The Impact of a Mediterranean Diet on the Gut Microbiome in Healthy Human Subjects: A Pilot Study

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Keywords

Microbiome · Microflora · Nutrition

Abstract

Background and Aims: Despite the reported salutary benefits of a Mediterranean diet (MD) on a wide variety of health conditions, the specific microbial changes associated with an MD within the gastrointestinal (GI) tract are not well studied. Specifically, although population and survey-based studies have shown microbial changes, there are no published data on how an MD alters the gut flora in a controlled setting. **Methods:** We recruited 10 healthy subjects, each of whom gave a stool sample at baseline and then was provided with prepared meals of a “typical” American diet; after 2 weeks, a second stool sample was collected. All subjects were then provided with prepared meals based on the MD for another 2 weeks, followed by a final stool sample collection. Stool samples were batch analyzed with DNA extraction, and sequencing libraries were generated. Measures of bacterial diversity, species richness, and enterotypes were performed. **Results:** All ten subjects tolerated the diets well. Bacterial diversity increased with an MD, as measured by alpha diversity via the Simpson index. Furthermore, there

were significant differences in 5 bacterial genera between the 2 diets. **Conclusion:** This small pilot study of controlled diets demonstrates that the MD can rapidly alter the gut microbiome in healthy subjects at the level of global microbial diversity and individual genera. These data confirm the findings of previous observational studies and establish the feasibility of conducting longer term studies on the impact of the MD on the flora of the GI tract and its relationship to digestive diseases.

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Introduction

The microbial population of the gastrointestinal (GI) tract is enormous, and our understanding of how it impacts the human body and disease is rapidly evolving. Trends predictive of health and disease have been identified in both specific communities of bacteria and the diversity of these communities [1]. Logically, habitual diet plays a major role in the microbial composition of the GI tract, and thus, the impact of diet on the microbiota has

Registry website: <https://clinicaltrials.gov/ct2/show/NCT03269032>.

been an attractive target for clinical research [2, 3]. The Mediterranean diet (MD) is an ancient dietary pattern that is consumed by a significant proportion of the world's population. The MD not only is known to have beneficial health effects – including reduction in risk for cardiovascular disease, type 2 diabetes, fatty liver disease, certain cancers, and neurodegenerative disorders [4] – but recently has been shown to impact microbial diversity and microbiome composition in observational studies in humans and diet modification studies in nonhuman primates [5–10].

The microbiome and its dysregulation have also been implicated in the etiology of digestive diseases, specifically the pathogenesis of irritable bowel syndrome (IBS), the most prevalent GI disorder [11–13]. Treatment of IBS poses many diagnostic and therapeutic challenges; however, recent evidence links adherence to an MD and improvement in IBS-type symptoms [14]. Therefore, delineating the changes in the microbiome caused by an MD is warranted. While prior studies have quantified changes in the microbiome as they relate to patient-reported diets, no human studies have specifically demonstrated how a controlled delivery of an MD can affect an individual's microbiome composition or diversity. Therefore, as a pilot study for future research on how the MD affects IBS, we performed a small dietary modification study to examine how a standardized MD changes the microbiota when fed after a baseline standardized “American” diet (AD). We hypothesized that the MD would produce more varied microbiota composed of favorable enterotypes than in an AD.

Methods

Study Details and Participants

We recruited 10 healthy individuals who were ≥ 21 years old and were not following any prescribed dietary regimens via posted notices. Respondents who reported that they were clinically healthy, had no active medical problems, and were not taking any prescribed or over-the-counter medications were scheduled for a screening visit at our medical center's Clinical Research Unit (CRU).

Study Design

At the screening visit, informed consent was obtained from each subject by a member of the study team. Subjects then had a nutritional consultation with the CRU research dietitian to review their dietary preferences, record any food allergies or intolerances, and review study procedures. Basal caloric expenditure was measured by indirect calorimetry using a metabolic cart in 8 of the subjects; in 2 subjects, basal caloric expenditure was estimated by

Table 1. Nutrient composition of each of the 2 week diets

Nutrient (% of total energy)	AD, %	MD, %
Carbohydrate/alcohol*	50	51
Protein	15	17
Total fat	35	32
Saturated	11.2	6.7
Monounsaturated	12.1	18.1
Polyunsaturated	8.2	4.7

* Alcohol (red wine, 5 ounces/day) included in MD only. MD, Mediterranean diet; AD, American diet.

the Harris-Benedict equation. A stool sample was collected or arranged during this visit. On a mutually agreeable date, subjects began an individually tailored isocaloric AD prepared in the CRU metabolic kitchen and provided as prepackaged trays and containers in soft coolers. Subjects returned to the CRU to collect food 3 times weekly and to assess adherence to the provided meals by CRU staff. Two weeks after starting the AD, the subjects returned to the CRU with a stool sample and then began the Mediterranean-style diet, provided as described for the AD, for the next 2 weeks. On day 28 of the study, subjects returned to the CRU to deliver a final stool sample. The nutritional composition of the diets and sample menus are given in Tables 1 and 2, respectively.

Gut Microbiota Measurement

Gut microbiome was examined as per our previously described methods [9, 15, 16]. In brief, the bacterial microbiome composition was analyzed in accordance with the Earth Microbiome Project benchmarked protocol (<http://www.earthmicrobiome.org>), by adopting a bar-coded high-throughput sequencing approach as described by Caporaso et al. [17]. The fecal samples were stored at -70°C as soon as they were delivered to the CRU. The DNA was extracted using the MoBio PowerFecal Kit (Qiagen, CA, USA) according to the manufacturer's instructions. The V4 hypervariable region of the 16S rDNA gene was PCR amplified using the universal primer pair 515F (bar-coded) and 806R. Both the forward and reverse primer sequences also contained appropriate Illumina adapters, primer pad, and 2-bp linker sequences, with forward primer containing a 12-bp error-correcting barcode unique to each sample to achieve multiplex capability. All primers were synthesized at the Integrated DNA Technologies (IDT Inc., San Jose, CA, USA). The PCR was performed, as described previously [9, 15, 18]. The resulting amplicons were cleaned up by using Agencourt® AMPure® XP magnetic purification beads (Beckman Coulter, Brea, CA, USA) and quantified by using a Qubit-3 Fluorometer (Invitrogen, Carlsbad, CA, USA) and dsDNA HS assay kit (Life Technologies, Carlsbad, CA, USA); the final amplicon library was made according to methods of Caporaso et al. [17]. The purified PCR products were pooled in equimolar concentrations and sequenced on one 2×300 -bp Illumina MiSeq run (Illumina Inc., San Diego, CA, USA) for paired-end sequencing. The sequencing quality control was executed with onboard MiSeq Control Software and MiSeq Reporter (Illumina Inc.), and the obtained sequences were de-multiplexed, quality-filtered, clustered, and taxonomically annotated against Greengenes reference database with

RDP Classifier using the Quantitative Insights into Microbial Ecology (<http://qiime.org/>) software package, as described previously [9, 15, 18, 19]. A 97% sequence similarity cutoff was used to cluster operational taxonomic units (OTUs). Negative controls were mock specimens that contained all reagents, including bar-coded primers but without any extracted DNA that went through PCR and sequencing procedures. To avoid the effect of DNA extraction or PCR conditions and primers on microbial community composition recovered by amplicon sequencing, all samples were batch-processed and analyzed to minimize such bias if any. A total of 1,365,088 sequences (minimum 21,793; maximum 72,742; median 43,184; standard deviation 12,957; reads per sample) were obtained after quality-filtering using default parameters. To avoid the bias of sequencing depths, the obtained sequences were rarefied to the lowest number (21,793 reads) of sequences for downstream analyses. To avoid the bias of sequencing errors or low-level contaminations, the OTUs with count <4 and prevalence less than 10% were removed from subsequent analyses. Of 4,795 total OTUs originally obtained, 3,457 OTUs were retained after these steps. The resultant data of taxon proportions were subjected to the total sum scaling, and the taxa with <1% mean relative abundance were further excluded from subsequent comparative analyses.

Data Analysis

The beta diversity of the microbiome was assessed using the Bray-Curtis dissimilarity index and was visualized with the principal coordinate analysis in R statistical software package (version 3.6.1; www.r-project.org). Difference in beta diversity was calculated by permutational multivariate analysis of variance, a permutation-based multivariate analysis of variance to a matrix of pairwise distance to assess the intergroup and intragroup distance. Alpha diversity indices were calculated within Quantitative Insights into Microbial Ecology. The random forest supervised learning model was applied on normalized data within R (model_randomForest; 70% training and 30% testing modules; trees = 500) to identify the major bacterial taxa whose abundance is affected by the intervention (OOB error: 0.5). Alpha diversity indices and the bacterial proportions were compared using the Kruskal-Wallis test followed by the Mann-Whitney multiple pairwise comparison test. Discriminative features, that is, the bacterial taxa, uniquely modulated by the dietary intervention and driving differences between different groups were identified by using the biomarker discovery algorithm (linear discriminatory analysis [LDA] effect size [LEfSe]) with an LDA score >3.0 and *p* value <0.05 [20]. Significance threshold was set at *p* < 0.05, unless otherwise specified.

Results

Demographic, anthropometric, and basal metabolic rate data for the 10 subjects are provided in Table 3. Six subjects were male, and 4 subjects were female; 7 subjects were Caucasian, and 3 subjects were Asian. Overall, there were no significant differences between ethnicity and gender. The average age of the subjects was 31.8 ± 3.3 years. The average BMI was 22.9 ± 0.9 at baseline and 22.8

Table 2. Sample menu for each of the 2 week diets

	MD		
	Day 1	Day 2	Day 3
Breakfast	Crème Brûlée French toast with syrup Sausage Orange juice	Ham and cheese breakfast casserole Cranberry Muffin Apple juice	Breakfast parfait Apple muffin
Lunch	Pimento BLT sandwich Pretzels Jello	Chicken and black bean burrito bowl Tortilla chips Lemon cookies	Pepperoni pizza Tossed salad
Dinner	Ham Macaroni and cheese Scalloped apples French bread	Sliced steak with mushrooms and red wine butter Roasted potato and corn medley French bread Sherbet	Chicken and broccoli Alfredo Garlic bread
Snack	Brownie bites Milk	Rice Krispie Treats	Cheese, grapes, and crackers hummus
			Pita chips, veggies, and hummus
			Snack mix
			Fruit and nuts

In addition to the foods provided above, subjects may have as many zero-calorie beverages (water, diet soda, unsweetened or artificially sweetened coffee/tea) and zero-calorie seasonings (salt, pepper, garlic powder, onion powder, herbs) as desired each day. MD, Mediterranean diet; AD, American diet.

Table 3. Demographic, anthropometric, and basal metabolic rate data for the 10 subjects

Subject	Age, years	Sex	Race	BMI Baseline	BMI Week 4	Resting metabolic rate (kcal)
1	60	F	Caucasian	22.2	21.6	1,104
2	26	M	Caucasian	23.9	23.5	1,912*
3	26	M	Caucasian	22.2	22.2	1,727
4	28	M	Caucasian	21.6	20.9	1,768
5	26	M	Asian	24.3	25.3	2,014*
6	26	M	Asian	20.5	20.6	1,417
7	33	M	Caucasian	24.4	23.5	1,694
8	33	F	Caucasian	29.4	29.6	1,414
9	25	F	Asian	19.9	20.3	1,177
10	35	F	Caucasian	20.4	20.2	1,303
Mean $\pm$ SE	31.8 $\pm$ 3.3	–	–	22.9 $\pm$ 0.9	22.8 $\pm$ 0.9	1,553 $\pm$ 99

BMI, body mass index. * Indicates RMR estimated from Harris-Benedict equation.

Fig. 1. Box plot showing the comparison of alpha diversity estimates by using a Simpson index at week 2 after the AD and at week 4 after the MD. MD, Mediterranean diet; AD, American diet.

± 0.9 at week 4. There was no significant change in BMI between the baseline visit and study completion at week 4. Average resting energy expenditure as determined by calorimetry or calculation of the resting metabolic rate

was 1,453 $\pm$ 99 kcal. Subjects reported good adherence to the diets; no significant adverse events were reported.

Using a Simpson index to evaluate the alpha diversity between the completion of the 2 standardized dietary interventions (week 2 vs. week 4), there was a significant increase in microbial diversity (Fig. 1). Furthermore, 5 key genera were identified as showing statistically significant signal differences via a random forest with a mean decrease in accuracy analysis between the 2 standardized dietary interventions (Fig. 2). Specifically, *Akkermansia*, *Lactococcus*, and *Lachnospira* increased, whereas *Coprococcus* and *SMB53* decreased. There were further signals within several other genera that did not meet statistical significance including a decrease in *Anaerostipes*, *Collinsella*, and *Eubacterium* as well as an increase in *Phascolarctobacterium* and *Faecalibacterium*. Evaluation with a Shannon index shows that between baseline and the post-AD stool, there was a trend toward less diversity of the microbiome; there was subsequently a trend toward greater diversity than at both baseline and post-AD after the MD intervention (Fig. 3).

Via use of a LefSe algorithm and LDA, classes that were statistically different with log >3 between the samples at week 2 (after AD) and week 4 (after MD) were identified. The phylotypes which were identified as key determinants of the increased diversity, as measured by the Simpson index, seen after consumption of the MD were identified as *Lachnospira*, *Sutterella*, *Burkholderiales*, *Alcaligenaceae*, and *Betaproteobacteria*, whereas *SMB53* was the sole statistically significant determinant of the signal seen after an AD. Figure 4 illustrates the LefSe and LDA. Figures 5 and 6 show the changes seen at the level of major bacterial phyla and genera and bacte-

Fig. 2. Plot of mean decrease in accuracy using a random forest classification to identify signatures among key genera among the participants at week 2 after the AD and at week 4 after the MD; 5 of which reached statistical significance at $p < 0.05$ (top 5). MD, Mediterranean diet; AD, American diet.

rial phenotypes, respectively. The trend seen within major phyla (Fig. 5) shows reduction in *Firmicutes*, increase in *Bacteroidetes*, and overall increased diversity on a phyla level, whereas differences at week 2 and week 4 between genera are more subtle with only slight increases in *Bacteroides*, *Faecalibacterium*, and *Lachnospiraceae*, while *Blautia* and *Ruminococcus* decreased (Fig. 5). The proportion of aerobic bacteria significantly increased from week 2 to week 4, and overall phenotype showed a trend toward less Gram-positive phenotypes (Fig. 6).

Discussion

Dietary modification of the microbiome has the potential to treat disease. Dietary patterns that are rich in variety are associated with a more diverse microbiome, 1 of the key characteristics in a healthy microbiota. Evidence suggests diet as the most important factor in the composition of the microbiome, but no controlled studies have examined specifically how an MD affects the human microbiome [21]. To the best of our knowledge, this study is therefore the first in examining, via high-through-

Fig. 3. Box plot showing the comparison of diversity estimates by using a Shannon index at baseline collection, week 2 after the AD and at week 4 after the MD. MD, Mediterranean diet; AD, American diet.

Fig. 4. LEfSe showing microbial classification of stool samples at week 2 after the AD and at week 4 after the MD. The cladogram on the left shows a phylogenetic classification of differences by each successive circle at the phylum, class, family, and genus level between the week 2 and week 4 stool collections. Highlighted elements in red indicate prominence at week 2 collection and in green indicate prominence in week 4 collection. Major differences noted in top middle with marked differences within *Sutterella* (c), *Alcaligenaceae* (d), and *Burkholderiales* (e). Each circle size represents the relative abundance within the sample. The LDA score is shown on the histogram to the right. MD, Mediterranean diet; AD, American diet; LDA, linear discriminatory analysis; LEfSe, linear discriminatory analysis effect size.

Fig. 5. Bar graph showing the major abundance of bacterial phyla and genera as measured among the study participants at week 2 after the AD and at week 4 after the MD. MD, Mediterranean diet; AD, American diet.

put DNA sequencing, the impact on the microbiome of an MD from both baseline and an AD with strictly controlled diets within a standardized setting among healthy adults.

Standardizing diets from an AD to an MD showed a significant rise in alpha diversity indicating a defined positive change in just 2 weeks (Fig. 1). This increased microbial diversity, or trend away from “dysbiosis,” contrasts results of survey-based and population studies, including 1 of the largest efforts, the NU-AGE project [5, 8, 22]. However, a decrease in the ratio of *Firmicutes*/*Bacteroidetes*

tes, the most prevalent phyla in the human gut, was seen with a standardized MD (Fig. 5) which mirrors changes seen in survey-based studies [5]. The *Firmicutes*/*Bacteroidetes* ratio has been linked to obesity with lower levels of *Bacteroidetes* associated with obesity and higher levels seen after successful weight loss [23]. Interestingly, similar to our study among healthy adult subjects, in a long-term standardized dietary intervention of MD versus an AD among nonhuman primates, both increased microbial diversity (via Shannon diversity index) and decreased *Firmicutes*/*Bacteroidetes* ratio were seen [9].

Fig. 6. Bar graph showing the major abundance of bacterial phenotypes as measured among the study participants at week 2 after the AD and at week 4 after the MD. MD, Mediterranean diet; AD, American diet.

While not reaching overall statistical significance, among these healthy individuals, a standardized AD showed a trend of decreasing diversity, while an MD increased diversity over both baseline and an AD via use of a Shannon index (Fig. 3). Despite this, statistically significant changes occurred among several identified genera. The marked increase in *Akkermansia* after MD follows recent evidence suggesting the benefit of these genera with respect to metabolism and glucose homeostasis, among other effects [24–27]. There were also trends toward a lower abundance of *Ruminococcus* and *Coprococcus* (Fig. 6), trends also seen in the aforementioned non-human primate study [9]. Furthermore, *Lachnospira* sp. were significantly higher after 2 weeks of an MD (Fig. 2), and evidence has suggested *Lachnospira* is beneficial in a variety of disease processes, including inflammatory bowel disease and asthma [28, 29].

However, despite the changes that were seen with this intervention, the short-term nature of this analysis (1 month of standardized dietary changes and just 2 weeks of MD) may limit appreciation of the long-term impact of MD as was suggested by Wu et al. [21]. However, this study implies that changes occur quickly to regimented dietary changes. The aforementioned population-based studies represent larger scale data to help guide conclusions; but dietary compliance in any study is challenging, particularly when no food is provided and when dietary trends are self-reported, as is often performed. Therefore,

the observed impacts of the controlled dietary intervention performed in this small study warrant further investigation to confirm the rapid change in alpha diversity.

Conclusion

The variety of positive outcomes seen in the literature with MD warrant further research to elucidate how this diet modifies disease. Data on the effect of fecal microbiota transplant in IBS patients as it relates to microbial alterations have shown mixed results, but targeted and standardized dietary therapy warrants further exploration [30, 31]. This interventional pilot study utilizing a regimented and controlled dietary intervention provides data to support MD as a mechanism to affect microbial alterations. These data will serve as a basis for further exploration of the impact of an MD on the microbiome as this relates to a variety of disease models, particularly IBS.

Statement of Ethics

The procedures were approved by the Wake Forest Health Sciences Institutional Review Board (IRB00039422) and registered with ClinicalTrials.gov (NCT03269032). Written informed consent was obtained for each participant.

Conflict of Interest Statement

There are no conflicts of interest to report.

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Author Contributions

J.R., F.W., R.N., H.Y., and R.W. performed literature search, data analysis, data interpretation, and writing. J.R., F.W., and R.W. performed study design, conceptualization, data curation, investigation methodology, project administration, supervision, and validation.

Data Availability Statement

Data cannot be shared for confidentiality reasons. Queries about the data should be directed to the corresponding author.

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