TITLE

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

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ABBREVIATIONS

ANCOM: analysis of composition of microbiomes, ASV: amplicon sequence variant, OTU: operational taxonomic unit, PERMANOVA: permutational multivariate analysis of variance

AUTHOR DISCLOSURES

RMO discloses grants and non-financial support from Almond Board of California for conducting the study. JD and ZL have no conflicts of interest.

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 that can favorably alter the gut microbiome.

Objective: The study aimed to examine the effects of 8 weeks of almond snacking on the gut (fecal) microbiome diversity and abundance compared to an isocaloric snack of graham crackers in college freshmen.

Methods: A randomized controlled, parallel-arm, 8-week intervention of 73 college freshmen (age: 18-19 years, 41 women and 32 men, BMI: 18-41 kg/m^2) with no cardiometabolic disorders was conducted. Participants were randomized into either an almond snack group (56.7 g/day; 364 kcal; n = 38) or graham cracker control group (77.5 g/day; 338 kcal/d; n =35). Stool samples were collected at baseline and 8 weeks after the intervention to assess primary microbiome outcomes i.e. 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 weeks decreased the abundance of *Bacteriodes fragilis*, a pathogenic bacterium by 48% (overall relative abundance, $P<0.05$). PERMANOVA analyses show significant time effects for the unweighted Unifrac distance and Bray Curtis beta-diversity methods ($P<0.05$, R-squared$\leq$3.1%). The dietary and clinical variables that were best correlated with the underlying bacterial community structure at week 8 of the intervention included dietary carbohydrate (% energy), dietary fiber (g), and fasting total and HDL cholesterol (model Spearman's rho=0.16, $P=0.01$).

Conclusions: Almond snacking for 8 weeks improved alpha-diversity compared to 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.

KEYWORDS

Adolescence; Amplicon sequence variants, ANCOM, Cardiovascular; Functional foods; Gut; Metabolism; Minority, Nutrients; Nuts

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 pro-inflammatory phenotype (2) compared to high gut microbiome diversity. Positive dietary 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 pistachio (42 g or 84 g) (10), different almond processed forms (42 g) (11), and walnut (42 g) (12) consumption over short periods (<3 weeks). Furthermore, whole almonds (56 g) and almond skin (10 g) supplementation for 6 weeks increased abundance of targeted bacterial species such as *Bifidobacterium* spp. and *Lactobacillus* spp. (13). In addition, walnut (43 g) consumption for 8 weeks 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 weeks of almond snacking (57g/day) on the gut microbiome abundance and diversity compared to an isocaloric snack of graham crackers in college freshmen. A secondary

analysis examined the association of dietary and clinical (anthropometric, glucoregulatory, 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 (18–19 years old, BMI: 18-41 kg/m^2) participating in a snacking intervention were recruited (15). The eligibility criteria were as follows: (a) 18-21 years of age, (b) newly enrolled, 1st-year college students with no nut allergies, (c) non-smokers, and (d) 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 (UC) Merced Institutional Review Board. The study is registered on ClinicalTrials.gov (registration number: NCT03084003).

Study design and protocol

The primary study was an 8-week randomized, controlled, parallel-arm intervention examining the effects of almonds vs. cracker snacking for 8 weeks on glucoregulatory profiles (15). The sample size calculations for the primary study were based on glucose and insulin profiles at the end of the 8-week intervention (15). For the present analysis, outcomes related to the gut microbiome were examined. In brief, participants were assigned into one of two study arms. Participants in the almond group (n=38) consumed 57 g/d (2 oz; 327 kcal; 14% carbohydrate (8g 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 weeks after the intervention and stored at -80 °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., Carlsbad, CA). The quality and quantity of the DNA was confirmed using a Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, DE). The 16S rRNA gene V4/V5 variable region PCR primers 530F/926R: GTGCCAGCMGCNGCGG / CCGTCAATTYYTTTRAGTTT with barcode on the forward primer were used in a 28 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. After amplification, PCR products are 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, Shallowater, TX, USA) on a MiSeq (Illumina, San Diego, CA).

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, Shallowater, TX, USA), 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 (features and associated representative sequences, ASVs), 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 Native 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, i.e. phyla, 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's log-ratios of observed abundances of a taxa relative to a predefined taxa with adjustment for multiple testing (here 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 sub-hypotheses (per ratio) rejected in comparison to 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). Since both the parametric and non-parametric tests gave similar results, we've 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/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's index, a measure of richness and evenness (23), and 4) Simpson's 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 non-phylogenetic Bray-Curtis dissimilarity, which quantifies differences between samples based on abundance/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 x snack on the distance/dissimilarity matrices were analyzed by the permutational, multivariate analysis of variance (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). 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) were 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). Input dataset for the environmental variables comprised of snack group (week 8 only), sex, body mass, body mass index (BMI), fat mass percent, waist circumference, systolic and diastolic blood pressures, fasting total, HDL, and LDL cholesterol, fasting triglycerides, fasting insulin, fasting glucose, total energy intake, dietary carbohydrate percent, dietary fat percent, dietary protein percent, dietary fiber, total saturated fat, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), oleic acid, linoleic acid, and alpha-tocopherol.

The best subsets of taxa and environmental variables at baseline and 8 weeks after the intervention were plotted as vectors along non-metric, multi-dimensional scaling (NMDS) plots and the significance of the individual variables on the 2D ordinations were assessed using vegan function envfit (27). It is important to note that that the results of envfit don't supersede the results of the BVSTEP and BIOENV analyses, but instead describe the individual contribution of the best model variables to the 2D ordinations.

RESULTS

Participant characteristics and findings from parent study

The participant 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 OGTT following almond consumption for 8 weeks compared to cracker consumption (15).

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

Firmicutes (64%), Bacteriodetes (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 weeks for order *RF39* (phylum Tenericutes) and decrease for family *S24.7* (phylum Bacteroidetes), and genera *Alistipes*, *Butyricimonas*, 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-week intervention (time x 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 to cracker group

Chao1 index, observed OTUs and Shannon index measures for the raw and rarefied abundances significantly increased (time effect, $P<0.05$), while Simpson's evenness measure for raw abundances significantly decreased (time effect, $P<0.05$) over the 8-week 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 OTU ($P<0.05$) 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 to the cracker group at week 8 of the intervention.

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

The principal coordinate 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** respectively. Procrustes analysis found that the raw vs. rarefied ordinations of weighted and unweighted Unifrac measures and Bray-Curtis dissimilarities were highly correlated (Procrustes correlation coefficient>0.94, $P<0.01$, **Supplemental Table 3**) indicating that the raw and rarefied matrices are similar.

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

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

The structural redundancy in the community structure is characterized by 40 to 55% of the identified microbial classes

The best correlated subset of taxa variables included 10 out of the 24 identified microbial classes at baseline (model Pearson's correlation coefficient=0.96, P=0.001, **Figure 3a**) and 13 out of 24 classes at week 8 (model Pearson's correlation coefficient=0.96, P=0.001, **Figure 3b**). Of notable interests 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 2D NMDS ordination at baseline was (individually) significantly associated with the Bacteroidia (Phylum Bacteriodetes), 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 2D NMDS ordination at week 8 of the intervention was (individually) significantly associated with the Bacteroidia (Phylum Bacteriodetes), 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's rho=0.17, P=0.014) and week 8 (model Spearman's rho=0.16, P=0.01) are depicted in **Figures 3c and 3d**. The subset of environmental variables with the highest correlation with the community structure were 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 2D NMDS ordination at week 8 of the intervention (R-squared=10%, P<0.05 envfit test, Supplemental Table 5**)**.

DISCUSSION

Almond snacking (57 g/day) for 8 weeks resulted in 3% greater quantitative alpha-diversity (Shannon index) and 8% greater qualitative alpha-diversity (Chao1 index) compared to isocaloric cracker snacking at the end of the 8-week 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 microbiome and carbohydrate metabolism may be associated. The fiber, monounsaturated fats, and polyphenols 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 (versus 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 weeks) (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-week 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-week 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 may promote greater stability of the microbiome in the long-term, thereby contributing to functional resilience against extreme stress and perturbations as is 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 as 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 enzymes activity (40). While enterotoxins produced by specific B. fragilis strains can cause gastrointestinal inflammation (41), other reports indicate that colonization of germ-free mice with polysaccharide (PSA) producing-B. fragilis can contribute to the maturation of the immune system (42). Although we did not assess inflammatory profiles or immune system markers in this study, Sugizaki and Naves (6) propose that the dietary fiber and polyphenol component of nuts may 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 approximately 40-50% of taxa at the class level. The taxa classes 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 relationship 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 may contribute to the modulation of bile acid, steroid, and dietary polyphenol metabolism (45).

Other favorable changes in the gut microbiome profile over the 8-week intervention involved a decrease in *Alistipes* (phylum Bacteriodetes), 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* (Bacteriodetes genera), was observed over 8 weeks. Although a decrease in butyric acid-producing potential is typically associated with cardiometabolic disorders (47), an increase in butyric acid producers of the order Bacteriodales was reported in murine models of colitis (48,49). As has been noted before, in times of physiological stress such as inflammation, there may 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 Bacteriodetes family *S24-7* decreased over 8 weeks, but 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 less than 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 since

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 may partly be related to the gut microbiome composition. In some studies, intrinsic factors outweighed dietary intervention and drove community structure (54), while in others, dietary factors that were significantly associated with inter-individual variation of gut microbiome outnumbered other intrinsic factors (55). The present study population was homogenous in terms of age (18-19 years), predominantly belonged to ethnic/racial minority groups, and were at low to moderate cardiometabolic risk (15). Hence, intra- and inter-individual factors such as genetics and 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 i.e. relative abundances across all taxa sum to one within a given sample. Since 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 Aitchson's 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 FDR, 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 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.

CONCLUSIONS

Almond snacking for 8 weeks 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.

AUTHOR RESPONSIBILITIES

JD and RMO designed and conducted the study, analyzed data and wrote the paper. ZL provided study design and sample processing support. All authors shared equal responsibility in writing the manuscript and of its final content. All authors read and approved the final manuscript.

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FIGURE LEGENDS

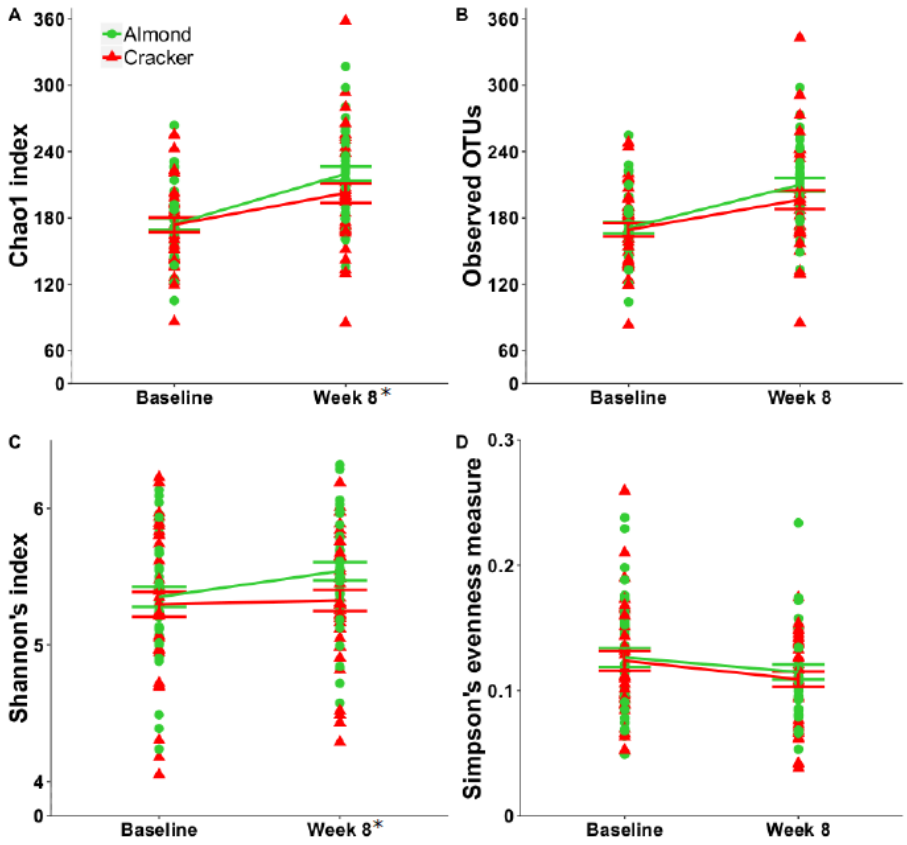

Figure 1: Alpha diversity indices (calculated from raw abundances) A) Chao1 index, B) Observed OTUs, C) Shannon's index and D) Simpson's evenness measure of college freshmen in the almond and cracker groups at baseline and week 8 of the intervention.

Values are individual data points representing each participant at baseline and week 8. Means ± 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.

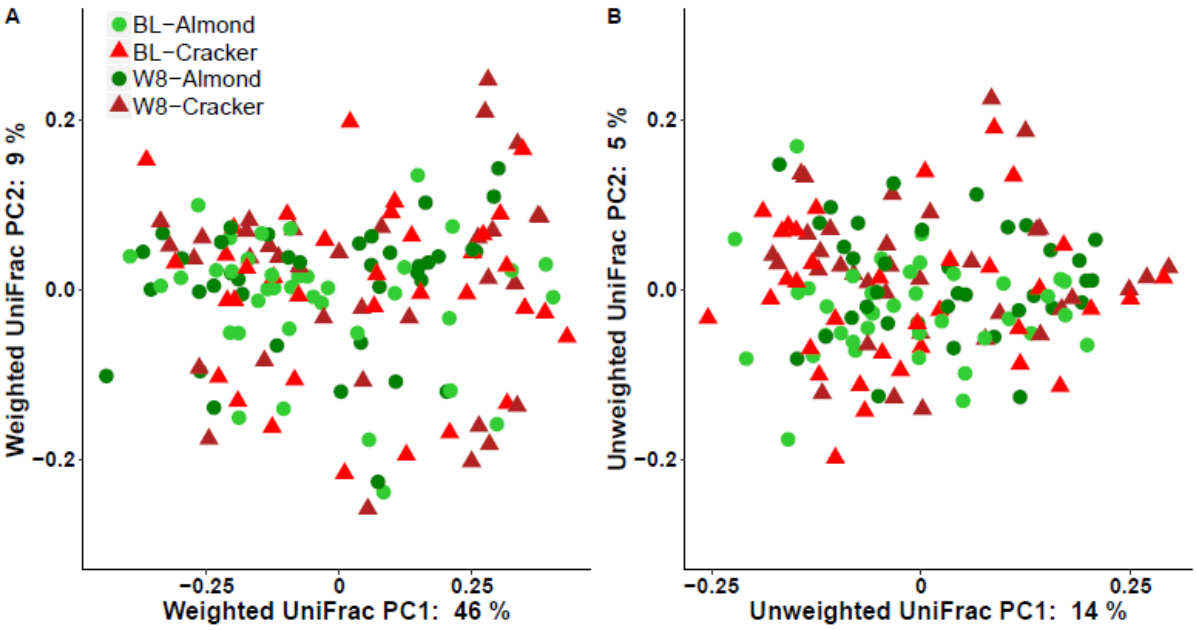

Figure 2: Principle coordinates analysis (PCoA) plot of beta-diversity measures (calculated from raw abundances) A) Weighted Unifrac and B) Unweighted Unifrac of college freshmen in the almond and cracker groups at baseline and week 8 of the intervention.

Almond: *n*=38, Cracker: *n*=35. PC-principal component, BL-baseline, W8-week 8.

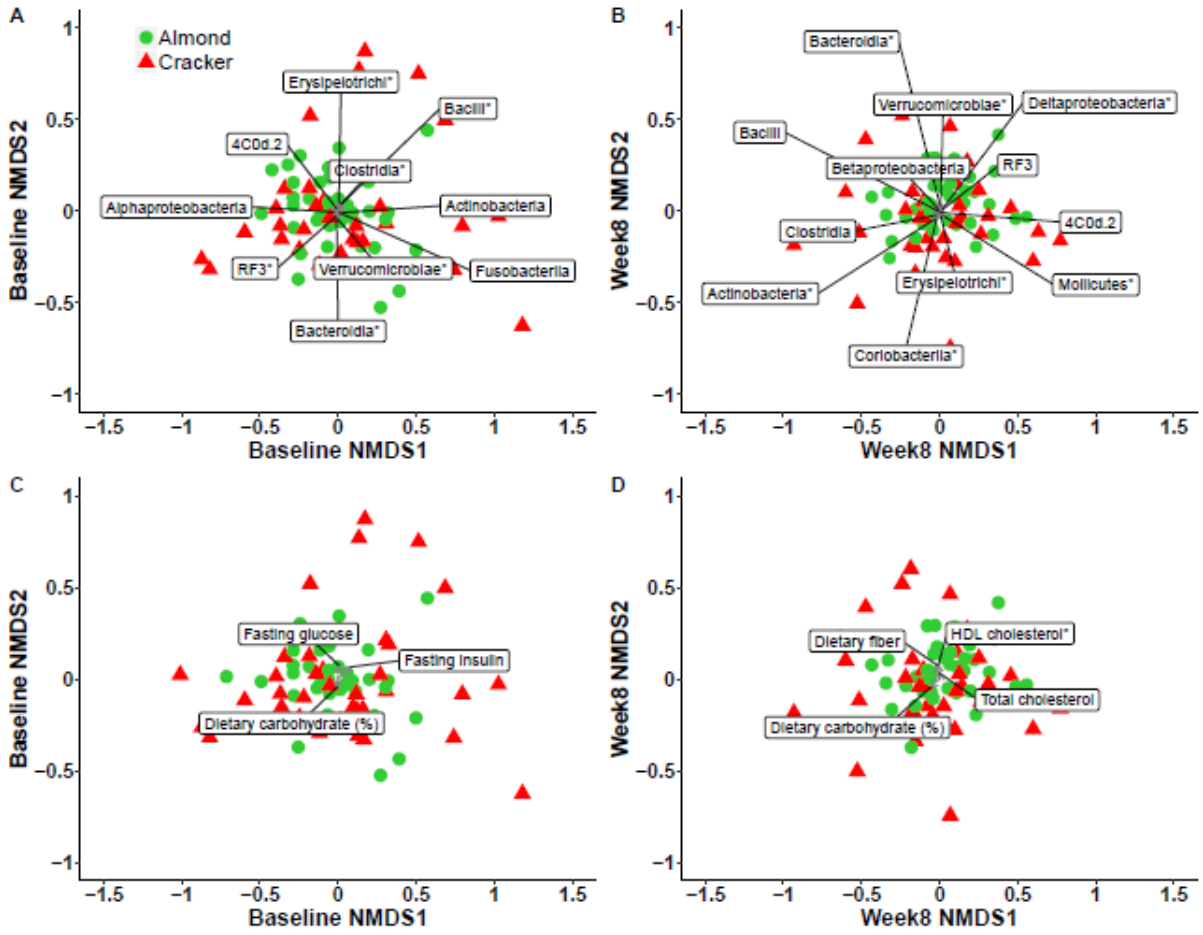

Figure 3: Non-metric multidimensional scaling (NMDS) plot of the Bray Curtis dissimilarities of college freshmen with best set of A) taxa variables at baseline, B) taxa variables at week 8, C) environmental variables at baseline and D) environmental variables at week 8.

NMDS baseline stress=0.176, NMDS week 8 stress=0.198. * P<0.05 from envfit. The arrow shows the direction of the (increasing) gradient, and the length of the arrow is proportional to the correlation between the variable and the NMDS score. Arrow lengths should not be compared across plots. p-phylum, c-class. Almond: *n*=38, Cracker: *n*=35.

Table 1: Analysis of composition of microbiomes (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 weeks after the intervention.

Taxa	Relative abundance (%)				W-taxa[1]		
	Baseline		Week 8				
	Almond (*n*=38)	Cracker (*n*=35)	Almond (*n*=38)	Cracker (*n*=35)	Time	Snack	Time x Snack
p__Tenericutes.c__Mollicutes.o__RF39	0.35±1.35[2]	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	1. W-taxa represent the number
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*	

ber of significant log-ratios for that taxa at the time, snack and time x snack levels. * denotes overall significance at a 60% cutoff i.e. 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 analysis of composition of microbiomes (ANCOM) framework.

2. 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. p-phylum, c-class, o-order, f-family, g-genus, s-species.