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Comparative metagenomic analysis of *Chanos chanos* gut metagenome in brackish and marine environments from selected aquacultures in the Philippines

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Abstract: The gut microbiome of milkfish *Chanos chanos* is of great interest due to the species' resilience in diverse aquatic environments around the country. It is a euryhaline species, capable of being cultured in a wide range of salinities, from freshwater to saltwater. This has likely affected the gut microbiome, a crucial physiological feature linked to the health and survival of the species. Few metagenomic studies have been done on its gut, with none comparing the environments they were sourced from. The study aims to compare and characterize the gut microbiome of the *Chanos chanos* in the Philippines, focusing on variation between compositional and functional diversity of brackish and marine environments. Gut samples collected from a brackish environment and a marine environment were collected and sequenced for analysis. Amplicon processing was performed with the DADA2 pipeline followed by taxonomic classification of identified bacterial ASVs. Diversity analyses and functional predictions of identified taxa were performed to compare samples from both environments. The microbiomes of brackish and marine *Chanos chanos* had greater diversity and evenness in brackish samples, while marine samples exhibited more homogeny, dominated by the genus Cetobacterium. Functional predictions show similar structure, with a stronger presence of xenobiotics metabolism in marine samples, while more minimal pathways such as glycan biosynthesis and metabolism of cofactors and vitamins had more presence among brackish samples. Statistical analyses revealed significant compositional differences between the metabolic functional diversity of samples from both environments. The implications of variation to nutrition and culture needs to be further explored.

Key Words: Gut Microbiome, Microbial Diversity, Functional Prediction, NGS, *Chanos chanos*

1. INTRODUCTION

The Philippines produced around 965,720 metric tons of fish in the third quarter of 2024 (PSA, 2024) and is expected to increase. Aquaculture systems with limited natural feed resources dedicate 60%-80% of their cost of production to feeds (Hasan, 2019) to ensure that the fish produced are of good commercial quality. Protein is the most expensive nutrient in the fish diet (Aragao et al., 2022). While there are a variety of protein sources for fish meal, such as plants, microalgae, and insects, fish meal is considered an ideal protein source because of its rich protein content, highly balanced amino acid profile, and good nutritive and palatable attributes (Serra et al., 2024). In 2022, 11% of fish caught are utilized in the production of fish meal and oil (FAO, 2024); and this increasing demand led to overexploitation of fisheries and destruction of aquatic ecosystems (Serra et al., 2024). Thus, there is great urgency in finding alternative sources to fish meals, which also possess similar nutritional attributes to fish meal, to ensure a sustainable supply of feed for the aquaculture industry as a whole.

At present, various studies explore alternative protein sources for fish feed, namely plant proteins, animal byproducts, insect meals, and marine algae (Serra et al., 2024), each with their advantages and drawbacks (Hua et al., 2019). Single cell proteins (SCP) refer to proteins extracted from pure or mixed cultures of microorganisms (Serra et al., 2024). Microbes considered as SCP sources are those that contain more than 300 g/kg of protein (dry weight) (Glencross et al., 2020). Studies have reported *Spirulina maxima* (Cyanobacteria), *Methylococcus capsulatus*, *Methylophilus methylotrophus*, and *Methylobacterium extorquens* (Proteobacteria) as efficient bacteria in producing SCP (Glencross et al., 2020). In the Philippines, microbes have also been utilized in aquafeeds such as the algal paste for milkfish larva called Juan Algae (PCAARRD, 2019), biofloc for fish cultured in tanks called Juan Biofloc (PCAARRD, 2016), and probiotics for aquaculture (PCIEERD, 2014).

There are over 500 different bacterial species that were reported to reside in the fish gastrointestinal tract, with Proteobacteria, Firmicutes, and Bacteroidetes comprising up to 90% of the bacterial population (Talwar et al., 2018). These microbes aid in various physiological processes in the fish such as nutrient absorption, digestion, growth, and immunity against diseases. Further, the type of diet and habitat also affects the fish gut microbiome (Talwar et al., 2018). Milkfish is a euryhaline species that can thrive in water with a wide range of salinities from freshwater to seawater. As an omnivore, milkfish harbors diverse microbes that produce enzymes to digest different kinds of nutrients (Hortillosa et al., 2021). In the study of Hassenruck et al. (2020), they also asserted that temperature affects the milkfish gut microbiome as they found out distinct differences in the bacterial population in the gut and the tank water. Other factors that may affect the gut microbiome include ecological influences (such as pollutants) and genetic factors (Yukgehnaish et al., 2020). To study the gut microbiome, metagenomics studies are employed, which relied on discoveries such as the 16S rRNA as a taxonomical marker for bacteria, polymerase chain reaction (PCR), and the advent of next-generation sequencing (NGS).

This study characterizes the gut microbiota of *Chanos chanos* among two common aquaculture environments: brackish and marine water. The main aim of the study was to compare taxonomic composition and functional pathways between the two groups, and determine key differences in their gut metagenome. Pertinent data such as diversity of microbial species as well as common and prevalent genera were analyzed to further describe the gut of *C. chanos in each environment.*

2. METHODOLOGY

2.1. Sample Collection

Chanos chanos samples were collected from two farms of different salinities: Minalin, Pampanga for brackish, and Bolinao, Pangasinan for marine

environments. Collected fishes were flash frozen and gut samples were immediately extracted and stored in dry ice during transit. They were then stored in -40 ℃.

Microbial DNA was isolated from gut samples using the QIAamp PowerFecal Pro DNA Kit(Cat no. 51804, Qiagen). Purity and concentration of DNA extracts was determined using the Omega Plate Reader (BMG Labtech) before being shipped for metagenomic sequencing. Samples were assessed for concentration and size during library preparation before being cleared for sequencing at Macrogen (Seoul, South Korea). Amplicons were sequenced using MiSeq Illumina producing paired-end reads.

2.2. Data Analysis

Amplicon reads were processed using the DADA2 pipeline(Callahan et al., 2016), with focus on the V1-V2 regions of the 16S sequences due to their better quality and suitability for gut microbiome analysis (Kameoka et al., 2021). Final amplicon sequences with trimmed reads were used for further downstream analysis.

After filtering, bacterial ASVs were classified to the Genus level using phyloseq functions(McMurdie & Holmes, 2013), with the Silva nr99 v138 database as reference(Quast et al., 2012). Some ASVs were tagged with specific species. ASVs attributed to Mitochondria, Chloroplast, or families with 1 representative only were excluded from analysis. Microbial diversity was analyzed using Shannon and Simpson indices. Prevalence of varying taxonomic levels and relative abundance at the phylum and genus level were also checked. Due to low volume of species tagging, species specific analysis was not performed.

Functional prediction was performed with PICRUSt2(Douglas et al., 2020) and classified using KEGG orthologs(Kanehisa & Goto, 2000). Composition of predicted functions was done with a focus on pathways classified under the general category of Metabolism: Carbohydrate Metabolism, Lipid Metabolism, Energy Metabolism, Biosynthesis of other secondary metabolites, Amino Acid metabolism, Xenobiotics biodegradation and metabolism, Metabolism of other amino acids, Glycan biosynthesis and metabolism, Metabolism of cofactors and vitamins, Metabolism of terpenoids and polyketides.

2.3. Statistical Analysis

Taxonomic assignment and functional diversity was analyzed compositionally as discussed by Gloor et al.(2017). PCA plots for taxonomic diversity and functional prediction diversity were generated using Bray-Curtis dissimilarity, and grouped based on closeness. Functional pathways were also analyzed using Aitchinson's distance statistics, and a PCA plot was generated for this as well. PERMANOVA was used to describe significant differences amongst environments between ASV abundance as well as functional composition. This was done using the adonis2() function from the vegan package (Oksanen et al., 2023) on R.

3. RESULTS AND DISCUSSION

Marine samples and brackish samples showed distinct characteristics in terms of taxonomic diversity. Furthermore, the similarity values were closest among members under the same environment. Diversity analyses between groups displayed more evenness and richness among brackish samples, and more homogeneity among marine samples (fig. 1).

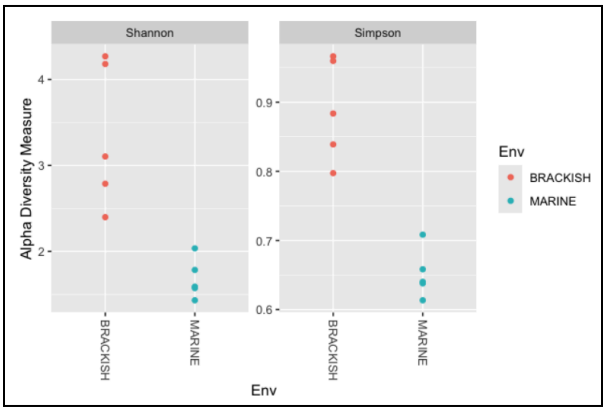

Fig. 1. Shannon & Simpson diversity indices between *Chanos chanos* gut microbiota in different environments.

This was further explored in the phylum and genus composition, wherein there was a higher variety

in major phyla and genera among brackish samples, while marine samples were heavily dominated by the genus *Cetobacterium*. This genus was also dominant in some brackish samples, but did not take as large of a majority in terms of bacterial composition (fig. 2). Other notable phyla include Actinobacteria and Cyanobacteria for brackish samples, and Firmicutes and Proteobacteria at a lesser prevalence in both environments.

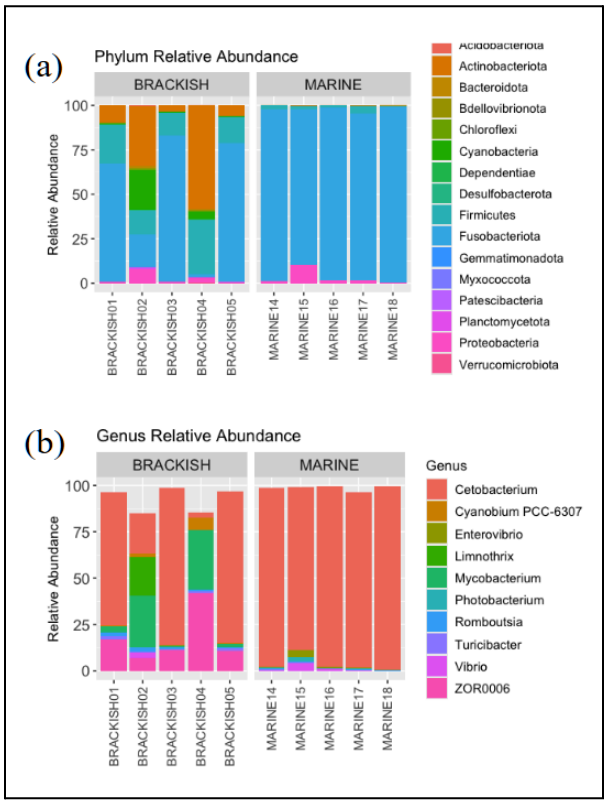

Fig. 2. Relative abundance of ASVs by top (a) phylum & (b) genus.

Functional pathways were shown to be dominated by metabolic process pathways. Composition of these metabolic pathways was similar, with the largest group being amino acid metabolism, 37.3% and 33.9% for brackish and marine respectively. Differences within the pathway compositions vary slightly between environments. However, some groups, such as glycan biosynthesis and metabolism of cofactors and vitamins are present in the brackish samples, while almost absent in marine(fig. 3).

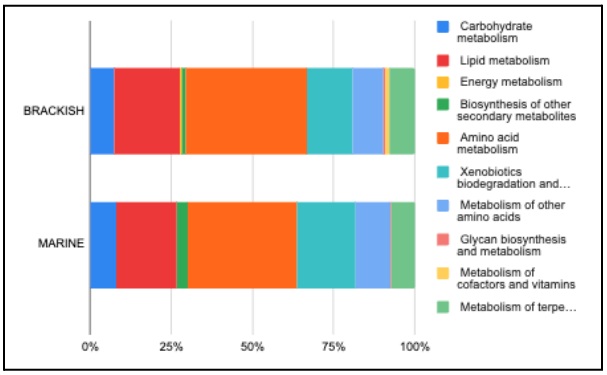

Fig. 3. Functional pathway composition of *Chanos chanos* gut microbiota by KEGG orthologs.

PERMANOVA showed strong significant differences ($p = 0.009$) between the abundance of ASVs between both groups. Functional composition also showed significant differences ($p = 0.006$). Thus, not only is there a variation in taxonomic diversity, but functional pathway preferences between environments. Principal component analysis on these factors further supported this statistic, as distinct groups were formed by environment.

The results show distinct differences between the environments where the *C. chanos* samples were collected from. Environmental changes are known to have an effect on the gut microbiomes of fish. Host factors such as fish taxon, feeding habits and trophic levels can significantly shape the microbiome of marine fishes (Huang et al., 2020) and freshwater fishes (Liu et al., 2016). However, it was also determined on a general scale that host habitat had the strongest effect in shaping the microbial community(Kim et al., 2021). The characteristics of the host's environment can affect the gut microbiome through dysbiosis, temperature, pH stress, and presence of other bacteria (Chen, 2022 & Yukgehnaish, 2020). This was described as dynamic, as dietary changes were shown to influence microbiomes as well for other species of fish (Ruiz et al., 2024). The data follows this trend, as the difference of sampling sites will offer distinct habitats, thus changing the microbiome itself.

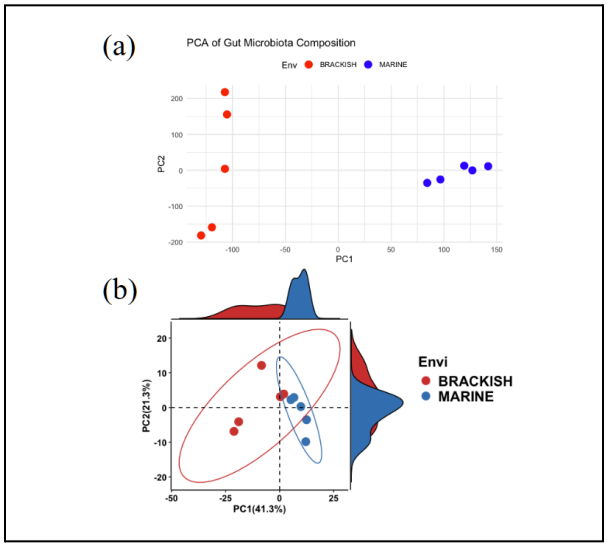

Fig. 4. Principal component analysis (PCA) of (a) microbiota composition [ggplot] and (b) functional pathway composition [ggpicrust2] by Bray-Curtis dissimilarity.

While there is a distinct lack of studies comparing the gut microbiota of *C. chanos* in different salinities or habitats, thermal stress has been identified as a significant effect on the species microbiome (Hassenrück et al., 2020). Changes in salinity, however, were found to have effect on the bacterial communities of the fish skin mucus (Krishnani et al., 2024), which would align with the current data.

Prominent bacteria found within the study align well with previous findings. Phyla such as Proteobacteria & Firmicutes, were present in other fish microbiota (Floris et al., 2024, Vala et al., 2024, Kim et al., 2021, Yi et al., 2019). Actinobacteria and Cyanobacteria were not as widespread but also recognized in these studies. The strong presence of Cetobacterium supplements studies showing prevalence in freshwater fish (Liu et al., 2016, Vala et al., 2024), amphibious mudskippers (Yi et al., 2019), and most importantly, juvenile *C. chanos* (Hassenrück, et al, 2020). Cetobacterium is a common core gut microbiome that is involved in various metabolic pathways such as amino acid production, carbohydrate metabolism, vitamin catabolism, etc.(Zhang et al., 2022). In addition, it has a role in glucose homeostasis (Wang et al., 2021), and vitamin B12 production (Qi et al., 2023). It was also discovered to serve various beneficial functions for other species: improves gut and liver health in common carp or *Cyprinus carpio* (Xie et al., 2021) and zebrafish or *Danio rerio* (Xie et al., 2022). Pathogen resistance was also observed in zebrafish, as well as within largemouth bass or *Micropterus salmoides* (Zhang et al., 2023). These benefits likely contribute to its prevalence in the gut microbiome of *C. chanos*.

4. CONCLUSIONS

This study reflects the first time that *C. chanos* gut microbiomes were compared across varying habitats with different salinities. The results of this study suggest that the gut microbiome of the *Chanos chanos* may change depending on the environment they are in. The identified taxa between environments had clear distinction in diversity and composition. While there were common prevalent groups identified, the abundance of these groups varied significantly. Differences in the composition of functional pathways in relation to metabolism were also significant, as the distribution of common KEGG subcategories varied between environments. This could be key for understanding selection of microorganisms within the gut, or their interaction within an environment or specific conditions.

Due to a possible influence of the environment towards the gut microbiome, analysis on the eDNA and other factors that may influence the surrounding microbiome should be studied. In relation to this, direct effects or relationships between these factors and the gut microbiome of *C. chanos* must be taken into account. The identified prominent taxa can be made a focus of future metabolism studies. Further implications on how this data affects health and nutrition or its impact on aquaculture needs to be explored.

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6. REFERENCES

Aragão, C., Gonçalves, A. T., Costas, B., Azeredo, R., Xavier, M. J., & Engrola, S. (2022). Alternative proteins for fish diets: Implications beyond growth. Animals, 12(9), 1211. https://doi.org/10.3390/ani12091211

Benjjneb. (n.d.). DADA2: High-resolution sample inference from Illumina amplicon data. Retrieved April 21, 2025, from https://benjjneb.github.io/dada2/tutorial.html

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. Nature methods, 13(7), 581-583

Chen, C. Z., Li, P., Liu, L., & Li, Z. H. (2022). Exploring the interactions between the gut microbiome and the shifting surrounding aquatic environment in fisheries and aquaculture: A review. Environmental Research, 214, 114202.

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., ... & Langille, M. G. (2020). PICRUSt2 for prediction of metagenome functions. Nature biotechnology, 38(6), 685-688.

Floris, R., Sanna, G., Teodora, S. C., Battaggia, G., De Pascale, F., Vezzi, A., & Fois, N. (2024). Microbial Communities Associated with the Intestinal Tract of Grey Mullets from a Mediterranean Aquatic Environment. Int J Aquac Fish Sci, 10(1), 009-019.

Food and Agriculture Organization. (2024, June 7). FAO report: Global fisheries and aquaculture production reaches a new record high. https://www.fao.org/newsroom/detail/fao-report-global-fisheries-and-aquaculture-production-reaches-a-new-record-high/en

Glencross, B. D., Huyben, D., & Schrama, J. W. (2020). The application of single-cell ingredients in aquaculture feeds—A review. Fishes, 5(3), 22. https://doi.org/10.3390/fishes5030022

Gloor, G. B., Macklaim, J. M., Pawlowsky-Glahn, V., & Egozcue, J. J. (2017). Microbiome datasets are compositional: and this is not optional. Frontiers in microbiology, 8, 2224.

Hassenrück, C., Reinwald, H., Kunzmann, A., Tiedemann, I., & Gärdes, A. (2020). Effects of thermal stress on the gut microbiome of juvenile milkfish (*Chanos chanos)*. Microorganisms, 9(1), 5.

Hortillosa, E. M., Amar, M. J. A., Nuñal, S. N., Pedroso, F. L., & Ferriols, V. M. E. N. (2021). Effects of putative dietary probiotics from the gut of milkfish (*Chanos chanos*) on the growth performance and intestinal enzymatic activities of juvenile Nile tilapia (Oreochromis niloticus). Aquaculture Research, 53(1), 98–108. https://doi.org/10.1111/are.15556

Hua, K., Cobcroft, J. M., Cole, A., Condon, K., Jerry, D. R., Mangott, A., Praeger, C., Vucko, M. J., Zeng, C., Zenger, K., & Strugnell, J. M. (2019). The Future of Aquatic Protein: Implications for Protein Sources in Aquaculture Diets. One Earth, 1(3), 316–329. https://doi.org/10.1016/j.oneear.2019.10.018

Huang, Q., Sham, R. C., Deng, Y., Mao, Y., Wang, C., Zhang, T., & Leung, K. M. (2020). Diversity of gut microbiomes in marine fishes is shaped by host‐related factors. Molecular ecology, 29(24), 5019-5034.

Kameoka, S., Motooka, D., Watanabe, S., Kubo, R., Jung, N., Midorikawa, Y., ... & Nakamura, S. (2021). Benchmark of 16S rRNA gene amplicon sequencing using Japanese gut microbiome data from the V1–V2 and V3–V4 primer sets. BMC genomics, 22(1), 527.

Kanehisa, M., & Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. Nucleic acids research, 28(1), 27-30.

Kim, P. S., Shin, N. R., Lee, J. B., Kim, M. S., Whon, T. W., Hyun, D. W., ... & Bae, J. W. (2021). Host habitat is the major determinant of the gut microbiome of fish. Microbiome, 9(1), 166.

Krishnani, K. K., Kailasam, M., Brahmane, M. P., Das, B. K., Sukhdhane, K., Panigrahi, A., ... & Ezhil, P. (2024). Unveiling the Effect of Stocking Density and Salinity on Mucus-Associated Greenwater Qualities, Physiology, and Growth Performance of Euryhaline Milkfish *Chanos Chanos*. Agricultural Research, 1-13.

Liu, H., Guo, X., Gooneratne, R., Lai, R., Zeng, C., Zhan, F., & Wang, W. (2016). The gut microbiome and degradation enzyme activity of wild freshwater fishes influenced by their trophic levels. Scientific reports, 6(1), 24340.

McMurdie, P. J., & Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS one, 8(4), e61217.

Oksanen, J., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., & O'Hara, R. (2023). vegan: R package for community ecologists: popular ordination methods, ecological null models and diversity analysis. 2013.

Philippine Statistics Authority. (2024). Fisheries situation report: July to September 2024. https://psa.gov.ph/system/files/fsd/Fisheries%20Situation%20Report%2C%20July%20to%20September%202024_0.PDF

Qi, X., Zhang, Y., Zhang, Y., Luo, F., Song, K., Wang, G., & Ling, F. (2023). Vitamin B12 produced by Cetobacterium somerae improves host resistance against pathogen infection through strengthening the interactions within gut microbiota. Microbiome, 11(1), 135.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., ... & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research, 41(D1), D590-D596.

Ruiz, A., Alós, J., Gisbert, E., Furones, D., & Viver, T. (2024). Long-term adaptation to dietary shifts of gut microbiota in gilthead seabream (Sparus aurata). Frontiers in Marine Science, 11, 1498892.

Serra, V., Pastorelli, G., Tedesco, A., Turin, L., & Guerrini, A. (2024). Alternative protein sources in aquafeed: Current scenario and future perspectives. Veterinary and Animal Science, 25, 100381. https://doi.org/10.1016/j.vas.2024.100381

Vala, F. D., Lende, S. R., Solanki, V. K., Jora, K., Desai, R., Sharma, P., ... & Mootapally, C. (2024). Melodious tuning of microbial dynamics in biofloc, cage, and pond culturing system: a study on Pangasius pangasius fish gut microbiome. Frontiers in Microbiology, 15, 1434312.

Wang, A., Zhang, Z., Ding, Q., Yang, Y., Bindelle, J., Ran, C., & Zhou, Z. (2021). Intestinal Cetobacterium and acetate modify glucose homeostasis via parasympathetic activation in zebrafish. Gut microbes, 13(1), 1-15.

Xie, M., Zhou, W., Xie, Y., Li, Y., Zhang, Z., Yang, Y., ... & Zhou, Z. (2021). Effects of Cetobacterium somerae fermentation product on gut and liver health of common carp (Cyprinus carpio) fed diet supplemented with ultra-micro ground mixed plant proteins. Aquaculture, 543, 736943.

Xie, M., Xie, Y., Li, Y., Zhou, W., Zhang, Z., Yang, Y., ... & Zhou, Z. (2022). Stabilized fermentation product of Cetobacterium somerae improves gut and liver health and antiviral immunity of zebrafish. Fish & Shellfish Immunology, 120, 56-66.

Yi, Y., Liang, L., Wang, Z., Ai, P., You, X., Bian, C., ... & Dong, B. (2019). A comparative metagenomics study on gastrointestinal microbiota in amphibious mudskippers and other vertebrate animals. Animals, 9(9), 660.

Yukgehnaish, K., Kumar, P., Sivachandran, P., Marimuthu, K., Arshad, A., Paray, B. A., & Arockiaraj, J. (2020). Gut microbiota metagenomics in aquaculture: factors influencing gut microbiome and its physiological role in fish. Reviews in Aquaculture, 12(3), 1903-1927.

Zhang, Z., Fan, Z., Yi, M., Liu, Z., Ke, X., Gao, F., ... & Lu, M. (2022). Characterization of the core gut microbiota of Nile tilapia (Oreochromis niloticus): Indication of a putative novel Cetobacterium species and analysis of its potential function on nutrition. Archives of Microbiology, 204(12), 690.

Zhang, Y., Qi, X., Zhang, Z., Jin, Z., Wang, G., & Ling, F. (2023). Effects of dietary Cetobacterium somerae on the intestinal health, immune parameters and resistance against Nocardia seriolae of largemouth bass, Micropterus salmoides. Fish & Shellfish Immunology, 135, 108693.