Effects of walnut consumption on colon carcinogenesis and microbial community structure

Masako Nakanishi¹, Yanfei Chen²,³, Veneta Qendro², Shingo Miyamoto¹, Erica Weinstock², George M. Weinstock² and Daniel W. Rosenberg¹

¹ University of Connecticut Health Center, Farmington, CT
² The Jackson Laboratory for Genomic Medicine, Farmington CT
³ State Key Laboratory for Diagnosis and Treatment of Infectious Disease, The First Affiliated Hospital, Zhejiang University, Hangzhou 310003, China

Running title: Walnuts inhibit colon tumorigenesis and alter microbial community structure

Keywords: Colon cancer, walnuts, cancer prevention, microbiome, azoxymethane

Financial support: American Institute for Cancer Research (AICR) (D.W. Rosenberg)

Corresponding author:
Daniel W. Rosenberg, Ph.D.
University of Connecticut Health Center
Center for Molecular Medicine
263 Farmington Ave.
Farmington, CT 06030-3101
Phone: 860-679-8794
Fax: 860-679-7639
e-mail: Rosenberg@uchc.edu

Conflict-of-interest statement: We have nothing to disclose.

Article type: Research Article

Word count: 5262

Total number of Figures: 6

Total number of Supplementary Tables: 2

Total number of Supplementary Figures: 4
Abstract

Walnuts are comprised of a complex array of biologically active constituents with individual cancer-protective properties. Here, we assessed the potential benefit of whole walnut consumption in a mouse tumor bioassay using azoxymethane (AOM). In study 1, a modest reduction (1.3-fold) in tumor numbers was observed in mice fed a standard diet (AIN-76A) containing 9.4% walnuts (15% of total fat). In Study 2, the effects of walnut supplementation were tested in the Total Western Diet (TWD). There was a significant reduction (2.3-fold; \( p \lt 0.02 \)) in tumor numbers in male mice fed TWD containing 7% walnuts (10.5% of total fat). Higher concentrations of walnuts lacked inhibitory effects, particularly in female mice, indicating there may be optimal levels of dietary walnut intake for cancer prevention. Since components of the Mediterranean diet have been shown to affect the gut microbiome, the effects of walnuts were therefore tested in fecal samples using 16S rRNA gene sequencing. Carcinogen treatment reduced the diversity and richness of the gut microbiome, especially in male mice, which exhibited lower variability and greater sensitivity to environmental changes. Analysis of individual operational taxonomic units (OTUs) identified specific groups of bacteria associated with carcinogen exposure, walnut consumption and/or both variables. Correlation analysis also identified specific OTU-clades that were strongly associated with the presence and number of tumors. Taken together, our results indicate that walnuts afford partial protection to the colon against a potent carcinogenic insult, and this may be due in part to walnut-induced changes to the gut microbiome.
Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths in the United States. Approximately 5% (1 in 18) of men and women will be diagnosed with this disease during the course of their lifetime. Several clinical and preclinical studies have identified risk factors that increase the likelihood for developing colorectal cancer. For example, obesity and diets high in red meat and fat are associated with increased risk (1-4). However, the consumption of nutritive foods, such as those comprising the Mediterranean diet, has become a popular approach to mitigating cancer risk (5). The Mediterranean diet is comprised mainly of vegetables, fruits, grains, legumes, olive oil, unsaturated fats and a moderate amount of red wine (6). These whole foods contain a variety of polyphenols and plant bioactive compounds that have modifying activities against inflammation, tumorigenesis and atherogenesis (6). While dietary patterns undoubtedly exert a powerful effect on the gut microbiome (7), there is accumulating evidence that among the beneficial effects of the Mediterranean diet is the ability to positively impact the composition of the gut microbiota (7, 8).

An important feature of the Mediterranean diet is the consumption of nuts. Nuts contain a large variety of beneficial bioactive components, including monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) and contain only low amounts of saturated fatty acids (SFA), as well as a number of phytochemicals (i.e. phenolic antioxidants), dietary fiber and minerals (9, 10). PUFAs, including omega-3 fatty acids, reduce inflammation and have also been shown to lower polyp burden in animal models of colon cancer (11). Among all the nuts from the tree nut family, walnuts (Juglans regia) are the most enriched in PUFAs, with the highest ratio of
omega-3:omega-6 (1:4.2).  Walnuts also contain high levels of \( \gamma \)-tocopherol, a form of vitamin E with proven anti-cancer benefit (9, 12). A number of studies have identified beneficial effects of walnut consumption in a variety of diseases, including heart disease, diabetes, neurological disorders and cancer (9).

Given the healthful composition of walnuts and the proven benefit of many of its individual components on cancer signaling pathways, here we sought to assess the potential benefit of whole walnut consumption in a well-established primary colon cancer model. In the present study, we performed two \textit{in vivo} studies using the organotropic colon carcinogen, azoxymethane (AOM) (13). The effects of walnuts on colon carcinogenesis were tested in two basal diets, AIN-76A and a recently developed experimental diet that represents the typical Western dietary pattern, referred to as the Total Western Diet (TWD) (14). To gain further insight into potential mechanisms by which walnuts may affect colon cancer development, fecal samples were collected to examine diet-induced changes to the community structure of the microbiome. Our results demonstrate for the first time that walnut consumption reduces colon tumor development, an effect that is more pronounced in male mice. Importantly, cancer protection is associated with significant alterations to the microbial community structure, which appeared to be associated with tumor suppression.

\textbf{Materials and methods}

\textbf{Animal treatment}

Male and female A/J mice were purchased from The Jackson Laboratory. Whole walnut halves were provided by the California Walnut Commission (Folsom, CA). The
amount of walnuts added to the diets was determined based on previous in vivo studies (15, 16). In Study 1, mice (4 week-old) were fed AIN-76A diet (Harlan Laboratories, Inc., Indianapolis, IN) supplemented with 0, 9.4, 14.1 or 18.8% of walnuts by weight, which are equivalent to 0, 15, 22.5 or 30.2% of energy from walnuts, respectively. In Study 2, mice (4 week-old) were fed the Total Western Diet (TWD: Harlan Laboratories) supplemented with 0, 3.5, 7 or 14% of walnuts by weight, which are equivalent to 0, 5.2, 10.5 or 21.4% of energy from walnuts, respectively. Macronutrient sources and fatty acid composition of AIN-76A and TWD diets are summarized in Supplementary Table 1 and Supplementary Table 2, respectively. Contents of the fat sources were proportionally lowered in each diet to compensate for the addition of walnuts. Walnuts were finely ground and added to each diet, which were prepared freshly each week. The diets and drinking water were given ad libitum.

Treatment with AOM and tissue processing

Five week-old mice received 6 weekly i.p. injections of AOM (Sigma-Aldrich); the first three doses were given at 5mg/kg b.w. and the last 3 doses at 10mg/kg body weight. This protocol reduces morbidity and allows animals to adapt to the treatment. Control mice were injected with vehicle alone (0.9% NaCl). Ten weeks after the last injection, mice were sacrificed, and colons were harvested for further analyses. Colons were flushed immediately with ice-cold PBS and excised longitudinally. Specimens were fixed-flat in 10% neutral-buffered formalin for 4 hours and stored in 70% ethanol. Tissues were Swiss-rolled, paraffin-embedded and sectioned at 5-μm thickness. Animal experiments were conducted after approval by the Animal Care Committee.
(ACC/IACUS) and Center for Comparative Medicine (CCM) at UCHC.

Quantification of lesions

Whole-mount colons were stained with 0.2% methylene blue and the number and size of aberrant crypt foci (ACF) and tumors were scored under a dissecting microscope. Colon tumor load per mouse was determined using tumor diameter to calculate the spherical tumor volume, \[ V=\frac{4}{3}\pi r^3. \]

Immunohistochemistry and immunofluorescence.

Tissue sections were deparaffinized, antigen-retrieved, blocked, and incubated with anti-\(\beta\)-catenin (1:100; Sigma-Aldrich), anti-Ki67 (1:600; Cell Signaling), anti-pStat3 (1:400; Cell Signaling) or anti-p21 (1:100; Santa Cruz Biotechnology). Sections were incubated with secondary antibody conjugated with the peroxidase micropolymers (Vector Laboratories Inc., Burlingame, CA), and signal was detected using DAB solution (Vector Laboratories). Tissues were counterstained with hematoxylin. Images were captured using QCapture PRO software (QImaging, Surrey BC, Canada).

Mouse colonoscopy

Mouse colonoscopy was performed using a modified Olympus human choledochoscope, consisting of an Olympus Exera CV-160 camera system with an Olympus CHF B160 camera unit with an insertion diameter of 3 mm as described previously (17). Mice were anesthetized by i.p. injection of Ketamine/Xylazine solution consisted of 0.6 ml ketamine (100 mg/ml), 0.4 ml xylazine (20 mg/ml) and 4 ml saline,
and the mixture was injected in a volume of ~8 μl per gram body weight, as described earlier (18). To clear intestinal contents, colons were flushed with sterile Hanks’ balanced salt solution using an 18-g gavage needle inserted to a depth of 4 cm. The tip of the endoscope was inserted slowly into the colon to a maximum depth of 4 cm. Number of colon tumors was scored during the procedure.

**Microbiome analyses**

Fecal samples were collected from 20 week-old mice, and stored at -80°C immediately after collection for subsequent microbiome analysis. Total bacterial DNA was extracted from fecal samples by using the Power Soil DNA Extraction kit (MoBio Laboratories, Carlsbad, CA, USA) according to manufacturer’s instructions. Bacterial 16S rDNA was amplified using the 27F/534R primer set (27F 5’-AGAGTTTGATCCTGGCTCAG-3’, 534R 5’-ATTACCGCGGCTGCTGG-3’). PCR reactions were performed using the Phusion High-Fidelity PCR Mastermix (Invitrogen, Carlsbad, CA, USA) with the following condition: 95 °C for 2 min (1 cycle), 95 °C for 20 s, 56 °C for 30s, 72 °C for 1 min (30 cycles). PCR products were purified using Agencourt AMPure XP beads (Beckman coulter, Brea, CA, USA) according to manufacturer’s protocol. DNA sequencing was conducted on an Illumina Miseq using paired end 300 base reads according to manufacturer’s protocols.

For the analyses, raw reads were filtered according to length and quality criteria. Filter-pass reads were assembled using Flash Assembly, for which the minimum overlap requirement is 30-bp and the maximum mismatch ratio is 10% (19). After assembly, chimeric sequences were removed using the USEARCH software based on
the UCHIME algorithm (20). Operational Taxonomic Units (OTUs) were selected using the \textit{de novo} OTU selection protocol with a 97\% similarity threshold. Taxonomy assignments of OTUs were performed by comparing sequences to RDP Classifier (cutoff=0.5). A total of 548,254 assembled reads were generated for the 54 samples. On average, 10,153 reads per sample with range from 4883 to 22182 were obtained. To normalize the sequence depth of each sample, 4,883 reads (the minimum sample number of reads) were randomly picked from each sample for further analysis. The R-package “Phyloseq” was used for alpha and beta diversity analysis (21). The 'reads' abundance was log-transformed for comparison between groups.

\textbf{Statistical analyses}

For tumor studies, statistical analyses were performed using GraphPad Prism V software (GraphPad Software, Inc., La Jolla, CA). Data are shown as mean ± SEM. \textit{p}-values were calculated by the Student’s \textit{t}-test or one-way ANOVA with Bonferroni’s multiple comparison tests where appropriate as indicated in the Figure legends. A \textit{p}-value less than 0.05 was considered statistically significant. For microbiome analyses, a two-sided Student’s \textit{t}-test was used for significance testing for normally distributed variables. The Mann-Whitney U-test was used for significance testing of variables that did not show a normal distribution. A Spearman Correlation Test was used for correlation analysis between gut bacteria and tumor number. The statistical tests and plotting were done in R with packages “plyr”, “ggplot2”.
Results

Effects of walnuts on colon carcinogenesis using a purified diet (Study 1)

In Study 1, we tested the effects of walnuts added to a purified diet, AIN-76A, at increasing concentrations of 0, 9.4, 14.1 and 18.8% by weight on colon carcinogenesis in A/J mice (Supplementary Table 1 and Fig. 1A). As shown in Fig. 1B, control mice showed significant weight gain at higher concentrations of walnuts. Mice that received AOM exhibited only a minimal increase in body weight gain that was similar between groups (Fig. 1B). Colonoscopy was performed 8 weeks after the last injection of AOM. As shown in Fig. 1C, tumor development was confirmed in the AOM-treated mice in the distal (~3 cm) portion of the colon. Tumor numbers were estimated by the colonoscopic images and showed a modest decline (33%, \( p=0.35 \)) in mice fed 9.4% walnuts (Fig. 1C).

At 10 weeks after the last dose of AOM, all mice were sacrificed and examined for the presence of ACF and tumors. The number and size of ACF in the colons was not significantly affected by dietary walnut consumption in both males and females (Supplementary Fig. S1A). As shown in Fig. 1D, the number, volume and size of colon tumors did not show significant differences between the walnut diet groups. However, there was a modest reduction in both the number (1.3-fold, \( p=0.15 \)) and volume (1.3-fold, \( p=0.37 \)) of tumors in mice that consumed the diet with 9.4% walnuts compared to mice on the control diet. Tumor protection was most pronounced in male mice, suggesting a gender-specific benefit of walnut consumption (Supplementary Fig. S1B). Furthermore, there was a modest, but non-significant trend towards increased tumor number and size at higher concentrations of dietary walnuts (14.1% and 18.8%) (Fig. 1D and Supplementary Fig. S1B). Overall, these results indicate that walnut...
supplementation may suppress both colon tumor initiation and promotion when consumed at lower concentrations, but these effects may be somewhat impaired by increased amounts of dietary walnuts. While AOM treatment induced the development of several distinct morphological subtypes of tumors (ACF, microadenomas and tumors), there was no obvious change in tumor morphology at 10 weeks after AOM in male mice fed 9.4% walnuts (Fig. 1E).

**Effects of walnuts on colon carcinogenesis using the TWD (Study 2)**

In Study 2, we used a Total Western Diet (TWD) as a base diet and tested for the efficacy of walnuts during AOM-induced colon carcinogenesis (Supplementary Table 2 and Fig. 2A). The TWD is a modified diet that contains fat from multiple sources, which can reflect consumption patterns of a typical American (14). All fat sources in the basal diet for each experimental group have been reduced proportionately to contain approximately 35% total fat by calories in the complete diet. The concentration of walnuts was adjusted to 0, 3.5, 7 and 14% (by weight), which included the recommended daily serving of walnuts, 56.6g (2 ounce) per day, based on a 2,000 total calorie diet (~18% by calories) (22).

As shown in Fig. 2B, body weight gain of vehicle-treated control mice was not affected by the addition of 7% walnuts (10.5% of total energy from walnuts) compared to controls. Similar to our findings in Study 1, the addition of walnuts to the TWD did not affect the number of colonic ACF when measured 10 weeks after AOM treatment (Supplementary Fig. S2). There was a modest, but non-significant reduction in the number of tumors in mice fed 7% walnuts compared to the 0% walnut group (1.4-fold,
Furthermore, when tumors were stratified by size, mice fed the 3.5% and 7% walnuts had a moderate shift towards smaller tumors (Fig. 2C).

Consistent with Study 1, the tumor suppressive effects of walnuts were significantly more pronounced in male mice (Fig. 3). As shown in Fig. 3A, males fed 7% walnuts showed a significant reduction in the number of tumors (2.3-fold, \( p=0.05 \)) and the size of tumors (1.6-fold, \( p=0.03 \), Fig. 3B) compared to mice fed 0% walnuts. Moreover, there was a significant reduction in the number of smaller tumors. As shown in Fig. 3C, 1 and 2-mm tumors were reduced by 73% and 50%, respectively, compared to the 0% walnut group. Surprisingly, these trends were not observed in female mice in each of the groups.

Histological examination of the tumor tissues confirmed that there were smaller tumors in male mice fed 7% walnuts, however, there were no significant differences in key markers involved in tumor promotion including Ki67, β-catenin, pStat3 and p21 (Supplementary Fig. S3). This observation suggested that other factors were involved in suppression of tumorigenesis.

**Walnuts consumption increases the richness and diversity of the gut bacterial community**

Based on accumulating evidence that the Mediterranean diet has beneficial effects on the gut microbiota, we next tested the possibility that walnut consumption might affect gut biodiversity and perhaps modify risks to the colon from carcinogen exposure. In the following analyses, we examined the bacterial DNA extracted from fecal samples collected from TWD-fed mice. As shown in Fig. 4A and
The number of observed OTUs and the Shannon index were higher with a diet supplemented with walnuts, suggesting that walnut consumption increased bacterial richness and diversity. This effect was independent of AOM treatment. In fact, carcinogen treatment was associated with a decline of microbial richness in male mice with 0% walnuts included in the diet (Fig. 4A).

To further examine changes to the microbial community structure, we performed diversity analysis using Nonmetric Multidimensional Scaling (NMDS). The NMDS plot showed a clear separation between male and female mice in the presence of AOM exposure and the absence of walnuts (Fig. 4C: males vs. females, AOM). It was apparent that AOM treatment specifically modified microbial diversity in male mice (Fig. 4C: AOM vs. control). However, the addition of walnuts to the diet eliminated this gender difference. Moreover, the microbial communities always clustered together following the inclusion of any concentration of walnuts added to the diet. These initial observations indicate that AOM treatment alone or dietary supplementation with walnuts causes significant alterations to the microbial community structure and that walnuts may exert a greater physiological impact to the male gut microbiome.

To examine the diversity of the bacterial community, we plotted the within-group distance for different concentrations of walnuts. The results showed that female mice had significantly higher values for both the AOM and saline control treatment groups than males, with the exception of mice in the AOM treatment group at a concentration of 7% walnuts (Fig. 4D: circle vs. triangle). The value was lower for both male and female mice at 7% walnuts. Similar to the results obtained for beta diversity analysis, male mice showed a significant difference by AOM treatment (Fig. 4D: triangle in AOM vs.
control), confirming that male mice were more sensitive to carcinogen treatment with respect to the overall phenotype of the microbiome. Finally, the addition of walnuts in the control group resulted in an increase in diversity in males but not females (Fig. 4D: control), again showing the greater responsiveness of males to this diet.

**Specific phylotypes were associated with walnut consumption and carcinogen exposure**

To identify how the key phylotypes of gut bacteria respond to dietary and carcinogen exposures, a cluster analysis of the top 100 OTUs (95% of the total reads) was performed and the data are presented as a heat-map (Fig. 5A). The analyses showed that the OTUs clustered into nine major phylogenetic clades (Fig. 5A: A-I). By comparing the abundance of each OTU clade between different groups, we identified clades that are specifically associated with carcinogen treatment (Clade A), walnut consumption (Clade B, C), and both variables (Clade E, F, and I). Representing 23.1% of the total reads that were generated, clade A is the most abundant group. Clade A consists entirely of one OTU (OTU 1) from the genus of *Akkermansia*. The abundance of this clade shows a significant increase in AOM-treated male mice compared to the NaCl-treated mice (Supplemental Fig. S4A).

Walnut consumption was associated with enrichment of Clade B, regardless of carcinogen exposure or the concentration of walnuts in the diet. For mice treated with AOM, the lowest abundance of Clade B was found in the 0% walnut group in both males and females (Supplemental Fig. S4B). The relative abundance of Clade C was
significantly lower in male mice treated with AOM compared to the vehicle-control group at the 0% walnut level (Supplemental Fig. S4C). However, Clade C was observed to repopulate to comparable levels found in the other groups by the addition of walnuts to the TWD. The increase in Clade C observed in the walnut groups was also observed in both male and female mice treated with AOM, suggesting that Clade C is directly associated with walnut consumption (Supplemental Fig. S4C). Clade E appeared to be strongly associated with AOM treatment as there were almost no reads identified in the vehicle-treated control mice (Fig. 5A & 5B). Moreover, the enrichment of Clade E in both male and female mice in the AOM-treatment groups was attenuated by the addition of walnuts to the diet. These observations indicate that Clade E is influenced by both carcinogen exposure and consumption of walnuts (Fig. 5B).

There was a phylotype that was significantly dependent upon the concentration of walnuts consumed. Clade F showed the lowest abundance in the 7% walnut group in both male and female mice treated with AOM (Fig. 5C). In the vehicle-controls, Clade F was further reduced to near zero in both male and female mice at the 7% walnut concentration. Similarly, a significant modification to the abundance of the phylotype at a walnut concentration of 7% was found in Clade I (Fig. 5D). Clade I was highly enriched at 7% walnuts in both male and female mice regardless of carcinogen treatment. These observations clearly indicate that dietary consumption of 7% walnuts is capable of significantly affecting the levels of specific bacteria groups; for example, reducing (Clade F) and increasing (Clade I) specific bacterial populations. Furthermore, these changes to bacterial populations are modified by either carcinogen exposure and/or by the presence of tumors within the colon.
OTU Clade F is associated with reduced colon tumor development

To further examine the potential influence of the gut microbiota on colon tumor formation, we performed a correlation analysis between OTU Clades and the number of tumors. As shown in Fig. 6, two OTU clades were correlated with tumor numbers. The abundance of OTU clade F was positively correlated with the number of tumors ($r=0.70; p=0.05$), especially with tumors of 1-mm diameter ($r=0.78; p=0.02$) (Fig. 6A & 6B). A negative correlation was observed between the number of tumors and the abundance of OTU clade I ($r=0.66, p=0.08$) (Fig. 6C). Furthermore, a stronger negative correlation was found between the abundance of OTU clade I and tumors of 2-mm diameter in size ($r=0.81, p=0.01$) (Fig. 6D). Taken together, these analyses suggest that a reduction in Clade F may be an important event that contributes to the suppression of tumor initiation. In addition, Clade I may be necessary for suppression of tumor promotion.
Discussion

The concept of exploiting whole foods for their chemopreventive benefit has gained significant traction in recent years (23). Whole foods are comprised of a wide array of beneficial nutrients that together may have additive or synergistic properties that contribute to reduced cancer risk. Walnuts may be a particularly attractive whole food approach for cancer prevention. They are readily available, widely consumed and contain an excellent profile of bioactive components that may exert complex and synergistic effects on tumorigenesis (16, 22, 24-30).

The present study has evaluated the impact of dietary walnut consumption on colon carcinogenesis using a well-established mouse cancer model. Importantly, we used two fundamentally distinct basal diets to evaluate the potential contribution of dietary patterns to the nutritive effects of walnuts. AIN-76A diet is a purified diet containing micronutrient profiles optimized for growth and fertility, and is widely used in preclinical research to provide “standardized” results among different animal experiments (31). On the other hand, as reported by Hintze et al. (14), TWD is formulated to recapitulate the typical American intake of macro- and micronutrients and can be used for dietary chemopreventive studies in rodent models of human disease. Compared to the AIN-76A diet, TWD contains less fat but uses a diverse set of sources to match patterns of fat consumption as reported by the National Health and Nutrition Examination Survey (NHANES), resulting a wide range of dietary fatty acids (14). Interestingly, the TWD diet alone did not cause a significant increase in tumor numbers compared to the AIN-76A diet, which is likely due to the potency of AOM treatment in strain A mice. As we have reported in previous studies (13), strain A mice are highly
sensitive to AOM-induced colon carcinogenesis, with up to 40 adenomas developing in the distal colon. On this potent cancer background the promotional effects of TWD are likely obscured.

Based on the two studies reported herein, walnut consumption at dietary concentrations of 7 and 9.4% by weight, equivalent to 10-15% of total caloric intake, afforded protection against colon cancer. These concentrations are equivalent to the recommended daily serving of walnuts, 56.6g (2 ounce) per day, based on a 2,000 total calorie diet (~18% by calories) (22). A higher walnut concentration added to the AIN-76A diet (18.8% by weight; 20-30% of total caloric intake) caused significant weight gain in control mice, but did not protect against AOM-induced colon tumor development (Fig. 1B & 3A). This result, although not statistically significant, suggests an optimal intake of walnuts for colon cancer protection. A higher walnut concentration added to the AIN-76A diet (18.8% by weight; 20-30% of total caloric intake) caused significant weight gain in control mice but did not protect against AOM-induced colon tumor development (Fig. 1B & 3A). This result, although not statistically significant, suggests an optimal intake of walnuts for colon cancer protection.

The concentration-dependent effect of walnuts in Study 1 may be related to a pronounced alteration in the levels of oleic acid that was reduced by ~50% at the highest concentration of walnuts (18.8%) (Supplementary Table 1). Oleic acid, also referred to as omega-9 fatty acid, is a major constituent of olive oil that has been shown to promote clearance of excess fatty acids to promote “weight loss” (32) and to possess anti-tumor activity (33). In a recent study, oleic acid was shown to inhibit TNF-α-induced COX-2 expression and PGE₂ production in a human glioblastoma cell line (34).
It was also reported that the levels of palmitoleic acid and oleic acid were significantly lower in colorectal cancer tissues (35). These results suggest that similar to omega-3 fatty acids, the levels of oleic acid may also be important for both weight control and cancer risk.

Importantly, our results indicate both concentration- and gender-specific effects of walnuts. As shown in Fig. 3, at optimal dietary concentrations of walnuts, male mice exhibited greater protection from colon cancer compared to females. This gender-related effect was observed when walnuts were incorporated into either the AIN-76A or TWD. Although 7% walnut consumption resulted in a 2.3-fold reduction in the number of tumors in male mice fed TWD, there was only a moderate effect on tumor volume, suggesting a more pronounced effect on tumor initiation in this carcinogenesis model.

The protection afforded by walnuts may have its basis in a number of nutritive components contained within the nut. For example, the omega-3 PUFAs are a major component of walnuts, and the long-chain omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to reduce adenomatous polyp number and size in patients with familial adenomatous polyposis (FAP) (36). Moreover, γ-Tocopherol is a potent anti-oxidant with anti-inflammatory activities and is found in significant concentrations in walnuts (12). Ju et al. (37) showed that dietary supplementation of 0.3% γ-Tocopherol-rich mixture (γ-TmT) on AOM/DSS-treated mice reduced inflammation and inhibited colon tumorigenesis by reducing the levels of PGE\textsubscript{2}, leukotriene B\textsubscript{4} and nitrotyrosine in the colon (37). Polyphenols in walnuts, mainly ellagitannins (ETs), also elicit anti-cancer properties. Dietary ETs are metabolized by gut flora to urolithin A & B (38). In a prostate cancer
cell line, urolithin A was reported to induce apoptosis, associated with a decrease in the anti-apoptotic protein BCL-2 with increased anti-proliferative p21 levels (39). Further molecular analysis on tumor suppressive mechanisms within the walnut-treated tissues is warranted to better understand the roles of these components.

Emerging evidence indicates profound effects of diet on the gut microbiota (reviewed in (40)). Dietary changes can cause dramatic and rapid alterations to bacterial community structure, leading to important alterations in the luminal formation of a wide range of microbial metabolites (41). Early stages of tumor development, particularly at the initiation phase, can be influenced by microbial community structure, mediated in part via the formation of key metabolic products (42). For example, bacteria utilize dietary fiber to produce short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate that have anti-inflammatory properties (reviewed in (43)). In fact, walnuts are rich in fiber (6.4%), providing a favorable luminal environment for producing the SCFAs. In the present study, we observed significant alterations to the structure of the gut microbial community by the addition of walnuts to the diet. At each concentration of walnuts tested, there was a marked increase in the diversity and richness of the gut microbiota. This finding underscores the beneficial effects of dietary walnut intake because low bacterial diversity in the gut has often been linked to human diseases, including obesity and inflammatory bowel disease (44, 45).

The walnut-supplemented diets influenced multiple phylotypes. For example, Akkermansia muciniphila is a mucin-degrading commensal bacterium that has been shown to exacerbate intestinal inflammation by disturbing mucosal barrier function (46). In fact, members of this genus have also been found at higher concentrations in CRC
patients (47) and in mice harboring AOM/DSS-induced colon tumors (48). As shown in Figure 5A, the *Akkermansia* genus was exclusively associated with mice exposed to AOM and harboring colon tumors (Clade A). Thus it is possible that *Akkermansia muciniphila* may play an as yet undefined role in colon tumor development. In contrast, we report that walnut consumption is associated with an increased abundance of bacteria belonging to the phylum *Furmicutes*, including *Lactobacillus*, *Clostridiales*, *Clostridium*, *Lachnospiraceae*, and *Ruminococcaceae*. *Furmicutes* are the most prevalent bacteria within both the human and rodent gut, and many members of this phylum tend to be reduced in CRC patients, as well as in the colons of tumor-bearing mice (49). In a recent study by Baxter *et al.* (49), fecal microbiota from CRC patients were transplanted into germ-free mice and tumors were then induced by treatment with AOM followed by DSS. Interestingly, the number of colon tumors that developed after 10 weeks was negatively correlated with the abundance of *Clostridiales*, including several members of the *Clostridium* Group XIVa (49). Moreover, *Lactobacillus* and *Ruminococcaceae* are probiotic species that are generally found at lower levels in both rodent tumor models and in CRC patients (50, 51). Consistent with these results, similar bacterial signatures were found to be strongly associated with carcinogen treatment (Fig. 5).

While it is clear that walnut consumption alone can re-shape the gut community structure into one that has potential anti-tumor properties, phylotypes associated with both carcinogen exposure and walnut consumption formed a distinct cluster. In addition to phylotypes associated with walnut consumption, many bacteria that belong to the phylum *Bacteroidetes* have also emerged within this group, including *Bacteroides*,
**Bacteroidales and Porphyromonadaceae.** *Bacteroidetes* are anaerobic bacteria and are present as the second largest bacterial population in the gut after *Furmicutes*. In fact, many members of the genus *Bacteroidales* are increased in both rodent models and in human subjects (52). Furthermore, a higher colonization rate of *Bacteroides* has been correlated with advanced CRC status, suggesting that some bacterial species in this genus may play a direct role in promoting colon carcinogenesis (53).

Our correlation analysis depicted in **Fig. 6** identified the presence of a potential microbial signature that could be directly associated with walnut consumption and colon cancer suppression in male mice. Mice harboring the lowest number of tumors tended to have a reduced abundance of the *Bactroidetes* and *Lachnospiraceaes* family. These changes were associated with an overpopulation of *Ruminococcaceae* and *Clostridium XIVa* genus. This bacterial signature is similar to that previously reported during inflammation-associated colon tumorigenesis in mice (49). This recent study also suggested that distinct bacterial communities would result in distinct luminal metabolite profiles, and that butyrate production by members of *Clostridium XIVa* could contribute to cancer protection (49). Butyrate is a major energy source for colonocytes and a critical mediator of the inflammatory response in the gut (54). It is also potent inhibitor of histone deacetylase (HDAC), which enhances apoptosis and suppresses intestinal inflammation (55, 56). During colon carcinogenesis, butyrate has been shown to inhibit cell proliferation (57). Interestingly, it was recently reported that walnuts were among the most highly effective nuts in generating butyrate during the fermentation process *in vitro* (58). Thus it is possible that dietary walnut intake can increase populations of
butyrate-producing bacteria, inducing a microbial community structure that is anti-tumorigenic within the gut.

The present study has further established the existence of marked differences in microbiome signatures between male and female mice. For example, male mice have a significantly lower overall bacterial diversity in the absence of walnut supplementation compared to female mice (Fig. 3). Upon walnut consumption, however, bacterial diversity increases in the male mice, ultimately achieving comparable levels that are present in females. These results suggest that male mice may be more sensitive to diet-induced changes, enabling rapid modifications to their microbial composition. In fact, gender-specific differences in disease susceptibility have been reported in relation to gut microbial community structure. For example, colonization of male commensal bacteria into female mice protected against Type 1 diabetes by changing the levels of testosterone and metabolic profiles generated by the microbiota (59). Alterations to the composition of the gut microbiome and bacterial metabolite profiles can further influence disease risk (60, 61). Therefore, it is possible that the male mice in our study underwent beneficial diet-induced changes to microbial composition, a change that contributed to the establishment of a protective luminal environment.

In summary, our results demonstrate that dietary walnut consumption can suppress colon carcinogenesis when provided at optimal concentrations in the diet, protection that is associated with both AIN-76A and a modified Western diet formulation. In addition, walnut intake significantly modifies the microbial community structure in the large intestine, potentially establishing a protective luminal microenvironment. Most importantly, we have identified a unique bacterial signature that is associated with tumor
suppression by walnut consumption. Additional studies are required to confirm these results and to elucidate the possible mechanisms by which walnut dietary supplementation may contribute to protection against colon cancer development.

Acknowledgments

We thank Nicole Horelik and Yuichi Igarashi for their technical assistance.

Grant Support

This study was financially supported by the American Institute For Cancer Research (D.W. Rosenberg).
References


56. Davie JR. Inhibition of histone deacetylase activity by butyrate. The Journal of nutrition. 2003;133:2485S-93S.

Figure Legends

**Figure 1:** Effect of walnut supplementation on AOM-induced colon tumor formation assessed by colonoscopy (Study 1). (A) Experimental design for Study 1 is depicted. Fecal samples were collected at the time point indicated (circled number). (B) Body weight change with walnut supplementation (0%, 9.4%, 14.4% and 18.8%) incorporated into AIN-76A diet, with or without AOM treatment. (C) Mouse colonoscopy identifying the presence and location of colon tumors at 8 weeks after AOM exposure is completed. The location of distal colon tumors are indicated by the arrows. Bar graph showing a quantification of tumor numbers visualized by colonoscopy (n=5,5,3,3,5 for
0% without AOM, 0%, 9.4%, 14.4%, 18.8% with AOM, respectively). (D) Colon tumor formation (number, volume and size) was evaluated 10 weeks after the last injection of AOM. (E) AOM treatment resulted in the development of small colonic lesions, including ACF (i) and microadenomas (ii). AOM-induced colon tumors exhibited polypoid (iii) or flat (iv) morphologies. ‘T’ and ‘N’ denote for tumor and normal, respectively. n=12, 12, 11, 14 in 0%, 9.4%, 14.4% and 18.8%, respectively. Bars indicate means ± S.E.M.

**Figure 2:** Effect of dietary walnut consumption on AOM-induced colon tumor formation (Study 2). (A) Experimental design for Study 2 is depicted. Fecal samples were collected at the time point indicated (circled number). (B) Body weight change with walnut supplementation (0%, 3.5%, 7.0 % and 14) incorporated into TWD, with or without AOM treatment. (C) Colon tumor formation (number, volume and size) was evaluated 10 weeks after the last injection of AOM. n=10 mice per group. Bars indicate means ± S.E.M.

**Figure 3:** Effect of walnut supplementation on AOM-induced colon tumor formation by gender (Study 2). Colon tumor formation in A/J mice fed TWD diet was enumerated by gender. Tumor number (A), volume (B) and size distribution (C) in males and females are shown. Bars represent the means ± S.E.M. n=5 mice per group. * Student’s t-test, *p*<0.05.

**Figure 4:** A comparison of alpha diversity dependent upon carcinogen exposure and walnut consumption (Study 2). Fecal samples collected from mice maintained
on TWD diet were analyzed. (A) Observed OTUs and (B) Shannon index. The alpha diversity was calculated after normalizing the sequence depth to the same level (4,883 reads per sample). (C) Non-metric multi-dimensional scaling (NMDS) plot of the 54 samples. For easier visualization of the samples from the different treatment groups, the NMDS plot was divided into two panels. The left panel includes samples treated with AOM, and the right panel includes control samples treated with NaCl. Other group information is indicated by color (red, female; navy, male) and shape (walnut level: circle, walnut 0%; triangle, walnut 3.5%; square, walnut 7%; cross, walnut 14%). (D) Comparison of within-group distance between the different treatment and diet groups. Data are shown as means ± SEM. Letters (L/M/H) denote significant differences as determined by one-way ANOVA between groups at different walnut level with carcinogen treatment. #, denotes significant difference when comparing the vehicle control group to the carcinogen-treated group at the same 0% walnut level and of the same gender. #, $p<0.05$, ##, $p<0.01$. *Indicates a significant difference between the 0% walnut group and the 7% walnut group, with the same gender and vehicle control treatment. *, $p<0.05$, **, $p<0.01$. &, denotes a significant difference between male and female mice when comparing carcinogen treatment at the same walnut concentration. &, $p<0.05$, &&, $p<0.01$.

**Figure 5:** Association of specific phylotypes with walnut consumption and carcinogen exposure (Study 2). (A) The OTU abundance was log-transformed to plot the cluster heat map after normalization to the same sequence depth. Cluster analysis was performed using Euclidean distance and Ward’s method. Rows indicate OTUs.
Columns indicate samples. Samples from different group were indicated by the color bar above the heat map (olive, NaCl-walnut 0%; silver, NaCl-walnut 7%; maroon, AOM-walnut 0%; red, AOM-walnut 3.5%; orange, AOM-walnut 7%; yellow, AOM-walnut 14%, navy, female; aqua, male). The heat map shows a gradient color scale from red, indicating value=0, to faint yellow, indicating value=8 (color key on the upper right). The samples from different group and gender were clearly separated. A total of nine OTU clades were observed and indicated by characters in the tree on the left. The abundance of major OTU clusters responding to carcinogen exposure and walnut consumption (the abundance of clades was log-transformed). (B) The abundance of OTU Cluster E in different groups. (C) The abundance of OTU Cluster F in different groups. (D) The abundance of OTU Cluster I in different groups. Differing letters (L/M/H) denote significant differences as determined by a one-way ANOVA among groups of AOM-treated mice fed different walnut concentrations. #, Denotes significant differences when comparing the vehicle-treated group to the carcinogen-treated group at the same 0% walnut level and gender-matched. #, p<0.05), ##, p<0.01). *, Indicates a significant difference between the 0% walnut group and 7% walnut group, gender-matched and vehicle-treated. *, p<0.05, **, p<0.01.

**Figure 6:** Identification of a specific bacterial signature that are strongly associated with tumor suppression by walnut consumption (Study 2). Correlation analysis OTU-Clade F and tumor numbers. (A) The abundance of OTU-Clade F and the total number of tumors. (B) Abundance of OTU-Clade F and number of tumors with a
diameter of 1 mm. (C) Abundance of OTU-Clade I and total number of tumors. (D) Abundance of OTU-Clade I and number of tumors with a diameter of 2 mm.
Figure 1

A. Study 1
- Age (wks)
  - 5 6 7 8 9 10 18 20
- AIN-76A with Walnuts
- AOM or NaCl (10mg/kg of b.w.)
- Colonoscopy
- Sacrifice
- n=2-3/group

B. % Body weight change
- 6% Walnuts - NaCl
- 10.8% Walnuts - NaCl
- 6% Walnuts - AOM
- 9.4% Walnuts - AOM
- 14.1% Walnuts - AOM
- 18.8% Walnuts - AOM

C. 0% Walnuts (NaCl)
- 18.8% Walnuts (AOM)

D. Number of Tumors
- Walnuts Concentration

E. ACF
- Microadenoma
- Tumors
- Tumors
Figure 2

A. Study 2

Age (wks)
5 6 7 8 9 10 11 13 16

TWD with Walnuts
AOM or NaCl
(5-10 mg/kg of b.w.)
Sacrifice
n=20/group

B. % Body Weight Change

Age (weeks)
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

C. Number of Tumors

Number of Tumors

0% 3.5% 7% 14%

Walnuts Concentration

Tumor Volume (mm^3)

0% 3.5% 7% 14%

Walnuts Concentration

Number of Tumors

0 2 4 6 8 10

Tumor diameter (mm)

0 1 2 3 4 5
Figure 3

A

Males

Females

Number of Tumors

Walnuts Concentration

0% 3.5% 7% 14%

B

Males

Females

Tumor Volume (mm³)

Walnuts Concentration

0% 3.5% 7% 14%

C

Males

Females

Number of Tumors

Tumor Diameter (mm)

0 1 2 3 4 5

0% 3.5% 7% 14%
Figure 6

(A) 

log(Number of tumors) vs. log(Abundance of OTU clade F)

$r=0.70, p=0.05$

(B) 

log(Number of tumors with 1mm diameter) vs. log(Abundance of OTU clade F)

$r=0.78, p=0.02$

(C) 

log(Number of tumors) vs. log(Abundance of OTU clade I)

$r=-0.66, p=0.08$

(D) 

log(Number of tumors with 2mm diameter) vs. log(Abundance of OTU clade I)

$r=-0.81, p=0.01$
Cancer Prevention Research

Effects of walnut consumption on colon carcinogenesis and microbial community structure

Masako Nakanishi, Yanfei Chen, Veneta Qendro, et al.

Cancer Prev Res  Published OnlineFirst May 23, 2016.

Updated version
Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-16-0026

Supplementary Material
Access the most recent supplemental material at:
http://cancerpreventionresearch.aacrjournals.org/content/suppl/2016/05/21/1940-6207.CAPR-16-0026.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.