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Influence of roasting on the chemopreventive potential of macadamia nuts

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Research

Author: Wiebke Schlörmann

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Wiebke Schlörmann [1,3 *], Tülin Dinc [1], Stefan Lorkowski [2,3], Michael Glei [1,3]

[1] Friedrich Schiller University Jena, Institute of Nutrition, Department of Nutritional Toxicology, Dornburger Straße 24, 07743 Jena, Germany
[2] Friedrich Schiller University Jena, Institute of Nutrition, Department of Nutritional Biochemistry and Physiology, Dornburger Straße 25, 07743 Jena, Germany
[3] Competence Cluster for Nutrition and Cardiovascular Health (nutriCARD), Halle-Jena-Leipzig, Germany

CORRESPONDENCE AUTHOR
Wiebke Schlörmann
Email: wiebke.schloermann@uni-jena.de
Tel: +49 3641 949694, fax: +49 3641 949672

CONFLICTS OF INTEREST
There are no conflicts of interest for any of the authors.

ABSTRACT

Background: Due to their content of dietary fiber, the consumption of macadamia nuts may help to reduce the risk for colon cancer development.

Methods: To examine potential chemopreventive effects of macadamia nuts and to study the influence of different roasting conditions (R), raw and roasted macadamia nuts (R1=138.9°C/25 min, R2=160.1°C/15 min and R3=170.7°C/13 min) were subjected to *in vitro* digestion and fermentation. LT97 colon adenoma cells were incubated with fermentation supernatants (FS) and cell growth and apoptosis as well as gene expression of *CAT*, *SOD2*, *GPx1* and *GSTP1* were examined.

Results: Macadamia nut FS decreased the growth of adenoma cells in a time- and dose-dependent manner. FS obtained from macadamia nuts significantly increased caspase-3 activity (up to 3.8-fold), particularly at a concentration of 5% in comparison to the respective blank control. In particular, FS obtained from raw macadamia nuts increased mRNA levels of *SOD2* (up to 1.6-fold) and *GSTP1* (up to 1.9-fold) in comparison to the fermentation blank control. The roasting process had no direct impact on the mentioned effects.

Conclusion: The present study indicates that macadamia nuts exhibit chemopreventive effects regarding the risk for colon cancer development which are largely unaffected by the state of roasting.

Keywords Apoptosis, colon cancer, dietary fiber, macadamia

1. INTRODUCTION

Cancer is a disease with increasing prevalence worldwide. The global incidence of new cancer cases is about 14.1 million, by which more than 8.2 million deaths occurred worldwide in 2012 [1]. For this reason, malignant neoplasm represent the most common cause of death beside coronary heart diseases [2], with colorectal cancer (CRC) being the second most common cause of cancer death in both men and women [3]. Unhealthy lifestyle has great influence on cancer risk. Tobacco use, overweight and physical inactivity are risk factors which are associated with cancer [4]. Especially in CRC dietary factors play an important role. Critical substances from the food can exert their effect on the large surface of the intestine. A continuing diet with low proportions of dietary fiber and rich in saturated fat can increase colon cancer risk. On the contrary, a healthy lifestyle and a diet rich in dietary fiber and unsaturated fat may contribute to the prevention of CRC [5, 6]. The recommended daily dietary fiber intake in Germany is about 30 g for men and women [7]. Attributable to the propitious composition of nuts, their daily consumption contributes to a healthy diet. Macadamia is one of the tree nuts which are a rich source of

monounsaturated fatty acids (MUFA) and dietary fiber [8, 9]. Therefore, the daily consumption of a sufficient quantity of macadamia nuts can be expected to impart health benefits to humans. It is already well established that macadamia nut intake has positive effects on the risk of developing coronary heart diseases due to the beneficial fatty acid pattern [8, 10]. With respect to the fiber content of the macadamia nut (6 g fiber in 100 g) [9], one could also expect positive effects on intestinal health.

Studies on the effects of dietary fiber on intestinal health have shown that fermentation end products of dietary fiber may contribute to potential chemopreventive effects [11, 12]. Fibers are almost indigestible and are fermented by the intestinal microbiota to short-chain fatty acids (SCFA), including butyrate which is particularly effective in chemoprevention. Dietary fiber also stimulates colorectal mucosal cell growth and reduces the risk of malignant transformation in the colon induced by harmful substances [13-15].

Until now, the number of studies dealing with macadamia nuts in the context of colon cancer prevention is limited. Therefore, the aim of the present study was to investigate possible chemopreventive effects of macadamia on colon cancer cells. Nuts, including macadamia nuts, are often consumed roasted but there is little information how the roasting process affects their potential chemopreventive effects. To study this, macadamia nuts were differentially roasted and were digested and fermented *in vitro* to obtain fermentation supernatants (FS). The FS were used to incubate LT97 colon adenoma cells to examine the impact on growth, apoptosis and the expression of antioxidant active enzymes and enzymes involved in biotransformation.

2. METHODS

2.1 Roasting

Macadamia nuts were roasted at laboratory scale in charges of 9 kg using a FRC-T.1 drum roaster (Probat-Werke von Gimborn Maschinenfabrik, Emmerich am Rhein, Germany) as described previously [16-18]. The following roasting conditions (R) were applied: R1=138.9°C/25 min, R2=160.1°C/15 min and R3=170.7°C/13 min. Macadamia nuts were stored vacuum-packed at 4 °C until use.

2.2 In vitro fermentation

Ground macadamia nuts (2 g) were subjected to a simulation of the gastrointestinal passage via *in vitro* digestion and fermentation as described in detail previously [18]. In brief, macadamia nuts were incubated with α-amylase for 5 min and pepsin for 2 h (37°C). Subsequently, the nuts were treated with an intestinal extract of pancreatin and oxgall and dialyzed under semi-anaerobic conditions (6 h, 37°C). Pre-digested samples were fermented *in vitro* using a feces inoculum of at least three healthy donors under anaerobic conditions (24 h, 37°C). Fermentation supernatants (FS) were obtained by centrifugation. A blank control without macadamia nuts (pure feces inoculum) was used as negative control of fermentation, while Synergy1® (oligofructose-enriched inulin, Beneo, Mannheim, Germany) served as positive control.

2.3 Cell culture

LT97 colon adenoma cells (a kind gift from Professor B. Marian, Institute for Cancer Research, University of Vienna, Austria) were prepared from colon microadenoma of a patient suffering from hereditary familiar polyposis [19], and represent an early stage of colon carcinogenesis. A detailed description of cell culture conditions is provided by Klenow et al. [20]. Authenticity of LT97 cells was checked via STR (short tandem repeat) profiling by the Leibnitz-Institute, German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

2.4 Growth inhibition

Time- and dose-dependent effects of macadamia nut FS on the growth of LT97 cells were examined using the DAPI (4`,6-diamidino-2-phenylindol) assay as described previously [18]. LT97 cells were treated with FS from raw and roasted macadamia nuts in concentrations of 2.5, 5, 10 and 20% for 24, 48 and 72 h. Subtoxic concentrations of macadamia nut FS were determined via nonlinear regression/one phase exponential decay from at least three independent experiments (GraphPad Prism®, GraphPad Software, San Diego, California, USA).

2.5 Apoptosis

Advanced apoptosis was determined via caspase-3 activity as described previously [18]. For this, LT97 cells were incubated with macadamia nut FS in concentrations of 2.5 and 5% as well as butyrate (4 mM) for 24 and 48 h. Levels of relative caspase-3 activity were calculated as fold change relative to the medium control, which was set to 1.

2.6 Expression of CAT, SOD2, GSTP1 and GPx1 mRNA

LT97 cells were treated with FS obtained from raw and roasted macadamia nuts and controls (blank, Synergy1®) in concentrations of 2.5% and 5% as well as 4 mM butyrate diluted in cell culture medium for 24 h. RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer`s instructions and concentrations were measured with a NanoDropND-1000 photometer (NanoDrop Technologies, Wilmington, Delaware, USA). 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, Germany). Subsequently, the reaction was stopped at 72°C for 15 min and remaining RNA was removed by RNaseH treatment (37°C, 20

min). After dilution of cDNA samples in RNase free water (1:50) the mRNA levels *CAT*, *SOD2*, *GSTP1* and *GPx1* were analyzed by qPCR and normalized to the geometric mean of *β-actin* and *GAPDH* as described previously [18].

2.8 Statistical analysis

Means and standard deviations of at least three independent experiments were calculated and 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).

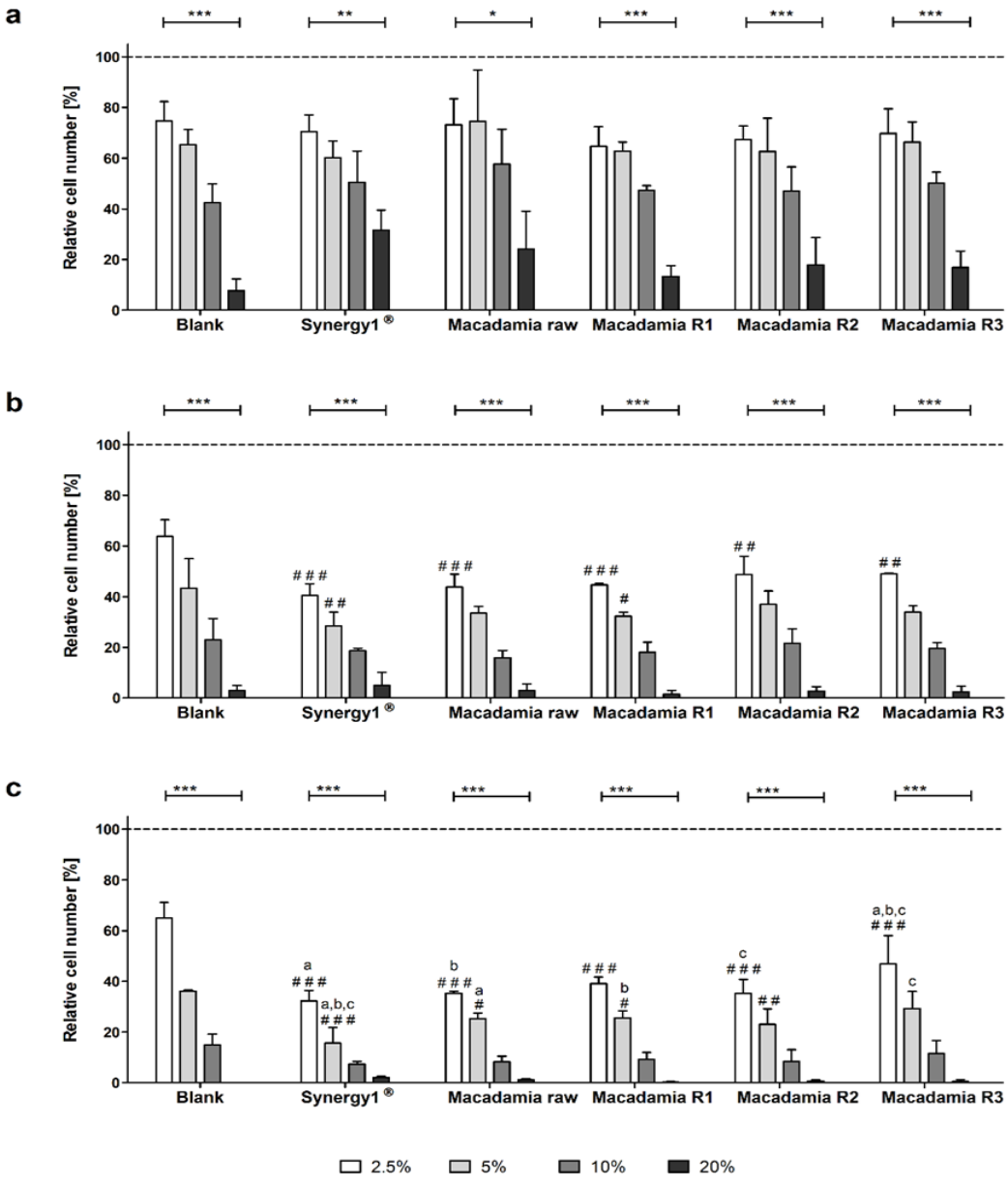

Figure 1: Growth inhibiting of LT97 colon adenoma cells after incubation with fermented samples of raw and roasted macadamia nuts (R1=138.9°C/25 min, R2=160.1°C/15 min and R3=170.7°C/13 min) and controls (blank, Synergy1®) in concentrations of 2.5–20% for a) 24 h, b) 48 h and c) 72 h (mean + SD, n = 3). Significant differences between blank and fermentation supernatants (FS) of Synergy1® or macadamia nuts ($^{\#}$ $p \leq 0.05$, $^{\#\#}$ $p \leq 0.01$, $^{\#\#\#}$ $p \leq 0.001$) and between FS ($^{a-c}$ $p \leq 0.05$, equal letters represent significant differences) were obtained by two-way Anova/Bonferroni post-test. Significant differences between different concentrations (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$) 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).

3. RESULTS

3.1 Macadamia nut FS induce growth inhibition

Treatment of LT97 colon adenoma cells with FS obtained from macadamia nuts and controls (blank and Synergy1®) for 24 h significantly and dose-dependently decreased cell growth. After incubation with 2.5 and 20% FS, average relative cell numbers ranged between 70.1% ± 7.6% and 18.6% ± 10.9 %, respectively (Figure 1a). After treatment for 48 h, cell growth inhibition was even more pronounced (Figure 1b). Incubation of LT97 cells with 2.5-20% FS obtained from the blank control resulted in relative cell numbers of 63.8% ± 6.6% and 2.9% ± 2.0 %, respectively, while treatment with 2.5 % FS obtained from raw and roasted macadamia nuts resulted in significantly lower cell numbers of 46.6 % ± 4.5% on average. These cell numbers were in the same range compared to the treatment with Synergy1®. Strongest growth inhibition could be observed after 72 h (Figure 1c). Treatment of cells with 2.5-20% FS obtained from raw and roasted macadamia nuts resulted in significantly lower relative cell numbers, particularly at lower concentrations (2.5 and 5 %), of 39.1% ± 7.3% and 0.6% ± 0.6% on average, respectively compared to the blank control. Again, growth inhibition induced by macadamia nut FS were comparable to the inhibition caused by Synergy1®.

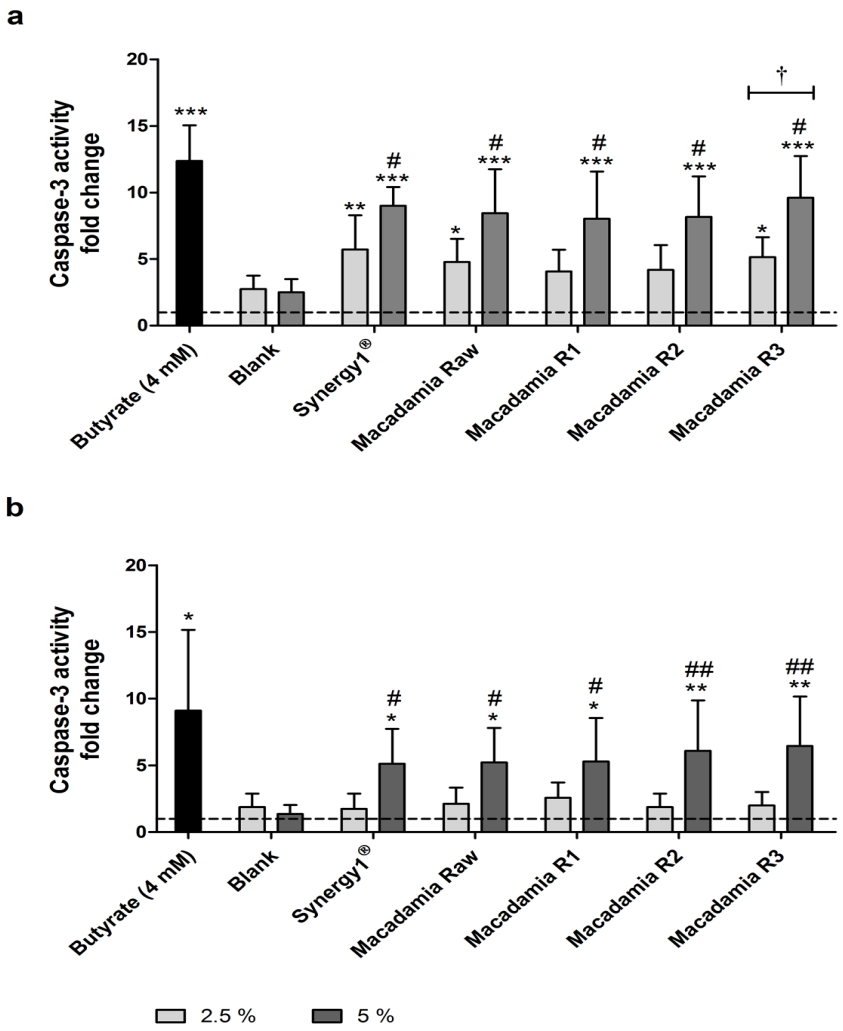

Figure 2: Caspase-3 activity in LT97 cells after incubation with fermentation supernatants (FS, 2.5 and 5%) of raw and roasted macadamia nuts (R1=138.9°C/25 min, R2=160.1°C/15 min and R3=170.7°C/13 min) and controls (4 mM butyrate, Synergy1®, blank) for a) 24 h and b) 48 h (mean + SD, n = 4). Values represent fold changes referring to the medium control (set as 1, dashed line). Significant differences compared to the medium control (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$) and to the blank control ($^{\#}$ $p \leq 0.05$, $^{\#\#}$ $p \leq 0.01$) were obtained by two-way Anova/Bonferroni post-test or unpaired Student`s t-test (butyrate vs. medium control). Significant differences between 2.5 and 5% were obtained by unpaired Student`s t-test († $p \leq 0.05$).

3.2 Macadamia nut FS induce apoptosis

Caspase-3 activity was significantly increased upon incubation with 2.5 and 5% FS obtained from raw and roasted macadamia nuts or Synergy® as wells as the positive control butyrate for 24 h (Figure 2a). In particular, 5% FS of macadamia lead to significantly higher caspase-3 activities (8.6 ± 3.0-fold, on average) compared to the respective blank control (2.5 ± 1.0-fold). Similar results could be obtained for 5% FS obtained from Synergy® which enhanced caspase-3 activity 9.0 ± 1.4-fold. Strongest induction of caspase-activity could be observed for butyrate which lead to an increase of 12.4 ± 2.7-fold. After 48 h, treatment of LT97 cells with 5% of macadamia FS (5.8 ± 3.1-fold, on average), Synergy® (5.1 ± 2.6-fold) or butyrate (9.1 ± 6.0 -fold) also resulted in increased caspase-3 activities compared to the medium and the respective blank control (1.4 ± 0.7-fold) (Figure 2b).

3.3 Macadamia nut FS modulate gene expression of CAT, SOD2 and GSTP1

Treatment of LT97 colon adenoma cells with 2.5 or 5% FS obtained from macadamia nuts roasted with R1 (2.2 ± 0.9-fold and 2.1 ± 0.9-fold, respectively) and R2 (2.6 ± 0.9-fold) resulted in significantly higher mRNA levels of *CAT* than the medium control (Figure 3a). In comparison, FS obtained from the blank control as well as butyrate, which served as positive control, were not able to induce *CAT* gene expression. In contrast, 5% FS of Synergy1® (3.8 ± 0.8-fold) significantly enhanced *CAT* mRNA levels compared to the medium and the respective blank control in a dose-dependent manner.

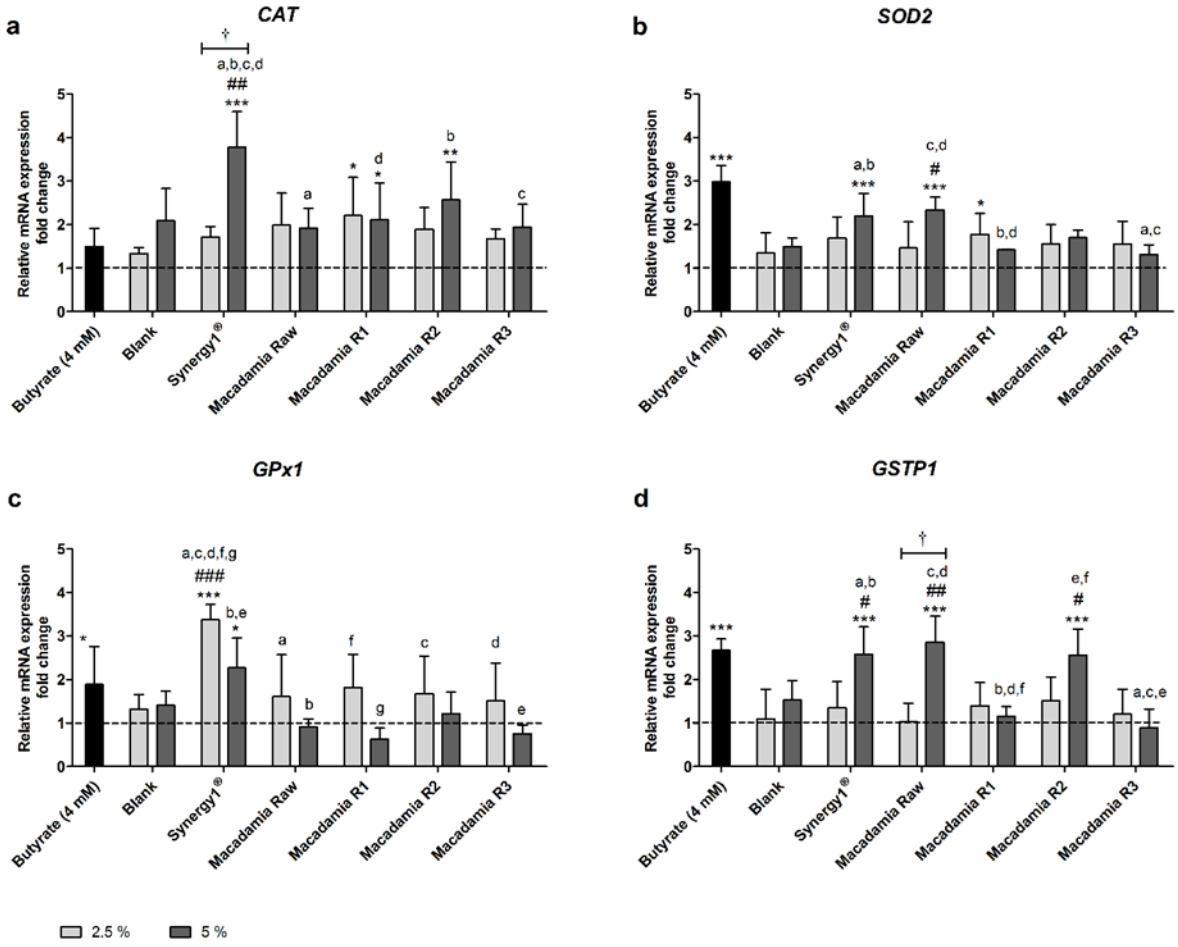

Figure 3: Relative mRNA expression of a) *CAT* (catalase), b) *SOD2* (superoxide dismutase 2), c) *GSTP1* (glutathione S-transferase P1), d) *GPX1* (glutathione peroxidase 1) in LT97 colon adenoma cells after incubation with fermentation supernatants (FS, 2.5 and 5%) of raw and roasted macadamia nuts (R1=138.9°C/25 min, R2=160.1°C/15 min and R3=170.7° C/13 min) and controls (4 mM butyrate, blank, Synergy1®) for 24 h (mean + SD, n = 3). Values represent fold changes referring to the medium control (set as 1, dashed line). Significant differences compared to the medium control (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), to the blank control ($^{\#}$ $p \leq 0.05$, $^{\#\#}$ $p \leq 0.01$, $^{\#\#\#}$ $p \leq 0.001$) and between FS ($^{a\text{-}g}$ $p \leq 0.05$, equal letters represent significant differences) were obtained by two-way Anova/Bonferroni post-test or unpaired Student`s t-test (butyrate vs. medium control). Significant differences between 2.5 and 5% were obtained by († $p \leq 0.05$).

Compared to the medium control, levels of *SOD2* mRNA were significantly increased by FS obtained from raw macadamia (FS 5%: 2.3 ± 0.3-fold) as well as roasted macadamia nuts (FS R1, 2.5%: 1.8 ± 0.5-fold) (Figure 3b). Treatment of LT97 cells with FS of raw macadamia also resulted in significantly higher *SOD2* mRNA levels compared to the respective blank control (1.5 ± 0.2-fold). Further, the controls butyrate (3.0 ± 0.4-fold) and 5% FS obtained from Synergy1® (2.2 ± 0.5-fold) significantly increased *SOD2* gene expression compared to the medium control.

Levels of *GPx1* mRNA were not increased in LT97 cells upon incubation with FS of raw or roasted macadamia nuts or the blank control (Figure 3c). In comparison, FS of Synergy1® (FS 2.5%: 3.4 ± 0.4-fold, FS 5%: 2.3 ± 0.7-fold) as well as butyrate (1.9 ± 0.9-fold) significantly enhanced *GPx1* gene expression compared to the medium control. Treatment with 5% of FS obtained from Synergy1® also resulted in significantly higher *GPx1* mRNA levels than the respective blank control (1.4 ± 0.3-fold).

The mRNA expression of *GSTP1* was significantly higher after treatment with FS (5%) obtained from raw (2.8 ± 0.6-fold) as well as roasted macadamia nuts (R2: 2.6 ± 0.6-fold) compared to the medium control as well as the respective blank control (1.5 ± 0.5-fold) (Figure 3d). This induction was like the increase caused by the FS of Synergy1® (2.6 ± 0.6-fold) or the positive control butyrate (2.7 ± 0.3-fold).

In general, no distinct effect of the roasting conditions of macadamia nuts on gene expression of *CAT*, *SOD2*, *GPx1* or *GSTP1* could be observed.

4. DISCUSSION

Due to their unique composition of fatty acids which is characterized by a high content of MUFA, the consumption of macadamia nuts can positively influence cardiovascular risk factors [10, 21]. Macadamia nuts also contain considerable amounts of dietary fiber which could also contribute to a healthy diet and may reduce the risk for colon cancer development. In general, studies indicated that consumption of nuts may reduce colon cancer risk [22, 23]. But until now, there is only limited information regarding the chemopreventive potential of macadamia nuts and there are no studies in which the impact of roasting on these effects has been investigated.

One mechanism to avoid the formation of cancer cells from already initiated cells is the inhibition of cell growth and an induction of apoptotic processes in these cells which is enabled by so-called suppressing agents [24]. Butyrate, one of the main end products of dietary fiber fermentation in the colon, has been extensively studied as an agent with such suppressing activity on colon adenoma or carcinoma cells [13-15, 24-26]. The formation of butyrate and other short-chain fatty acids was also identified recently in FS obtained from nuts including macadamia nuts [27]. Butyrate could therefore be mainly responsible for growth inhibitory effects of macadamia FS observed in the present study. These results are in line with other studies demonstrating growth inhibition of LT97 colon adenoma [18] or HT29 colon carcinoma cells [28] by FS obtained from different raw nut varieties including macadamia. Growth inhibition of colon adenoma or cancer cells could also be shown for FS obtained from other dietary fiber rich sources such as bread [29] or wheat aleurone [30], respectively. In the present study, the roasting process had no distinct effect on the growth inhibitory potential of macadamia nuts, indicating that growth inhibition is caused by substances resulting from fermentation of macadamia nut samples such as butyrate which is unaffected by an upstream heat treatment of the nuts. One mechanism by which butyrate in macadamia FS may cause growth inhibition is by induction of apoptosis which could be mediated by several ways such as histone deacetylase inhibition [14, 24, 26], activation of the death receptor 5 [31], TGF-β1 [32], the JNK MAP [33] and mitochondrial pathways [34], as well as the induction of the WNT pathway [35]. In addition, also propionate [36] or secondary bile acids such as deoxycholic acid [37] may contribute to the growth inhibitory effects.

The pro-apoptotic action of macadamia FS was confirmed in the present study by the increase of capsase-3 activity in LT97 cells. These results are in line with former studies which revealed induction of apoptosis in LT97 cells by FS obtained from different raw nut varieties including macadamia nuts [18], or other dietary fiber sources producing butyrate as fermentation end product [29, 30, 38]. Again, no direct impact of different roasting conditions on apoptotic potential of fermented macadamia nuts could be observed in the present study. These results are in line with a recent study investigating the influence of different roasting conditions on chemopreventive effects of almonds [39].

Another fundamental mechanism of chemoprevention is the reduction of toxification and induction of detoxification, e. g. by antioxidant effects or by preventing the formation of carcinogens or reactive oxygen species by so-called “blocking agents” [24]. Therefore, the induction of gene expression of enzymes involved in detoxification as part of the antioxidant defense system such *CAT*, *SOD2* and *GPx1* as well as a part of biotransformation such as *GSTP1*, respectively, by macadamia nut FS was analyzed in the present study. The results demonstrate that macadamia FS induced the expression of *SOD2* and *GSTP1* compared to the blank control which lacks a fermentable dietary fiber source. Furthermore, *CAT* expression was significantly induced by FS obtained from macadamia nuts in comparison to the medium control whereas the blank control did not induce *CAT* expression. These results are in line with a recent study investigating the induction of these genes by FS obtained from different nut

varieties including macadamia nuts [18]. In the latter study, macadamia FS also significantly enhanced *CAT* mRNA levels in comparison to the blank control which might be the result of the use of different feces donors during fermentation or a different response of the cells to the incubation with macadamia FS or butyrate since the latter failed to induce *CAT* expression in the present study. These results also indicate that butyrate again might be mainly responsible for the induction of the examined genes without a definite impact of the roasting conditions applied to macadamia nuts prior to fermentation. This "blocking agent"-activity of butyrate, e. g. by inducing the expression of genes of biotransformation such as GSTs, probably mediated by its function as histone deacetylase inhibitor, has already been well described in earlier studies [24, 26].

Taken together, the present study revealed for the first time that macadamia nuts exhibit chemopreventive effects by inhibiting the growth of colon adenoma cells which is mediated at least partly by the induction of apoptotic processes as well as by inducing genes of enzymes involved in detoxification. This induction of detoxifying enzymes could prevent extensive formation of reactive oxygen species and further DNA damage in colon adenoma cells.

In conclusion, the results from the present study indicate that a regular consumption of raw and also roasted macadamia nuts may contribute to a healthy diet and may reduce the risk for colon cancer development.

AUTHORS' CONTRIBUTIONS

MG, WS and SL designed the study. TD performed experimental work. WS and TD were responsible for data evaluation and statistical analyses. WS and DT wrote and MG and SF co-wrote the manuscript. MG and SL reviewed the manuscript. All authors read and approved the manuscript.

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Contact Us:

SIFT DESK ,

Deerpark Dr, #75, Fullerton,CA,92831 United States. E-mail: helpdesk@siftdesk.org

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