

Microbiome, Metagenomics, Metaproteomics, and Xenometabolomics

# Almond Snacking for 8 wk Increases Alpha-Diversity of the Gastrointestinal Microbiome and Decreases *Bacteroides fragilis* Abundance Compared with an Isocaloric Snack in College Freshmen

Jaapna Dhillon,<sup>1</sup> Zhaoping Li,<sup>2</sup> and Rudy M Ortiz<sup>1</sup><sup>1</sup>School of Natural Sciences, University of California, Merced, CA, USA and <sup>2</sup>Center for Human Nutrition, David Geffen School of Medicine, Department of Medicine, University of California Los Angeles, California, CA USA

## ABSTRACT

**Background:** Changes in gut microbiota are associated with cardiometabolic disorders and are influenced by diet. Almonds are a rich source of fiber, unsaturated fats, and polyphenols, all nutrients that can favorably alter the gut microbiome.

**Objectives:** The aim of this study was to examine the effects of 8 wk of almond snacking on the gut (fecal) microbiome diversity and abundance compared with an isocaloric snack of graham crackers in college freshmen.

**Methods:** A randomized, controlled, parallel-arm, 8-wk intervention in 73 college freshmen (age: 18–19 y; 41 women and 32 men; BMI: 18–41 kg/m<sup>2</sup>) with no cardiometabolic disorders was conducted. Participants were randomly allocated to either an almond snack group (56.7 g/d; 364 kcal; n = 38) or graham cracker control group (77.5 g/d; 338 kcal/d; n = 35). Stool samples were collected at baseline and 8 wk after the intervention to assess primary microbiome outcomes, that is, gut microbiome diversity and abundance.

**Results:** Almond snacking resulted in 3% greater quantitative alpha-diversity (Shannon index) and 8% greater qualitative alpha-diversity (Chao1 index) than the cracker group after the intervention ( $P < 0.05$ ). Moreover, almond snacking for 8 wk decreased the abundance of the pathogenic bacterium *Bacteroides fragilis* by 48% (overall relative abundance,  $P < 0.05$ ). Permutational multivariate ANOVA showed significant time effects for the unweighted UniFrac distance and Bray–Curtis beta-diversity methods ( $P < 0.05$ ;  $R^2 \leq 3.1\%$ ). The dietary and clinical variables that best correlated with the underlying bacterial community structure at week 8 of the intervention included dietary carbohydrate (percentage energy), dietary fiber (g), and fasting total and HDL cholesterol (model Spearman rho = 0.16;  $P = 0.01$ ).

**Conclusions:** Almond snacking for 8 wk improved alpha-diversity compared with cracker snacking. Incorporating a morning snack in the dietary regimen of predominantly breakfast-skipping college freshmen improved the diversity and composition of the gut microbiome. This trial was registered at clinicaltrials.gov as NCT03084003. *Curr Dev Nutr* 2019;3:nzz079.

## Introduction

Specific gastrointestinal (gut) microbiome profiles are associated with obesity and cardiometabolic disorders (1). For example, an overweight phenotype and glucose intolerance are characterized by an increase in pathogenic bacteria and a decrease in anti-inflammatory and butyrate-producing bacteria (1). In addition, low gut microbiome diversity indicated by the number of operational taxonomic units (OTUs) is associated with greater adiposity (2), insulin resistance (2), dyslipidemia (increase in triglycerides and decrease in HDL cholesterol) (3), and a proinflammatory phenotype (2) compared with high gut microbiome diversity. Positive dietary



**Keywords:** adolescence, amplicon sequence variants, ANCOM, cardiovascular, functional foods, gut, metabolism, minority, nutrients, nuts

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Supplemental Figures 1–5 and Supplemental Tables 1–5 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/cdn>.

Address correspondence to JD (e-mail: [jdhillon5@ucmerced.edu](mailto:jdhillon5@ucmerced.edu), or [djaapna@gmail.com](mailto:djaapna@gmail.com)).

Abbreviations used: ANCOM, analysis of composition of microbiomes; ASV, amplicon sequence variant; CSS, cumulative-sum-scaled; NMDS, nonmetric, multidimensional scaling; OTU, operational taxonomic unit; PCoA, principal coordinates analysis; PERMANOVA, permutational multivariate ANOVA.

modulation of the gut microbiome by consuming diets rich in prebiotics (1), probiotics (1), and plant-based foods (4) can improve biomarkers of cardiometabolic health (5).

Nuts such as almonds are rich sources of fiber, unsaturated fats, and polyphenols (6), all nutrients that can favorably alter the gut microbiome (7–9). Differences in relative abundance of specific bacterial taxa have been observed with varying doses of almonds and pistachios (42 g or 84 g) (10), different almond processed forms (42 g) (11), and walnut (42 g) (12) consumption over short periods (<3 wk). Furthermore, whole almonds (56 g) and almond skin (10 g) supplementation for 6 wk increased abundance of targeted bacterial species such as *Bifidobacterium* spp. and *Lactobacillus* spp. (13). In addition, walnut (43 g) consumption for 8 wk increased the abundance of butyric acid-producing species (14). However, little is known about the effects of longer-term almond consumption in young adults.

We have previously demonstrated the effects of almond snacking on cardiometabolic profiles in college freshmen (15). As an extension of that study, we evaluated the impact of 8 wk of almond snacking (57 g/d) on the gut microbiome abundance and diversity compared with an isocaloric snack of graham crackers in college freshmen. A secondary analysis examined the association of dietary and clinical (anthropometric, gluco-regulatory, and cardiovascular) variables with the microbiome community structure. We also highlight issues with the use of common statistical frameworks in nutrition research that do not account for compositional constraints while analyzing differential abundance of microbiome data.

## Methods

### Participants

Seventy-three (41 women and 32 men) young adults (aged 18–19 y; BMI: 18–41 kg/m<sup>2</sup>) participating in a snacking intervention were recruited (15). The eligibility criteria were as follows: 1) aged 18–21 y; 2) newly enrolled, first-year college students with no nut allergies; 3) nonsmokers; and 4) no diagnosed endocrine or cardiometabolic disorders. Participants were recruited via public advertisements. Participants who met eligibility criteria provided written, informed consent prior to commencement of study visits. All procedures involving human subjects were approved by the University of California Merced Institutional Review Board. The study is registered on clinicaltrials.gov (registration number: NCT03084003).

### Study design and protocol

The primary study was an 8-wk randomized, controlled, parallel-arm intervention examining the effects on gluco-regulatory profiles of almonds compared with cracker snacking (15). The sample size calculations for the primary study were based on glucose and insulin profiles at the end of the 8-wk intervention (15). For the present analysis, outcomes related to the gut microbiome were examined. In brief, participants were assigned into 1 of 2 study arms. Participants in the almond group ( $n = 38$ ) consumed 57 g/d [2 oz; 327 kcal; 14% carbohydrate (8 g fiber), 74% fat, 13% protein] of whole, dry-roasted almonds. Participants in the cracker group ( $n = 35$ ) consumed 5 sheets (77.5 g/d) of graham crackers [325 kcal; 74% carbohydrate (2.5 g fiber), 20% fat, 6% protein] and were asked to avoid all nuts, seeds,

and nut-containing products. Stool samples were collected in sterilized collection containers at baseline and 8 wk after the intervention and stored at  $-80^{\circ}\text{C}$ . Anthropometric, biochemical, and dietary data were collected and analyzed as described previously (15).

### DNA extraction and sequencing

DNA from homogenized stool samples was extracted using the MoBio power soil DNA isolation kit (MoBio Laboratories, Inc). The quality and quantity of the DNA was confirmed using a Nanodrop 1000 (Thermo Fisher Scientific). The 16S rRNA gene V4/V5 variable region PCR primers 530F/926R, GTGCCAGCMGC-NGCGG/CCGTCAATYYTTTRAGTTT, with barcode on the forward primer, were used in a 28-cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen) under the following conditions:  $94^{\circ}\text{C}$  for 3 min, followed by 28 cycles of  $94^{\circ}\text{C}$  for 30 s,  $53^{\circ}\text{C}$  for 40 s, and  $72^{\circ}\text{C}$  for 1 min, after which a final elongation step at  $72^{\circ}\text{C}$  for 5 min was performed. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Sequencing was performed at MR DNA ([www.mrdnalab.com](http://www.mrdnalab.com)) on a MiSeq (Illumina).

### Sequence quality control

The raw sequence data from the Illumina platform were converted into forward and reverse read files using the FASTQ processor ([www.mrdnalab.com](http://www.mrdnalab.com)), which were then imported to QIIME 2, an open-source microbiome analysis platform (16) for further analysis. The paired-end sequences were demultiplexed, and then denoised, dereplicated, and merged with the DADA2 quality control package (17) in QIIME2. The amplicon sequence variants (ASVs: features and associated representative sequences) resulting from DADA2, are the final products used in all downstream analyses. ASVs are higher-resolution artifacts than commonly used operational taxonomic units (OTUs) (18). A total of 3259 ASVs were detected after demultiplexing and DADA2 quality control.

### Taxonomic analysis

The ASVs were assigned taxonomy in QIIME2 using a naive Bayes classifier, which was trained on the 530–926 region of the Greengenes 13\_8 database reference sequences clustered at 99% sequence similarity (19). The differential abundance of the ASVs at the different taxonomic levels, namely, phylum, class, family, order, genus, and species, was analyzed using the analysis of composition of microbiomes (ANCOM) statistical framework in R (version 3.5.2) (20). The ANCOM framework uses standard statistical tests to compare Aitchison log-ratios of observed abundances of a taxon relative to a predefined taxon with adjustment for multiple testing (here the Benjamini–Hochberg procedure) (20). For example, testing the effect of snack group on a specific feature, when a total of 5 features are observed, would involve computation of 4 log-ratios for that feature, and the snack effect  $P$  value for each ratio would need to be adjusted for multiple testing of 4 ratios. The overall statistical significance of the snack effect would then be determined by the number of subhypotheses (per ratio) rejected compared with a cutoff value (set at 0.6). We included the following statistical tests within the flexible ANCOM framework: 1) linear mixed model analysis with time and group as fixed factors, and participant as a random effect; and 2) nonparametric analysis of time and group

effects using the nparLD package in R (21). Because both the parametric and nonparametric tests gave similar results, we have reported only the parametric results. The ANCOM framework was also modified to test for effects of BMI category and sex, as well as pairwise comparisons for significant interaction effects with adjustment for multiple comparisons using the Bonferroni procedure. Only taxa prevalent in at least 25% of the samples were included in the analysis to avoid confounding results due to low-frequency taxa (20).

### Alpha- and beta-diversity analysis

Alpha-diversity measures were assessed on the raw abundances and abundances of sequences rarefied to an even sequence depth of 8268 sequences per sample in QIIME2 (Supplemental Figure 1). The following alpha-diversity measures were assessed: 1) Chao1 index, a measure of species richness that is particularly useful for low-abundance datasets (22); 2) observed OTUs, a measure of the number of distinct features; 3) Shannon index, a measure of richness and evenness (23); and 4) Simpson evenness measure, a measure of how well represented a species is (24). The alpha-diversity measures were analyzed in R using linear mixed-model analysis with time and snack as fixed factors and participant as a random effect. Analyses were adjusted for baseline when baseline values had a significant effect on the model.

Phylogenetic beta-diversity measures such as weighted UniFrac (quantitative, i.e., weighs branches of phylogenetic tree based on abundance) and unweighted UniFrac (qualitative, i.e., fraction of unique branches in phylogenetic tree) (25), and nonphylogenetic Bray–Curtis dissimilarity, which quantifies differences between samples based on abundance per count (26), were assessed on the raw, rarefied, and cumulative-sum-scaled (CSS) datasets. The ordinations of the matrices obtained from the raw, rarefied, and CSS datasets were compared using Procrustes analysis in R (27). The effects of time and time  $\times$  snack on the distance/dissimilarity matrices were analyzed by the permutational multivariate ANOVA (PERMANOVA) (28,29) framework in the vegan package of R (27).

### Taxa and environmental associations with community structure

The structural redundancy in community composition was quantified through the BVSTEP (30) analysis in the sinkr package of R (31). The input dataset for the taxa variables consisted of the ASV abundances (at the class level). The best set of environmental (i.e., clinical and dietary) variables associated with the community structure (i.e., ASVs abundance) was deduced by computing the maximum (rank) correlation of all environmental variables with Bray–Curtis dissimilarities using the BIOENV (32) analysis in the vegan package of R (27). The significance for the BVSTEP and BIOENV procedures was assessed using the Mantel test (27). The input dataset for the environmental variables comprised: snack group (week 8 only), sex; body mass; BMI; fat mass percentage; waist circumference; systolic and diastolic blood pressures; fasting total, HDL, and LDL cholesterol; fasting triglycerides; fasting insulin; fasting glucose; total energy intake; dietary carbohydrate percentage; dietary fat percentage; dietary protein percentage; dietary fiber; total saturated fat; MUFAs; PUFAs; oleic acid; linoleic acid; and  $\alpha$ -tocopherol.

The best subsets of taxa and environmental variables at baseline and 8 wk after the intervention were plotted as vectors along nonmetric, multidimensional scaling (NMDS) plots, and the significance of the

individual variables on the 2-dimensional ordinations was assessed using vegan function envfit (27). It is important to note that the results of envfit do not supersede the results of the BVSTEP and BIOENV analyses, but instead describe the individual contribution of the best model variables to the 2-dimensional ordinations.

## Results

### Participant characteristics and findings from parent study

The participants' demographic, clinical, and dietary characteristics have been described in detail previously (15). The parent study exploring the glucoregulatory and cardiometabolic outcomes demonstrated a smaller decline in HDL cholesterol but similar reductions in fasting glucose and LDL cholesterol, and greater postprandial insulin sensitivity during the oral-glucose-tolerance test following almond consumption for 8 wk compared with cracker consumption (15).

### ANCOM results of selected taxa prevalent in at least 25% of the samples

*Firmicutes* (64%), *Bacteroidetes* (29%), and *Actinobacteria* (4%) were the most dominant bacterial phyla in this study population at baseline. The ANCOM results depict an overall significant time effect ( $P < 0.05$ ) indicating an increase over 8 wk for order RF39 (phylum *Tenericutes*) and decrease for family S24.7 (phylum *Bacteroidetes*), and genera *Alistipes*, *Butyrivimonas*, and *Odoribacter* (all in phylum *Bacteroidetes*), and an increase for genus *Lachnospira* (phylum *Firmicutes*) (Table 1). In addition, *Bacteroides fragilis* decreased significantly ( $P < 0.05$ ) in the almond group over the 8-wk intervention (time  $\times$  snack effect,  $P < 0.05$ ; Table 1). The relative abundance percentages of all selected taxa at the different taxonomic levels are shown in Supplemental Table 1.

### Almond group had greater alpha-diversity at week 8 compared with the cracker group

Chao1 index, observed OTUs, and Shannon index measures for the raw and rarefied abundances significantly increased (time effect,  $P < 0.05$ ), whereas the Simpson evenness measure for raw abundances significantly decreased (time effect,  $P < 0.05$ ) over the 8-wk intervention (Figure 1, Supplemental Table 2). The almond group had significantly greater baseline-adjusted Chao1 index and Shannon index ( $P < 0.05$ ) for raw and rarefied abundances and observed OTUs ( $P < 0.05$ ) measures for rarefied abundances at week 8 (Figure 1, Supplemental Table 2). The rarefaction curves for Chao1 index, observed OTUs, and Shannon index (Supplemental Figures 2–5) confirm the greater diversity, at different sequencing depths, in the almond group compared with the cracker group at week 8 of the intervention.

### Procrustes analysis indicated that the raw and rarefied datasets were highly correlated

The principal coordinates analysis (PCoA) plots of the weighted and unweighted UniFrac measures of the raw data at baseline and week 8 of the intervention are depicted in Figure 2. Procrustes analysis found that the raw compared with rarefied ordinations of weighted and unweighted UniFrac measures and Bray–Curtis dissimilarities were highly correlated (Procrustes correlation coefficient  $>0.94$ ;  $P < 0.01$ ;

**TABLE 1** ANCOM results of selected taxa prevalent in at least 25% of the samples obtained from college freshmen in the almond and cracker groups at baseline and 8 wk after the intervention

Taxa	Relative abundance (%) <sup>1</sup>				W-taxa <sup>2</sup>		
	Baseline		Week 8		Time	Snack	Time x snack
	Almond (n = 38)	Cracker (n = 35)	Almond (n = 38)	Cracker (n = 35)			
p_Tenericutes.c_Mollicutes.o_RF39	0.35 ± 1.35	0.49 ± 2.35	0.54 ± 1.35	0.87 ± 2.92	10*	0	0
p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_S24.7	0.06 ± 0.2	0.11 ± 0.35	0.08 ± 0.25	0.04 ± 0.08	26*	0	0
p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Rikenellaceae.g_Alistipes	4.33 ± 5.22	2.63 ± 3.4	2.41 ± 3.09	2.07 ± 3.19	36*	0	0
p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Odoribacteraceae.g_Butyricimonas	0.08 ± 0.19	0.12 ± 0.48	0.06 ± 0.16	0.03 ± 0.1	37*	0	0
p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Odoribacteraceae.g_Odoribacter	0.25 ± 0.36	0.2 ± 0.34	0.09 ± 0.14	0.11 ± 0.19	39*	0	0
p_Firmicutes.c_Clostridia.o_Clostridiales.f_Lachnospiraceae.g_Lachnospira	0.08 ± 0.12	0.1 ± 0.18	0.16 ± 0.17	0.1 ± 0.19	45*	0	0
p_Proteobacteria.c_Betaproteobacteria.o_Burkholderiales.f_Alcaligenaceae.g_Sutterella	0.19 ± 0.37	0.08 ± 0.15	0.71 ± 1.61	0.13 ± 0.33	4	38*	0
p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Bacteroidaceae.g_Bacteroides.s_fragilis	0.66 ± 1.22**	0.66 ± 1.6	0.34 ± 0.76	0.76 ± 1.61	3	0	68*

<sup>1</sup>Values presented are means ± SDs of relative abundance percentages of participants in the almond and cracker groups at baseline and week 8 of the intervention.

\*\*Significantly different from week 8. ANCOM, analysis of composition of microbiomes; c, class; f, family; g, genus; o, order; p, phylum; s, species.

<sup>2</sup>W-taxa represent the number of significant log-ratios for that taxon at the time, snack, and time × snack levels. \*Denotes overall significance at a 60% cutoff, that is, at least 60% of the log-ratios were significant (i.e.,  $P < 0.05$ ) after adjusting for multiple testing of log-ratios using the Benjamini–Hochberg procedure. Analysis was a linear mixed model analysis within the ANCOM framework.

Supplemental Table 3) indicating that the raw and rarefied matrices are similar.

#### PERMANOVA analysis indicated differences in beta-diversity over time for the raw and rarefied datasets

PERMANOVA analyses showed significant, albeit small, time effects for the unweighted UniFrac distance and Bray–Curtis dissimilarity matrices of the raw and rarefied datasets ( $P < 0.05$ ;  $R^2 \leq 3.1\%$ ; Supplemental Table 4), which are not visible in the 2-dimensional PCoA plots (Figure 2). There were no significant effects of snack and time × snack on any distance/dissimilarity matrices. The best subset of taxa contributing to the community structure at baseline and week 8 is explored in the section below.

#### The structural redundancy in the community structure was characterized by 40–55% of the identified microbial classes

The best correlated subset of taxa variables included 10 of the 24 identified microbial classes at baseline (model Pearson correlation coefficient = 0.96;  $P = 0.001$ ; Figure 3A) and 13 of 24 classes at week 8 (model Pearson correlation coefficient = 0.96;  $P = 0.001$ , Figure 3B). Of notable interest are the absence of *Fusobacteria* and *Alphaproteobacteria* from the week 8 model, and the presence of *Coriobacteria* and *Mollicutes* in the week 8 model. The 2-dimensional NMDS ordination at baseline was (individually) significantly associated with the *Bacteroidia* (phylum *Bacteroidetes*), *Bacilli*, *Clostridia*, and *Erysipelotrichi* (phylum *Firmicutes*), RF3 (phylum *Tenericutes*), and *Verrucomicrobiae* (phylum *Verrucomicrobia*) classes ( $P < 0.05$  envfit test; Figure 3A, Supplemental Table 5). The 2-dimensional NMDS ordination at week 8 of the intervention was (individually) significantly associated with the *Bacteroidia* (phylum *Bacteroidetes*), *Erysipelotrichi* (phylum *Firmicutes*), *Coriobacteria* and *Actinobacteria* (phylum *Actinobacteria*), *Mollicutes*

(phylum *Tenericutes*), *Verrucomicrobiae* (phylum *Verrucomicrobia*), and *Deltaproteobacteria* (phylum *Proteobacteria*) classes ( $P < 0.05$  envfit test; Figure 3B, Supplemental Table 5).

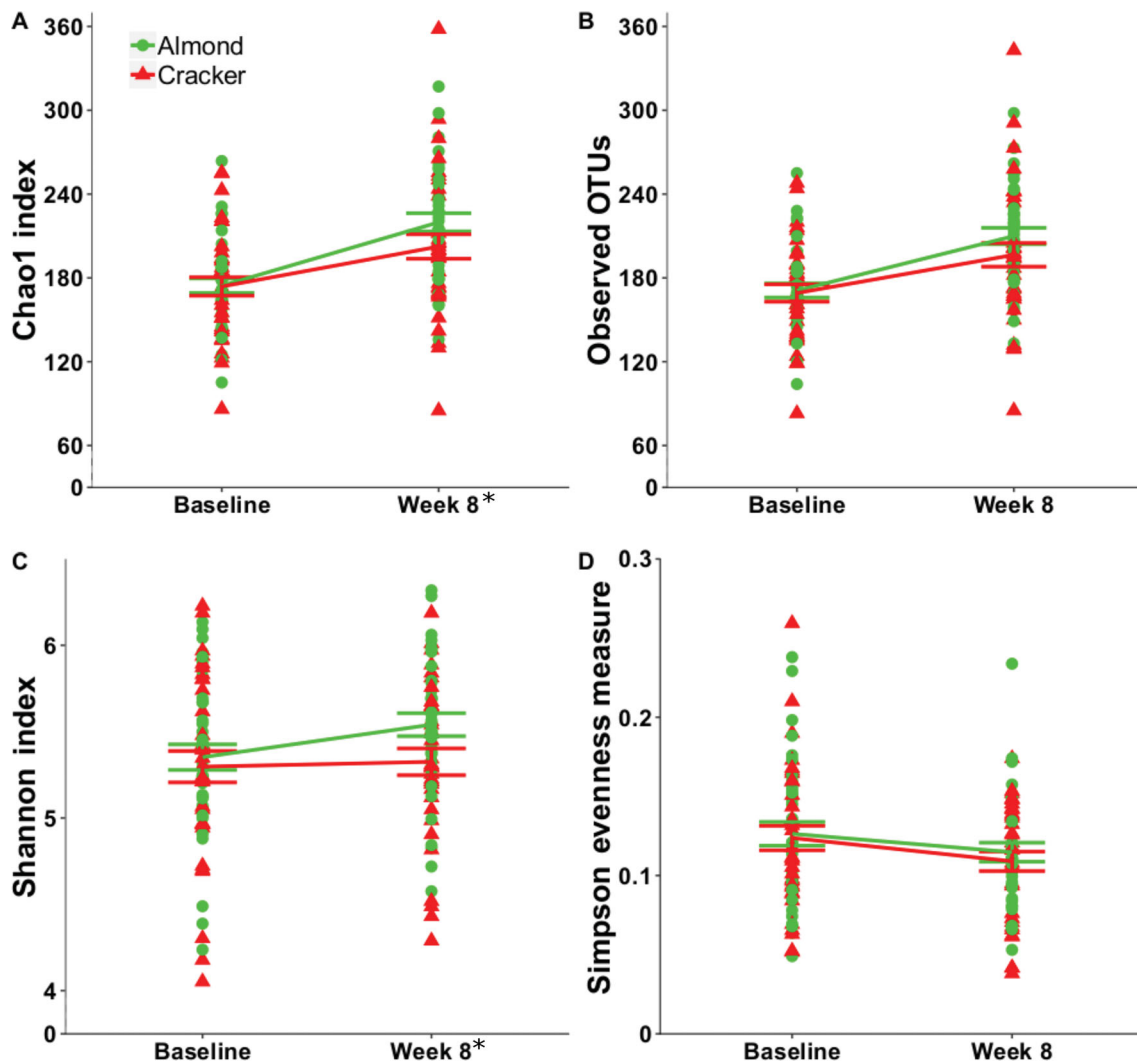
#### Dietary and clinical variables contributed to a small amount of variation in the community structure

The best correlated subset of environmental variables at baseline (model Spearman rho = 0.17;  $P = 0.014$ ) and week 8 (model Spearman rho = 0.16;  $P = 0.01$ ) is depicted in Figure 3C,D. The subset of environmental variables with the highest correlation with the community structure comprised dietary carbohydrate (%), fasting glucose, and fasting insulin at baseline (Figure 3C, Supplemental Table 5) and dietary carbohydrate (%), dietary fiber, and fasting total and HDL cholesterol at week 8 (Figure 3D, Supplemental Table 5). Moreover, fasting HDL cholesterol demonstrated a statistically significant association with the 2-dimensional NMDS ordination at week 8 of the intervention ( $R^2 = 10\%$ ;  $P < 0.05$  envfit test; Supplemental Table 5).

#### Discussion

Almond snacking (57 g/d) for 8 wk resulted in 3% greater quantitative alpha-diversity (Shannon index) and 8% greater qualitative alpha-diversity (Chao1 index) compared with isocaloric cracker snacking at the end of the 8-wk intervention. In addition, almond snacking decreased overall *B. fragilis* relative abundance by 48%. The snacking intervention increased alpha-diversity over time, but the unique nutrient profile of almonds had a greater impact on alpha-diversity than graham crackers. Increased bacterial richness is associated with favorable health outcomes (2). In this same cohort of study participants, glucose tolerance and postprandial insulin sensitivity were improved with almond snacking (15), suggesting that improved gut



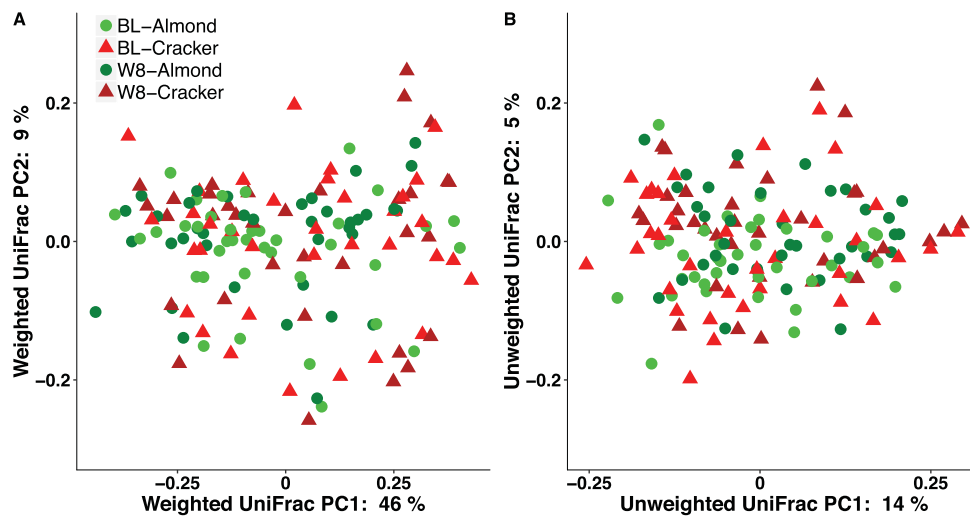


**FIGURE 1** Alpha-diversity indices (calculated from raw abundances). (A) Chao1 index, (B) observed OTUs, (C) Shannon index, and (D) Simpson evenness measure of college freshmen in the almond and cracker groups at baseline and week 8 of the intervention. Values are individual data points representing each participant at baseline and week 8. Means  $\pm$  SDs of the 2 snack groups at baseline and week 8 are also plotted. Analyses were conducted using 1) linear mixed model with snack (almond or cracker) as between-subject factor, time (baseline or week 8) as within-subject factor, and participant as random factor; and 2) analysis of covariance with baseline value as covariate and snack as between-subject factor. \*Snack effect (adjusting for baseline value),  $P < 0.05$ . Almond:  $n = 38$ , cracker:  $n = 35$ . OTU, operational taxonomic unit.

microbiome and carbohydrate metabolism could be associated. The fiber, monounsaturated fat, and polyphenol content of almonds are likely responsible for the greater alpha-diversity. The beneficial effects of dietary fiber on modulating the gut microbiome diversity are well characterized (33). Monounsaturated fats (compared with saturated fats) are associated with promoting a positive gut microbiome profile as well (34). Polyphenols in almond skins are partially bioavailable (35), and the unabsorbed polyphenols are metabolized by specific colon microbiota into absorbable metabolites (8). Sustained consumption of polyphenol-rich diets can stimulate the growth of beneficial bacteria (8), thereby promoting greater diversity as well.

Other nut studies have either not assessed (13) or have not detected any significant differences in alpha-diversity (10–12, 14). Three of

those studies were short-term (<3 wk) (10–12), suggesting that the intervention duration was not sufficient to induce significant changes in species richness as seen in other short-term dietary interventions (36, 37). Conversely, an 8-wk walnut intervention did not alter alpha-diversity; however, phylogenetic beta-diversity (between-sample diversity) was different by 5% between the walnut and control diets (14). In the present study, the 8-wk intervention can only explain, at most, 3% of the variance in beta-diversity with no differential effect of snack group. However, the beta-diversity was positively correlated with alpha-diversity ( $P < 0.01$ , data not shown) suggesting that beta-diversity increased over time as well. Increased microbiome diversity could promote greater stability of the microbiome in the long term, thereby contributing to functional resilience against extreme stress and



**FIGURE 2** Principal coordinates analysis plots of beta-diversity measures (calculated from raw abundances). (A) Weighted UniFrac, and (B) unweighted UniFrac of college freshmen in the almond and cracker groups at baseline and week 8 of the intervention. Almond:  $n = 38$ , cracker:  $n = 35$ . BL, baseline; PC, principal component; W8, week 8.

perturbations as purported by the classical ecological resilience theory (38, 39).

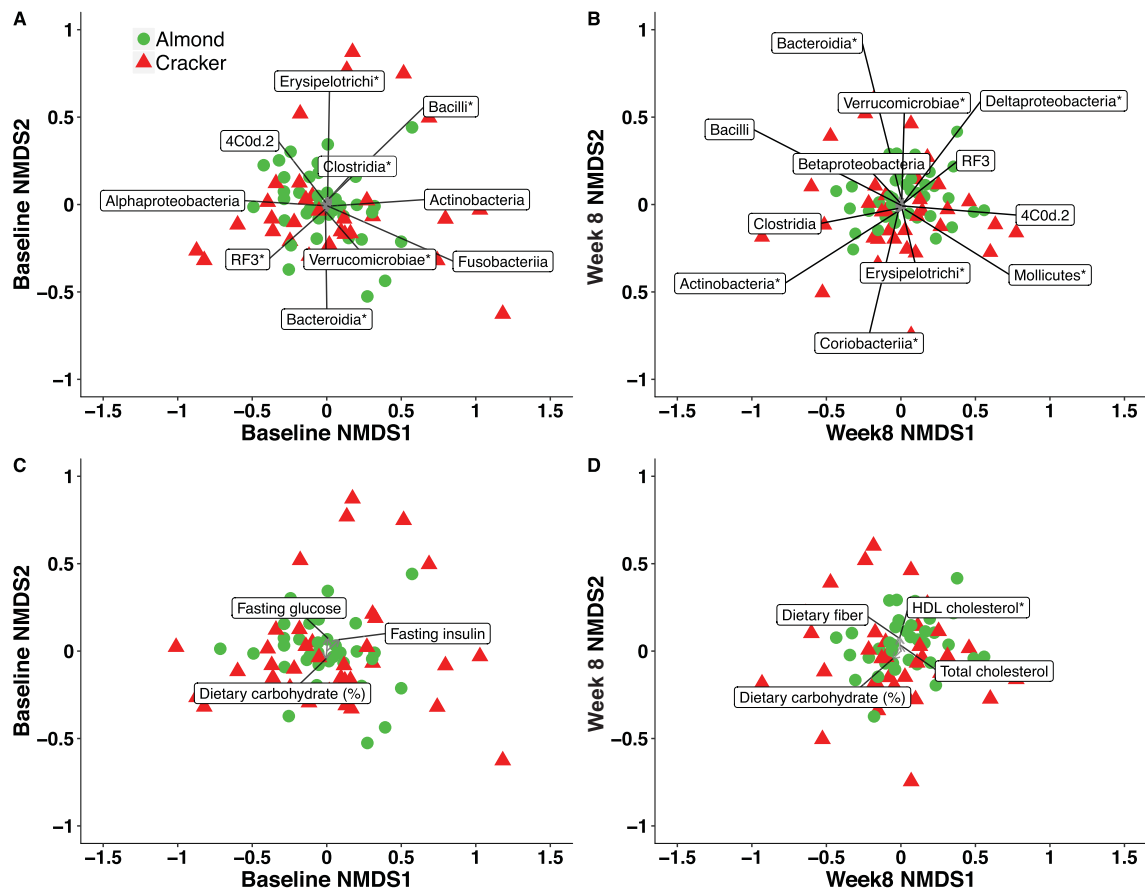
Another important finding of the present study was the decrease in *B. fragilis* with almond consumption. *B. fragilis* is an anaerobic pathogen that is most frequently isolated from clinical specimens and is considered the most virulent *Bacteroides* species because of its: 1) adhesion properties that facilitate adherence to host tissues, 2) lipopolysaccharide capsule that protects it from the host's immune system, and 3) histolytic enzyme activity (40). Although enterotoxins produced by specific *B. fragilis* strains can cause gastrointestinal inflammation (41), other reports indicate that colonization of germ-free mice with polysaccharide-producing *B. fragilis* can contribute to the maturation of the immune system (42). We did not assess inflammatory profiles or immune system markers in this study; however, Sugizaki and Naves (6) propose that the dietary fiber and polyphenol component of nuts can promote a homeostatic intestinal state via host-microbe interactions.

Over 90% of the variation in the bacterial community structure pre- and post-intervention was explained by ~40–50% of taxa at the class level. The taxa characterizing the structural redundancy were mostly similar at both time points of the intervention with the notable contributions of classes *Mollicutes* (phylum *Tenericutes*), which was predominantly composed of the order RF39, and *Coriobacteriia* (phylum *Actinobacteria*), which was mostly composed of the family *Coriobacteriaceae* at week 8 of the intervention. The relation between bacteria in the order RF39 and the host's metabolism is not defined. However, twin studies demonstrated that an increased abundance of RF39 was associated positively with a lean phenotype (43) and negatively with metabolic syndrome (44). The *Coriobacteriaceae* in the gut could contribute to the modulation of bile acid, steroid, and dietary polyphenol metabolism (45).

Other favorable changes in the gut microbiome profile over the 8-wk intervention involved a decrease in *Alistipes* (phylum *Bacteroidetes*), which is indicative of a shift away from an animal-based diet,

and increase in *Lachnospira* (phylum *Firmicutes*), which are pectin degraders (46). Additionally, a decrease in the butyric acid producers, *Odoribacter* and *Butyricimonas* (*Bacteroidetes* genera), was observed over 8 wk. Although a decrease in butyric acid-producing potential is typically associated with cardiometabolic disorders (47), an increase in butyric acid producers of the order *Bacteroidales* was reported in murine models of colitis (48, 49). As noted before, in times of physiological stress such as inflammation, there can be an increased reliance of colonocytes on butyric acid for energy, hence promoting the growth of butyric acid producers (48). Thus, it can be postulated that a decrease in stress can reduce the reliance on butyric acid producers. In addition, the abundance of uncultured *Bacteroidetes* family S24-7 decreased over 8 wk, but this has not been well studied in humans. However, murine studies report conflicting effects of diets on S24-7 in obese and diabetes models (50–52).

Dietary components such as carbohydrate and fiber, and cardiometabolic markers such as total and HDL cholesterol, explained <3% of the variation in the bacterial community composition at week 8 of the intervention in this cohort. The association of carbohydrate and fiber intake with the underlying community structure is not surprising because these are the principal energy sources for bacteria (53). Additionally, we have demonstrated that incorporating a morning snack into the diet of these predominantly breakfast-skipping college freshmen improved fasting glucose, and total and LDL cholesterol, with a protective effect of almond consumption on HDL cholesterol (15). Hence, changes in cholesterol profiles could partly be related to the gut microbiome composition. In some studies, intrinsic factors outweighed dietary intervention and drove community structure (54), whereas in others, dietary factors that were significantly associated with interindividual variation of gut microbiome outnumbered other intrinsic factors (55). The present study population was homogeneous in terms of age (18–19 y), predominantly belonged to ethnic/racial minority groups, and was at low to moderate cardiometabolic risk (15). Hence, intra- and inter-individual factors such as genetics and



**FIGURE 3** Nonmetric multidimensional scaling (NMDS) plot of the Bray–Curtis dissimilarities of college freshmen with best set of (A) taxa variables at baseline, (B) taxa variables at week 8, (C) environmental variables at baseline, and (D) environmental variables at week 8. NMDS baseline stress = 0.176, NMDS week 8 stress = 0.198. \* $P < 0.05$  from envfit. The black line shows the direction of the (increasing) gradient, and the length of the line is proportional to the correlation between the variable and the NMDS score. Line lengths should not be compared across plots. Almond:  $n = 38$ ; cracker:  $n = 35$ .

environment (43) could have a greater influence on the gut microbiome composition.

Developing statistical models for analyzing differential abundance of taxa is an active area of research. Compositional data such as relative abundances exist in the simplex rather than Euclidean space, that is, relative abundances across all taxa sum to 1 within a given sample. Because standard statistical tests such as  $t$  test, ANOVA, linear regression, and others do not account for the simplex nature of compositional data, they should not be directly used for analyzing relative abundance data. The ANCOM method is a highly sensitive statistical framework that uses Aitchison log-ratios to test for differences in the mean abundances of log-transformed taxa, which can be used to draw inferences regarding relative abundance differences in the ecosystem (20) (represented by the gut here). The ANCOM method controls the false discovery rate, while maintaining high power (20, 56), and can incorporate standard statistical tests within the framework.

Strengths of the study included: 1) excellent participant compliance (15), 2) the use of high-resolution ASVs, and 3) the incorporation of the sensitive and powerful ANCOM method for analyzing differential abundance. Limitations included: 1) the lack of a “no snack” group

to capture changes in the gut microbial profile in response to the students’ regular diet, and 2) the use of 16S rRNA profiling instead of shotgun sequencing. However, the 16S taxonomic resolution concerns are partly mitigated by sequencing ASVs (57) of the V4 and V5 variable regions.

In conclusion, almond snacking for 8 wk led to greater alpha-diversity than the isocaloric cracker group in college freshmen. In addition, almond snacking decreased the relative abundance of *B. fragilis*, a commonly isolated pathogenic bacterium. In general, incorporating a morning snack in the dietary regimen of predominantly breakfast-skipping college freshmen improved the diversity and composition of the gut microbiome.

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