DNA was obtained *via* reverse transcription of 1.5 µg total RNA in a 20 µl reaction mix (42°C, 50 min) using the SCRIPT Reverse Transcriptase kit (Jena Bioscience, Jena, Germany). The samples were heated to 72°C for 15 min to stop the reaction and remaining RNA was removed by treatment with RNaseH (37°C, 20 min). For further experiments, cDNA samples were diluted (1:50) in RNase free water. The mRNA expression of antioxidant and phase II enzymes (*CAT*, *SOD2*, *GPx1* and *GSTP1*) was analyzed by RT-qPCR as described previously using the GoTaq® qPCR Master Mix (Promega, Mannheim, Germany) and the iCycler iQ Real time PCR Detection System (Bio-Rad Laboratory, Munich, Germany) as well as gene specific primers (23). The expression of *CAT*, *SOD2*, *GPx1* and *GSTP1* was normalized to the geometric mean of two reference genes *β-actin* and *GAPDH* based on the equation of Pfaffl *et al.* (29) and expressed as fold change (fc).

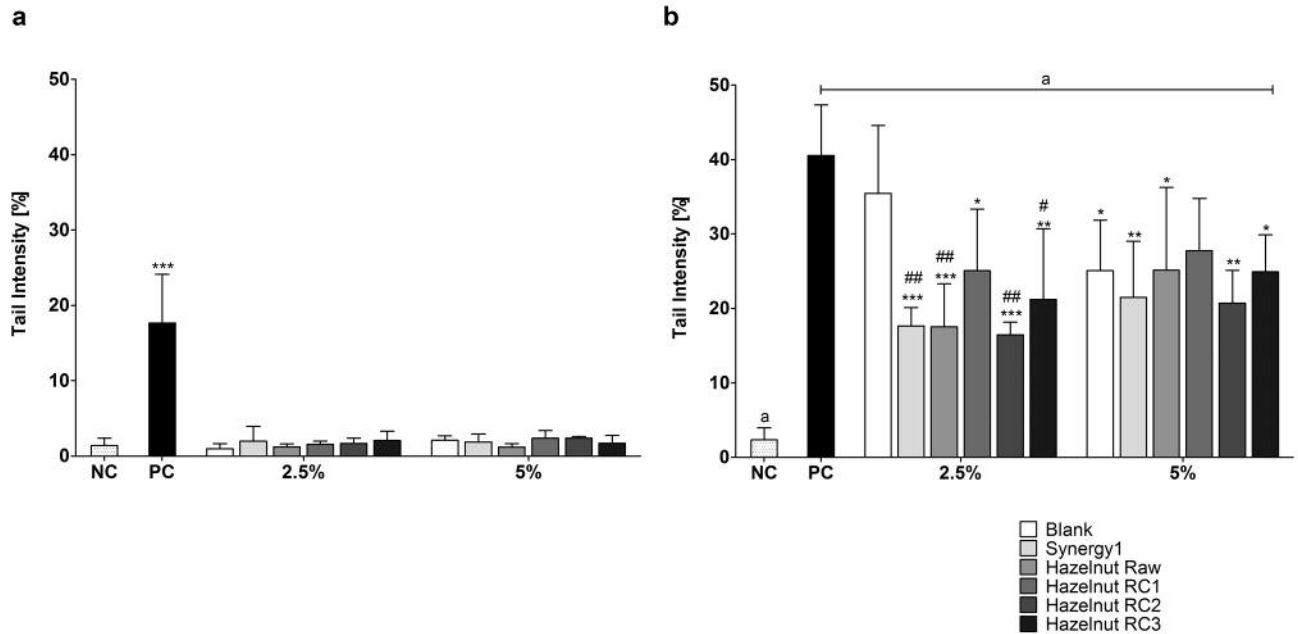


Figure 1. Genotoxic (a) and antigenotoxic (b) effects of fermentation supernatants (FS, 2.5 and 5%) of raw and roasted hazelnuts (RC1=140.6°C/25 min, RC2=155.1°C/20 min and RC3=180.4°C/21 min) and controls (blank, Synergy1®) for 1 h in LT97 colon adenoma cells (mean±SD, n=3). Significant differences between FS from raw and roasted hazelnuts as well as Synergy1® and the positive control (PC, 75 μ M H₂O₂, * p ≤0.05, ** p ≤0.01, *** p ≤0.001), the negative control (NC, PBS, ^a p ≤0.05) and the blank control ([#] p ≤0.05, ^{##} p ≤0.01) were obtained by two-way Anova/Bonferroni post-test.

Determination of cell growth. To analyze growth inhibitory effects of hazelnut FS, LT97 colon adenoma cells were treated with different concentrations of FS from raw and roasted hazelnuts (2.5, 5, 10 and 20%) for 24, 48 and 72 h. The DAPI (4',6-diamidino-2-phenylindol) assay was used to determine the time- and dose-dependent effects on growth of LT97 cells as described previously (30).

Detection of apoptosis. LT97 cells were grown to a confluence of about 70% and treated with different concentrations (2.5 and 5%) of FS from raw and roasted hazelnuts and controls as well as butyrate (4 mM) for 12 and 24 h. Quantification of early apoptotic cells was performed via 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) according to the manufacturer's instructions. In addition, caspase-3 activity as marker of advanced apoptosis was analyzed in LT97 cells treated with hazelnut FS and fermentation controls (2.5 and 5%) for 24 and 48 h as well as butyrate (4 mM) as described by Borowicki *et al.* (31). Relative caspase activities were calculated as fold changes on the basis of the medium control, which was set to 1.

Statistical analysis. Means and standard deviations of three independent experiments were calculated. Statistical differences were analyzed by one- or two-way ANOVA including Bonferroni post-test or Student's *t*-test for comparison of two groups using GraphPad Prism® version 5 for Windows (GraphPad Software, San Diego, California, USA).

Results

Determination of genotoxic and antigenotoxic effects. Genotoxic and antigenotoxic effects of FS from raw and differentially roasted hazelnuts in LT97 colon adenoma cells were determined via Comet Assay. With an average tail intensity of 1.8±0.5% hazelnut FS proved to be non-genotoxic (Figure 1a). These results were comparable to tail intensities determined for cells treated with FS from the blank control (1.6±0.8%), FS from Synergy1® (1.9±0.1%) as well as the negative control (PBS, 1.4±0.9%). In comparison, the tail intensity was significantly higher after treatment with the positive control (17.7±6.4%). These results indicate, that no DNA damage was induced by hazelnut FS and that the roasting process had no impact on a potential genotoxicity of hazelnut FS. Co-incubation of LT97 cells with FS from raw and roasted hazelnuts and controls together with H₂O₂ reduced the levels of H₂O₂-induced DNA damage (Figure 1b). Especially, treatment with 2.5% FS from Synergy1® (17.7±2.5%), hazelnut raw (17.6±5.7%) as well as RC2 (16.5±1.7%) and RC3 (21.2±9.5%) resulted in significantly reduced levels of DNA damage compared to the positive control (40.5±6.8%) and the FS from the blank control (35.4±9.1%). Significantly lower tail intensities compared to the positive control could also be observed after treatment

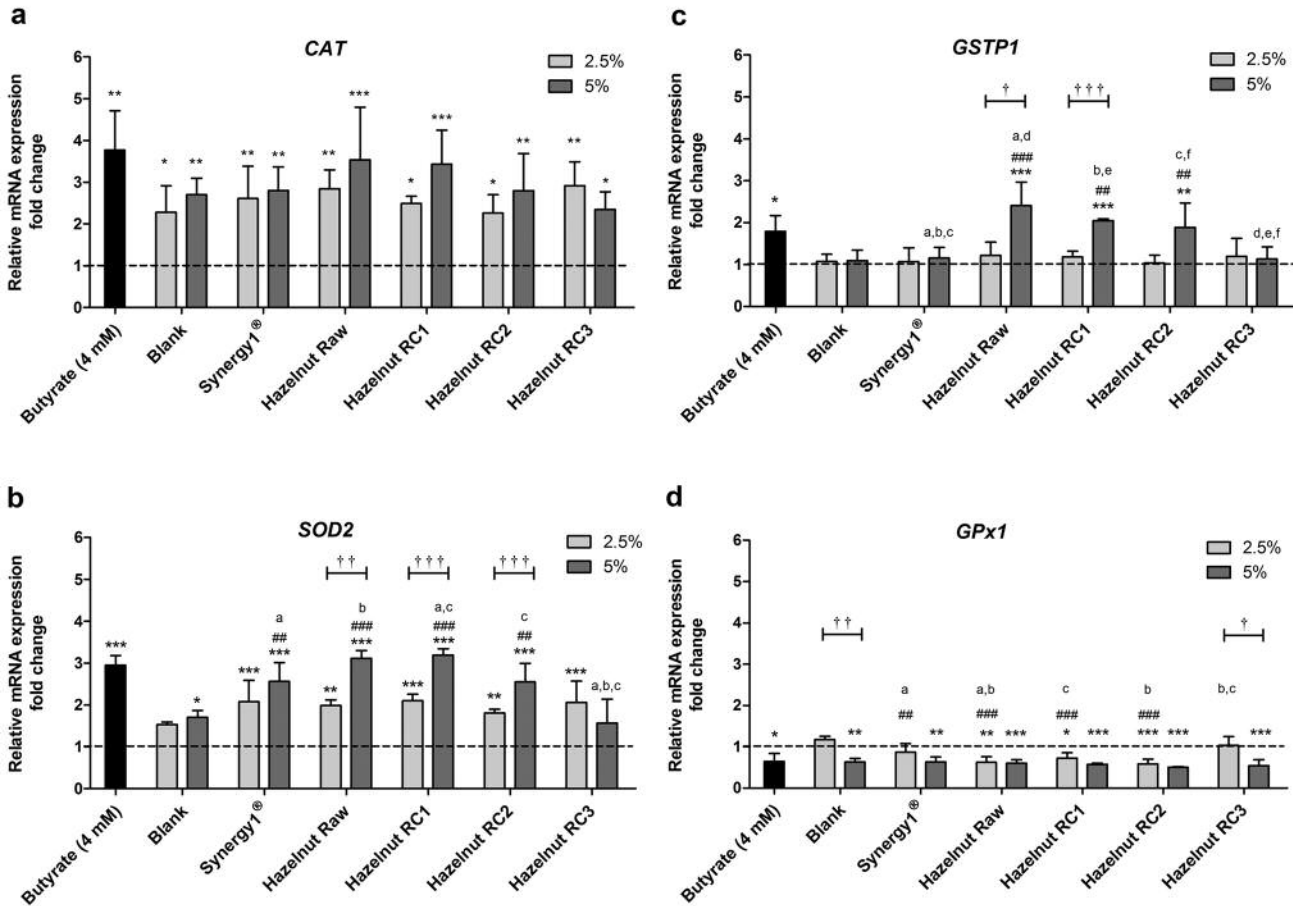


Figure 2. Relative mRNA expression of a) *CAT* (catalase), b) *SOD2* (superoxide dismutase 2), c) *GSTP1* (glutathione *S*-transferase *P1*), and d) *GPx1* (glutathione peroxidase 1) in LT97 colon adenoma cells after incubation with fermentation supernatants (FS, 2.5 and 5%) of raw and roasted hazelnuts (RC1=140.6°C/25 min, RC2=155.1°C/20 min and RC3=180.4°C/21 min) and controls (4 mM butyrate, blank, Synergy1®) for 24 h (mean±SD, n=3). Values represent fold changes on the basis of a medium control (set as 1, dashed line). Significant differences compared to the medium control (*p≤0.05, **p≤0.01, ***p≤0.001), to the blank control (##p≤0.01, ###p≤0.001) and between FS (a-fp≤0.05, equal letters represent significant differences) were obtained by two-way Anova/Bonferroni post-test. Significant differences between 2.5 and 5% were obtained by unpaired Student's *t*-test (†p≤0.05, ††p≤0.01, †††p≤0.001).

with 5% FS from raw and roasted hazelnuts (23.0±2.6% on average, except RC1) as well as the FS of the blank (25.1±6.8%) and FS of the Synergy1® control (21.5±7.5%).

Modulation of selected target genes in LT97 cells by hazelnut FS. Expression levels of mRNA of *CAT*, *SOD2*, *GPx1* and *GSTP1* were examined in LT97 cells treated with FS from raw and differentially roasted hazelnuts and controls using RT-qPCR. A significant induction of *CAT* mRNA levels in comparison to the medium control (set as 1) could be measured after treatment with FS from raw and roasted hazelnuts (FS 2.5% fc: 2.5±0.5, FS 5% fc: 3.0±0.9, on average) and FS from blank (FS 2.5% fc: 2.3±0.6, FS 5% fc: 2.7±0.4) as well as Synergy1® (FS 2.5% fc: 2.6±0.8, FS

5% fc: 2.8±0.6) (Figure 2a). Butyrate (4 mM), which served as a positive control, also significantly increased levels of *CAT* mRNA (fc: 3.8±0.9) in a similar manner. Levels of *SOD2* mRNA were also significantly induced by all hazelnut FS (FS 2.5% fc: 2.0±0.3, FS 5% fc: 2.6±0.8, on average) and FS obtained from Synergy1® (FS 2.5% fc: 2.1±0.5, FS 5% fc: 2.6±0.4) as well as butyrate (fc: 2.9±0.2) in comparison to the medium control (Figure 2b). In particular, treatment with 5% FS from raw hazelnuts (fc: 3.1±0.2), RC1 (fc: 3.2±0.1) and RC2 (fc: 2.6±0.4) as well as Synergy1® resulted in dose-dependently and significantly higher *SOD2* mRNA levels compared to the blank control (fc: 1.7±0.2). These *SOD2* mRNA levels were also significantly higher compared to the treatment with FS

obtained from the most intensively roasted hazelnuts (FS RC3 5% fc: 1.6 ± 0.6). Similar results could be obtained for *GSTP1* mRNA expression (Figure 2c). Here, treatment with 5% FS from raw (fc: 2.4 ± 0.6) and roasted hazelnuts RC1 (fc: 2.0 ± 0.0) and RC2 (fc: 1.9 ± 0.6) led to a significant increase of *GSTP1* mRNA levels compared to the medium control as well as the respective FS from the blank control (fc: 1.1 ± 0.3), Synergy1[®] (fc: 1.2 ± 0.3) and the most intensively roasting condition RC3 (fc: 1.1 ± 0.3). In general, the induction of *GSTP1* by hazelnut FS was comparable to the induction caused by butyrate (fc: 1.8 ± 0.4). In contrast to all other genes, *GPx1* mRNA expression was significantly reduced by almost all FS, especially after treatment with 5% FS from raw and roasted hazelnuts (fc: 0.6 ± 0.1 , on average), butyrate (fc: 0.6 ± 0.2), Synergy1[®] (fc: 0.6 ± 0.2) and also the blank control (fc: 0.6 ± 0.1) (Figure 2d). Treatment of LT97 cells with 2.5% FS from Synergy1[®] (fc: 0.9 ± 0.2) and particularly FS from raw (fc: 0.6 ± 0.1) and roasted hazelnuts RC1 (fc: 0.7 ± 0.1) and RC2 (fc: 0.6 ± 0.1) resulted in significantly reduced *GPx1* levels compared to the respective blank control (fc: 1.2 ± 0.1) as well as FS RC3 (fc: 1.0 ± 0.2).

Inhibition of LT97 cell growth by hazelnut FS. Treatment of LT97 cells with FS from raw and roasted hazelnuts as well as controls led to a significant reduction of cell growth in a time- and dose-dependent manner (Figure 3). After 24 h, the average LT97 cell number was already significantly reduced to $63.2 \pm 4.0\%$ and $48.5 \pm 5.0\%$ upon treatment with 2.5 and 20% FS from raw and roasted hazelnuts, respectively in comparison to the medium control which was set to 100% (Figure 3a). In comparison, especially FS from Synergy1[®] exhibited stronger growth inhibitory effects which ranged from $62.3 \pm 6.1\%$ to $11.4 \pm 10.8\%$ after treatment with 2.5 to 20%, respectively. The strongest growth inhibitory potential of hazelnut FS were detectable after 48 and 72 h. Treatment of LT97 cells with 2.5-20% hazelnut FS for 48 h resulted in an average growth reduction ranging between $44.3 \pm 6.2\%$ and $11.5 \pm 4.1\%$ (Figure 3b). Almost similar cell numbers could be measured after treatment with FS (2.5-20%) from Synergy1[®] ($48.3 \pm 1.7\%$ to $1.9 \pm 1.1\%$), while the blank FS reduced cell growth in the range from $62.6 \pm 1.5\%$ and $3.1 \pm 1.7\%$ (2.5 and 20%, respectively). In addition, treatment with 2.5% and 5% FS from Synergy1[®] resulted in significantly lower cell growth (14.4% and 12.6%, respectively) than the respective blank control. Similar results were obtained after treatment with 2.5% and 5% FS from hazelnuts which led to significantly lower cell numbers ($18.3 \pm 3.1\%$ and $12.3 \pm 1.4\%$, respectively) than the respective blank control. Comparable results were obtained after treatment with 2.5% and 5% FS from Synergy1[®] and hazelnuts for 72 h, which also led to significantly lower cell numbers in comparison to FS from the respective blank

control (Figure 3c). In general, cell growth at this time point ranged between $64.0 \pm 3.4\%$ and $0.7 \pm 0.6\%$ after treatment with FS blank (2.5-20%), between $52.7 \pm 4.6\%$ and $1.8 \pm 0.7\%$ after treatment with FS Synergy1[®] (2.5-20%) and between $49.4 \pm 6.4\%$ and $1.9 \pm 1.8\%$ on average after treatment with hazelnut FS (2.5-20%). In general, no differences between raw or roasted nuts could be observed regarding their growth inhibitory potential.

Induction of apoptosis in LT97 cells by hazelnut FS. Examination of early apoptotic processes mediated by fermented hazelnut samples *via* annexin V-FITC/7-AAD staining and flow cytometry revealed that FS from roasted hazelnuts RC1 (FS 5%: $7.9 \pm 3.3\%$) and RC2 (FS 2.5%: $6.7 \pm 2.7\%$) were able to significantly enhance the number of apoptotic cells after treatment for 12 h (Figure 4a) in comparison to the medium control ($2.4 \pm 1.3\%$) as well as the respective FS from the blank control (FS 2.5%: $3.1 \pm 1.6\%$, FS 5%: $3.8 \pm 0.3\%$) and Synergy1[®] (FS 2.5%: $2.9 \pm 0.5\%$, FS 5%: $4.1 \pm 2.3\%$). The increase of early apoptotic cells was more pronounced after 24 h and largely dose-dependent (Figure 4b). At this time point, mainly 5% FS from raw and roasted hazelnuts ($9.6 \pm 0.9\%$, on average), the blank control ($6.8 \pm 3.1\%$) and Synergy1[®] ($6.6 \pm 1.0\%$) as well as butyrate ($5.8 \pm 0.8\%$), which served as positive control, significantly increased the number of early apoptotic cells in comparison to the medium control ($2.9 \pm 1.0\%$). In addition, 2.5% and 5% FS from roasted hazelnuts RC1 and RC2 also significantly enhanced the number of apoptotic cells compared to the respective blank control.

In addition, caspase-3 activity as a marker of advanced apoptosis was significantly enhanced in a dose-dependent manner in LT97 cells after treatment with FS from raw and roasted hazelnuts (FS 2.5% fc: 3.3 ± 0.8 , FS 5% fc: 6.4 ± 1.4 , on average) as well as Synergy1[®] (FS 2.5% fc: 3.4 ± 0.4 , FS 5% fc: 5.8 ± 0.6) and butyrate (fc: 6.5 ± 0.9), especially after 24 h (Figure 5a). In contrast, FS from the blank control was not able to induce caspase-3 activity (FS 2.5% fc: 1.4 ± 0.2 , FS 5% fc: 1.7 ± 0.2) resulting in significantly increased caspase-3 activities after treatment with all fermented hazelnut samples (except FS RC3 2.5%) in comparison to the FS of the blank. Levels of caspase-3 activity were significantly higher after treatment with hazelnut FS from the most intense roasting condition than after incubation with FS from raw or more weakly roasted hazelnuts RC1. Treatment of LT97 for 48 h resulted in lower caspase-3 activities compared to the 24 h treatment (Figure 5b). But, butyrate (fc: 4.9 ± 1.0) and 5% FS from hazelnuts (fc: 4.4 ± 1.0 , on average) and Synergy1[®] (fc: 4.3 ± 1.3) were able to significantly enhance caspase-3 activity compared to the medium and also to the respective blank control (fc: 0.9 ± 0.3), whereas in general no induction of caspase-3 was detectable for treatment with 2.5% FS.

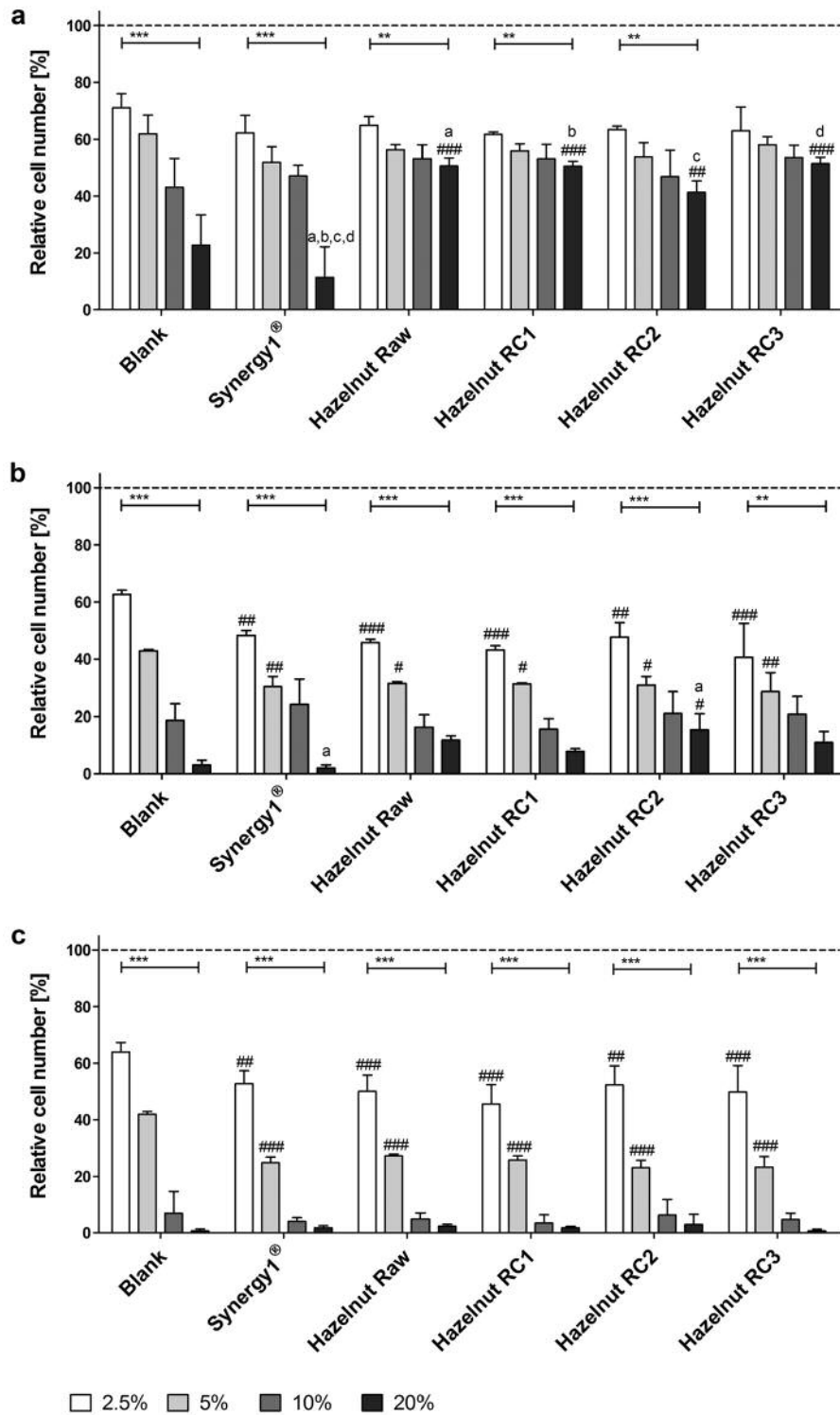


Figure 3. Growth inhibition of LT97 colon adenoma cells after incubation with fermented samples of raw and roasted hazelnuts (RC1=140.6°C/25 min, RC2=155.1°C/20 min and RC3=180.4°C/21 min) and controls (blank, Synergy1[®]) in concentrations of 2.5-20% for a) 24 h, b) 48 h, and c) 72 h (mean±SD, n=3). Significant differences between blank and fermentation supernatants (FS) of Synergy1[®] or hazelnuts (#p≤0.05, ##p≤0.01, ###p≤0.001) were obtained by two-way Anova/Bonferroni post-test. Significant differences between different concentrations (**p≤0.01, ***p≤0.001) were obtained by one-way Anova/Bonferroni post-test. All fermentation samples were significantly different compared to the medium control which was set to 100% (dashed line).

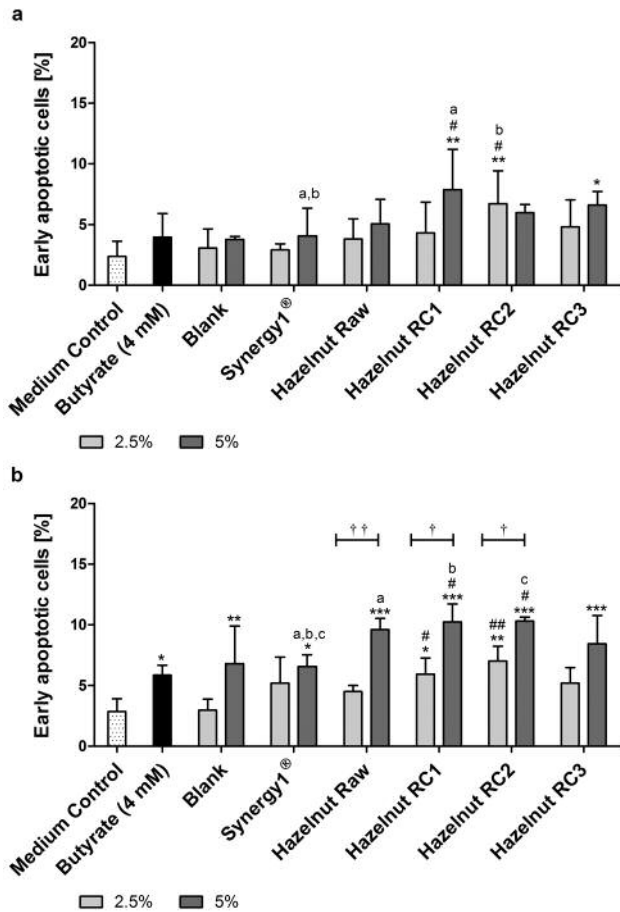


Figure 4. Number of early apoptotic LT97 cells in percent after incubation with fermentation supernatants (FS, 2.5 and 5%) of raw and roasted hazelnuts (RC1=140.6°C/25 min, RC2=155.1°C/20 min and RC3=180.4°C/21 min) and controls (4 mM butyrate, Synergy1[®], blank) for a) 12 h and b) 24 h (mean±SD, n=3). Significant differences compared to the medium control (* p ≤0.05, ** p ≤0.01, *** p ≤0.001), to the blank control (# p ≤0.05, ## p ≤0.01) and between FS (a - c p ≤0.05, equal letters represent significant differences) were obtained by two-way-Anova/Bonferroni post-test. Significant differences between 2.5 and 5% were obtained by unpaired Student's t -test († p ≤0.05, †† p ≤0.01).

Discussion

The influence of different roasting conditions on potential chemopreventive effects of *in vitro* digested and fermented hazelnuts regarding colon cancer development was examined in the present study. An initial step of chemoprevention is the reduction of potential carcinogens like reactive oxygen species (ROS) that can cause DNA damage in colon cells (32). The results from the present study demonstrate that FS from hazelnuts are able to reduce the level of DNA damage

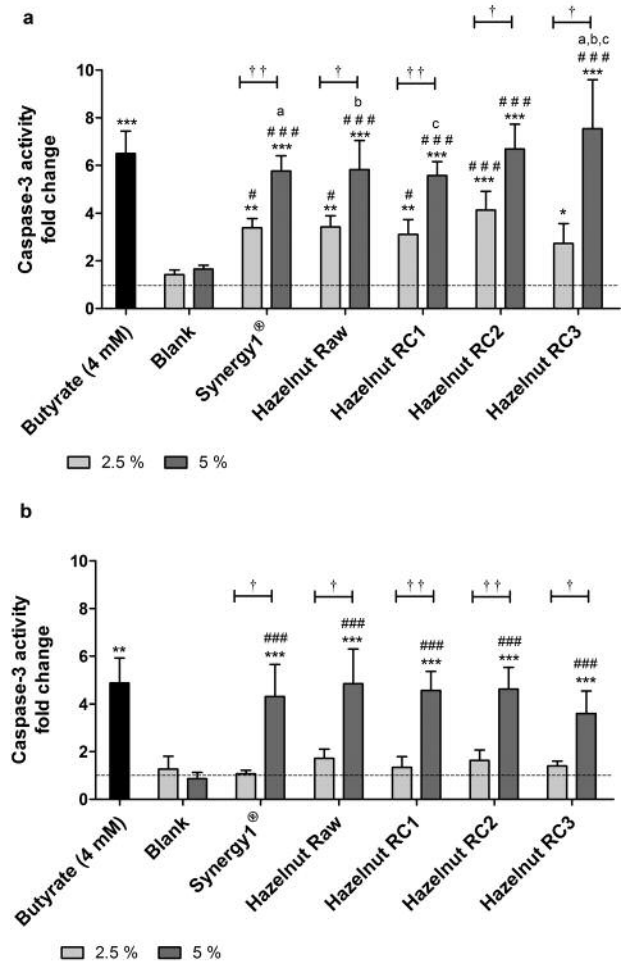


Figure 5. Caspase-activity in LT97 cells after incubation with fermentation supernatants (FS, 2.5 and 5%) of raw and roasted hazelnuts (RC1=140.6°C/25 min, RC2=155.1°C/20 min and RC3=180.4°C/21 min) and controls (4 mM butyrate, Synergy1[®], blank) for a) 24 h and b) 48 h (mean±SD, n=3). Values represent fold changes on the basis of a medium control (set as 1, dashed line). Significant differences compared to the medium control (* p ≤0.05, ** p ≤0.01, *** p ≤0.001), to the blank control (# p ≤0.05, ## p ≤0.01) and between FS (a - c p ≤0.05, equal letters represent significant differences) were obtained by two-way Anova/Bonferroni post-test. Significant differences between 2.5 and 5% were obtained by unpaired Student's t -test († p ≤0.05, †† p ≤0.01).

in LT97 colon adenoma cells challenged with H₂O₂. The reduction of DNA damage after short-term treatment could be the result of antioxidant active compounds from hazelnuts or metabolites formed during fermentation. In general, nuts and especially hazelnuts are rich in bioactive phytochemicals like phenolic acid, proanthocyanidins, flavonoids and α -tocopherol, which each exert antioxidant activities (2). Studies indicate that roasting of nuts (33) or hazelnuts (34), respectively, is associated with a loss of these bioactive compounds resulting in lower antioxidant capacities. In the

present study, no distinct influence of the roasting process could be observed regarding the reduction of DNA damage. This is in line with results from a former study showing relative stable hydrophilic antioxidant capacities for hazelnuts after roasting with different time and temperature ranges (18).

Protection from excessive ROS accumulation is also mediated by antioxidant and phase II enzymes. The present study showed that hazelnut FS are able to significantly induce gene expression of such enzymes like *CAT*, *SOD2* and *GSTP1* in LT97 colon cells. Especially, *SOD2* and *GSTP1* were inducible by hazelnut FS in comparison to the blank control, whereas *GPx1* was mostly significantly down-regulated. Similar results were obtained in a recent study which analyzed the expression of these enzymes in LT97 cells after treatment with FS from different raw nut varieties (23). Levels of mRNA after treatment with hazelnut FS were similar to that after butyrate treatment, indicating that the induction of these enzymes may be mainly mediated by this fermentation product of hazelnut dietary fiber. In a recent study we have demonstrated the production of short chain fatty acids (SCFA) and especially butyrate in fermented nut samples (35). Former studies also indicated that butyrate as the key fermentation product of dietary fiber is responsible for the induction of genes encoding *CAT*, *GSTs* or *SOD* (36-39). An induction of *CAT* and *GSTP1* in HT29 colon carcinoma cells upon incubation with fermentation products from fiber rich sources like wheat aleurone was also observed by Stein *et al.* (40). The induction of antioxidant and phase II enzymes by butyrate may be mainly mediated by its function as histone deacetylase inhibitor (38, 39, 41) or *via* association with increased levels of NF-E2-related factor 2 (42). This transcription factor, which can be activated by many dietary compounds such as flavonoids and polyphenols, regulates the expression of many antioxidant and phase II enzymes *via* antioxidant response elements (ARE) (32). Therefore, in addition to butyrate, other hazelnut compounds like flavonoids or polyphenols may contribute to the induction of antioxidant and phase II genes. Metabolites of phenolic compounds resulting from fermentation in the colon might also induce antioxidant or phase II enzymes. Metabolites of quercetin and chlorogenic acid/caffeic acid (3,4-dihydroxyphenylacetic acid and 3-(3,4-dihydroxyphenyl)-propionic acid, respectively) for example, induced *GSTT2* gene expression in LT97 cells and also reduced cumene hydroperoxide-induced DNA damage (43).

The impact of hazelnut FS on gene expression of *SOD2*, *GSTP1* and *GPx1* was lower for FS resulting from hazelnuts which were roasted with the most intense condition (RC3). This reduction could be due to the loss of antioxidants like phenolic compounds which could activate ARE. Such antioxidants are predominantly located in the pellicle of hazelnuts, which can be removed upon roasting with high roasting temperatures and duration (34, 44).

Mechanisms of secondary chemoprevention include the induction of apoptosis and reduction of proliferation of initiated cells (39). The present study demonstrates that fermented hazelnuts are able to significantly reduce the growth of LT97 adenoma cells in a time- and dose-dependent manner independently of the roasting process. These results are confirmed by former studies, which revealed growth inhibitory effects of FS from different raw nut varieties including hazelnuts in LT97 (23) and HT29 cells (24). In addition, the FS from other dietary fiber sources like wheat aleurone (31, 45) or bread (30) also exhibited growth-inhibitory potential on LT97 cells. These growth inhibitory effects of nuts and other dietary fiber rich foods are to a large part mediated by metabolites like butyrate and to some extent also propionate, which are formed during fermentation of dietary fiber (30, 41, 45, 46). In a recent study we have confirmed that fermentation of hazelnuts results in the formation of these SCFA (35). In contrast, the blank control representing the pure feces matrix, contained only minor amounts of SCFA or butyrate, respectively. Here, the growth inhibitory effects might be mediated by high concentrations of bile acids like deoxycholic acid measured especially in the FS blank (35, 47).

The present study also revealed an induction of apoptosis in LT97 cells by FS from raw and roasted hazelnuts as shown by an increase in early apoptotic cells and an induction of caspase-3 activity. These apoptotic effects may be mainly responsible for the observed growth inhibition triggered by hazelnut FS. Similar results were obtained in a recent study with FS from different raw nut varieties including hazelnuts (23). In addition, results from other studies show an induction of apoptosis in HT29 and LT97 cells by FS from different dietary fiber rich sources (30, 31, 45). The apoptotic effects, caused by hazelnut FS were similar to or even higher than levels observed for butyrate or the fermentation control Synergy1[®], indicating that butyrate is mainly responsible for the induction of apoptosis. Butyrate can exhibit pro-apoptotic potential *via* several mechanisms including its function as a histone deacetylase inhibitor (38, 39, 41), activation of the death receptor 5 (48), TGF- β_1 (49), the JNK MAP (50) and mitochondrial pathways (51), as well as the induction of the WNT pathway (52). In addition to butyrate, several compounds can be formed during *in vitro* digestion and fermentation of hazelnuts, which may contribute to the apoptotic potential such as phenolic acids, flavonoids, lignans or phytosterols (33, 53-55).

In summary, the results from the present study demonstrate the chemopreventive potential of *in vitro* digested and fermented hazelnuts. While genes involved in elimination of ROS or carcinogens are only induced by raw or mildly roasted hazelnuts, the inhibition of cell growth as well as the induction of apoptosis in colon adenoma cells was not

affected by the roasting process. The proof that roasting has no concrete diminishing effect on chemopreventive properties of hazelnuts is an important finding since hazelnuts are often consumed roasted.

In conclusion, the consumption of raw and also moderate-roasted hazelnuts might be associated with a lower risk for colon cancer development.

Conflicts of Interest

The Authors declare that they have no competing interests.

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