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Changes in the gut microbial communities following addition of walnuts to the diet

Lauri O. Byerley[a,*], Derrick Samuelson[b], Eugene Blanchard IV [c], Meng Luo[c], Brittany N. Lorenzen[a], Shelia Banks[a], Monica A. Ponder[d], David A. Welsh[b,c], Christopher M. Taylor[c]

[a]Department of Physiology, School of Medicine, Louisiana State University Health Sciences Center, 1901 Perdido St., New Orleans, LA 70112
[b]Department of Internal Medicine, School of Medicine, Louisiana State University Health Sciences Center, 1901 Perdido St, New Orleans, LA 70112
[c]Department of Microbiology, Immunology and Parasitology, School of Medicine, Louisiana State University Health Sciences Center, 1901 Perdido St., New Orleans, LA 70112
[d]Department of Food Science and Technology, 401-D HABB1, Virginia Tech, Blacksburg, VA, United States 24061

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Abstract

Walnuts are rich in omega-3 fatty acids, phytochemicals and antioxidants making them unique compared to other foods. Consuming walnuts has been associated with health benefits including a reduced risk of heart disease and cancer. Dysbiosis of the gut microbiome has been linked to several chronic diseases. One potential mechanism by which walnuts may exert their health benefit is through modifying the gut microbiome. This study identified the changes in the gut microbial communities that occur following the inclusion of walnuts in the diet. Male Fischer 344 rats (n=20) were randomly assigned to one of two diets for as long as 10 weeks: (1) walnut (W), and (2) replacement (R) in which the fat, fiber, and protein in walnuts were matched with corn oil, protein casein, and a cellulose fiber source. Intestinal samples were collected from the descending colon, the DNA isolated, and the V3-V4 hypervariable region of 16S rRNA gene deep sequenced on an Illumina MiSeq for characterization of the gut microbiota. Body weight and food intake did not differ significantly between the two diet groups. The diet groups had distinct microbial communities with animals consuming walnuts displaying significantly greater species diversity. Walnuts increased the abundance of *Firmicutes* and reduced the abundance of *Bacteriodetes.* Walnuts enriched the microbiota for probiotic-type bacteria including *Lactobacillus*, *Ruminococcaceae,* and *Roseburia* while significantly reducing *Bacteroides* and *Anaerotruncus*. The class *Alphaproteobacteria* was also reduced. Walnut consumption altered the gut microbial community suggesting a new mechanism by which walnuts may confer their beneficial health effects.

Keywords: Walnut; Gut microbiome; Diet; Bacterial diversity; Probiotics; Prebiotics

1. Introduction

Several epidemiologic studies have linked eating tree nuts, such as walnuts, to living a longer, healthier life [1–3]; however, the mechanism by which nuts impart this benefit has not been identified. Eating walnuts has been associated with a reduced the risk of cardiovascular disease in humans [4], slowing the rate of tumor growth in mice [5,6], and maintaining brain health during aging [7].

Walnuts have been labeled a “superfood” because they are rich in the omega-3 fatty acid, alpha-linolenic acid (ALA), as well as phytochemicals, antioxidant polyphenols, and fiber [8]. Which of these components imparts the health benefits associated with eating walnuts is not clear. Walnuts are one of the few foods that are rich in ALA. Also, walnuts contain approximately double the concentration of phenols compared to other fruits and vegetables [9,10] and have one of the highest concentration of antioxidants [11,12]. Dietary fiber content is 6–7% [13,14], but the polysaccharide composition of the fiber has not been well studied.

The importance of the gut microbiome on human health has been demonstrated recently in several studies. The presence of distinct bacterial communities is linked to a number of chronic diseases including heart disease [4], cancer [6], and brain health [7]. Clearly, diet composition influences the relative abundance of bacterial communities present in the gut [15]. Nakanishi et al. [16] showed using a mouse colon carcinogenesis model that inclusion of walnuts in the diet may partially protect against colon cancer and suggest a possible mechanism may be the changing the gut microbiome. Mice with the lowest number of tumors had a lower abundance of the *Bacteriodetes* and *Lachnospiraceases* bacterial families and a greater abundance of *Ruminococcaceae* and the *Clostridium XIVa* species subcluster.

One mechanism by which walnuts may exert their health benefit is through modulating the gut microbiome. The goal of this study was to determine if the inclusion of walnuts in the diet changed the gut microbiome and identify the changes in the gut microbial

Abbreviations: W, Walnut diet; R, Replacement diet.

* Corresponding author at: Department of Physiology, School of Medicine, 1901 Perdido St., Louisiana State University Health Sciences Center, New Orleans, LA 70112-1393. Tel.: +1 704 340 4482.

E-mail addresses: lbyerl@lsuhsc.edu (L.O. Byerley), dsamu2@lsuhsc.edu (D. Samuelson), eblan2@lsuhsc.edu (E. Blanchard), mluo2@lsuhsc.edu (M. Luo), bnlorenzen@gmail.com (B.N. Lorenzen), sheliadarjean@gmail.com (S. Banks), mponder@bt.edu (M.A. Ponder), dwelsh@lsuhsc.edu (D.A. Welsh), ctay15@lsuhsc.edu (C.M. Taylor).

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communities that occurred leaving future studies to determine if this is a mechanism by which walnuts confer their health benefit.

2. Materials and methods

2.1. Study design

This study was approved by the Institutional Care and Use Committee at the Louisiana State University Health Sciences Center (LSUHSC) in New Orleans, LA. Mature rats weighing more than 250 g were studied. Upon arrival at the LSUHSC vivarium, 20 male Fischer 344 rats were group housed for 1 week and maintained on rat chow (Harlan, Madison, WI) to allow them to adjust to their new environment. After, each rat was weighed and randomly assigned to one of two diet groups: (1) walnut (W), or (2) replacement (R). The diets are described under "Diets" and in Table 1. For the remainder of the study, each rat was singly housed, weighed daily and fed daily their assigned diet. The animals were sacrificed 6 or 10 weeks later, and at the time of sacrifice, fecal samples were collected aseptically from the descending colon, immediately frozen in liquid nitrogen and stored at −80°C until DNA isolation.

2.2. Diets

The diets were identical to the diets previously reported by Hardman et al. [10]. This diet is based on the AIN-76 diet. Approximately 11% by weight ground walnut per 100 g diet was added. Since walnuts contain protein, fat, carbohydrate and fiber, these macronutrients were adjusted in the replacement diet that contained no walnuts (Table 1) using the values for walnuts found in the USDA nutrient database [14]. Corn oil and alphacel fiber were used to adjust the fat and fiber content, respectively, of the replacement diet. The protein content was matched by increasing the casein in the replacement diet. All ingredients except the sugar, corn oil, and walnuts were purchased from Dyets (Bethlehem, PA, USA). The sugar and corn oil were purchased from a local grocery store in bulk (Albertsons, Mandeville, LA, USA). Shelled, whole walnuts were graciously provided by the California Walnut Commission (Folsom, CA, USA). To prevent deterioration once received, the walnuts were vacuumed-sealed in 1-kg bags and stored in a walk-in cooler maintained at −4°C. Each diet was made in small batches. At the time the diet was made, the walnuts were ground to a fine state and mixed with the rest of the ingredients in an industrial sized mixer (Hobart, Troy, OH). When the diet was the consistency of cookie dough, it was rolled, vacuum-sealed in small batches and frozen at −20°C until fed to the animals. At the time of feeding, the diet was thawed, cut into 1-in. cubes, weighed and given to the animal. Every 2 days, fresh diet was provided. The diets were analyzed for protein, fat, carbohydrate, fiber, ash and moisture content by Covance Laboratories (Madison, WI, USA). The total calorie content of each diet was determined by multiplying each macronutrient by its appropriate kcal/g.

Table 1
The composition of the walnut and replacement diet

Ingredient	Walnut [1] Percent by weight	Replacement Percent by weight
Casein [a]	18.3	20
Sucrose [b]	45	45
Corn starch [a]	13.5	15
Cellulose [a]	4.8	5
Choline bitartrate [a]	0.2	0.2
DL-methionine [a]	0.3	0.3
Mineral mix [c]	3.5	3.5
Vitamin mix [d]	1	1
Ground walnuts [e]	11.1	0
Corn oil [a]	2.63	10
Content determined by chemical analysis [m]		
Protein [f] (g/100 g)	15.6	15.5
Fat [g] (g/100 g)	4.3	5.8
Crude Fiber [h] (g/100 g)	3.67	2.7
Moisture [i]	16.2	15.7
Ash [j]	2.2	2.17
Mathematically derived from chemical analysis		
Carbohydrate [k] (g/100 g)	61.7	60.9
Total Energy Content (Cal/100 g) [l]	348	358
Omega 6/Omega 3 ratio	4.5/1	23.3/1

[1] 18% of calories from walnut.
[a] Dyets, Bethlehem, PA, USA.
[b] Flavorite, Eden Prairie, MN, USA.
[c] AIN-76, Dyets, Bethlehem, PA, USA.
[d] AIN-76A, Dyets, Bethlehem, PA, USA.
[e] Donated California Walnut Commission, Folsom, CA, USA.
[f] Measured by Dumas method, *Official Methods of Analysis of AOAC INTERNATIONAL*, 18th Ed., Methods 968.06 and 992.15, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005) (Modified).
[g] Quantitated by Soxhlet, *Official Methods of Analysis of AOAC INTERNATIONAL*, 18th Ed., Methods 960.39and 948.22. AOAC International, Gaithersburg, MD, 2005 (Modified).
[h] Quantitated by *Official Methods of Analysis of AOAC INTERNATIONAL (2005)* 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, Official Method 962.09.
[i] Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 925.09 and 926.08, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified).
[j] *Official Methods of Analysis of AOAC INTERNATIONAL*, 18th Ed., Method 923.03, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified).
[k] Calculated by difference.
[l] Calculated from values in United States Department of Agriculture, "Composition of Foods" Agricultural Handbook, No. 8, pp. 159–160, (1975).
[m] Covance Laboratories, Madison, WI.

2.3. DNA isolation and PCR amplification

Total DNA was extracted from approximately 0.25 g of feces using a protocol developed by the Louisiana State University School of Medicine Microbial Genomics Resource Group (http://metagenomics.lsuhsc.edu/mgrg), as previously published [17]. Briefly, DNA was isolated using the QIAamp DNA Stool Kits (Qiagen, Germantown, MD, USA) modified to include bead-beating and RNAase treatment steps.

2.4. Sequencing

The V3-V4 hypervariable region of 16S rRNA gene was PCR amplified using V3F = CCTACGGGAGGCAGCAG and V4R = GGACTACHVGGGTWTCTAAT primers, Illumina adaptors and molecular barcodes [18]. Illumina indexes were ligated onto each sample and samples were multiplexed for sequencing on a single Illumina MiSeq run using the Illumina V3 600-cycle sequencing kit (Illumina, San Diego, CA, USA) in paired-end mode as previously published [17].

2.5. Quality filtering/picking

Due to persistent read quality issues with the reverse sequencing reads from Illumina V3 sequencing kits, the forward reads files were processed through the UPARSE pipeline [19] and reverse reads were discarded. Reads were truncated to a uniform length of 280 bp and reads with quality scores less than 16 were filtered out. The UPARSE pipeline steps described by Edgar were performed in sequence and OTU clusters were formed at 97% with chimeric OTUs removed from the data. After quality filtering, reads were analyzed using QIIME 1.9.0 [20].

2.6. Microbial community analysis

A total of 20 samples were included in the QIIME analysis with read counts ranging from 14,628 to 90,465 with an average read count per sample of 56,041. Alpha rarefaction was performed at a level of 14,600 reads to include all samples.

2.7. Statistical analysis

Alpha rarefaction plots were produced by plotting the number of sequences in a sample against several different diversity metrics, for example, Shannon, Simpson, and Chao1. Beta diversity was determined by principal coordinate analysis using both unweighted and weighted UniFrac metrics. Emperor 3D viewer was used to visualize the plots. Statistical difference was determined using SAS software (Cary, NC, USA) or GraphPad Prism 6 (La Jolla, CA, USA). Student's *t* test was used to determine statistical significance between two groups using $P<.05$ as a cutoff. Mann–Whitney–Wilcoxon was used to determine significant differences for specific microbial communities between each diet and any *P*-value less than .05 is shown. The *P*-value was not corrected for multiple comparisons; instead, the actual value was reported. Data are presented as a mean ± S.E.M. In Table 2, only significantly different organisms present in five or more animals are shown.

Potential microbial functions were identified by PICRUst v0.9.0 (http://picrust.github.io/picrust/) [21]. Following PICRUst analysis the potential microbial functions associated with walnut consumption were identified by LEfSe (Linear Discriminant Analysis Effect Size) (http://huttenhower.sph.harvard.edu/lefse/) as described elsewhere [22]. An LDA score was generated using linear discriminate analysis for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. LDA is a classification method that searches for linear combinations of variables (predictors) that best separate two classes (walnut vs. replacement diet).

3. Results

3.1. Animal weight and food intake

Average body weight did not differ significantly between the two diet groups at the start of the study (data not shown). Regardless of

Table 2
Relative abundance significantly different between walnut diet and replacement diet

Taxa	Wilcoxon *P* value	Walnut			Replacement		
		Average (%)	SD	# of Rats	Average (%)	SD	# of Rats
Higher abundance walnut diet vs. *replacement diet*							
Firmicutes							
p__Firmicutes	0.007	67.39	9.03	10	55.52	6.80	10
p__Firmicutes;c__Bacilli	0.004	2.60	3.59	10	0.46	0.43	10
p__Firmicutes;c__Bacilli;o__Lactobacillales	0.011	1.84	3.78	10	0.26	0.23	10
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae	0.003	1.42	2.70	10	0.15	0.19	10
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	0.003	1.42	2.70	10	0.15	0.19	10
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus;s__	0.004	1.33	2.56	10	0.15	0.19	10
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus;s__reuteri	0.003	0.090	0.145	9	0.003	0.003	7
p__Firmicutes;c__Bacilli;o__Turicibacterales	0.034	0.751	0.636	9	0.192	0.228	7
p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae	0.034	0.751	0.636	9	0.192	0.228	7
p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae;g__Turicibacter	0.034	0.751	0.636	9	0.192	0.228	7
p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae;g__Turicibacter;s__	0.034	0.751	0.636	9	0.192	0.228	7
p__Firmicutes;c__Clostridia	0.026	64.705	10.89	10	54.93	5.769	10
p__Firmicutes;c__Clostridia;o__Clostridiales	0.026	64.705	10.89	10	54.93	6.769	10
p__Firmicutes;c__Clostridia;o__Clostridiales;Other	0.017	15.62	5.354	10	9.832	2.962	10
p__Firmicutes;c__Clostridia;o__Clostridiales;Other;Other	0.017	15.615	5.354	10	9.832	2.962	10
p__Firmicutes;c__Clostridia;o__Clostridiales;Other;Other;Other	0.017	15.615	5.354	10	9.832	2.962	10
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae;g__	0.017	0.009	0.007	8	0.004	0.013	2
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae;g__;s__	0.017	0.009	0.007	8	0.004	0.013	2
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Moryella	0.017	0.336	0.246	10	0.097	0.086	10
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Moryella;s__	0.017	0.336	0.246	10	0.097	0.086	10
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Roseburia	0.026	0.093	0.08	10	0.041	0.062	9
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Roseburia;Other	0.026	0.093	0.080	10	0.042	0.062	10
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__[Ruminococcus]	0.021	0.158	0.147	10	0.066	0.046	10
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;Other	0.016	0.225	0.137	10	0.099	0.054	10
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira	0.045	11.547	5.534	10	6.732	1.724	10
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira;Other	0.005	1.0367	0.799	10	0.458	0.121	10
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira;s__	0.031	10.511	4.892	10	6.273	1.644	10
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus;Other	0.003	0.142	0.190	9	0.004	0.003	9
Actinobacteria							
p__Actinobacteria;Other	0.045	0.030	0.035	10	0.007	0.009	10
p__Actinobacteria;Other;Other	0.045	0.030	0.035	10	0.007	0.009	10
p__Actinobacteria;Other;Other;Other	0.045	0.030	0.035	10	0.007	0.009	8
p__Actinobacteria;Other;Other;Other;Other	0.045	0.030	0.035	10	0.007	0.009	8
p__Actinobacteria;Other;Other;Other;Other;Other	0.045	0.030	0.035	10	0.007	0.009	8
Cyanobacteria							
p__Cyanobacteria;c__Chloroplast	0.024	0.005	0.006	6	0.0004	0.001	1
p__Cyanobacteria;c__Chloroplast;o__Streptophyta	0.024	0.005	0.006	6	0.0004	0.001	1
p__Cyanobacteria;c__Chloroplast;o__Streptophyta;f__	0.024	0.005	0.006	6	0.004	0.001	1
p__Cyanobacteria;c__Chloroplast;o__Streptophyta;f__;g__	0.024	0.005	0.004	6	0.0004	0.001	1
p__Cyanobacteria;c__Chloroplast;o__Streptophyta;f__;g__;s__	0.024	0.005	0.006	6	0.0004	0.001	1
Lower abundance walnut diet vs. *replacement diet*							
Bacteroidetes							
p__Bacteroidetes	0.007	23.56	6.94	10	34.19	7.40	10
p__Bacteroidetes;c__Bacteroidia	0.007	23.08	6.92	10	33.46	7.40	10
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales	0.007	23.08	6.92	10	33.46	7.40	10
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae	0.002	9.99	4.58	10	20.29	5.81	10
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	0.002	9.99	4.58	10	20.29	5.81	10
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__	0.005	7.05	3.20	10	15.10	5.58	10
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	0.002	9.988	4.583	10	20.286	5.811	10
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;Other	0.038	2.332	2.035	10	4.219	2.009	10
Firmicutes							
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae	0.029	0.002	0.003	3	0.007	0.007	9
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae;g__Granulicatella	0.029	0.002	0.003	3	0.007	0.007	9
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae;g__Granulicatella;s_	0.029	0.002	0.003	3	0.007	0.007	9
p__Firmicutes;c__Clostridia;Other	0.009	0.0004	0.001	2	0.002	0.002	8
p__Firmicutes;c__Clostridia;Other;Other	0.009	0.0004	0.001	2	0.002	0.002	8
p__Firmicutes;c__Clostridia;Other;Other;Other	0.009	0.0004	0.001	2	0.002	0.002	8
p__Firmicutes;c__Clostridia;Other;Other;Other;Other	0.009	0.0004	0.001	2	0.002	0.002	8
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia	0.036	0.012	0.027	6	0.055	0.101	9
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia;Other	0.044	0.009	0.021	6	0.016	0.022	9
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus	0.026	6.405	3.471	10	12.349	6.495	10
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus;s__eutactus	0.014	3.484	3.075	10	10.136	6.873	10
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__[Ruminococcus];s__gnavus	0.021	0.158	0.147	10	0.066	0.046	10
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__;s__	0.045	2.348	1.302	10	3.434	1.478	10
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__	0.045	2.348	1.302	10	3.434	1.478	10
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Anaerotruncus	0.003	0.006	0.005	8	0.034	0.035	9
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Anaerotruncus;s__	0.003	0.006	0.005	8	0.034	0.035	9
p__Firmicutes;c__Erysipelotrichi	0.045	0.044	0.028	10	0.102	0.074	10
p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales	0.045	0.044	0.028	10	0.102	0.073	10
p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	0.045	0.044	0.028	10	0.102	0.074	10

Table 2 (*continued*)

Taxa	Wilcoxon *P* value	Walnut			Replacement		
		Average (%)	SD	# of Rats	Average (%)	SD	# of Rats
p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Allobaculum	0.022	0.008	0.013	5	0.055	0.065	9
p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Allobaculum;s__	0.022	0.008	0.013	5	0.055	0.065	9
p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;Other;Other	0.011	0.001	0.003	1	0.008	0.010	7
Proteobacteria							
p__Proteobacteria;c__Alphaproteobacteria	0.0004	0.049	0.039	10	0.390	0.264	10
p__Proteobacteria;c__Alphaproteobacteria;o__RF32	0.001	0.043	0.039	10	0.385	0.268	10
p__Proteobacteria;c__Alphaproteobacteria;o__RF32;f__;g__	0.001	0.043	0.039	10	0.385	0.268	10
p__Proteobacteria;c__Alphaproteobacteria;o__RF32;f__;g__;s__	0.001	0.043	0.039	10	0.385	0.268	10
p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae	0.043	0.003	0.006	3	0.007	0.013	6
Tenericutes							
p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales	0.007	0.008	0.012	6	0.115	0.153	9
p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales;f__Anaeroplasmataceae;g__Anaeroplasma;s__	0.007	0.008	0.012	6	0.115	0.153	9
Cyanobacteria							
p__ Cyanobacteria	0.014	0.121	0.071	10	0.334	0.303	10
p__Cyanobacteria;c__4C0d-2	0.011	0.116	0.072	10	0.334	0.303	10
p__Cyanobacteria;c__4C0d-2;o__YS2;f__;g__;s__	0.011	0.116	0.072	10	0.334	0.303	10
p__Cyanobacteria;c__4C0d-2;o__YS2;f__	0.011	0.116	0.072	10	0.334	0.303	10
p__Cyanobacteria;c__4C0d-2;o__YS2;f__;g__	0.011	0.116	0.072	10	0.334	0.303	10

diet consumed, the animals grew at a similar rate (0.91±0.1 g/day). This indicates that the addition of walnuts to the diet did not increase body weight more than the replacement diet. At the time of sacrifice, the animals weighed 340±24 g walnut diet and 340±24 g replacement diet (Fig. 1A).

The composition of the two diets is shown in Table 1. The replacement diet was slightly higher in calories than the walnut diet (walnut: 3.48 kcal/g vs. replacement: 3.57 kcal/g, *P*=.96). To make up for the difference in calories, the animals eating the walnut diet consumed *ad libitum* slightly more food (W: 15.4±2.6 g/day vs. R: 14.9±2.0 g/day, *P*=.96) so caloric intake was remarkably similar and not significantly different between the two diet groups throughout the study (Fig. 1B).

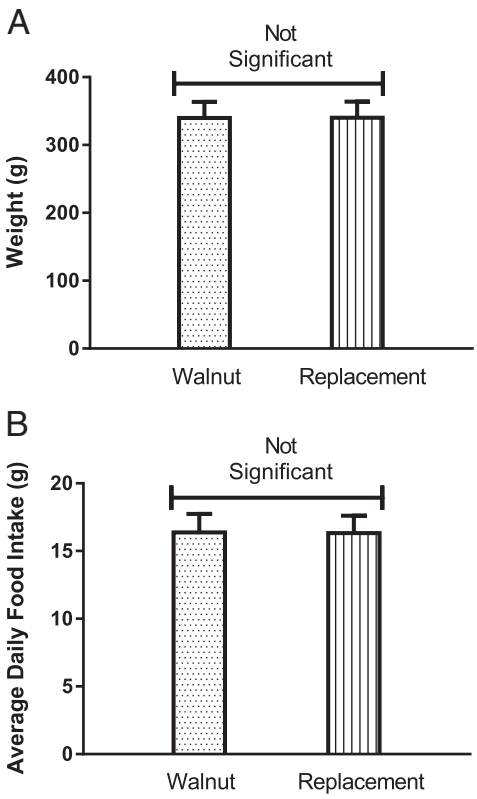


Fig. 1. Body weight (1A) and daily food intake (1B) for the two diet groups. Body weight and food intake did not differ significantly between the two groups.

3.2. Gut microbiome

The alpha diversity for walnut and replacement diets is shown in Fig. 2. Adding walnuts to the diet significantly increased bacterial diversity measured by Shannon's (*P*=.018) and Simpson's (not shown, *P*=.013) indices. However, Chao1 diversity was not different between the two groups. Thus, there was a significant increase in community evenness (Shannon's and Simpson's) for those animals eating the walnut diet compared to the replacement diet, but not in richness (Chao1, *P*=.77).

Beta diversity (Principal Coordinate Analysis plots) for walnut and replacement diets are shown in Fig. 3. As demonstrated by unweighted UniFrac analysis, clear, distinct clustering was observed between the two diet groups. Beta diversity for walnut and replacement diets were significantly different using both unweighted (*P*=.0003) and weighted UniFrac analysis (data not shown, *P*=.002).

Fig. 3B rotates the plane, keeping PC1 in the "Y" axis position and exchanging the PC2 and PC3 between the "X" and "Z" axis. By rotating the plane, it becomes clearly evident that there are three rats which group together by beta diversity metrics, two of which were from the walnut group (one 6-week and one 10-week sacrifice) and one of which was from the replacement group (6-week sacrifice). There is no

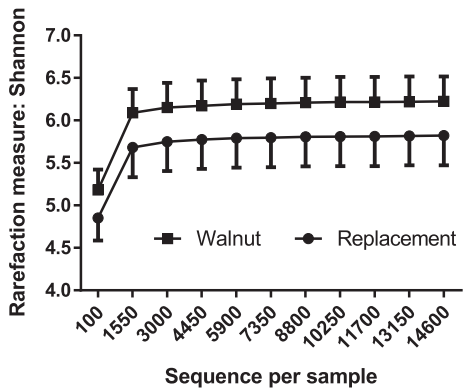


Fig. 2. Alpha diversity (within a community) of the gut microbiome shown using Shannon analysis. The addition of walnuts significantly increased (*P*=.018) the diversity evenness of the gut microbial community.

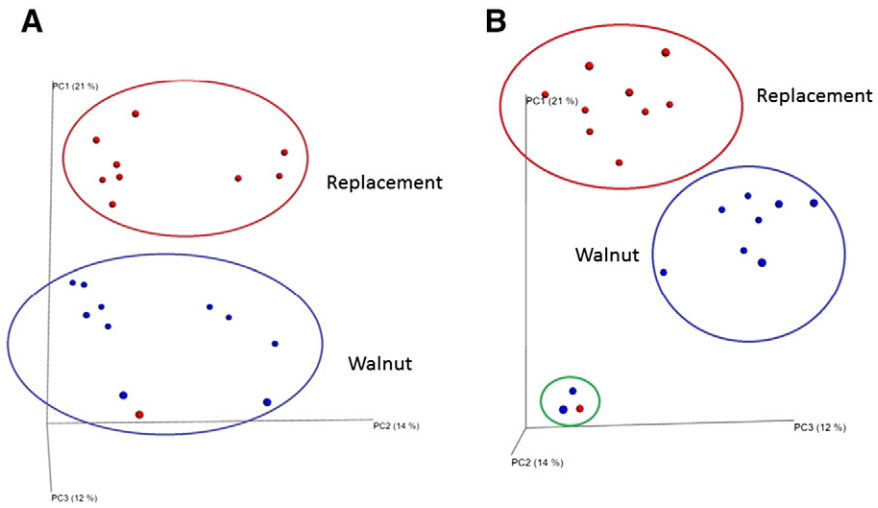


Fig. 3. Beta diversity (between communities) of the gut microbial communities. The principle coordinate analysis (PCoA) plot based on unweighted (shown in the figure) UniFrac distances showed two distinct gut microbial communities (replacement diet red circles, walnut diet blue circles) (Fig. 3A). Although Fig. 3A suggests one outlier from the walnut group in the replacement group, rotating the axis shows clearly three outliers (Fig. 3B) – two from the walnut diet and one from the replacement diet.

clear explanation for the overlap of these three animals. Each animal was individually housed and fed separately.

The changes in operational taxonomic units for the bacterial phyla are shown in Fig. 4A (walnut diet) and B (replacement diet). The pie charts in Fig. 4A and B demonstrate that the addition of walnut to the diet changed the bacterial communities present in the descending colon. At the phylum level, the abundance of *Firmicutes* and *Bacteriodetes* were significantly different between the two diets. As expected, the preponderance of bacteria belonged to these two phyla made up more than 90% of the bacteria present in the lower colon. While Fig. 4A and B show that the walnut group had no *Lentisphaerae*, there was one animal in the replacement diet with organisms from this phyla, and there was no significant difference between the two diets. Fig. 4C shows the ratio of *Firmicutes* to *Bacteriodetes* in the walnut and

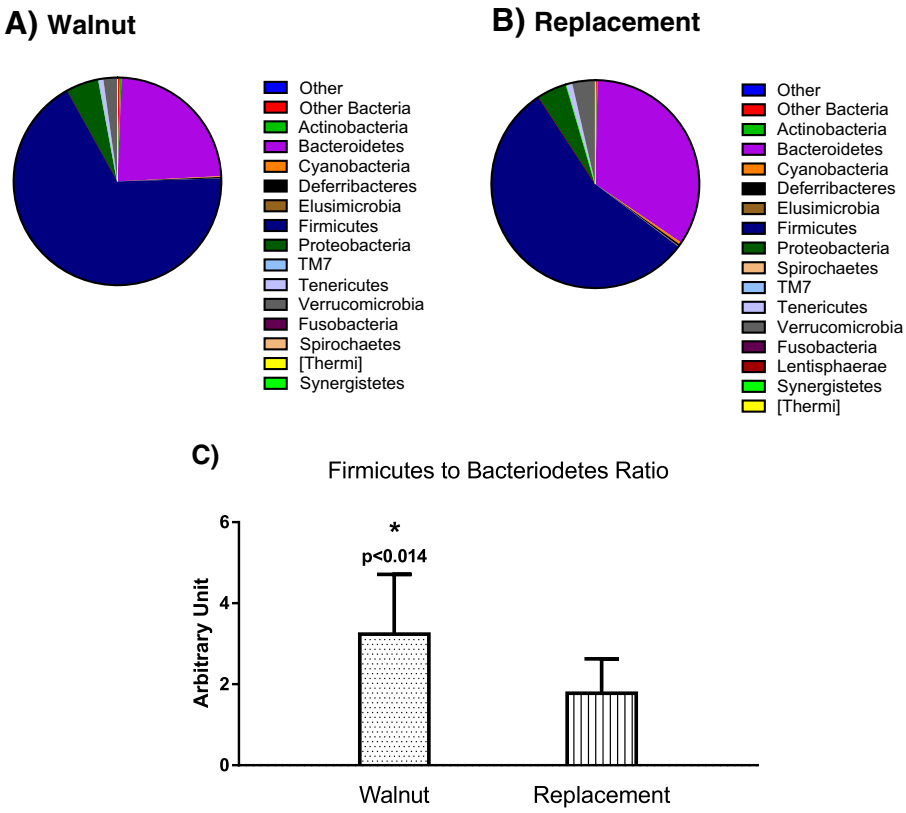
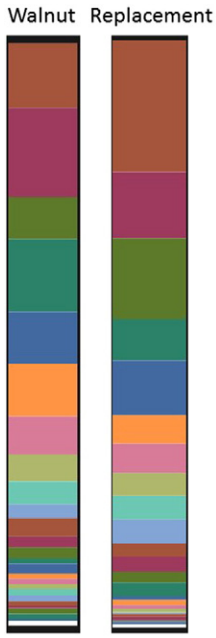


Fig. 4. Relative abundance of the bacterial phyla between the walnut and replacement diet. Relative abundance was calculated from the relative abundance of 16S rRNA gene sequences assigned to each bacterial community using the Greengenes database. Fig. 4A shows the changes at the phyla level for the walnut diet and Fig. 4B shows the phyla changes for the replacement diet. Only *Firmicutes* and *Bacteriodetes* were significantly changed, and the ratio of *Firmicutes* to *Bacteriodetes* is shown in Fig. 4C.



Bar Color	Taxonomy	Walnut %	Replacement %	P-value
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	11.10%	21.60%	0.001
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__	14.60%	10.80%	
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus	6.90%	13.00%	0.029
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira	12.00%	6.70%	0.044
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__S24-7;g__	8.30%	8.90%	
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus	8.60%	4.70%	
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;Other	6.30%	4.80%	
	p__Firmicutes;c__Clostridia;o__Clostridiales;Other;Other	4.40%	3.70%	
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__	3.80%	3.90%	
	p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;g__Akkermansia	2.30%	3.90%	
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__SMB53	3.10%	2.00%	
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__	1.70%	2.50%	
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;Other	1.90%	1.80%	
	p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__Bilophila	0.90%	2.30%	
	p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;Other	1.70%	0.60%	
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides	0.90%	0.90%	
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;g__rc4-4	0.90%	0.60%	
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae;g__Dehalobacterium	0.80%	0.60%	
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__	1.10%	0.20%	0.004
	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	1.00%	0.20%	
	p__Tenericutes;c__Mollicutes;o__RF39;f__;g__	0.80%	0.50%	
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;Other;Other	0.50%	0.70%	
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__;g__	0.90%	0.10%	0.032
	p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae;g__Turicibacter	0.90%	0.20%	0.027
	p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__Sutterella	0.40%	0.40%	

Fig. 5. The top 25 most abundant bacteria in genus. The two columns on the left graphically represent the data shown in the table. The taxa in the boxes are shown in the same descending order as the table.

replacement diet fed rats. The animals that ate walnuts had a significantly greater (>1.8-fold) ratio of *Firmicutes* to *Bacteriodetes* when compared to the replacement diet.

The 25 most abundant bacteria communities for each diet at the genus level are shown in Fig. 5. The 25 predominant microbes at the genus level were derived from five different phyla, seven different classes, nine different orders, and 17 different families. *Bacteroides* and *Coprococcus* were significantly more abundant after eating the replacement diet while *Oscillopira*, *Lachnospiraceae*, and *Turicibacter* were significantly more abundant after long-term, continuous consumption of walnuts.

Table 2 lists the significant shifts in the relative abundance of various bacteria following long-term continuous consumption of modest amounts of walnuts daily. Animals consuming walnuts had a greater relative abundance of the phyla *Firmicutes* and the smaller communities of *Actinobacteria* and *Cyanobacteria*. Although an increase in the *Firmicutes* phyla was observed, within the phyla particular taxa increased and decreased. Within the *Firmicutes* phyla, significant changes in the *Bacilli, Erysipolotrichi,* and *Clostridia* were observed. The *Bacilli* class includes the *Lactobacillus* family, which produce lactic acid. The species *L. Reuteri* had a three-fold higher relative abundance following walnut consumption. In addition, *Turicibacteriaceae* increased approximately three-fold. The *Lactobacillales* order also contains the family *Carnobacteriaceae* whose relative abundance significantly decreased.

Both increases and decreases were observed in the relative abundance of specific members of *Clostridia*, which is known for its butyrate-production. Increases were seen in Oscillospira, *Moyella*, *Roseburia*, *Peptococeaceae,* and *Ruminacoccaecea*. Alternatively, some members of this class were reduced by the addition of walnuts to the diet. These included *Anaerotruncus*, *Dehalobecteriaciae*, *Blautia* and *Coprococus*. The relative abundance of *Erysipelotrichi* class decreased.

The *Cyanobacteria* phyla also saw increases and decreases in the relative abundance of specific members. The *Streptophyta* order increased more than tenfold while the 4COD-2 decreased almost three-fold. *Bacteroidetes*, *Proteobacteria* and *Tenecutes* were significantly reduced

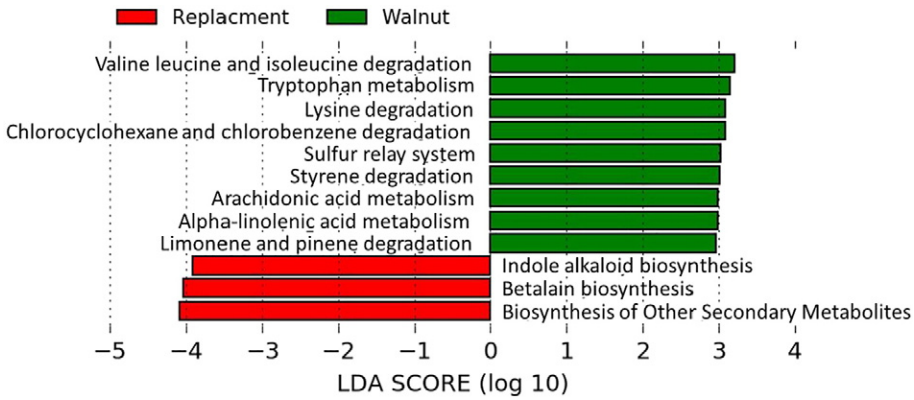


Fig. 6. Inferred functional capacity of the microbial communities associated with walnut and replacement diet determined by linear discriminative analysis (LDA) effect size (LEfSe) analysis of KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. Positive LDA scores are enriched in animals eating the walnut diet (green bars) while negative LDA scores are enriched in those animals eating the replacement diet (red bars).

following walnut consumption. At the genus level, the reductions in *Bacteroides*, which make up a substantial portion of this phylum, was more than two-fold. Within the *Proteobacteria* family, *Alphaproteobacteria* and *Gammaproteobacteria* saw reductions. Members *Anaeroplamalaes* and ML615j-28 were reduced within the *Tenericutes* phyla.

3.3. Predicted metagenome inference

Fig. 6 shows the different inferred functional capacities ranked by effect size associated with the bacterial communities present in the colon of animals eating the walnut (green) or replacement diet (red). Nine pathways were more dominant when walnuts were included in the diet. Three pathways involved amino acid metabolism and two pathways focused on omega-3 and omega-6 fatty acid metabolism. Three pathways were more dominant following long-term continuous consumption of the replacement diet. Two of these pathways involved products synthesized from amino acids. The amino acid tryptophan is implicated in both diets but different metabolic pathways. For the animals eating walnuts, the pathways for producing tryptophan products such as serotonin were more prominent while tryptophan pathways involved with indole alkaloid biosynthesis were predominant in the replacement diet animals. Thus the relative abundance of bacterial communities significantly altered the inferred functional capacity of the microorganisms in the gut.

4. Discussion

The walnut and replacement diets have several notable differences. The fiber, protein, fat and carbohydrate found in walnuts were substituted to make the replacement diet eucaloric and similar in macronutrient content. It was not the intent of this study to determine the impact of individual constituents but to examine walnuts as a whole since humans eat whole walnuts. First, Alphacel, a 99% cellulose based fiber, was used to replace the fiber in walnuts as it is readily available and the type of fiber present in walnuts has yet to be identified. Second, walnuts contain a mixture of polyunsaturated and monounsaturated fats and are one of the few plant foods that contain the anti-inflammatory omega-3 fatty acids. The fat content of the replacement diet was corn oil, which is high in polyunsaturated fatty acids and lack omega-3 fatty acids. Third, casein was the protein source for both diets, and this was increased slightly in the replacement diet. There have been several reviews recently published that have discussed the role of individual nutrients on gut microbiome composition [15]. Most likely these macronutrients and fiber were involved in producing the unique bacterial signature observed for with walnut diet.

Deep 16S rDNA sequencing found significant differences in the gut microbial communities of rats administered walnuts compared to rats consuming the replacement diet. There was a clear, distinct separation between the two diets with walnuts significantly increasing community diversity driven by an increase in evenness of bacterial species. These same changes were observed by Nakanishi et al. who fed walnuts to mice [16]. Also, De Filippo et al. [23] found higher microbial diversity in children from Burkina Faso who ate a diet higher in whole grains, legumes and vegetables compared to European children whose diet contained more animal-based foods. However, children who consumed 1.5 oz. of almonds or an equivalent amount of almond butter for 3 weeks did not change their gut microbial diversity as measured by Shannon's or Simpson's [24].

Walnut consumption shifted the predominant microbe phyla from *Bacterodites* to *Firmicutes*. Other studies have shown a greater relative abundance of *Firmicutes* in the young, but that the predominance of this phylum declines, while the abundance of *Bacterodites* increases, with age [25]. Shifts in these two phyla have been associated with obesity, as well. Generally, obese individuals have a greater abundance of *Firmicutes* and lower amount of *Bacteriodetes* although these changes may be related more to a high fat, obesogenic diet than excessive adipose [26]. The fat content of the diet used in this study was approximately 5%, very low compared to the high fat (>40%) used to induce obesity in laboratory animals. Based on our current understanding of these phyla shifts, the increased abundance of *Firmicutes* microbes seen when walnuts are incorporated continuously long-term into the diet may not be perceived as beneficial, but the animals consuming walnuts had greater microbial diversity than those animals on the replacement diet. Microbial diversity has been associated with better health outcomes, and this shift may be more important than the relative abundance of the *Firmicutes* and *Bacteriodetes* phyla. Low bacterial diversity has been linked to obesity and inflammatory bowel disease [27,28].

The major shift within the phylum *Bacteriodetes* was a decrease in the genus *Bacteroides*. A reduction in *Bacteroides* and increase in *Firmicutes* has been observed in response to the addition of whole grains to the diet [29]. Very few studies have investigated the effect of tree nuts on the gut microbiome. Burns et al. [24] found no changes at the phylum and family level following the addition of 1.5 oz. almonds to the diet for 3 weeks while Ukhanova et al. [30] found significant changes at the phylum and genus level when twice the dose of almonds was provided. Although walnuts and almonds are both considered tree nuts, they are distinctly different in composition. Walnuts contain less fiber but more phytochemicals/antioxidants and omega-3 fatty acids. Given this, a differential effect on the gut microbial community is not surprising.

Very little is known about the impact of nuts on the gut microbiome, but the available evidence strongly suggests that tree nuts alter gut microbial communities. Two human studies have been published on almonds [24,30]. Only one report has examined the impact of walnuts on the gut microbiome, and this study used a carcinogenesis model to produce colon cancer [16]. In humans, Ukhanova et al. [30] found almonds significantly modulated the microbiota at the phylum and genus levels and increased the relative abundance of butyrate producers, but not the number of lactate producers (*Lactobacillidus* or *Bifidobacteria*). Burns et al. [24] found almonds only modified the gut microbiome at the genus level. Several genera were altered but only one change was similar to walnuts; *Turicibacter* increased. One notable difference between the two almond studies was the dose of almonds consumed each day: 3 oz/day [30] vs. 1.5 g/day [24], respectively.

Nakanishi et al. [16] fed three levels of walnuts, 5.2%, 10.5% and 21.1% of total calories to mice chemically induced to grow colon cancers. They reported an increase abundance of *Firmicutes*, including *Lactobacillus*, *Clostridiales*, *Clostridium*, *Lachnospiraceae* and *Ruminococcaceae*. We found a similar bacterial signature except *Clostridium* was not significantly different between the two groups in the current study. There are several plausible reasons for this as there may have been an interaction between diet and the carcinogenesis model. Nakanishi et al. [16] found carcinogen treatment reduced microbe diversity and richness of the gut, so this could be one plausible explanation, as xenobiotics can alter the relative abundance of gut bacteria [31]. A second explanation could be a difference in the animal species. Nakanishi's model used mice while our study used rats. Finally, the bacterial signature observed in Nakanishi's study may be the result of inflammation-associated with colon tumorigenesis because changes have been reported by others studying colon carcinogenesis [32]. The animals in our study were healthy without known pathology.

Gut microbes produce many lipids with biological activity. For example, *Lactobacillus* and *Bacteroides* mediate synthesis of secondary bile acids and important components of lipid transport [33]. Walnuts increased both *Lactobacillus* and *Bacteroides* after long-term continuous consumption compared to the replacement diet.

Prebiotics are dietary substances that selectively promote proliferation and/or activity of "beneficial" colonic bacteria. Typically targeted are the genera *Bifidobacterium* and *Lactobacillus,* but there are several emerging probiotic candidates: *Ruminococcus bromii*, *Roseburia intestinalis*, *Eubacterium rectale* and *Faecalibactruim prausnitzii* [34,35]. Adding walnuts to the diet increased *Lactobacillus*, *Ruminococcus*, and *Roseburia* suggesting a prebiotic role for walnuts; some part of the walnut escaped assimilation in the small intestine and was fermented in the colon or events in the upper tract migrated downstream, positively altering the composition of the gut microbiome.

The addition of walnuts to the diet shifted the relative abundance of the inferred functional capacities of the microbial communities. Twelve KEGG metabolic pathways were affected. Further studies targeted at understanding these changes are needed since it is not clear if these changes are important for the microbes to flourish when walnuts are added to the diet and if there is an added host benefit. The greater functional capacity to degraded branch chain amino acids was suggested by the shift in relative abundance of microbes in the animals eating walnuts. Most likely this is related to the shifts in relative abundance of the microbes and their associated metabolic capacities. The diets were matched for protein content (Table 1) and the amino acid composition was similar (data not shown). Metabolomic studies have recently suggested branch chain amino acids may play a role in type 2 diabetes, fatty acid metabolism, and immunity [36–38].

Functional capacity for tryptophan metabolism was also increased with long-term consumption of walnuts in the diet. Tryptophan catabolism has been implicated in modulating the delicate balance between the immune system's response to pathogens and non-harmful antigens [39]. Also, the tryptophan metabolism pathway is known for serotonin, melatonin and niacin synthesis. Several recently published studies have linked gut microbes to brain health. Yano et al. showed that microbes indigenous to the gut can regulate host serotonin biosynthesis [40]. Several studies have been published suggesting walnuts can improve brain functions [7]. The connection between our observation and these other studies needs further investigation.

Both arachidonic and alpha-linolenic acid metabolism pathways were increased by continuous walnut consumption. Walnuts are an excellent source of omega-3 fatty acids, particularly alpha-linoleic acid. The KEGG arachidonic acid metabolism pathway involves the production of eicosanoids, for example, prostaglandins, prostacyclin, thromboxanes and 5-HETE, leukotrienes, 15-HPETE, 12-HETE, hepoxillins and anandamide. These are inflammatory-modulating molecules. At the same time, the metabolic pathways for alpha-linolenic acid are also more prevalent. Omega-3 fatty acids are generally considered anti-inflammatory. This KEGG pathway also produces a number of other molecules, like volicitin, but the importance of these has not been clearly delineated.

The functional capacity of microbes to degrade limonene and pinene was significantly greater in those animals consuming walnuts. Both these compounds are pheromones emitted by plants. Limonene gives lemons their characteristic smell while pinene is the most dominant volatile emitted by walnut trees [41]. Wang et al. recently showed that *Cyanobacteria* have enhanced limonene production [42] and several members of these phyla were significantly more abundant following long-term continuous consumption of walnuts.

5. Conclusion

In summary, we show that walnuts change the bacterial communities found in the descending colon. We propose that reshaping of the gut microbe community may play a physiological role in promoting walnut's health benefits and this needs further exploration.

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Authors' contributions

LOB designed the study and oversaw its conduct in the Animal Resource Center and her laboratory. MAP and SB provided the preliminary data that encouraged this study. BL completed the day to day care of the animals and the laboratory assays. ML completed the amplicon sequencing using fecal DNA isolated in LOB's laboratory by SB and BL. CMT and EB performed the bioinformatics analysis. CMT, DAW, and EB advised on the bioinformatics analysis. DS complete the metagenomics analysis. LOB completed the other data analysis. LOB, CMT and DS wrote the manuscript and DAW and MAP provided their expertise comment, as well as interpretation of the data, during the preparation of this manuscript. All authors read and approved the final manuscript.

Conflicts of interests

The authors declare that they have no competing interests.

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