Primary source of dietary protein is correlated with differences in the intestinal microbiome diversity

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SUMMARY
In spite of the recent popularization of the vegan diet, there is currently a deficit of studies that compare the impact and associated health benefits of dietary protein source, one of the main differentiators between vegan and non-vegan diets, on the intestinal microbiome. The objective of this study was therefore to correlate observed changes in the intestinal microbiome to changes in dietary protein sources. We hypothesized that changes in dietary protein source are associated with changes in the composition of the intestinal microbiome. The participant changed their diet over four separate one-week periods to include plant proteins only, plant proteins and dairy proteins, animal proteins only, and finally animal proteins and dairy proteins. The participant followed their respective dietary treatments for one week. Bacterial 16S dual-index sequencing and bioinformatic analysis identified seven genera and two families of bacteria. Of these, Bifidobacterium, Bacteroides, Lactobacillum, and Clostridium exhibited population trends correlated with changes in diet. Moreover, a comparison of α-diversity indicated a statistically significant change over the course of the experiment. Although the results of this experiment cannot be generalized due to the sample size, the hypothesis of this study was validated: changes in dietary protein source are correlated with changes in the diversity of the intestinal microbiome. However, we recommend further research into the impacts of variance in dietary protein source on the microbiome, especially by implementing longer-term dietary treatments.

INTRODUCTION
The recent popularization of the vegan diet as being a healthier, more environmentally sustainable alternative to modern non-vegan diets has mandated critical evaluations of both, including their impacts on the physiological health of an individual (1, 2). Given the extent to which the intestinal microbiome is implicated in maintaining homeostasis, changes to it as a result of the aforementioned diets have been a frequent subject of research (3). Interestingly, key differences between both diets have been observed to have significant modulatory impacts on the microbiome. The most apparent difference between the two is animal-product consumption: the vegan diet is defined by an abstinence from animal-based products like meat and dairy, while non-vegan diets often include some form of animal products (4, 5). Furthermore, the vegan diet generally has higher fiber content than most popular non-vegan diets (6).

While fiber has been extensively researched in relation to gut flora, macronutrients like proteins have been relatively overlooked (7). Dietary protein source varies significantly between the vegan and non-vegan diets: plant proteins and animal proteins respectively (5). However, comparatively little is known about changes in the microbiome associated with such changes in dietary protein source. Furthermore, there is currently a deficit in comparative studies that consider the merits of vegan and non-vegan diets in relation to one another. Most research done into the impacts of both diets only consider the diets in isolation (8). While numerous studies claim that vegan diets promote a stable microbial gut composition, more studies comparing vegan and non-vegan diets are needed to validate these assumptions.

To that end, our study compared the impacts of a “lean-protein” based diet—which includes chicken and fish—with the impacts of a vegan diet, on the intestinal microbiome. Hence, the hypothesis of this study is that changes in dietary protein source are associated with changes in the diversity of the intestinal microbiome. In order to test this hypothesis, the participant followed four dietary treatments that limited their intake of protein to either plant protein or animal protein and took stool samples at three points during each treatment to evaluate the changes in their microbiome. While this study validated this hypothesis, we specifically observed increases in the diversity of the microbiome during the animal protein dietary treatment phases, which contrasts with the findings of previous studies on the impacts of the vegan diet on the microbiome. Hence, our findings underscore the need for further research into the differential impacts of differing dietary protein sources on gut flora, as well as increased participant diversity in microbiome studies.

RESULTS
The sole participant followed four different dietary treatments for one-week periods: plant protein only, plant and dairy protein, animal protein only, and animal and dairy protein. The components of the participant’s normal diet outside of this experimental setting, and the dietary treatments followed for this experiment, have been tabulated (Table 1). The participant collected three fecal samples during each dietary treatment period, leaving two days between each sample collection date, and we sequenced the samples using 16S dual-index sequencing to identify the genera and abundance of bacteria present in each sample.

We identified seven genera of bacteria: Bifidobacterium, Coriobacterium, Bacteroides, Lactobacillum, Clostridium, Selenomonas, and Mollicutes (Figure 1). We also
identified bacteria within the families *Erysipelotrichales* and *Betaproteobacteria*, but in insufficient abundances to identify specific genera (Figure 1). Subsequently, we calculated Shannon Diversity Indices, an α-diversity metric that quantifies the diversity of species within a singular sample, for each of the obtained samples (Figure 2). There was a net increase in the derived Indices values, from 1.45 to 1.60, indicating that the recorded diversity of the participant’s microbiome had increased over the course of the experiment. We observed a statistically significant difference in α-diversity between all four dietary treatment groups (Friedman test, \( p = 0.0421 \)). Although we also compared the composition of samples taken at corresponding times during each dietary treatment using Manhattan Distances (a β-diversity metric) and tabulated the resulting values, there were no observed significant differences between the corresponding samples (Mann-Whitney-Wilcoxon Test, \( p = 0.18684 \)) (Table 2). Similarly, we could not identify any statistically significant changes in the percent abundances of specific genera, including *Lactobacillum* and *Bacteroides* (Figures 3, 4).

### DISCUSSION

This experiment supported our hypothesis; changes in dietary protein source are correlated with changes in the diversity of the intestinal microbiome. The net increase in microbial diversity over the course of the experiment was of particular note, as existing literature indicates that plant-based diets are associated with higher gut flora diversity than the Western diet. Moreover, the magnitude of increase in diversity between each sample was relatively higher during the animal protein dietary treatment phase than during the plant protein dietary treatment phase.

The marked increase in microbial diversity observed as the dietary treatments transitioned from solely plant protein (i.e., the vegan diet) to animal and dairy protein (i.e., the Western diet) contrasted the findings of existing literature (Figure 2). Specifically, it opposed the conclusion that decreased diversity in gut flora is a significant consequence of the Western diet (9). In addition, the lack of discernible trends in the β-diversity of the obtained samples reflected a key potential limitation of this study: the long-term stability and resilience of the participant’s intestinal microbiome is unknown. Higher stability and resilience are associated with higher microbial diversity, but establishing a baseline level of microbial diversity against which the diversity of the participant’s intestinal microbiome could be compared was evidently unfeasible. The aforementioned lack of significant trends in β-diversity could be attributed to the resilience of the participant’s microbiome to changes in composition in response to short-term dietary changes. The composition of the participant’s microbiome potentially did not change significantly over each dietary treatment, but the diversity did...
is a β-diversity metric used to compare the compositional similarity corresponding samples taken during each dietary treatment. The dietary treatments.

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However, previous experiments indicate that the abundance of protein source. In this study, for instance, there was an observed net decline in the abundance of Bacteroides during the animal protein diet phases of the experiment (Figure 3). However, previous experiments indicate that the abundance of Bacteroides typically increases in subjects following a primarily animal-based diet. This contrast could be due to differing abundances of protein; in animal-based diets, the relative amount of protein consumed daily would likely exceed that of the amount consumed daily during the animal-protein dietary phases of the experiment. As Bacteroides are known to be involved in both amino-acid fermentation and carbohydrate degradation, the observed decline during the dietary treatments that were likely lower in carbohydrates and protein overall is logical (11).

Additional observations of trends that apparently contrast existing literature include the divergence in abundances of bacteria of the phyla Firmicutes and Bacteroidetes from the expected trend in their abundances. Existing literature holds that increased consumption of plant-based nutrients is associated both with an increased abundance of Firmicutes, and increased production of Short-Chain Fatty Acids like butyrate by bacteria in this phylum (12). This could explain the slight increase in Lactobacillum, for instance, observed over the course of the plant-protein diets, due to the relatively higher rate of plant-based nutrients during those diets relative to the animal-protein diets (Figure 4). However, the reasons behind the rapid fluctuation in Lactobacillum abundance observed during the animal-protein diets are less evident. Further complications arise with the marked decrease in Bacteroidetes bacteria (Bacteroides specifically) over the course of the animal-protein diets, which sharply contrasts with a previous observation that Bacteroides abundance is positively associated with diets rich in animal protein (Figure 3) (2). However, Tomova et al. noted that this observation was made in individuals who followed a long-term diet rich in animal proteins and saturated fats (2). Given the short-term nature of the animal-protein dietary treatments, it is possible that the observed decrease in Bacteroides during those treatments was anomalous and would have been reversed if the duration of those treatments had been extended.

This particular trend also reflects an aforementioned limitation of this experimental design: the inability to conduct comparative analysis. The participant’s normal diet diverges from the average diet of an individual living in a Western country, the U.S. specifically, as meat was not a major component of the participant’s normal diet, or of the participant’s immediate family’s normal diet (13). Though intended to replicate the Western diet, it is likely that the animal protein treatments (both with and without dairy) likely contained less protein overall, and more closely resembled a generalized omnivorous diet. Moreover, as the implemented sources of plant protein, such as beans and legumes, were rich in both fiber and protein, it is possible that the plant protein dietary treatments had greater fiber content than the animal protein dietary treatments. Similarly, it is highly likely that the plants consumed during the animal protein dietary treatments had plant protein as well, albeit smaller amounts than typical animal proteins and saturated fats (2). Given the short-term change significantly over the entire sample collection period (Figure 2).

While short-term dietary changes have been demonstrated to reproducibly induce significant changes in gut microbial composition, the studies that demonstrated those changes implemented dietary shifts of relatively greater magnitude than the dietary shifts implemented here (10). The primary reason for the difference in magnitude is that this study intended to isolate one specific component of diet, dietary protein source, leaving the others consistent and thereby limiting the magnitude of the dietary shift. Future study of the correlation between dietary protein source and gut microbial composition, therefore, should consider lengthening the time that the subjects follow each dietary treatment to a great extent. If feasible, moreover, measures to ensure that the other components of the subjects’ diets are kept constant across each treatment should be taken. For instance, logging the grams of fiber consumed each day would allow for fiber content to be controlled across the different diets.

Another consideration for future study would be identifying with greater certainty the specific taxonomic groups that displayed changes in abundance correlated with changes in dietary protein source. In this study, for instance, there was an observed net decline in the abundance of Bacteroides during the animal protein diet phases of the experiment (Figure 3). However, previous experiments indicate that the abundance of Bacteroides typically increases in subjects following a primarily animal-based diet. This contrast could be due to differing abundances of protein; in animal-based diets, the relative amount of protein consumed daily would likely exceed that of the amount consumed daily during the animal-protein dietary phases of the experiment. As Bacteroides are known to be involved in both amino-acid fermentation and carbohydrate degradation, the observed decline during the dietary treatments that were likely lower in carbohydrates and protein overall is logical (11).

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<table>
<thead>
<tr>
<th>Samples Compared</th>
<th>Manhattan Distance</th>
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<tbody>
<tr>
<td>Plant Protein Only vs. Animal Protein Only</td>
<td>0.0988</td>
</tr>
<tr>
<td># 1 - # 7</td>
<td>0.0937</td>
</tr>
<tr>
<td># 2 - # 8</td>
<td>0.1694</td>
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<td>Plant + Dairy Protein vs. Animal + Dairy Protein</td>
<td>0.2447</td>
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<tr>
<td># 4 - # 10</td>
<td>0.1529</td>
</tr>
<tr>
<td># 5 - # 11</td>
<td>0.0648</td>
</tr>
<tr>
<td># 6 - # 12</td>
<td>0.0648</td>
</tr>
</tbody>
</table>

Table 2. Manhattan distances for corresponding samples across dietary treatments. Calculated Manhattan Distances between the corresponding samples taken during each dietary treatment. The samples were serially numbered from #1 to #14. Manhattan Distance is a β-diversity metric used to compare the compositional similarity of two samples.

Figure 3. Abundance of bacteria of the genus Bacteroides declined overall but did not significantly change with protein source. Abundance of bacteria of the genus Bacteroides in each of the samples taken over the course of the experiment. The samples are serially num-bered on the graph from #1 to #14. Source of protein was changed for the single participant over four separate weeks, and 16S sequencing was performed on 3 samples taken throughout each diet change (for a total of 12 samples).
increase in microbial diversity during the animal protein dietary treatments, as opposed to the plant protein dietary treatment. In addition, this contrast underscores the need for gut flora studies to be conducted with participants from a wider range of ethnicities, due to the significant differences in dietary patterns observed between ethnic groups, which could lead to significant differences in gut flora (19). As the participant in this study was of South Asian descent, their habitual dietary patterns prior to the experiment, which significantly influenced their baseline microbiome, likely diverged widely from members of other ethnic communities. As such, future gut microbial studies should prioritize sample diversity, to better guarantee the generalizability of their obtained results.

Figure 4. Abundance of bacteria of the genus Lactobacillum increased overall but did not significantly change with protein source. Abundance of bacteria of the genus Lactobacillum in each of the samples taken over the course of the experiment. The samples are serially numbered on the graph from #1 to #14. Source of protein was changed for the single participant over four separate weeks, and 16S sequencing was performed on 3 samples taken throughout each diet change (for a total of 12 samples).

MATERIALS AND METHODS

Participant

The sole participant was a 16 year old male of South Asian descent, who gave informed consent to participate. Informed consent was also provided by the parents of the participant. 12 OMNigen GUT liquid buffer kits and OM-AC1 toilet accessories, provided by DNA Genotek, were used to store the collected fecal samples.

Methodology

The participant's diet was changed to include various dietary protein sources: plant proteins, animal proteins and dairy proteins (isolated due to being a potentially confounding factor) (20). During week 1, plant proteins were the only protein source. In week 2, the participant ate both plant and dairy protein sources. For week 3, only animal protein was eaten, and in week 4 both animal and dairy protein sources were eaten. Given that the microbiome has been observed to change significantly over the timeframe of a week, the sample-collection period was divided into 4 one-week intervals (10). Three samples were taken during each interval, using OM-AC1 toilet accessories, and stored in OMNigen GUT liquid buffer kits (DNA Genotek, cat. # OM-200) (21).

In order to keep the protein sources constant across the first week of sample collection, all dairy and meat products were removed from the participant's diet. Moreover, one week before the beginning of this experiment, all sources of dairy were removed from the participant's diet to ensure that there would be no residual dairy proteins that could contaminate the first dietary treatment. As substitutes, plant milks (specifically almond milk and oat milk) and plant milk-based products (Silk Almondmilk Yogurt Alternative) were used due to their lack of dairy proteins. Similarly, all meat-based products were substituted for plant-based alternatives (Beyond Meat and Don Lee Farms Chipotle Black Bean Burgers Don Lee Farms). For the latter half of the sample collection period, however, which involved animal proteins and dairy proteins, all sources of plant protein were stricken from the participant's diet. This included the aforementioned plant-based meat alternatives, as well as beans, nuts and other protein-rich legumes. For the animal protein phase of sample collection, these were replaced with chicken and fish-based foodstuffs.

Fecal samples were taken every three days, amounting to three samples per dietary treatment, and stored in liquid buffer kits due to refrigeration constraints. After the sample collection period elapsed, the collected samples were sent to the University of Minnesota's Genomics Center for 16S dual-index sequencing (similar to 16S amplicon sequencing).
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References


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