Characterizing the Intestinal Microbiome of Fibromyalgia Patients

Bryant Stewart MLT (ASCP)^{CM}, Joshua Slagowski MLT (ASCP)^{CM}, Bradley Boman MLT (ASCP)^{CM}, Kendal Beazer MHA, BS MLS (ASCP)^{CM}

Abstract

Fibromyalgia (FM) is a painful and debilitating condition that affects an estimated 2 to 4% of American adults. It is characterized by diffuse bilateral chronic pain, fatigue, sleep disturbances, and impaired cognitive function. Interactions between the hypothalamic-pituitary-adrenal axis, communities of intestinal flora, and the central nervous system have been investigated as potential contributing factors to a variety of pathologies, including auto-immune, rheumatological, and psychiatric diseases. A recent investigation of the intestinal microbiomes of female FM patients and controls found characteristic alterations in intestinal flora at the genus and species levels. This study characterized the gut microbiomes at the genus level of nine FM patients, along with ten control-group individuals. Prepared stool samples were analyzed by sequencing the 16S rRNA genes of bacteria present using the MinION portable nanopore sequencer. Although there were no significant differences between the FM and control groups in the diversity of their intestinal microbiomes as measured by Shannon diversity scores (Mann-Whitney U = 38, p = 0.596), the control group's microbiota included three additional genera at an abundance greater than 1%.

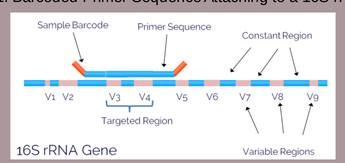
Introduction

Fibromyalgia (FM) is a chronic syndrome of widespread body aches, fatigue, tender points on both sides of the body, sleep disturbance, and cognitive dysfunction that negatively affects the quality of life of 2% to 4% of adults. The etiology of fibromyalgia is complex and poorly understood; as a result, it is difficult to treat. Recent investigations of the interactions between the intestinal microbiome (the bacteria, fungi, and viruses living within the large intestine) and the central nervous system have uncovered connections between these miniature ecosystems and a number of conditions, such as autism, diabetes, and irritable bowel disease.

A recent (2019) Canadian study explored the microbiome of seventy-seven female fibromyalgia patients and found that these patients had an "altered" set of bacteria in comparison with seventy-nine healthy control individuals. This variance was better explained by FM-related variables than by any other variable in the study, suggesting that a similar "dysbiosis" might exist for other individuals with FM. This study investigated the intestinal bacteria of a group of nine FM patients and ten control patients with the hypothesis that microbiome diversity and differential abundances of bacterial genera would be significantly different between the groups.

Although the composition of intestinal bacterial microbiomes can be sampled by sequencing the whole genomes of bacteria found in stool samples, this is costly and time-consuming. By targeting the gene for 16S rRNA (for which bacterial genera and species have unique DNA), this process can be accomplished more economically than by examining whole genomes. Additionally, multiple samples can be pooled and sequenced simultaneously in real-time by attaching unique "barcodes" to the primers used to amplify the DNA in each sample, in a process called "multiplexing".

Figure 1. Barcoded Primer Sequence Attaching to a 16S rRNA Gene



The pooled samples in this study were sequenced using a small device called the MinION, which was developed by Oxford Nanopore Technologies to make such sequencing capabilities available at a lower cost to small laboratories.

Methodology

Recruitment and Specimen Collection

Nine FM patients were recruited from various sources: flyers were displayed at clinics specializing in pain management, social media outreach was done by sharing posts to a Fibromyalgia support group and the MLS department Facebook page, and participants were also recruited by word-of-mouth. In order for an individual with FM to participate in the study, they were required to have been diagnosed by a licensed physician. Ten additional control participants were recruited to match experimental demographics of gender and age. Exclusion criteria for both groups included presence of infection or use of antibiotics within the last two months. Recruitment kits were assembled containing an Informed Consent form and questionnaire, a Parapak Stool Sample Collection tube, a disposable commode to facilitate sample collection, and contact information for members of the research team. Participants filled out the forms and collected the sample at home. The samples were stored at -80 C until preparation for analysis at the Weber State University Medical Laboratory Science department.

DNA Extraction

Bacterial DNA was extracted from the samples using specialized spin column tubes and reagents from the Qiagen PowerFecal Pro kit, which removed substances and enzymes that could inhibit the barcoding and amplification process.

Library Preparation

Once the DNA was extracted and purified, it was processed for analysis using Oxford Nanoporetech's 16S Barcoding Kit. Sample DNA along with nuclease-free water were prepared and placed in PCR tubes along with Taq 2X Master Mix. Nucleotide barcodes were added to each patient sample. The DNA was amplified in a twenty-five cycle process of denaturation at 90 C, annealing at 55 C, extension at 65 C, and finally held at 4 C. The samples were then transferred to 1.5 mL DNA LoBind Eppendorf tubes and purified using AMPure XP beads and a magnetic rack. 10 µL of the eluate was then preserved in LoBind tubes. 2 µL of each sample was analyzed on an Epoch 2 microplate spectrophotometer to establish purity and concentration. The barcoded samples were pooled to a total concentration of 50 - 100 fmoles along with 1 µL of Oxford's Rapid Sequencing Adapter.

MinION Sequencing

The pooled samples were subsequently analyzed on the MinION device. A specialized flow-cell was primed using a flush buffer, while the pooled sample library was mixed with a sequencing buffer, loading beads, and nuclease-free water in preparation for analysis. After pipetting 200 µL of the priming mix into the flow cell, 75 µL of sample was then injected in a dropwise fashion. The sample was drawn through the MinION's nanopores as the base-pair sequence of each barcoded 16S rRNA gene was determined and identified in real-time using Oxford's Guppy algorithms. The resulting data was uploaded into the EPI2ME cloud-based bioinformatics tool where the number of reads of each bacterial genus and species was broken down for each sample. A resulting taxonomic tree of the intestinal bacterial microbiome of each participant was visualized, along with the reads and taxonomic trees of the FM group and control group.

Results

Nineteen people participated in the study, with nine Fibromyalgia patients and ten control individuals. Eight of the participants were male and eleven were female; the Fibromyalgia group included three males and six females, while the control group consisted of five males and five females. The average age of the participants was 39 years old; in the fibromyalgia group it was 43, and in the control group it was 37. The pooled sample library resulted in 99,710 sequence reads with an average accuracy of 88%.

Data and Discussion

A comparison of the taxonomic trees of the two groups highlights three genera present in the control group over 1% abundance that are not present at those levels in the FM group: *Oscillibacter, Clostridium, and Romboutsia.*

Figure 2. Taxonomic Trees of the Control and FM Group

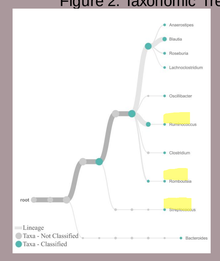


Fig. 2a. Control Group

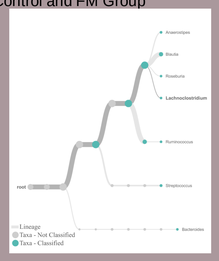


Fig. 2b. Fibromyalgia Group

Though not statistically significant, stacked bar graphs and a heat map of the relative abundance of the top genera found within the two groups makes some of the differences more easily visible, allowing the generation of future research questions.

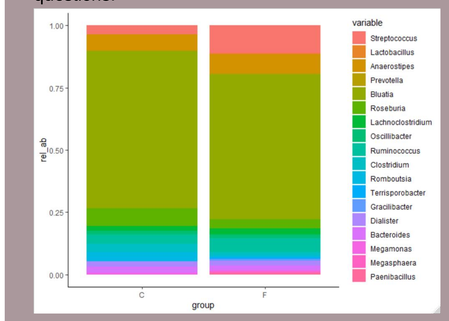


Figure 3. Stacked Bar Graphs of the Relative Abundance of Genera Found Within the Two Groups

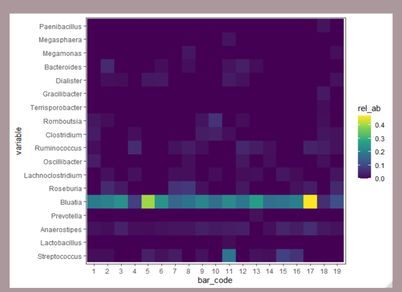


Figure 4. Heat map of relative abundance data. Bar codes 1-10 represent control group, 11-29 represent the Fibromyalgia group.

The Shannon diversity index, a measure of the richness and evenness of taxa within a microbiome, was calculated for each individual, and the distribution of scores of the two groups was plotted. Both distributions were bimodal, with a large spike in density of genera visible in the control group, as seen in Figure 5.

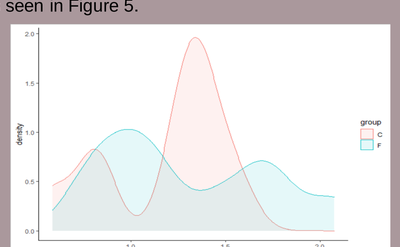


Figure 5. Figure 5. Distribution of Shannon Diversity Scores of the FM and Control Groups (*Mann-Whitney U = 38, p* = 0.596).

Conclusion

- Microbiome research offers a new perspective on disease processes, attempting to understand and account for the interactions between humans and the microorganisms living on and around them.
- Although a previous, larger study found significant differences in the diversity and relative abundance of bacteria in individuals with FM versus healthy controls, this study was unable to do so. This is may be due to limitations in sample size and the technology available to conduct this study in a small laboratory.
- Though statistically insignificant, the presence of three additional genera above the 1% abundance threshold in the control group—*Oscillibacter, Clostridium, and Romboutsia*—raises intriguing questions for future investigation.
- The combination of 16S rRNA analysis of bacteria and multiplexing capability, along with the availability of portable sequencing technology such as the Oxford Nanopore Technologies MinION, make this kind of investigation much more achievable on a small scale.

Recommendations

Considering the difficulty of recruiting the necessary number of participants, as well as the technological barriers present, future studies should secure larger grants in order to incentivize participants and ensure access to more sensitive testing methods. The relative paucity of male participants in FM studies must be overcome, as well as the representation of a more racially and ethnically diverse sample.

The impact of diet on the microbiome of FM patients, including the use of probiotics, should also be investigated. The 2019 Canadian study used detailed measures to track the diet of participants, and a similar method should be employed in future studies. Additionally, the impact of medications on the microbiome should be taken into account.

Acknowledgements

We would like to thank the Ralph Nye Charitable Foundation for funding this project, as well as our faculty at the MLS Department at Weber State University. We especially would like to thank our mentors Kendal Beazer and Dr. Matthew Nicholaou for their expertise in the field of microbiology and molecular techniques, as well guidance with data analysis. We would also like to thank Kent Criddle for excelling in his role as Lab Manager and for patiently helping us acquire the needed supplies to carry out this research.

WEBER STATE UNIVERSITY
Dumke College of Health Professions
DEPARTMENT OF MEDICAL LABORATORY SCIENCES