Colon Cancer Prevention with Walnuts: A Longitudinal Study in Mice from the Perspective of a Gut Enterotype–like Cluster

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ABSTRACT

There is limited understanding of how walnut consumption inhibits the development of colorectal cancer. A possible mechanism may involve alterations to the gut microbiota. In this study, the effects of walnut on gut microbiota were tested in a mouse tumor bioassay using the colonotropic carcinogen, azoxymethane (AOM) added to the total Western diet (TWD). 16S rRNA pyrosequencing identified three enterotype-like clusters (E1, E2, and E3) in this murine model. E1, E2, and E3 are associated with AOM exposure, walnut consumption, and TWD diet, respectively. E2 and E3 showed distinct taxonomic and functional characteristics, while E1 represented an intermediate state. At the family level, E1 and E3 were both enriched with *Bacteroidaceae*, but driven by two different operational taxonomic units (OTU; OTU-2 for E1, OTU-4 for E3). E2 was overrepresented with *Porphyromonadaceae* and *Lachnospiraceae*, with OTU-3 (family *Porphyromonadaceae*) as the "driver" OTU for this cluster. Functionally, E3 is overrepresented with genes of glycan biosynthesis and metabolism, xenobiotic metabolism, and lipid metabolism. E2 is enriched with genes associated with cell motility, replication and repair, and amino acid metabolism. Longitudinally, E2 represents the gut microbial status of early life in these mice. In comparison with E1 and E3, E2 is associated with a moderate lower tumor burden ($P = 0.12$). Our results suggest that walnuts may reduce the risk of colorectal cancer within a Western diet by altering the gut microbiota. Our findings provide further evidence that colorectal cancer risk is potentially modifiable by diet via alterations to the microbiota.

Introduction

Colorectal cancer is the third most common form of cancer worldwide and overall causes more than 690,000 deaths each year (1). The adoption of a Westernized lifestyle, increased consumption of high fat and high sugar diets have contributed to the increased incidence of colorectal cancer (2). Several large-scale epidemiologic studies have shown an inverse association between nut and seed consumption and the incidence of colorectal cancer (3, 4). In particular, walnuts, which contain a wide variety of constituents such as omega-3 fatty acids, phytosterols, and antioxidants, are among the most widely consumed of all commercially grown tree nuts in the world. Walnuts added to the diet of mice have been shown to inhibit colorectal cancer growth in part by suppressing angiogenesis and altering miRNA expression profiles (5, 6).

A growing body of evidence indicates that colorectal cancer arises from a step-wise disturbance of the composition of the gut microbiota, induced by food components or diet, plus genetic alterations in oncogenes and tumor-suppressor genes (7–10). The composition of the microbiota has been shown to change during different stages of the carcinogenic process (11). For example, the microbiome in patients with colorectal cancer is often enriched in proinflammatory opportunistic pathogens and microbes that are associated with metabolic disorders, such as *Streptococcus, Escherichia coli, Bacteroides fragilis*, and *Enterococcus faecalis*, and depleted in butyrate-producing bacteria, such as *Roseburia, Clostridium, Faecalibacterium*, and *Bifidobacterium* (12–14). Several species of bacteria have garnered attention for their associations with, and potential roles in colorectal cancer, such as *Fusobacterium nucleatum* (15). The possible mechanisms include alterations in gut microbiota that may result in the onset of inflammation and the synthesis of chemical carcinogens (e.g., acetaldehyde and N-nitroso compounds) directly within the gut lumen (16, 17).

Advances in DNA-sequencing technology have enabled the collection of high-dimensional data from microbial

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communities on an unprecedented scale. A common data structure obtained from microbiota sequencing is a sample-by- operational taxonomic units (OTU) abundance matrix. A typical analytic plan often includes the analysis of taxon relative abundances, α-diversity, and β-diversity. However, given the complexity of the gut microbiota, when multiple factors must be analyzed simultaneously, the application of this methodology provides inherent challenges for the stratification of individual factors (18). Clustering represents another kind of exploratory and unsupervised data analysis approach. Community clusters characterized, by differences in the abundance of signature taxa referred to as enterotypes, were first reported almost a decade ago in human studies (19). Although the concept of human enterotypes has been somewhat controversial, enterotype-like clusters provides an attractive pathway for understanding complex microbial data with the convergence of multiple influence factors and/or time points (20–22).

Specific groups of bacteria associated with carcinogen exposure and walnut consumption had been identified in our previous study (23). In this analysis, our goal was to obtain a more holistic and dynamic picture of associations among walnuts, carcinogen exposure, Western diet, and gut microbiota. The effects of walnut consumption on gut microbiota were tested in the A/J mouse model of colorectal cancer using a typical Western-style diet, the total Western diet (TWD; ref. 24). Enterotype-like clustering was used to stratify the influence of a variety of factors. Our results demonstrate that walnut consumption leads to a distinct enterotype-like cluster, which is associated with a moderate reduction of colon tumor development in A/J mice.

Materials and Methods

Animals

This study was conducted on strain A mouse (A/J, 4 weeks old) purchased from the Jackson Laboratory. Upon arrival at University of Connecticut Health Center (UCHC; Farmington, CT), all mice were maintained on a TWD (Harlan Laboratories, Inc.; ref. 24). Macronutrient sources and fatty acid composition of TWD diets are summarized in Supplementary Table S1. To induce colon cancer, mice (5-week-old) received 6 weekly intraperitoneal injections of azoxymethane (AOM, Sigma-Aldrich); the first three doses were given at 5 mg/kg of body weight and the last three doses at 10 mg/kg of body weight. Control mice were injected with vehicle control (0.9% NaCl) using the same volume. Body weights were recorded once per week. Both male and female mice were used in the study. All mice were maintained in a light-cycled, temperature-controlled room and allowed free access to drinking water and diet. Ten weeks after the last injection of AOM, mice were euthanized under inhaled CO_2 anesthesia. All colons were collected and slit open longitudinally for tumor enumeration. Whole-mount colons were stained with 0.2% methylene blue and the number of colon tumors was scored under a dissecting microscope. The animal experiments were approved by the UCHC Center for Comparative Medicine (CCM). The detailed experimental design is summarized in **Table 1**. Fecal samples were collected at 6, 11, 13, 16, and 20 weeks of age. For time points at 6-week-old and 11-week-old, fecal samples of only 2 mice (1 male and 1 female) from each group were collected due to technical problems. All animal experiments were conducted under an animal protocol (101369-0519) approved on May 31, 2016 by the CCM at the UCH, and were performed in strict accordance with all Institutional Animal Care and Use Committee guidelines.

The amount of walnut added to the diets was determined on the basis of previous *in vivo* studies (5, 25). Two servings of walnuts per day in humans usually provide approximately 376 calories, or 18.8% of a 2,000 calorie/day diet (5). For AOM-treated mice, four levels of walnut supplement (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) were included in the diet. Two levels of walnut supplement (0% and 7% of walnuts by weight, which are equivalent to 0% or 10.5% of energy from walnuts, respectively) were included in the diet for the NaCl-treated control group. The amount of other macronutrients was adjusted accordingly to keep the balance of total energy intake with different walnut levels (Supplementary Table S1). The walnut-supplemented TWD was given to mice at fixed amount for a week, considering a mouse will eat 3 g of the diet per day (3g/mouse/day). We found that mice (all groups) ate almost all of the food in 7 days, therefore we can assume that mice were getting similar amount of calories.

DNA extraction and 16S rDNA sequencing

Fresh fecal samples were stored at −80°C immediately after collection. Total bacterial DNA was extracted from fecal samples by using the Power Soil DNA Extraction Kit (Mo Bio Laboratories) according to the manufacturer's instructions. Bacterial 16S rDNA was amplified using the 27F/534R primer

Table 1. Experiment design.

	Carcinogen treatment	Walnut levels	Number of mice	Gender (F/M)	Fecal sampling points
Group 1	AOM	0%	20	10/10	6/11/13/16/20-week-old
Group 2	AOM	3.5%	20	10/10	6/11/13/16/20-week-old
Group 3	AOM	7%	20	10/10	6/11/13/16/20-week-old
Group 4	AOM	14%	20	10/10	6/11/13/16/20-week-old
Group 5	NaCl	0%	10	5/5	6/11/13/16/20-week-old
Group 6	NaCl	7%	10	5/5	6/11/13/16/20-week-old

set (27F 5′-AGAGTTTGATCCTGGCTCAG-3′, 534R 5′-ATTACCGCGGCTGCTGG-3′). A PCR reaction was performed using Fusion high-fidelity PCR Master Mix (Invitrogen) with the following condition: 95°C for 2 minutes (one cycle), 95°C for 20 seconds/56°C for 30 seconds/72°C for 1 minute (30 cycles). PCR products were purified using Agencourt AMPure XP Beads (Beckman Coulter) according to the manufacturer's protocol. Pyro-sequencing was conducted on an Illumina Miseq 2*300 platform according to the standard protocol.

Bioinformatic and statistical analysis

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% (26). After assembly, chimeric sequences were removed using the Usearch software based on the Uchime algorithm (27). A total of 4,907,318 assembled reads were generated for the 319 samples, on average 15,383 reads per sample with range from 4,269 to 40,479. Then, sequences are clustered into bins called OTUs based upon similarity. Typically, OTU clusters are defined by a 97% identity threshold of the 16S gene sequences to distinguish bacteria at the genus level (28). OTU was picked using *de novo* OTU picking protocol with a 97% similarity threshold. Taxonomy assignment of OTUs was performed by comparing sequences to RDP classify (cutoff = 0.5). Enterotype-like clustering was performed in R with package "BiotypeR" on Jensen–Shannon distance for the OTU-level relative abundance profile (19). The optimal number of clusters was chosen based on Calinski–Harabasz (CH) values, which evaluate the cluster validity based on the average between- and within-cluster sum of squares. The phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) software package was used to infer the metagenomic content based on the taxonomy and abundance of each OTU (29). To determine metabolic features that were differentially abundant either between clusters, linear discriminant

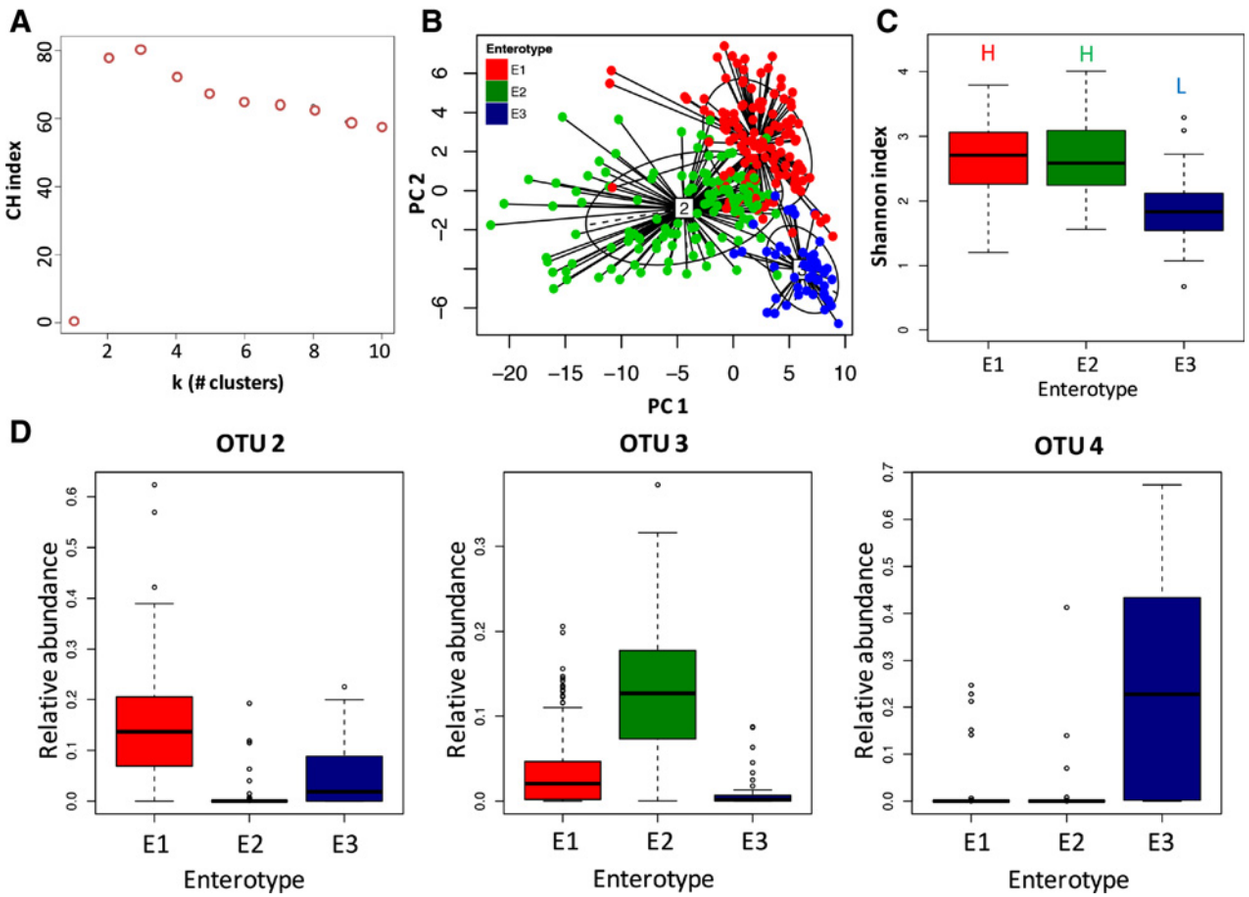

Figure 1.

Identification of enterotype-like clusters in mice gut microbiota. **A,** Selection of optimal number of clusters by CH index suggested an optimal number of three enterotype-like clusters. **B,** Between-class principal coordinate (PC) analysis of mice gut microbiota showed clear separation of the three enterotype-like clusters. **C,** Comparison of microbial diversity by Shannon index among enterotype-like clusters. **D,** Relative abundance of "driver" OTUs in each enterotype-like cluster. Boxes represent the interquartile range between the first and third quartiles, with a line at the median. Circles denote outliers of the group. Letters above the box indicated significant difference with $P < 0.05$.

analysis effect size (LEfSe) was applied (30). The R package "phyloseq" was used for α-diversity analysis (31). To compare the α-diversity, relative abundance of "driver" OTUs, relative abundance of taxa, and tumor burden among different clusters, one-way ANOVA with Tukey honestly significant difference (HSD) test was used. The statistical tests and plotting were done in R with package "plyr" and "ggplot2".

Results

Three enterotype-like clusters identified in mice colorectal cancer model

To stratify the influence of multiple factors, enterotype-like clustering was used to identify clusters based on bacterial community composition. On the basis of the partitioning around medoids method using Jensen–Shannon distance for the OTU-level relative abundance profile, three distinct enterotype-like clusters (E1, E2, and E3) were observed with the highest CH value, as the optimal number of clusters (**Fig. 1A**). The three enterotype-like clusters were visualized by between-class analysis and showed clear separation (**Fig. 1B**). The microbial diversity of E1 (mean ± SD, 2.65 ± 0.54) and E2 (mean ± SD, 2.69 ± 0.57) were significantly higher than E3 (mean ± SD, 1.86 ± 0.49), as estimated by the Shannon index ($P < 0.01$; **Fig. 1C**).

We summarized the top 10 OTUs in each cluster (supplementary Table S2). The cumulative total ratio of the top 10 OTUs in E1, E2, and E3 is 65%, 64%, and 82%, respectively. This might also suggest that the community structure of E1 and E2 were more scattered and diverse than E3, as revealed by the Shannon index results. At the OTU level, all three clusters were dominated by OTU-1 from the bacterial genus *Akkermansia*. The OTUs with the greatest contribution to the formation of individual clusters are defined as "driver" OTUs (19). OTU-2, OTU-3, and OTU-4 were found to be the "driver" OTUs of E1, E2, and E3, respectively (**Fig. 1D**). Taxonomically, OTU-2 and OTU-4 are both associated with genus *Bacteroides*, while OTU-3 is from bacterial family *Porphyromonadaceae*.

Compositional analysis of enterotype-like clusters

To identify signature taxa within each cluster, we tested for significant differences in abundance among taxa displaying

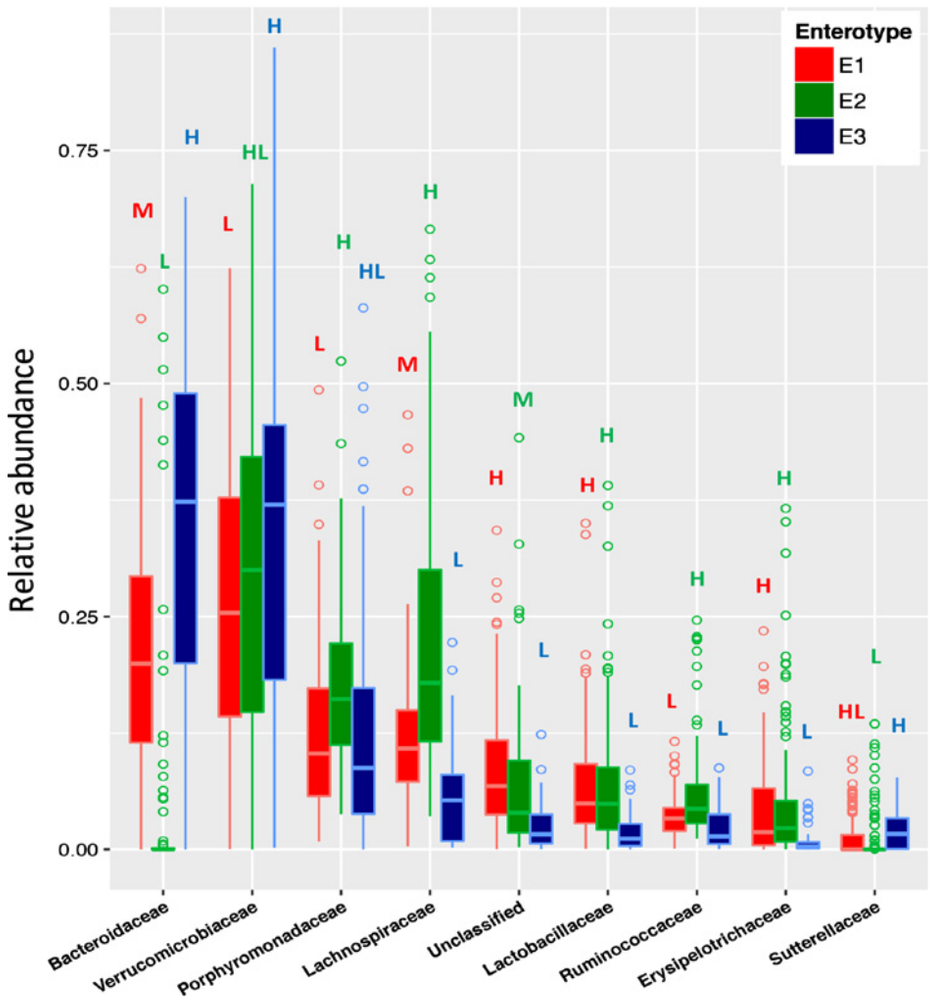

Figure 2.
Differentially abundant bacterial families among enterotype-like clusters. Boxes represent the interquartile range (IQR) between the first and third quartiles, with a line at the median. Circles denote outliers of the group. Letters above the box indicated significant difference with $P < 0.05$.

>1% abundance across the entire dataset (Supplementary Table S3). One-way ANOVA with Tukey HSD test was used to compare taxonomic differences among the three enterotypes generated from all the fecal samples. Major differences were observed between E2 and E3, while E1 represents an intermediate state. At the phylum level, microbiota was distributed across four bacterial phyla, *Bacteroidetes, Firmicutes, Proteobacteria*, and *Verrucomicrobia*. The highest ratio of *Bacteroidetes* was observed in E3, followed by E1, then E2. For the phylum *Firmicutes*, the highest relative abundance is in E2, with the middle level in E1, and lowest level in E3. The ratio of *Verrucomicrobia* is lowest in E1, and highest in E3. At the family level (**Fig. 2**), E3, dominated by *Bacteroidaceae*, was also enriched for *Verrucomicrobiaceae*, as well as *Sutterellaceae* ($P < 0.05$). E2, dominated by *Verrucomicrobiaceae*, exhibited relative enrichment of *Lachnospiraceae*, as well as *Porphymonadaceae, Lactobacillaceae, Ruminococcaceae*, and *Erysipelotrichaceae* ($P < 0.05$). Although E1 and E3 shared substantial taxonomic overlap, E1 was uniquely enriched for *Lactobacillaceae* and *Erysipelotrichaceae* ($P < 0.01$).

Functional analysis of enterotype-like clusters

Bacterial metagenomes were predicted with PICRUSt. Functional categories associated with each cluster were identified by LEfSe analysis (**Fig. 3**). E2 exhibited a relative high abundance of functional categories that were associated with cell motility, genetic information processing pathways (e.g., replication and repair, and translation), and environmental information processing (e.g., membrane transport). Metabolism pathways, such as glycan biosynthesis and metabolism, xenobiotic biodegradation and metabolism, lipid metabolism, and metabolism of cofactors and vitamins were found to be more dominant in E3. Similar to the taxa comparison results, E1 also showed an intermediate state between E2 and E3 at the functional level. Only one pathway involved in metabolism of other amino acids was found to be more prominent in E1.

Factors influence the formation of enterotype-like clusters

Enterotype-like cluster distribution among groups at the age of 20 weeks is presented in **Fig. 4**. Only two clusters (E1 and E3) could be identified in groups without the addition of walnuts (**Fig. 4**). It is possible that walnuts added to the diet have converted the gut microbiota from E3 to E2. For the non-AOM–treated control groups, structure of microbiota is shaped as E2 by walnuts, when comparing the 0% and 7% walnut groups. This trend can also be observed in the AOM-treated groups, wherein E2 gradually replaced E3 as the dietary walnut levels increase. With increasing concentrations of walnuts in the AOM-treated groups, more gut microbiota is changed from E3 to E2, whereas the ratio of E1 remains constant. When comparing the carcinogen-treated group and the noncarcinogen–treated group, both at a 7% walnut concentration, an increase of E1 was observed in the carcinogen-treated group, which also indicates that carcinogen treatment helps to stabilize the gut microbiota as E1. Overall, these data suggest that E1 and E2 are associated with carcinogen treatment and walnuts, respectively.

Longitudinal analysis of the enterotype-like cluster distribution

To characterize the dynamic process of gut microbiota over time, fecal samples were collected at an interval of 3 or 4 weeks from 6-week-old mice until 20 weeks of age (**Fig. 5**). The frequency of occurrence of these enterotype-like clusters varied among time points. At 6-week-old, the microbiota from each treatment group showed a community structure consistent with the E2 enterotype-like cluster. For mice without

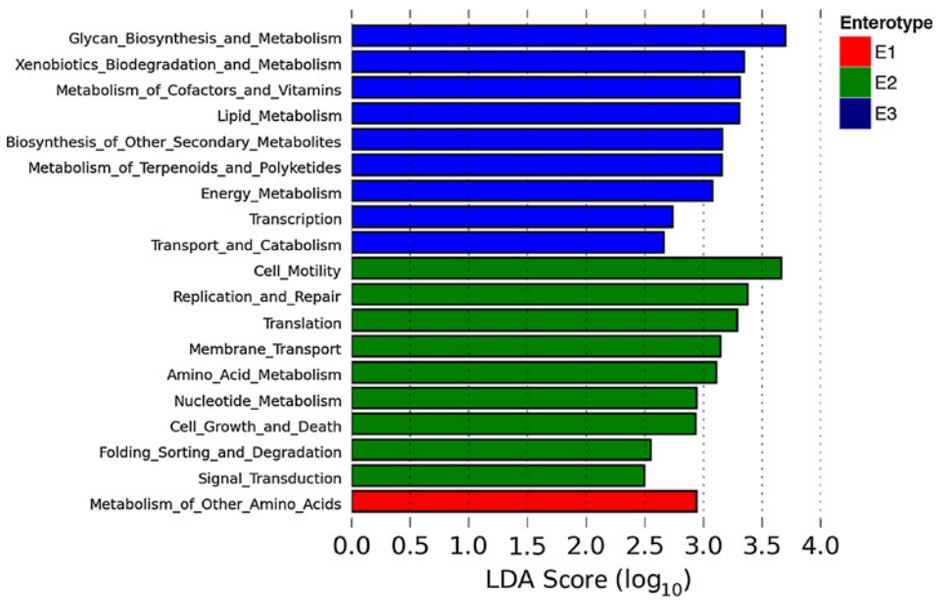

Figure 3.
Differentially abundant bacterial functional categories in enterotype-like clusters based on LEfSe analysis.

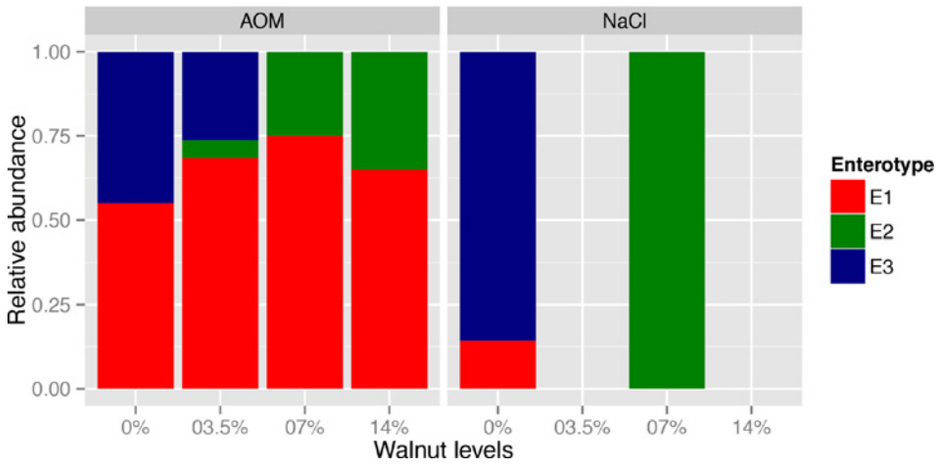

Figure 4.
The frequency of occurrence of enterotype-like cluster among different treatments at 20 weeks of age.

carcinogen treatment, at the 7% walnut concentration, the gut microbiota stabilized as E2 across each of the time points. The distribution of enterotype-like clusters then becomes steady from 16-week-old, which might indicate that the gut microbiota has developed a mature composition after that timepoint. For mice fed the highest levels of walnuts (14% of walnuts by weight), from 13 weeks to 20 weeks of age, the frequency of occurrence of E2 gradually decreased from 75% (13 weeks of age) to 60% (16 weeks of age), finally reaching 40% by 20 weeks of age (Supplementary Table S4). The results showed that with the carcinogen-treatment time elongating and the tumor enlarging, the effect of walnuts on gut microbiota decreased. Significant gender-related differences in the composition of the gut microbiota in response to walnuts were observed in the previous study (23). In this analysis, the observed enterotype-like cluster distribution is generally similar between male and female mice (Supplementary Fig. S1).

Association between enterotype-like clusters and tumor burden

When comparing the number of tumors among different enterotype-like clusters, there is a moderate decrease of tumor numbers in E2 than in E1 and E3 (11.8 ± 6.5 in E2 vs. 15.9 ± 7.5 in E1, and 15.9 ± 6.9 in E3; $P = 0.12$; **Fig. 6**).

Discussion

There is growing evidence that the intestinal microbiota is a key determinant in the development of colorectal cancer, and in some cases may provide a potential target for anticancer agents. A number of studies have recently shown that walnut consumption is associated with a reduced risk of colorectal cancer (5, 6, 23). In this study, potential mechanisms for the tumor-protective properties of walnuts on colorectal cancer described earlier (23) have been explored in greater detail. Using enterotype-like cluster analysis, we have found that carcinogen treatment, walnut consumption, and TWD are associated with three distinct enterotype-like clusters, E1, E2, and E3, respectively. E2 is associated with a slight decrease in colon tumor numbers. Our results suggest that adding walnuts to a Western-type formulated diet might shape the microbiota toward a distinct community structure that harbors a lower risk of colorectal cancer.

There have been several studies showing the influence of walnut consumption on gut microbial community structure. Nakanishi and colleagues found the abundance of OTUs from *Porphyromonadaceae*, *Ruminococcaceae*, *Lachnospiraceae*, and *Lactobacillus* increased in response to the addition of walnuts in the diet (23). Byerley and colleagues later showed that rats consuming walnuts display significantly greater species diversity. And at the family level, walnuts are associated with increase in the abundance of *Lactobacillaceae, Lachnospiraceae*, and *Ruminococcaceae*, and decrease in the abundance of *Bacteroidaceae* (32). These results from animal studies indicate that walnuts consumption enriched probiotic-type bacteria like *Lactobacillus* and *Ruminococcaceae*, and reduced *Bacteroides*. The probiotic-type bacteria can ferment complex dietary residues in to short chain fatty acids (SCFA). SCFAs are the preferred energy sources for colonocytes, and could maintain mucosal integrity, and suppress inflammation and carcinogenesis (10). Although murine models showed consistent results of walnut-related microorganisms, different results were reported in human studies. Bamberger and colleagues conducted a large scale, randomized, controlled trail in healthy Caucasian subjects to confirm the effect of walnut-enriched diet on gut microbiome. The abundance of *Ruminococcaceae* and *Bifidobacteria* increased significantly, while *Clostridium* species *cluster XIVa* species (*Blautia* and *Anaerostipes*) decreased during walnuts consumption. However, unlike our results, they found significant lower levels of *Lachnospiraceae* species after walnuts consumption (33). Holscher and colleagues reported walnuts consumption resulted in higher relative abundance of *Faecalibacterium, Clostridium, Dialister*, and *Roseburia* and lower relative abundances of *Ruminococcus, Dorea, Oscillospira*, and *Bifidobacterium* (34). The possible explanation for the inconsistent results of human studies is that factors that might influence the gut microbiota are hard to control in human studies. Thus, additional *in vitro* and *in vivo* research is

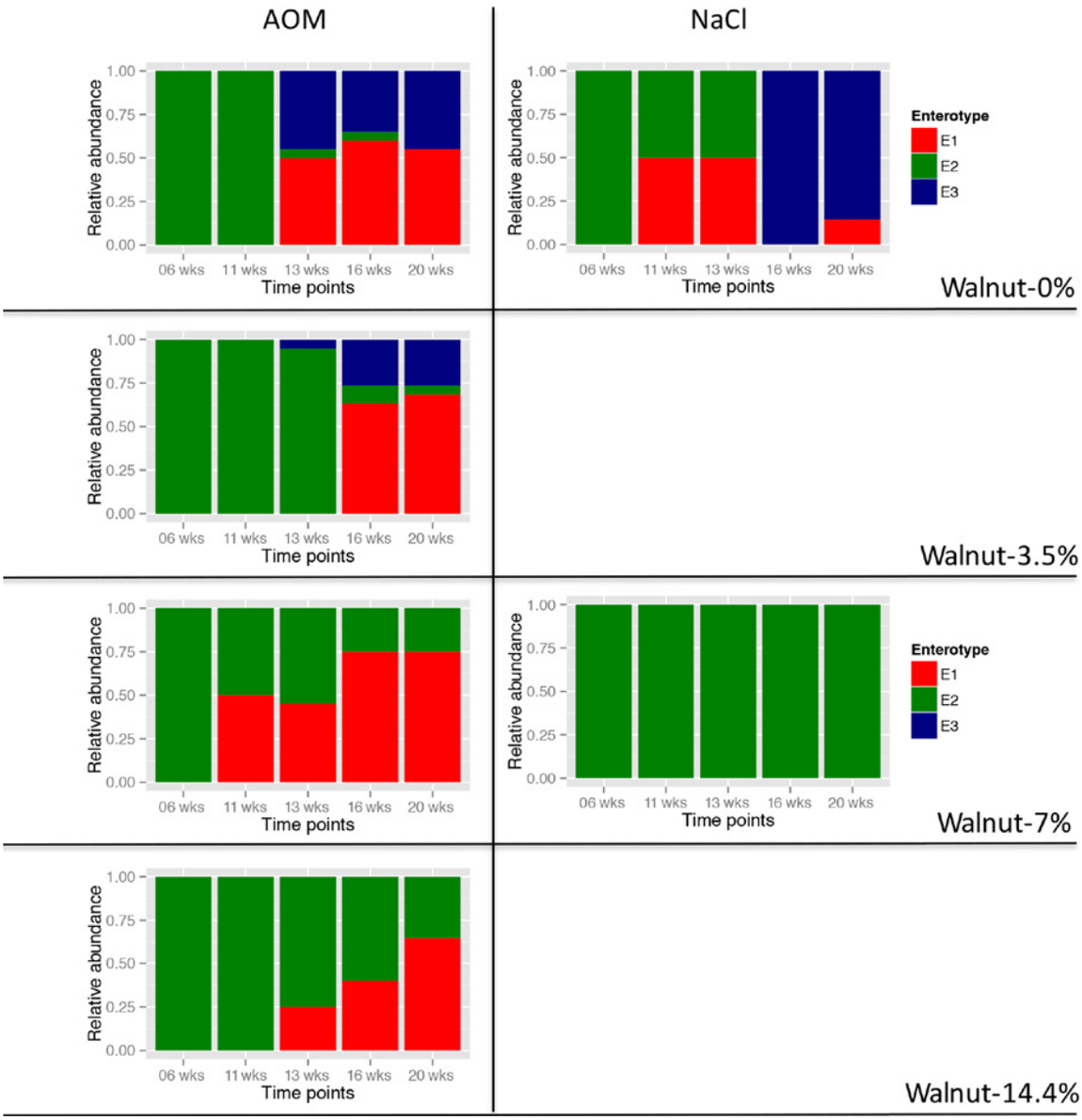

Figure 5.
The frequency of occurrence of enterotype-like cluster over time at different groups.

necessary to determine whether walnuts caused a common change regardless of species.

Host–microbial ecosystems are complex and dynamic. Longitudinal studies of the microbiota can help to elucidate the forces that shape and sustain the community. A total of five different time points, ranging from 6-week-old to 20-week-old, were included, which showed a dynamic process of gut microbiota in response to different treatments. As can be observed in **Fig. 5**, E2 might indicate the microbial structure of early life. Food ingredients are one of the major drivers of gut microbiota during early life. *Bifidobacterium*, favoring milk oligosaccharide fermenters, is the main component of infant microbiota when diet is almost exclusively milk (35). Weaning and introduction of solids foods triggers an increase in abundance of microbes that can utilize polysaccharides not digested by host enzymes, including *Bacteroides, Clostridium*, and *Ruminococcus*, with a decrease of *Bifidobacterium* and *Enterobacteriaceae* (36). There are differences of dominant bacterial taxa in early life of murine models and human. Caruso and colleagues demonstrated that dominant bacterial families in feces of 3-week-old mice included *Porphymonadaceae, Lachnospiraceae, Prevotellaceae*, and *Ruminococcaceae* (37). These were exactly the enriched bacterial families in E2. The exact reasons that walnuts consumption

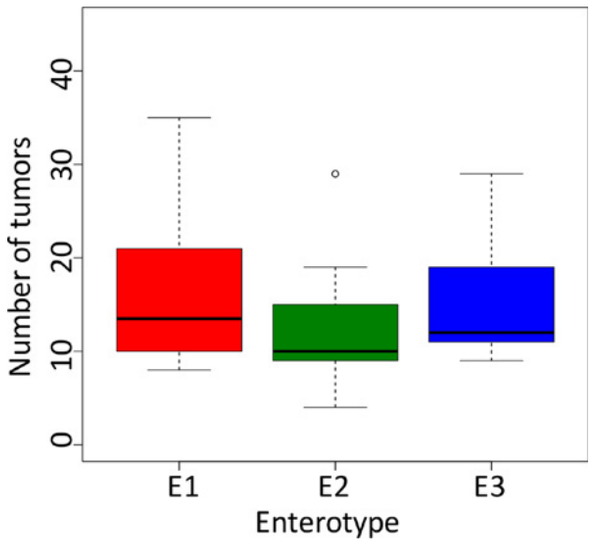

Figure 6.
Comparison of tumor burden among enterotype-like clusters. wks, weeks.

shapes the gut microbiota toward early life state were not clear. Walnuts are a rich source of nutrients including plant protein, fiber, and monounsaturated fatty acids (38). Walnuts and breast milk may share some nutrients like omega-3 polyunsaturated fatty acids. Previous studies reported microbial changes in the gut after omega-3 PUFA supplementation, including an increase in *Lachnospiraceae* with a decrease in *Bacteroides* (39, 40).

Comparative functional analysis with PICRUSt indicated microbial features modified in E2 included altered potential for amino acid metabolism and bacterial pathogenesis, specifically cell motility and signal transduction pathways. It is interesting to observe enrichment of functional genes related to pathogenesis in enterotype-like cluster associated with walnuts. One possible explanation is that the nutrients of the walnuts-added diet or early life both are relatively simple. The gut microorganisms need to compete with each other for the same kind of nutrients, which may consequently lead to an overrepresentation of these genes related to microbial mobility. However, if the overrepresentation of these pathogenic genes could easily lead to infections is not clear. Further researches are still warranted.

Because of the close correlation between colorectal cancer and Western diet, and to simulate real conditions, all the mice were fed a base Western diet in this research. For mice without walnuts and carcinogen treatment, most of the fecal microbiota fell into E3, which suggests TWD is associated with a community structure as E3. Western diet (high in animal protein and fat and low in fiber) is usually associated with reduced diversity of gut microbiota in human studies (41, 42). These findings are also confirmed in our study, which showed that E3 has relatively lower microbial diversity. Several studies have revealed that high animal protein and high saturated fat intake would result in disproportionately more propionate and acetate producing species, including *Bacteroides* and *Enterobacteriales* (43, 44). The study of Wu and colleagues found that enterotype driven by *Bacteroides* was associated with high fat/low fiber diet in human population (45). In the murine model, a significant positive correlation was observed between *Lachnospiraceae* and the percentage of plant-derived food sources (46). In-line with these previous results, E3 showed relative higher abundance of *Bacteroidaceae*, with lower relative abundance of *Lachnospiraceae*. Accordingly, microbial features modified in E3 conditions included altered potential for glycan biosynthesis and metabolism, lipid metabolism, and energy metabolism, which suggest the major role of microbiota in such nutritionally adequate environments is to harvest energy.

Abundant researches had examined the gut dysbiosis during the process of colorectal cancer. Thus, it is not our main purpose to characterize the microbial communities shaped directly by carcinogen treatment. However, some tendencies can still be observed. Carcinogen treatment with AOM is associated with enterotype-like cluster E1 (**Fig. 4**). In comparison with E2 and E3, E1 had moderate decrease of *Verrucomicrobiaceae* and *Porphyromonadaceae* (**Fig. 2**). This was in-line with a recent mice study with a similar carcinogen treatment. They found that tumor-bearing mice showed decreases in OTUs affiliated with members of the *Porphyromonadaceae* families (47). At the family level, E1 showed a kind of middle state between E2 and E3 (**Fig. 2**). However, the difference might mostly be on OTU level, because our clustering analysis was based on OTU profile, and E1 and E3 both showed enrichment of family *Bacteroidaceae* (**Fig. 2**), but were driven by two different OTUs (**Fig. 1**). Besides, the ratio of unclassified reads at the family level was the highest in E1 than in other clusters, which might also indicate OTUs of unknown taxa origins contribute to the formation of this cluster.

We observed a moderate decrease, but not statistical significant, of tumor numbers in E2 than in E1 and E3. It is speculated that walnuts have suppressive potential on colon cancer, but cannot absolutely inhibit the development of colorectal cancer. Without AOM treatment, fecal microbiota can totally be shaped to E2 by walnuts. For those AOM-treated mice, walnuts can only reverse the effect of TWD on microbiota, changing E3 to E2, while the frequency of occurrence of E1 on different walnuts level kept stable. So, the influence of AOM on gut microbiota is difficult to be reversed by walnuts consumption. Our results might suggest that colorectal cancer suppressive effects of walnuts would be more beneficial for people on Western diet. This conclusion is supported by Knudson's two-hit hypothesis, which suggests that host factors play a decisive role in the predisposition to carcinogenesis, and a second environmental hit can lead to uncontrolled cellular proliferation (48). Factors responsible for causation of colorectal cancer include dietary, geographic, and genetic factors (49). Walnuts could partially reverse the carcinogen effect

of dietary effect. The antitumor effects would be more obvious on people with high risk Western diet.

There are several limitations of this study. First, no dietary controls were included in this study. Future studies with other diets as controls are needed to check if walnuts could reduce the risk of colorectal cancer on other diets. Second, how the results here are relevant to the human population is not clear. Human studies with large sample size are needed in the future. Third, this research explored the influence of several different factors on gut microbiota. Because interactions might exist among these factors, which would also influence the results. Future researches using multiple technologies, like germ-free animals and metabolomics analysis, could be combined to verify the results here.

In summary, the study here compared the influence of three factors (carcinogen treatment with AOM, TWD diet, and walnuts consumption) on gut microbiota in A/J mice. Using enterotype-like clustering analysis, we found that the three factors could drive gut microbiota toward three enterotype-like clusters. Mice with E2 induced by walnut consumption showed relative lower colon tumor burden. The alteration of gut microbiota is likely responsible for the protective effect of walnut on colorectal cancer. It is suggested that modulation of gut microbiota using dietary intervention, such as walnuts may be effective in colon cancer prevention strategies. More future researches are needed to identify the specific biochemical that contributes to the process.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Nakanishi, D.W. Rosenberg, G.M. Weinstock
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