



The difference in the diversity of gut microbes in healthy and ADHD-positive zebrafish (*Danio rerio*)



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Abstract

Since the 1960s, the zebrafish (*Danio rerio*) has become increasingly frequent in scientific research, one reason being that their genomic composition is very similar to humans. Research in recent years has also confirmed a link between gut microbe and attention deficit hyperactivity disorder (ADHD) in humans. For this project, we aim to study the difference in the diversity of of gut microbe in healthy zebrafish and ADHD-positive zebrafish. We expect our research results to indicate similar outcomes as the previous studies that have been done on humans, which in turn can further validate the zebrafish as a relevant model [1] for scientific research as well as contribute to translational studies between humans and zebrafish in the future.

Introduction



In recent years, numerous studies have been done on humans to suggest that there is a direct correlation between gut microbiota and ADHD [2]. It is also known that zebrafish share about 70% of the same genes with humans, including up to 84% of genes associated with human disease. However, what remains unknown is whether zebrafish, an organism so similar to humans, also showcases a link between ADHD and the presence of specific gut microbiota associated with the disease. For this reason, we set up a research project aiming to identify the difference in diversity of gut microbiomes between healthy and ADHD-positive zebrafish. We hypothesize that the ADHD-positive zebrafish will have different gut microbiota from the healthy zebrafish and that the types of the microbes found in the ADHD-positive zebrafish may be similar to those found in the guts of humans with ADHD.

To carry out our project, we will be using DNA barcoding to extract and amplify the CO1 barcode region to confirm the species of the zebrafish. Then, we will use the Qiagen Powersoil Pro Kit to extract bacterial genomic DNA from gut samples of healthy zebrafish and ADHD-positive zebrafish. We will then use Oxford Nanopore Technologies to amplify bacterial 16S and sequence barcode libraries to make it easier to identify the difference in bacterial microbes found in the guts of the healthy and ADHD-positive zebrafish.



Materials & Methods

We were lucky enough to acquire 6 healthy (Wildtype) and 6 ADHD-positive zebrafish (Per1bMutants) from a local lab in Soochow University. The guts of the respective zebrafish were then dissected and put into microcentrifuge tubes labelled EAW-001, EAW-002, and so on.

We then used the Qiagen Powersoil Pro Kit to extract the fish and bacterial genomic DNA. We then used PCR to target the CO1 barcode region for the fish to confirm the species, and the 16S barcode region for the bacteria found in the gut of the fish. Gel electrophoresis was performed to confirm a successful PCR in each case.

After that, we used the 16S barcoding kit with Oxford Nanopore to generate the barcodes through PCR. Then, we sequenced the 16S DNA libraries using a flongle and MinION sequencer. We then used the EPI2ME 16S workflow to analyze the microbiomes in our fish samples.

The results and the figures are shown on the right.

Data and Analysis

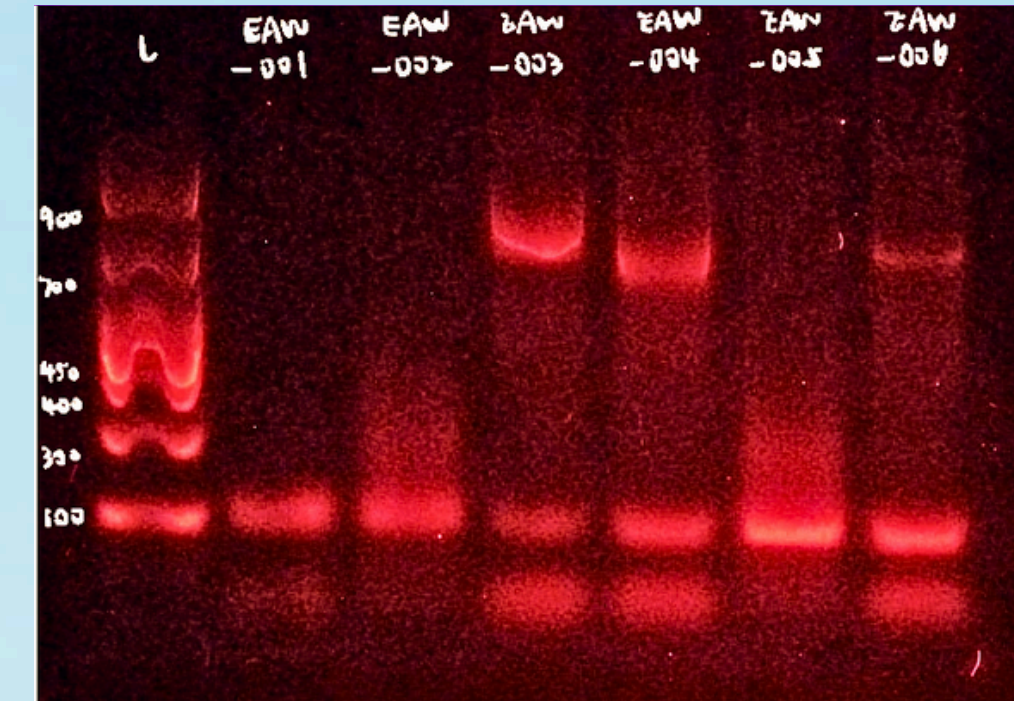


Fig.1 Gel results for EAW samples to confirm CO1 amplification for fish samples

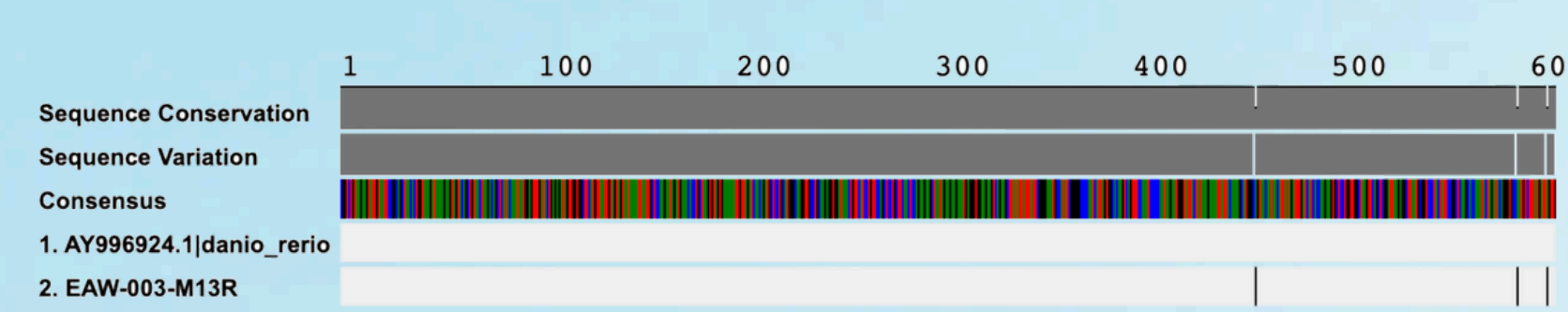


Fig.2 MUSCLE alignment of successful CO1 sequence using DNA Subway

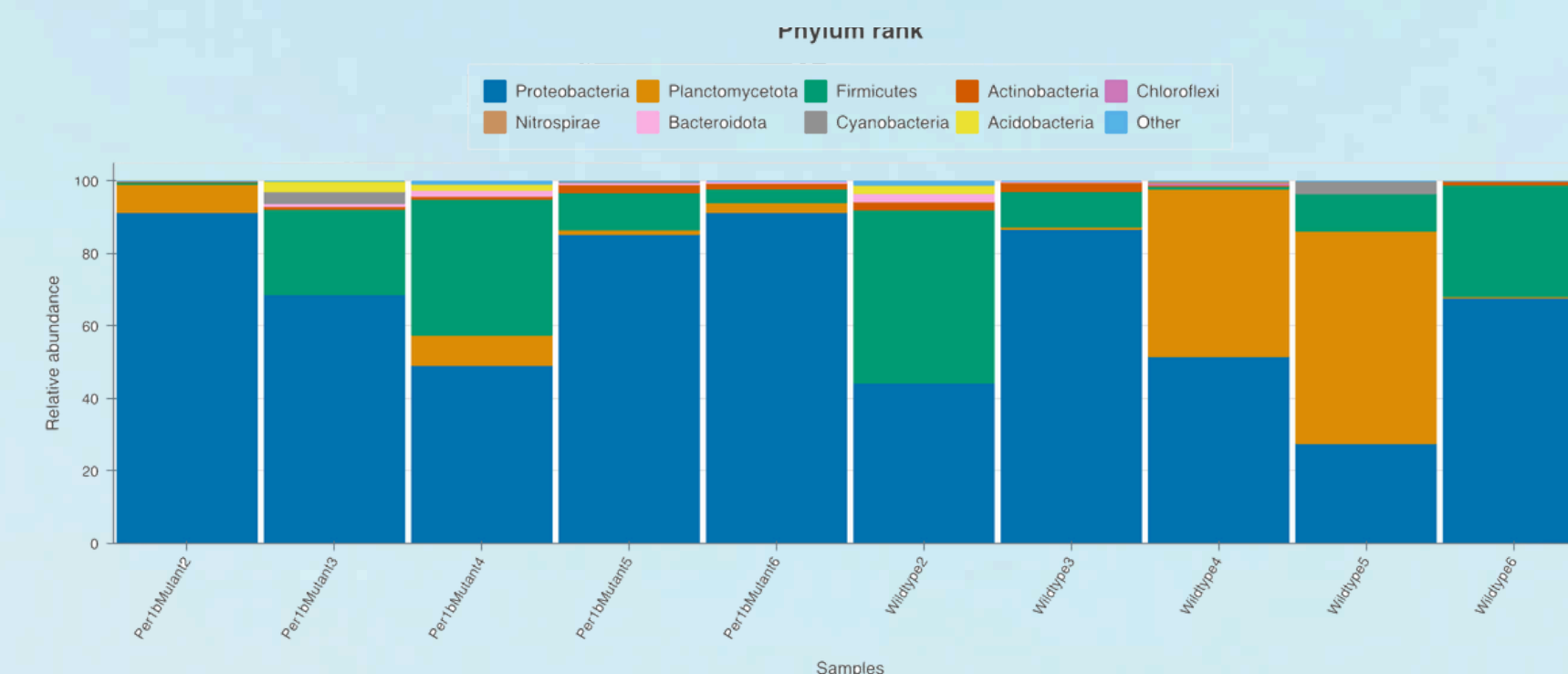


Fig.3 Differential abundance results displaying bacterial phyla

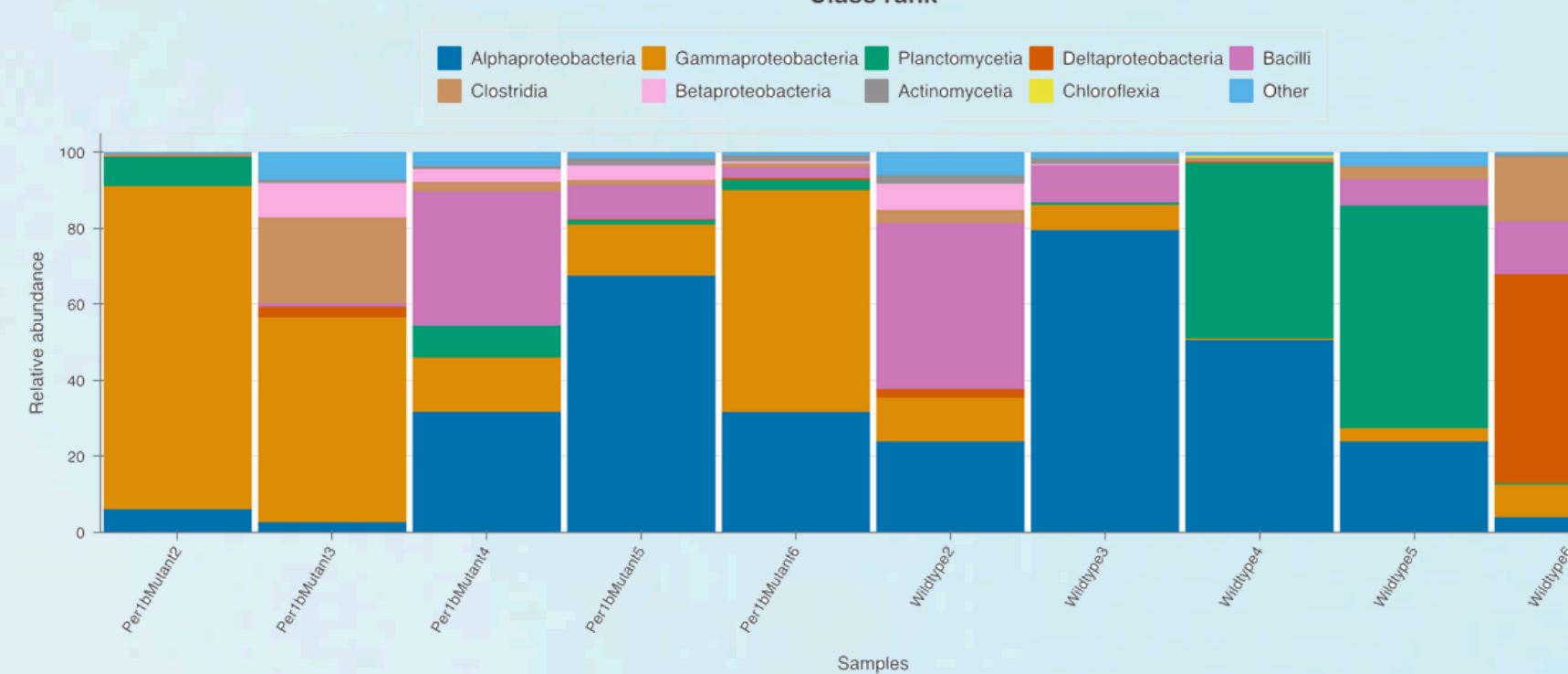


Fig.4 Differential abundance results displaying bacterial class

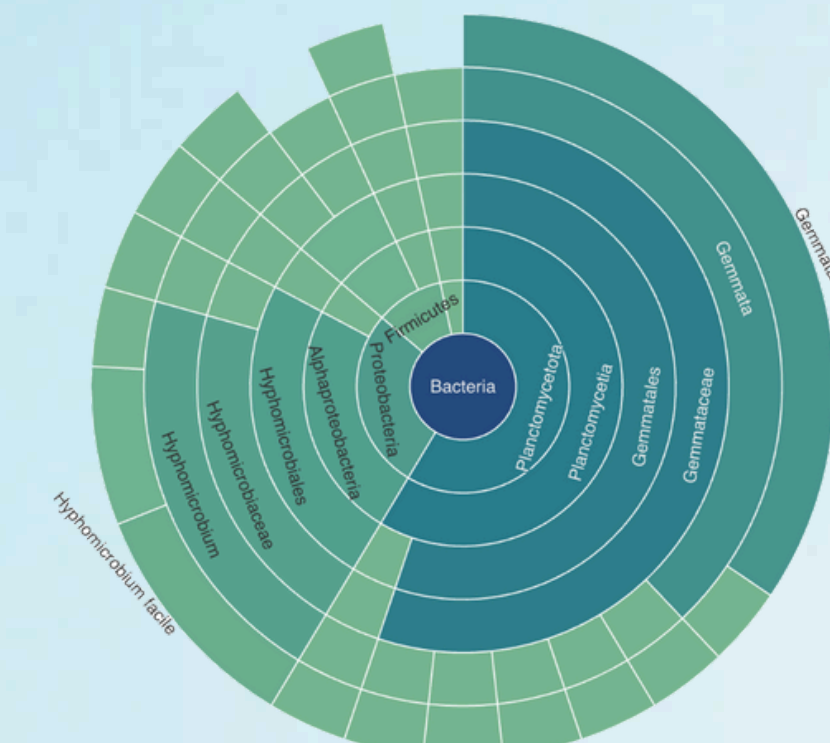


Fig.5 Sunburst data of Wildtype5

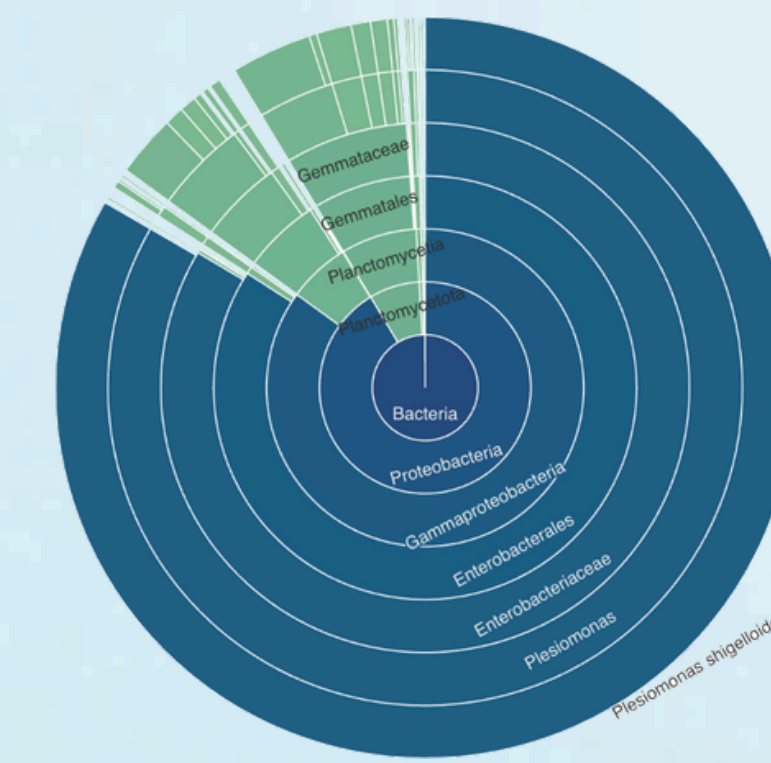


Fig.6 Sunburst data of Per1bMutant2

Results

	Proteobacteria	Firmicutes	Planctomycetota
Wildtypes	less (27.6-67.8%)	much more (10.3-47.8%)	more (46.2-58.6%)
Per1bMutants	more (68.7-91.4%)	more (10.3-37.5%)	less (1.2-8.3%)

Fig.1 shows results of our gel electrophoresis run to confirm the species of zebrafish. Although many of our samples were not ideal, EAW-003 shows the successful isolation of zebrafish DNA.

Fig.2 shows the successful EAW BLASTN results compared to the barcode of *Danio rerio* from GenBank. Even though only the reverse of EAW-003 successfully generated a barcode, it still positively identifies the gut sample of *Danio rerio*.

Differential abundance results in Fig.3 and Fig.4 showcase diverse gut microbes in Wildtypes (healthy) and Per1bMutant (ADHD). Proteobacteria, including Gammaproteobacteria in Fig.4, is more common in Per1bMutants compared to wildtypes, indicating that the presence bacteria in the phylum Proteobacteria may have a positive correlation to the disease. Apart from that, Firmicutes were also highly represented in wildtypes, but also relatively common in the mutants, suggesting that the Firmicutes bacteria doesn't play an important role in gut microbes in relation to ADHD. Additionally, it is also evident that Planctomycetota is higher in wildtypes, implying a negative correlation between Planctomycetota bacteria in the gut and ADHD.

A comparison of Wildtypes and Per1bMutants are summarized in Fig.5 and Fig.6, respectively.

Discussion

Our results for the gut bacteria found in the ADHD-positive zebrafish show some similarities with the gut bacteria found in ADHD-positive humans. For example, certain genera of proteobacteria, a bacteria associated with dysbiosis (an imbalance of microbial species and a reduction in microbial diversity within certain bodily microbiome), was linked to elevated levels of ADHD in humans. This was also the case for our mutant fish samples (Fig.3). The figure shows that the percentage of Proteobacteria (dark blue) was overall higher in the Per1bMutant samples than the Wildtype samples. This is further exemplified in Fig.4, where the orange blocks representing Gammaproteobacteria, a species under the phylum Proteobacteria, is evidently more common in the Per1bMutant samples than the Wildtype samples.

Another example was the presence of Planctomycetota bacteria in our Wildtype samples. It is known that although they are not predominant, Planctomycetota bacteria can be found in the gut of healthy individuals. Known for their unique metabolic capabilities, they also contribute to the overall microbial diversity. This is clear on our graph, as you can tell that the blocks in orange are a lot more prevalent in our Wildtype samples than our Per1bMutant samples. (Fig. 3).

Another common bacteria mentioned in research articles was Bacteroides [2]. These types of bacteria are found to be significantly elevated in ADHD, suggesting a central role in the ADHD gut microbiome. However, the data generated by our run did not include any of these types of bacteria, leading us to think that the bacteroid is more commonly associated with humans than zebrafish, regardless if they are diseased or not.

Future prospects and Conclusion

If we were to continue projects like this in the future, we would aim to collect a different part of the fish when verifying the species, as we realized the main reason our gel run was largely unsuccessful was because the zebrafish's diet consisted of other fish such as larvae and so the CO1 barcode region of those fish were also amplified as well during the PCR.

Just like we hypothesized in the abstract, many of the results for the gut microbiota we found in the ADHD-positive fish were the same to those found in humans, including proteobacteria and planctomycetot bacteria. These outcomes further corroborate the zebrafish as a model organism for scientific research, which we hope can help translational projects between humans and zebrafish in the future.

References

- [1] Wang, L., Liu, F., Fang, Y., Ma, J., Wang, J., Qu, L., Yang, Q., Wu, W., Jin, L., & Sun, D. (2023). Advances in Zebrafish as a Comprehensive Model of Mental Disorders. *Depression and Anxiety*, 2023, 1–48.
- [2] Cickovski, T., Kalai Mathee, Aguirre, G., Gorakh Tatke, Hermida, A., Narasimhan, G., & Stollstorff, M. (2023). Attention Deficit Hyperactivity Disorder (ADHD) and the gut microbiome: An ecological perspective. *PLoS ONE*, 18(8), e0273890–e0273890.

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