16s rRNA Sequencing of the fecal sample of mice receiving antibiotics treatment

Xinyi Zhang CID: 02020037

Introduction

The gastrointestinal tract of animals, including humans and mice, harbors a variety of microbiomes consisting of bacteria, archaea, and viruses, collectively known as the microbiota (Jarett et al., 2021). The gut microbiota serves essential functions in immunity, digestion, behavior, and more. However, the composition of the microbiota can be influenced by the administration of antibiotics (Patangia et al., 2022), which in turn can affect the disease predisposition, and its resistance to gastrointestinal pathogens like *Clostridioides difficile*. Therefore, it is crucial to investigate microbiota composition changes for the animal health.

16s rRNA sequencing is a powerful tool in microbiome studies, involving sequencing of the characteristic ribosomal RNA biomarker gene in bacteria. 16s rRNA sequencing technique such as Illumina MiSeq allows scientists to amplify the 16s rRNA and learn the composition of the bacterial community in the microbiota and bacteria with unique 16s rRNA by detecting Amplicon Sequence Variants (ASV). Hence, this technique can detect microbiota structure alteration after antibiotics treatment. This report will be based on the analysis of the 16s rRNA of mouse microbiota to infer the impact of the antibiotics Ciprofloxacin (CIP), Clindamycin (CLI), and Metronidazole (MTZ) on the microbiota composition.

Methods

The dataset consists of mouse faecal samples before and after antibiotics treatment (Schubert, Sinani & Schloss, 2015). Firstly, DNA is extracted, and the V4 region of the 16S rRNA gene is amplified and sequenced using Illumina MiSeq. The whole pipeline is performed by R language (R Core Team, 2022). The pre-processing is performed by the DADA2 package (Callahan et al., 2016), starting with measuring quality scores for forward and reverse reads. Then, data is trimmed if quality scores drop below 30 for both forward and reverse reads. The following merging process generates denoised sequences by aligning the forward and reverse reads and removing unmatched reads; also, nearly 3% chimeric sequences are deleted. Hence, DADA2 generates a frequency table of ASVs occurred in each sample and assigns taxonomy to identified ASVs in sequence reads.

The statistical analysis pipeline aims to examine the microbiota shift following antibiotic administration. Firstly, beta diversity is measured to assess community similarities and dissimilarities using DESeq2 (Love, Huber & Anders, 2014), followed by hierarchical clustering providing information about sample relationships. Principle Coordinates Analysis (PCoA) displays sample relatedness through dimension reduction by Phyloseq. Alpha diversity measured microbiome diversity, including richness estimation and diversity. The following taxonomic summaries included rows representing >5% of individual samples. To investigate significance, betadisper (Oksanen et al., 2022) is used to check for the homogeneous dispersion assumption, and hence the permutational ANOVA (PERMANOVA) with adonis is used to determine if microbiota change is significant. To further identify which ASVs are mainly contributing to the microbiota shift, differential abundance analysis is performed using DESeq2 to identify significantly different ASVs.

Results and Discussion

After treatment with CIP, CLI, and MTZ antibiotics, there are notable changes in the faecal microbiome. In particular, Figure 1 boxplot indicate that CIP does not significantly affect the proportion of each phylum, but CLI and MTZ treatments lead to significant decline *Bacteroidota* percentages (from 80% for CLI and 75% for MTZ) to nearly 0% and a noticeable increase in the proportion of *Proteobacteria* in the microbiota from 0% to 87% for CLI and 70% for MTZ. Other phyla do not show considerable alterations.

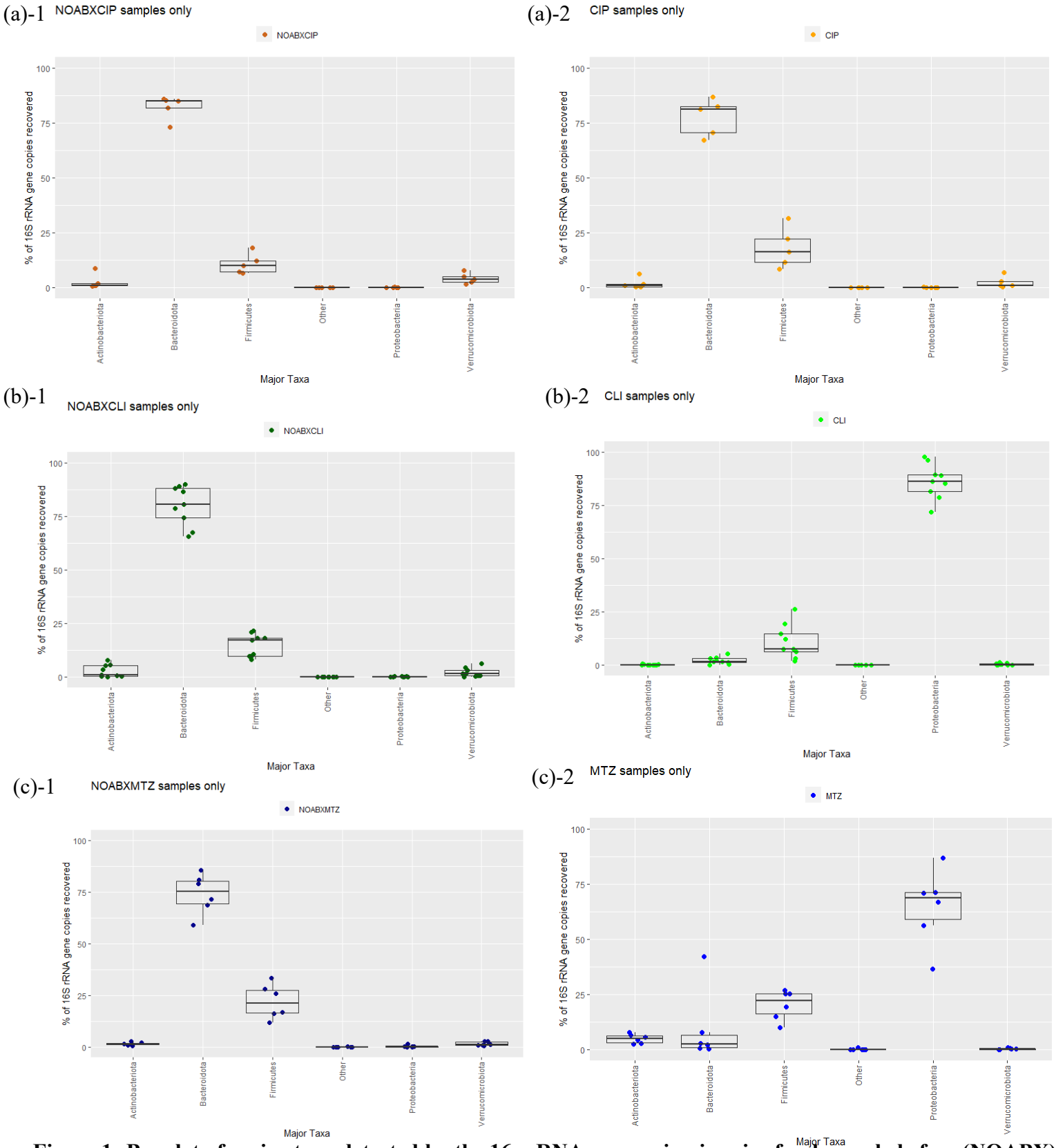

Figure 1: Boxplot of major taxa detected by the 16s rRNA sequencing in mice fecal sample before (NOABX) and after receiving antibiotics CIP, CLI, and MTZ. After receiving: (a) CIP: no significant changes in the proportion of each taxa; (b) CLI: *Proteobacteria* replaces *Bacteroidota* as the taxa that occupies the largest proportion of the microbiota; (c) MTZ: *Proteobacteria* replaces *Bacteroidota* as the taxa that takes the largest proportion of the microbiota.

To further analyze if there is any difference between pre-antibiotics and post-antibiotics treatment, Principal Coordinates Analysis (PCoA) is performed to inform the similarities and dissimilarities between the groups by reducing dimensions. From Figure 2 (a), CIP and NOABXCIP seem pretty similar. CLI and NOABXCLI or MTZ and NOABXMTZ, on the other hand, shows dissimilarities, supporting the results in Figure 1.

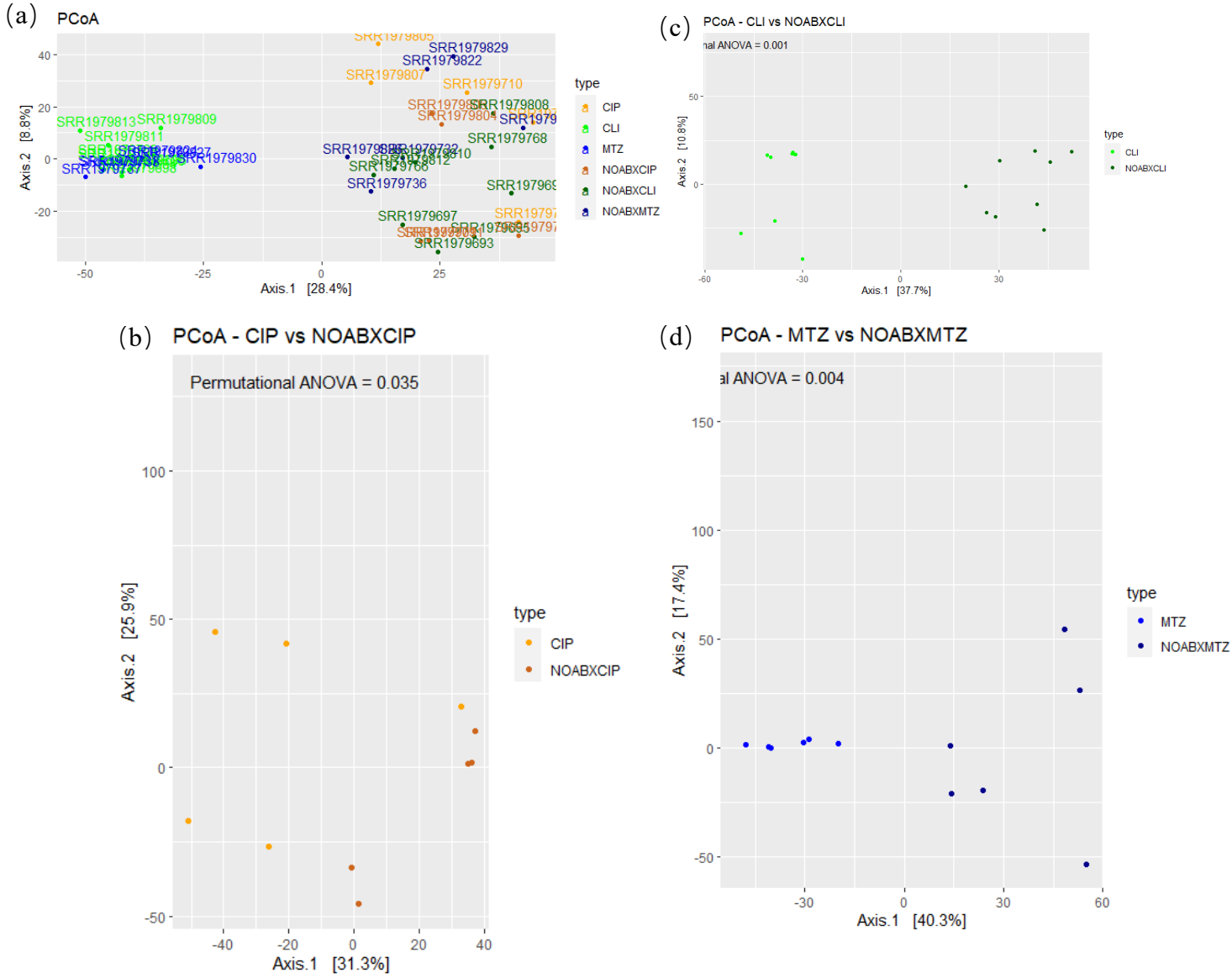

Figure 2: PCoA plots of the samples before (NOABX) and after receiving three antibiotics. a) PCoA plot for all the samples; b) PCoA plot for antibiotics CIP; c) PCoA plot for antibiotics CLI; d) PCoA plot for antibiotics MTZ

Betadisper test by ANOVA is firstly performed to check the sample homogeneity level. The p-value for CIP vs. NOABXCIP is 0.07415, CLI vs. NOABXCLI is 0.9052, and MTZ vs. NOABXMTZ is 0.009341. Under the 0.05 significance level, MTZ-treated samples show significant change in dispersion pattern, suggesting that PERMANOVA results for MTZ may be unreliable. The CIP and CLI, on the other hand, observes the PERMANOVA assumption of homogeneous dispersion.

Therefore, PERMANOVA testing is conducted to assess statistical differences between the groups, considering the homogeneity of dispersion. The p-values obtained are 0.035 for CIP vs. NOABXCIP, 0.001 for CLI vs. NOABXCLI, and 0.004 for MTZ vs. NOABXMTZ, all indicating significant difference (p-value<0.05). However, because MTZ shows difference in dispersion, the interpretation of the PERMANOVA must be cautious. Thus, PCoA plots for each group comparison should be plotted (Figure 2(b)-(d)). Plots clearly demonstrates distinctions between pre- and post-antibiotic treatments for CIP and CLI, plus spatial disparities between NOABXMTZ and MTZ. The plots once again prove the statistical difference between groups.

DESeq2 analysis identifies specific ASVs and genera that contribute to the differences in microbiota structure between the samples. For NOABXCIP and CIP, the difference is attributed to ASV35 from the genus *Lachnospiraceae* NK4A136 group.

On the other hand, CLI and MTZ antibiotics create difference in the microbiota due to over 10 ASVs (CLI: ASV_1,2,6,13,17,21,23,41,45,52,55,64,67; MTZ: ASV_1,2,13,14,19,23,35,38,41,51,52,57,64,67,88,90,98,106). These ASVs belong to genera including *Escherichia-Shigella*, *Bacteroides*, *Alistipes*, *Colidextribacter*, and *Incertae Sedis*. Notably, *Escherichia-Shigella* (ASV-1) is from the phylum *Proteobacteria*, while *Bacteroides* (ASV-2) and *Alistipes* (ASV-13) are from the phylum *Bacteroidota*. Hence, these genera might explain the decrease in the *Bacteroidota* and the increase in *Proteobacteria* observed from Figure 1.

The changes in microbiota composition can have implications for gut protection. The decrease in *Bacteroidota*, the most abundant phylum in the gut (36.5%) (Tian et al., 2022), following CLI or MTZ treatment may weaken polysaccharides metabolism, pathogens defense, and nutrient supply to other microbes (Zafar & Saier, 2021). Increased *Proteobacteria* levels is a flag to intestinal diseases, such as Inflammatory Bowel Disease (IBD) (Rizzatti et al., 2017). Correlation analysis found significant elevation in *Escherichia-Shigella*, which is also identified in CLI and MTZ treated mice faecal samples, in patients with Crohn's Disease (Hu et al., 2022). Additionally, decline in *Bacteroides* from *Bacteroidota* is also observed in IBD patients (Zhou & Zhi, 2016). *Proteobacteria* can also impact gut microbial biodiversity, gradually affecting the whole microbiota (Rizzatti et al., 2017). In summary, CLI and MTZ antibiotics pose a higher risk to the microbiota and health status than CIP due to the high proportion of *Proteobacteria* and low percentage of *Bacteroidota*.

While 16s rRNA sequencing is very powerful, it has limitations. One is the biases during sequencing, where specific genes may be amplified more efficiently due to factors like GC content or primer affinity, leading to result deviations. Although 16s rRNA sequencing can identify the phylum or genus of the microbes, related bacteria share similar sequences and similar variable regions. 16s rRNA sequencing might fails to differentiate closely related species (Muhamad Rizal et al., 2020). Thus, more

datasets on the taxonomy should be provided to learn the microbiota. The taxonomy assignment relies on the quality of dataset. Careful comparisons between datasets should be done before referencing. In addition, 16s rRNA sequencing cannot provide detailed information regarding the microbes it identified. For example, information regarding the functions and metabolic activities can be included.

In conclusion, the antibiotics CIP does not impact the gut microbiota greatly, while CLI and MTZ show significant differences in the composition, especially the proportion of *Bacteroidota* and *Proteobacteria*. These differences are attributed to ASVs. CLI and MTZ antibiotics might even cause health issues such as IBD. The 16s rRNA sequencing provides insights into the microbiota structure but can be improved regarding the accuracy, taxonomy assignment, and level of detail provided.

Reference

Hu, J., Cheng, S., Yao, J., Lin, X., Li, Y., Wang, W., Weng, J., Zou, Y., Zhu, L. & Zhi, M. (2022) Correlation between altered gut microbiota and elevated inflammation markers in patients with Crohn's disease. *Frontiers in Immunology*. 13. https://www.frontiersin.org/articles/10.3389/fimmu.2022.947313.

Love, M.I., Huber, W. & Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*. 15 (12), 550. doi:10.1186/s13059-014-0550-8.

Muhamad Rizal, N.S., Neoh, H., Ramli, R., A/L K Periyasamy, P.@ R., Hanafiah, A., Abdul Samat, M.N., Tan, T.L., Wong, K.K., Nathan, S., Chieng, S., Saw, S.H. & Khor, B.Y. (2020) Advantages and Limitations of 16S rRNA Next-Generation Sequencing for Pathogen Identification in the Diagnostic Microbiology Laboratory: Perspectives from a Middle-Income Country. *Diagnostics*. 10 (10), 816. doi:10.3390/diagnostics10100816.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., ... & Wagner, H. (2022). vegan: Community Ecology Package. R package version 2.5-7. URL https://CRAN.R-project.org/package=vegan

Rizzatti, G., Lopetuso, L.R., Gibiino, G., Binda, C. & Gasbarrini, A. (2017) Proteobacteria: A Common Factor in Human Diseases. *BioMed Research International*. 2017, 9351507. doi:10.1155/2017/9351507.

R Core Team (2022). R: A language and environment for statistical computing. *R Foundation for Statistical Computing*. Vienna, Austria. URL https://www.R-project.org/.

Schubert, A.M., Sinani, H. & Schloss, P.D. (2015) Antibiotic-Induced Alterations of the Murine Gut Microbiota and Subsequent Effects on Colonization Resistance against Clostridium difficile. *mBio*. 6 (4), e00974. doi:10.1128/mBio.00974-15.

Tian, Z., Pu, H., Cai, D., Luo, G., Zhao, L., Li, K., Zou, J., Zhao, X., Yu, M., Wu, Y., Yang, T., Guo, P. & Hu, X. (2022) Characterization of the bacterial microbiota in different gut and oral compartments of splendid japalure (Japalura sensu lato). *BMC Veterinary Research*. 18 (1), 205. doi:10.1186/s12917-022-03300-w.

Zafar, H. & Saier, M.H. (2021) Gut Bacteroides species in health and disease. *Gut Microbes*. 13 (1), 1848158. doi:10.1080/19490976.2020.1848158.

Zhou, Y. & Zhi, F. (2016) Lower Level of Bacteroides in the Gut Microbiota Is Associated with Inflammatory Bowel Disease: A Meta-Analysis. *BioMed Research International*. 2016, 5828959. doi:10.1155/2016/5828959.