Malaysian Applied Biology
https://doi.org/10.55230/mabjournal.v54i4.3011

Research Article

# Gut Microbiota Diversity Between Normal And Moribund Orange-Spotted Grouper *Epinephelus coioides* in the Merbok River Using 16S rRNA Gene Amplicon Sequencing

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**ABSTRACT**

This study was conducted in the Merbok River, northwest Peninsular Malaysia, which is exposed to annual episodes of pollution. The microbiota of foregut, midgut and hindgut were analyzed to identify differences in the gut microbiome between three normal and two moribund orange-spotted grouper, *Epinephelus coioides*. Live specimens were collected two weeks after an episode of mass mortality. The 16S amplicon sequencing targeting the V3-V4 highly variable region revealed similar community richness among samples with common dominant phyla, including Proteobacteria, Actinobacteria, Tenericutes, Cyanobacteria and Acidobacteria. Significantly higher abundance of phylum Cyanobacteria, genus *Sphingomonas* and species *Eubacterium coprostanoligenes* in normal guts was likely beneficial in maintaining gut health. Moribund guts were enriched with opportunistic pathogens, including *Escherichia-Shigella*, *Clostridium sensu stricto 1* and *Shewanella.* In particular, moribund hindguts displayed a reduced Firmicutes-to-Proteobacteria ratio and significant enrichment of *Photobacterium damselae* subsp. *damselae,* indicating dysbiosis and predicted to associate with skin conditions. Higher abundance of *Photobacterim* in moribund fish hindguts may also correlate with the increased chitinolytic activity and altered metabolic pathways. The outcomes of the study provide fundamental insights into the roles of gut microbiota in regulating the orange-spotted grouper's survival in a polluted environment.

**Key words:** Aquaculture, grouper, intestinal, fish, next generation sequencing, probiotic

## INTRODUCTION

The orange-spotted grouper, *Epinephelus coioides*, is a highly economical cultured marine fish in China and Southeast Asian countries for its fast growth performance, high nutritional value and delicate taste (Ranjan, 2017). Regarded as one of the most favoured seafoods, the aquaculture industry of this teleost species has become widely expanded to meet the high market demand. However, the rapid development of intensive aquaculture activities exposes the groupers to various stressors, such as pollution, potentially increasing susceptibility to diseases, threatening the grouper aquaculture industry (Xu & Li, 2021; Duan *et al*., 2023).

The fish gut is a complex ecosystem, colonized by specific microbiota including aerobes, facultative anaerobes and obligate anaerobe bacteria (Egerton *et al*., 2018). This microbial composition may be modulated by host-related factors and habitats; consequently, substantial taxonomic variability has been commonly found in the gut microbiomes of fish and other animal models (Huang *et al*., 2020; Kim *et al*., 2021). Recent research has also shown that the gut functional communities are shaped by multiple factors (Parata *et al*., 2020; Karlsen *et al*., 2022). For instance, energy metabolism is particularly enriched in herbivorous and zooplanktivorous fish gut, whereas lipid and glycan metabolisms are more active in zoobenthivorous and piscivorous fish gut (Huang *et al*., 2020). The fish gut microbiome is of utmost importance to facilitate the host adaptation and acclimation process, and plays a critical role in maintaining the well-being of the host (Lai *et al*., 2020). For instance, several strains, including *Tenacibaculum dicentrarchi* and *Aliivibrio* sp., in the salmon distal gut are associated with health deterioration (Bozzi *et al*., 2021).

The Merbok River has been known to host various aquaculture activities, including the farming of fish and oysters (Lim *et al*., 1995). The river has been classified as polluted for decades (Lim *et al*., 1995) and the water quality categorized as Class III (polluted) in accordance with the National Water Quality Standards (NWQS). This was based on the National Environmental Quality Report for the period 2016-2019 at Petani River, an annual monitoring station of the Department of Environment, located within the Merbok River Basin (around 5 km away from the specimen collection site). High levels of pollution indices, such as Biochemical Oxygen Demand (BOD), Ammoniacal Nitrogen (AN) and Suspended Solid (SS) were recorded, attributed to

***Article History***
Accepted: 13 September 2025
First version online: 21 December 2025
***Cite This Article:*** Lau, C.C., Mohd Nor, S.A., Mohd Noor, M.E., Afiqah-Aleng, N., Tan, M.P., Ng, S.K., Sung, Y.Y., Munir, M.B. & Danish-Daniel, M. 2025. Gut microbiota diversity between normal and moribund orange-spotted grouper *Epinephelus coioides* in the Merbok River using 16S *rRNA* gene amplicon sequencing. Malaysian Applied Biology, 54(4): 68-77. https://doi.org/10.55230/mabjournal.v54i4.3011

anthropogenic activities and weathering at the upper catchment area (Ismail & Ibrahim 2015; Msahir Atshan *et al*., 2020). In this present study, an investigation was conducted at a local farm located near the Semeling Bridge at the downstream of the Merbok River in 2019. The farm practices traditional fish farming, where fish fingerlings are outsourced and reared to adult size in cages along the Merbok River. Three normal fish and two moribund fish were collected two weeks post-episode of mass-mortality to investigate any differences in composition and functional communities in the foregut, midgut and hindgut microbiomes. The findings provide insights into the relationship between gut microbiome and the health state of the host fish that inhabit polluted rivers.

## MATERIALS AND METHODS

### Fish intestine sample collection

Five adult specimens weighing 500-700 g were obtained from a local fish farm in Merbok, northwest Peninsular Malaysia (5.684035, 100.469320), in December 2019 (Figure 1a). Due to an episode of mass mortality that occurred two weeks before specimen collection (personal communication), only two live moribund specimens (M1, M2; *n*=2) that appeared inactive with body covering by white skin patches and lesions (Figure 1b-c) could be collected. The normal (H1, H2, H3; *n*=3) specimens, however, showed normal swimming behaviours without any skin conditions. Each individual was euthanized by standard procedures with 100 mg/L of MS-222 (Sigma, USA), and dissection was performed after 10 min of inactivity. The foregut (f), midgut (m) and hindgut (h) tissues were collected and preserved in RNAlater® (Thermo Fisher, USA). The gut specimens were kept in -80°C until further use.

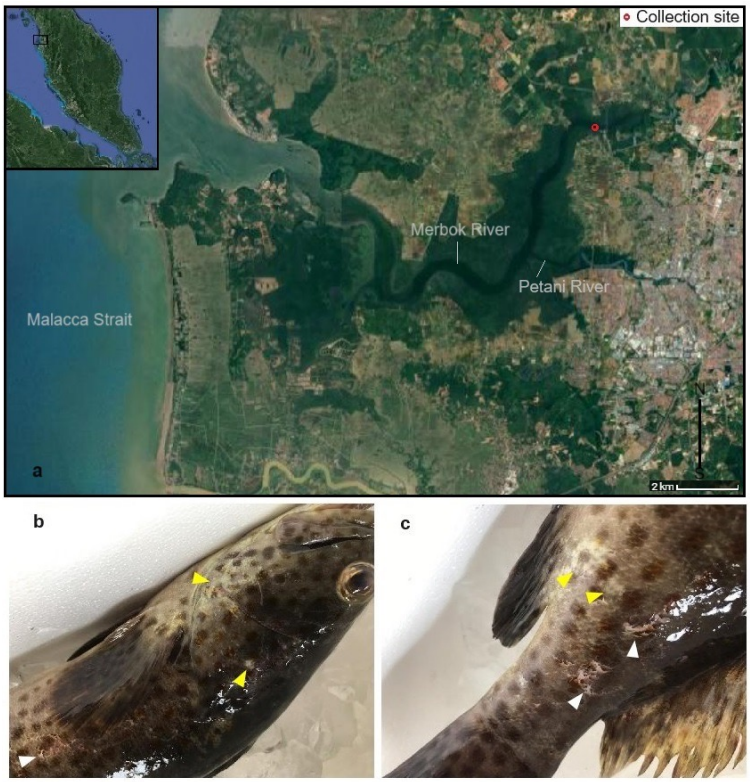


**Fig. 1.** (a) Collection site at Merbok River (red dot). The inset shows the location of the Merbok River (black box) with reference to Peninsula Malaysia. The map is modified from Google Maps. (b-c) Commercial fish farmers classify the farmed orange-spotted groupers into a moribund group based on the presence of white patches (yellow arrowheads) and c) skin lesions (white arrowheads).

### Genomic extraction

Total DNA was extracted using a conventional phenol-chloroform extraction protocol. Each gut tissue specimen (1 $cm^3$) was cut into small pieces and separately homogenized in 300 µL of TE buffer (pH 8.0). The tissue-containing buffer was boiled for 5 min, and 300 µL of phenol was added after the buffer solution had cooled to room temperature. The solution was vigorously mixed and centrifuged at 12,000 × *g* for 5 min. The supernatant was then mixed with an equal volume of chloroform and centrifuged at 12,000 × *g* for 1 min. The upper aqueous layer was collected, and 2× volume of absolute ethanol was added. The mixture was spun at 12,000 × *g* for 15 min, and the supernatant was discarded. The DNA pellet was washed with 70% ethanol. The DNA pellet was then air-dried and rehydrated in 50 µL of TE buffer (pH 8.0) and stored in -20ºC until further use.

### 16S rRNA gene amplicon sequencing

The V3-V4 variable region of the 16S rRNA gene was amplified using a degenerate primer pair, 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') containing 8 bp sequence unique barcodes. The PCR reaction mixture consists of 1× TransStart® FastPfu buffer, 5 mM dNTPs, 4 µM of each forward and reverse primer, 1

U of TransStart® FastPfu DNA Polymerase (TransGen, China) and 10 ng of DNA template. The PCR was performed as follows: initial denaturation at 95°C for 2 min, followed by 25 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, and a final extension at 72°C for 5 min. The PCR amplicons were purified by AxyPrep DNA Extraction kit (Axygen Biosciences, USA) and quantified using QuantiFluor™ ST (Promega, USA). The purified amplicons were then paired-end sequenced on the Illumina MiSeq platform to generate 250 bp reads.

### Bioinformatics and data analysis

The raw paired-end reads, now deposited into the NCBI SRA database under BioProject accession number PRJNA1048110, were imported and processed in the QIIME pipeline (v1.9.1). These raw reads were merged into raw tags using FLASH (Magoč and Salzberg 2011) and quality-filtered by Trimmomatic (Bolger *et al*., 2014). Chimeric sequences were identified and removed by UCHIME (Edgar *et al*., 2011) to obtain effective tags. Effective tags with ≥97% similarity were clustered into the same operational taxonomic unit (OTU) with UPARSE (v7.1)(Edgar 2013). The taxonomic assignment was conducted by RDP Classifier (http://rdp.cme.msu.edu/) against the public databases, including SILVA (SSU123) (Quast *et al*., 2012), RDP (Cole *et al*., 2009) and Greengenes (DeSantis *et al*., 2006) with a confidence threshold set at 0.7. The alpha diversity indices (Chao, Ace, Shannon & Simpson), sequencing depth index (the Good's coverage) and the rarefaction curve were generated by MOTHUR (Schloss *et al*., 2009). Statistical differences in the indices were analyzed using Welch's t-test at $p<0.05$ in Microsoft Excel.

Unweighted and weighted UniFrac Principal Coordinate Analysis (PCoA) were used to compare the microbial community composition of the normal and moribund groups in different gut sections. The microbial composition was analyzed for statistical significance at the phylum and genus levels based on Welch's t-test at $p<0.05$ in Microsoft Excel. Microbiome Multivariable Association with Linear Models (MaAsLin2) analysis was performed to identify microbial genera with significantly different abundances ($p<0.05$, FDR<0.25) between normal and moribund groups at different gut sections (Mallick *et al*., 2021). Differences in microbial functional abundances between normal and moribund groups were predicted using PICRUSt2 (Douglas *et al*., 2020), with statistical significance determined by the Tukey-Kramer post hoc test ($p<0.05$) in STAMPP (Parks *et al*., 2014).

## RESULTS

### Fish gut microbiome complexity

A total of 15 datasets containing the bacterial V3-V4 region of the 16S rRNA gene were obtained from the three gut sections (foregut, midgut & hindgut) of five fishes (three normal & two moribund). The number of reads from each of the 15 samples ranged between 50,794 to 74,515, comprising a total of 591 identified OTUs that were classified into 23 bacterial phyla. The community richness and diversity indices (Table 1) between both experimental groups did not differ significantly ($p>0.05$), implying that the gut microbial complexity in the three gut sections was similar. The Good's coverage of all samples was above 0.99, indicating that nearly all bacteria were captured (Table 1). This was further supported by the saturation rarefaction curves of all specimens (Supplementary Figure S1), demonstrating sufficient sequencing depth for all libraries.

**Table 1.** An overview and alpha-diversity indices in foregut, midgut and hindgut samples of normal and moribund experimental groups. Moribund fishes: M1, M2; Normal fishes: H1, H2, H3.

| Section | Group | Replicate | Read | Good's coverage | Community Richness | | Community Diversity | |
|---|---|---|---|---|---|---|---|---|
| | | | | | OTU | Chao1 | Shannon | Simpson |
| Foregut | M | M1-f | 72228 | 0.9998 | 164±28[a] | 180±28[a] | 2.23±0.49[a] | 0.25±0.15[a] |
| | | M2-f | 53333 | 0.9996 | | | | |
| | H | H1-f | 69568 | 0.9998 | 190±49[a] | 198±46[a] | 2.70±0.78[a] | 0.19±0.16[a] |
| | | H2-f | 50169 | 0.9998 | | | | |
| | | H3-f | 73904 | 0.9999 | | | | |
| Midgut | M | M1-m | 73803 | 0.9994 | 130±4[a] | 171±8[a] | 1.57±0.43[a] | 0.39±0.14[a] |
| | | M2-m | 63624 | 0.9994 | | | | |
| | H | H1-m | 68759 | 0.9998 | 163±29[a] | 192±29[a] | 2.09±0.64[a] | 0.30±0.13[a] |
| | | H2-m | 55153 | 0.9996 | | | | |
| | | H3-m | 56944 | 0.9992 | | | | |
| Hindgut | M | M1-h | 70798 | 0.9995 | 134±11[a] | 150±13[a] | 2.51±0.08[a] | 0.16±0.02[a] |
| | | M2-h | 66356 | 0.9998 | | | | |
| | H | H1-h | 56819 | 0.9999 | 141±58[a] | 149±57[a] | 1.99±0.51[a] | 0.31±0.18[a] |
| | | H2-h | 72577 | 0.9998 | | | | |
| | | H3-h | 57764 | 0.9998 | | | | |

M, moribund. H, normal. Different letters indicate a significant difference ($p<0.05$) among means within the same row.

### Microbial composition in the fish gut

The qualitative (unweighted) (Figure 2a) and quantitative (weighted) (Figure 2b) PCoA plots demonstrated that normal and moribund specimens did not form distinct clusters in foregut and midgut sections. In contrast, two groups were clearly separated in hindguts, suggesting that bacterial profiles differed between normal and moribund hindguts. A total of 23 bacterial phyla were identified from the 15 gut specimens combined, with Proteobacteria, Actinobacteria, Tenericutes, Cyanobacteria, Acidobacteria, Firmicutes, Fusobacteria, Bacteroidetes, Deinococcus-Thermus and Dependentiae being the ten most abundant phyla (Figure 3).

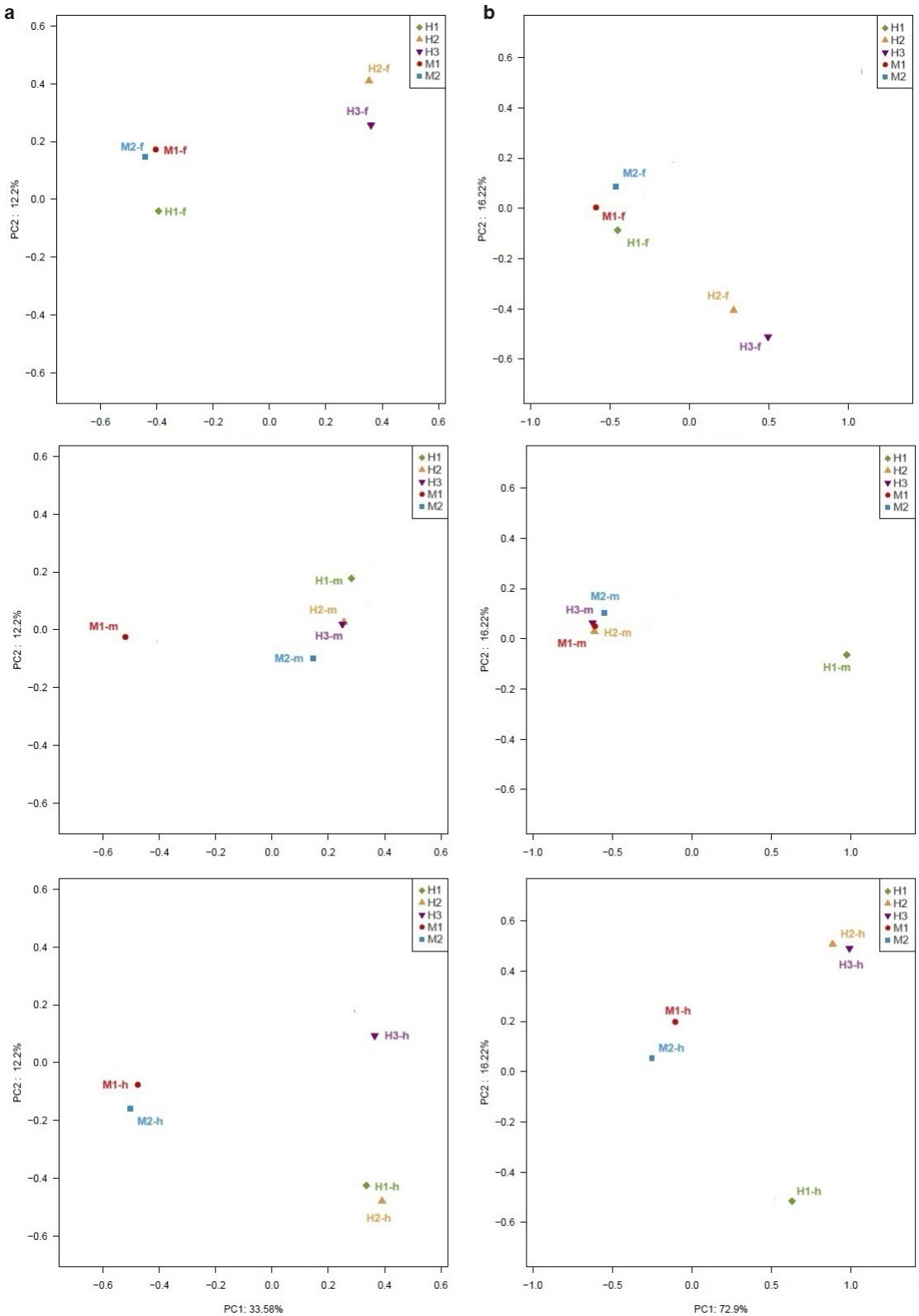


**Fig. 2.** (a) Unweighted and (b) weighted PCoA plots of microbial community composition between normal and moribund foreguts (top), midguts (middle) and hindguts (bottom). Normal and moribund groups formed distinct clusters in hindguts but not in foreguts and midguts. Moribund fishes: M1, M2; Normal fishes: H1, H2, H3.

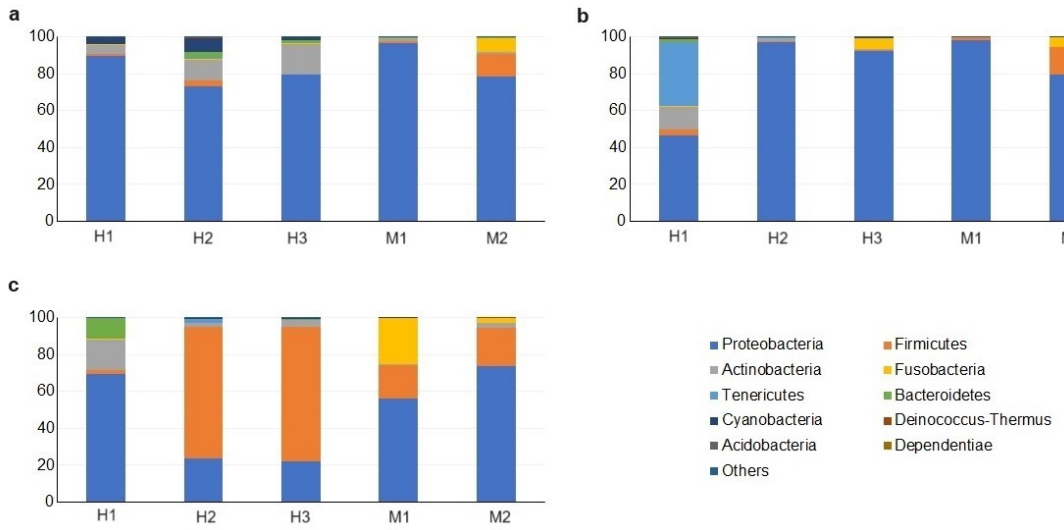


**Fig. 3.** The top 10 microbial phyla identified from (a) foregut, (b) midgut and (c) hindgut of normal and moribund fishes. Moribund fishes: M1, M2; Normal fishes: H1, H2, H3.

Proteobacteria were the dominant phylum in both the foregut and midgut, accounting for more than 78% of the total relative abundance in both groups. In normal foreguts, the next most abundant phyla were Actinobacteria (10.75 ± 5.78%) and Cyanobacteria (4.24 ± 3.17%), whereas in moribund foreguts, Firmicutes (6.59 ± 7.85%) and Fusobacteria (3.98 ± 5.60%) were more prominent (Figure 3a). However, no significant differences were detected at the phylum level in the foregut between the two experimental groups. The top three phyla in normal midguts were Proteobacteria (78.77 ± 27.74%), Tenericutes (11.66 ± 20.13%) and Actinobacteria (4.65 ± 6.23%), whereas the three most abundant phyla in moribund midguts were Proteobacteria (89.02 ± 13.01%), Firmicutes (7.89 ± 9.24%) and Fusobacteria (2.75 ± 3.86%) (Figure 3b). Cyanobacteria was found to be significantly higher ($p<0.05$) in normal midguts than in moribund midguts. (Figure 3c). The microbial composition at the phylum level differed in normal hindguts, where the top three phyla were Firmicutes (48.95 ± 40.26%), Proteobacteria (38.26 ± 26.81%) and Actinobacteria (7.45 ± 7.78%). Meanwhile, the Firmicutes-to-Proteobacteria ratio was lower in moribund hindguts, where Proteobacteria (64.92 ± 12.72%) remained the top phylum, followed by Firmicutes (19.48 ± 1.29%) and Fusobacteria (13.72 ± 15.92%). The abundance of Dependentiae was significantly enriched ($p<0.05$) in normal hindguts as compared to moribund hindguts.

A total of 365 genera were identified across the 15 gut specimens. Genera with lower relative abundance (ranked above the top 30) were grouped under the category 'Others' (Figure 4). The most abundant genera in normal foreguts were classified under *Photobacterium*, *Halomonas* and *Curvibacter* that accounted for 59.10% of the bacterial community (Figure 4a). However, in moribund foreguts, *Photobacterium* and *Vibrio* made up 67.55% of the total abundance. *Brevundimonas* and *Microbacteriaceae_* uncultured were significantly more abundant ($p<0.05$) in normal foreguts. *Photobacterium* and *Vibrio* remained the most dominant genera in midguts of both groups, making up 56.31% in the normal group and 87.19% in the moribund group (Figure 4b). No significantly different dominant genus was detected between normal and moribund midguts. In hindguts, the dominant genera in the normal group were *Bacillus* (44.11 ± 38.18) and *Halomonas* (16.85 ± 18.19), while *Photobacterium* (36.09 ± 6.56) and *Vibrio* (15.13 ± 6.39) were predominant in the moribund group (Figure 4c).

In addition, a significantly higher ($p<0.05$) abundance of genus *Photobacterium,* including *P. aphoticum* and *P. damselae* subsp. *damselae*, was only observed in moribund hindguts (Figure 5), but not in foreguts and midguts.

MaAslin2 was employed to identify the bacterial genera that were potentially associated with the health deterioration in moribund fishes (Figure 6). Several genera were enriched in moribund fish, including *Escherichia-Shigella* in midguts and *Clostridium sensu stricto 1*, G*rimontia Romboutsia* and *Shewanella* in the hindguts. *Vermiphilaceae* and *Eubacterium coprostanoligenes* group were identified to be significantly enriched in normal hindguts.

**Predictive -functional profiles of gut microbial community**

Comparison of fish gut microbiome functional profiles between normal and moribund fishes revealed 15, 6 and 28 significantly distinct ($p<0.05$) functions in the foregut, midgut and hindgut, respectively (Figure 7). Multiple degradation pathways of aromatic compounds were more active in normal foreguts, including catechol degradation, 4-methylcatechol degradation (ortho cleavage), aromatic compounds degradation via β-ketoadipate, phenylacetate degradation I (aerobic), superpathway of phenylethylamine degradation, superpathway of salicylate degradation and toluene degradation III (aerobic) (via p-cresol) (Figure 7a). The tRNA charging activity seems to be significantly more active ($p<0.05$) in moribund midguts. It is worth noting that three pathways, L-rhamnose degradation II, superpathway of L-threonine metabolism and superpathway of L-tryptophan biosynthesis, were not detected in normal midguts (Figure 7b). Meanwhile, microbial functional groups that carry out energy-related metabolisms, such as TCA cycle VII and VIII, aerobic respiration I and various amino acid biosynthesis pathways, were far more abundant in normal hindguts, whereas in moribund hindguts, carbohydrate degradation and lipid biosynthesis were more active (Figure 7c)

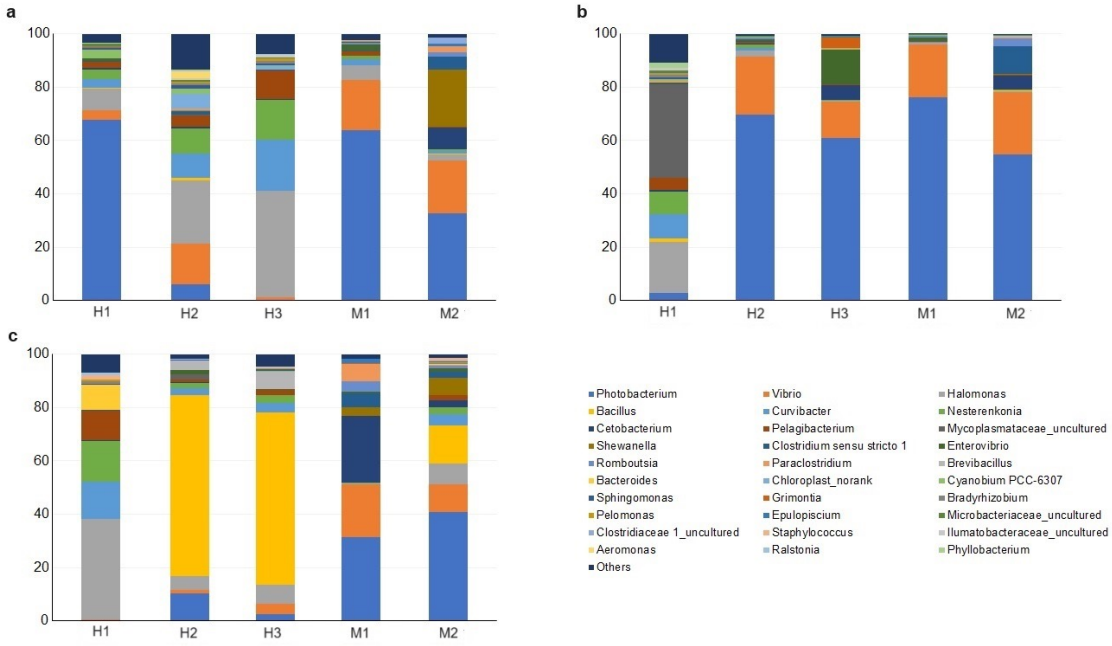


**Fig. 4.** The top 30 microbial genera identified from (a) foregut, (b) midgut and (c) hindgut of normal and moribund fishes.

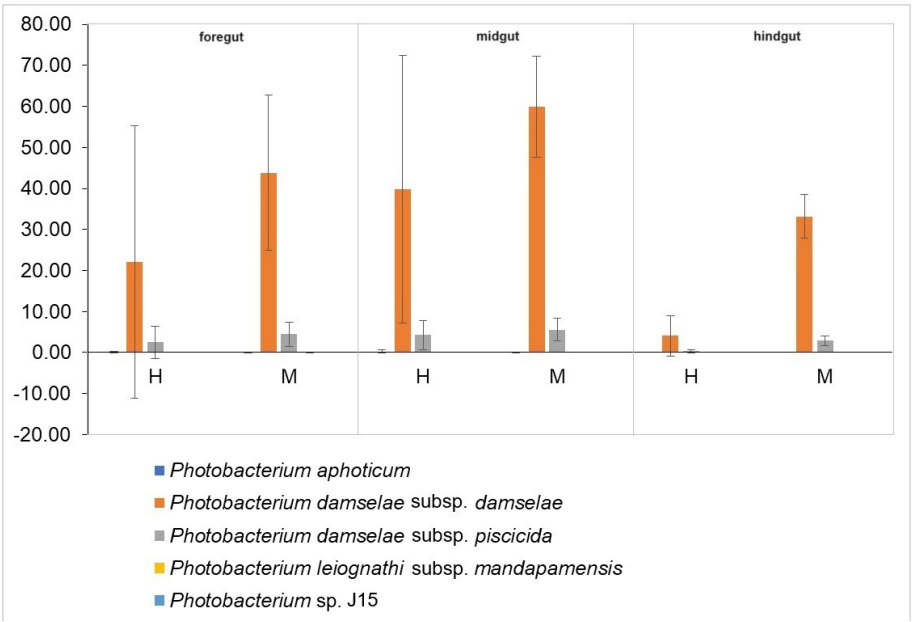


**Fig. 5.** The relative abundance of *Photobacterium* spp. in normal and moribund fish guts. An asterisk indicates a significant difference flagged by Welsh's t-test, $p<0.05$. H, normal; M, moribund.

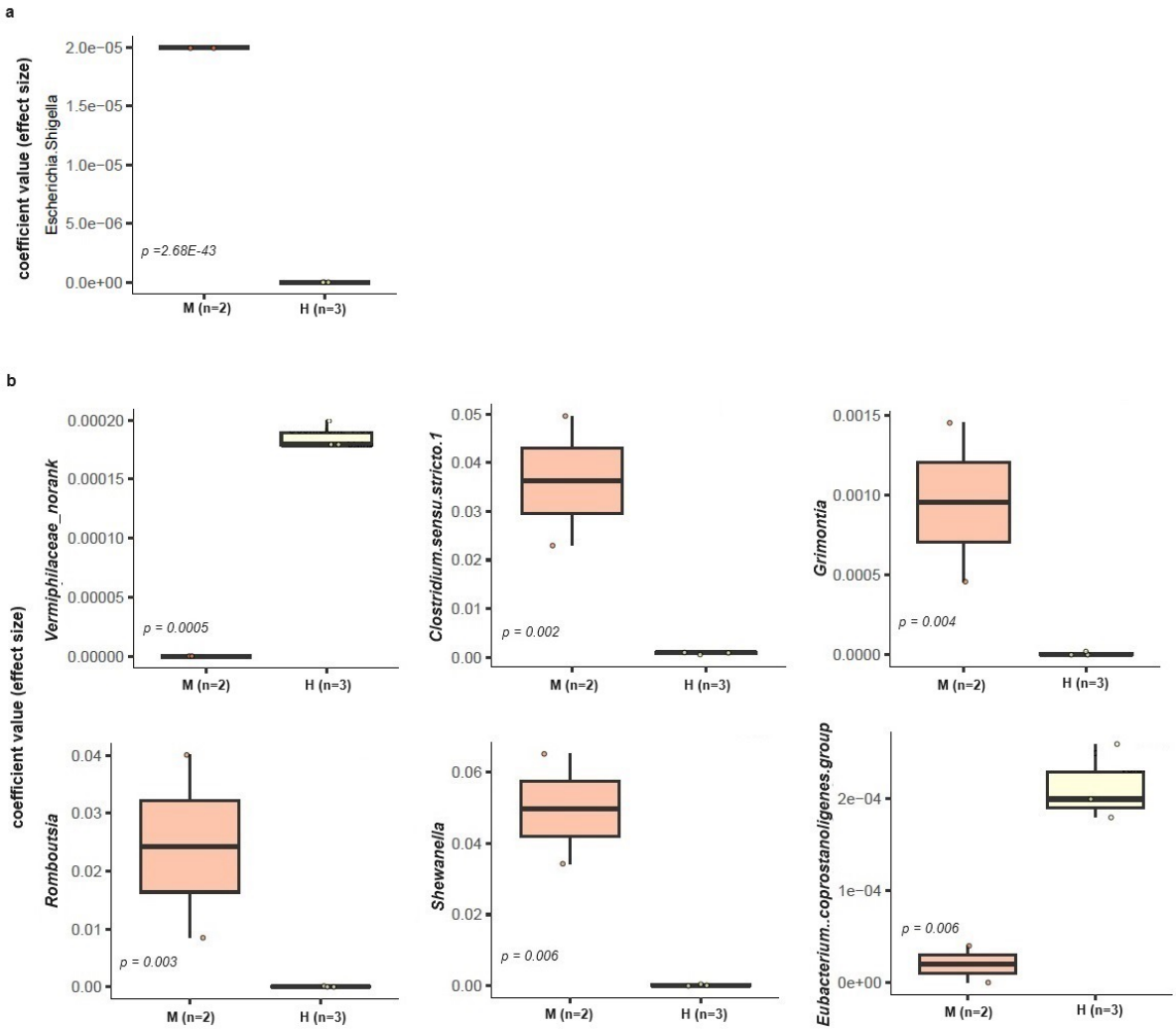


**Fig. 6.** Significantly enriched ($p<0.05$ and FDR<0.25) bacterial genera identified between normal and moribund (a) midguts and (b) hindguts based on MaAsLin2

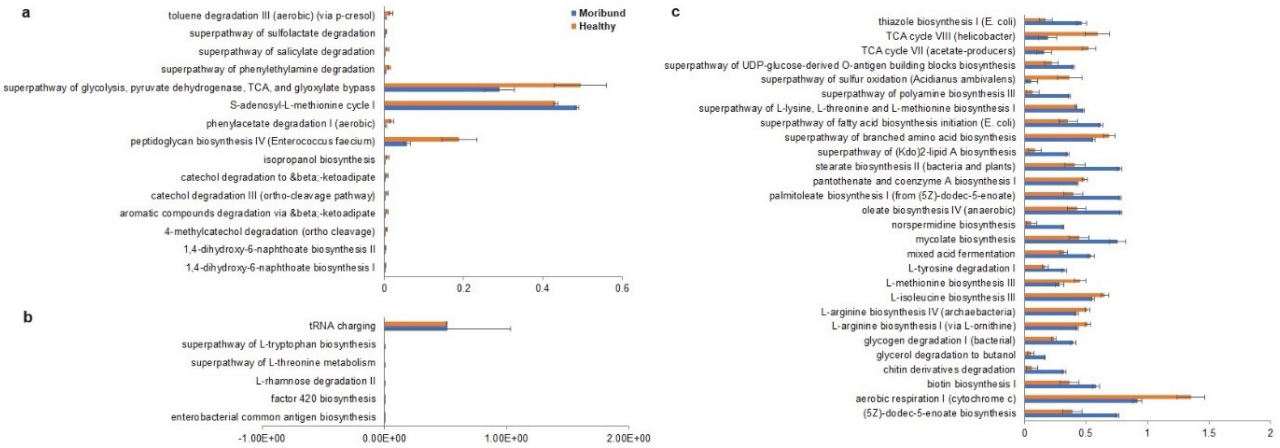


**Fig. 7.** Significant differences in predicted microbial functional abundances between normal and moribund fishes in the (a) foregut, (b) midgut and (c) hindgut identified by Tukey-Kramer post hoc test at $p<0.05$.

## DISCUSSION

Gut microbial communities are known to influence the physiology of the host fish, which they serve as the "hidden organ", regulating the nutrient absorption, homeostasis and immunity while residing in the host gut (Liu *et al*., 2016; Butt & Volkoff, 2019; Yukgehnaish *et al*., 2020; Kakakhel *et al*., 2023). Their association with the fitness of host fish has been previously reported in various fish species, including damselfish and cardinalfish (Parris *et al*., 2016).

In the present study, we explored the microbial diversity in different gut sections, as each section plays specialized functional roles owing to its unique cell structures (Salleh *et al*., 2019) and gene expression profiles (Martin *et al*., 2016). Foregut and midgut are where enteric digestions occur, while the hindgut, with its larger lumen, is important for enteric immune functions

(Buddington *et al*., 1997; Egerton *et al*., 2018; Salleh *et al*., 2019). Thus, the microbial compositional differences among these gut locations revealed in the present study were presumably due to gut functional division, as described in the rainbow trout (Betiku *et al*., 2023). However, irrespective of the health state, the most abundant phyla in all gut sections were Proteobacteria, Actinobacteria, Firmicutes, Fusobacteria and Tenericutes, known to be the common groups in fish gut (Wang *et al*., 2018).

Hindgut is considered a vital structure in fish immunity (Salleh *et al*., 2019), and this is true in our findings, where significant microbiota variation between normal and moribund fish was revealed mainly in moribund hindguts. The analysis outcome in hindguts cued several reasons for the health deterioration. First of all, the reduced Firmicutes-to-Proteobacteria ratio in hindguts may indicate dysbiosis (Wang *et al*., 2020). This is in consistent with a recent study (Duan *et al*., 2023) which had reported that hybrid groupers had lower Firmicutes-to-Proteobacteria ratio when exposed to pollutants and exhibited depression in physiological homeostasis, as observed in moribund hindguts in this study (Figure 7c). Dysbiosis in fish gut microbiota is a known consequence of environmental pollution due to anthropogenic activities (Kakakhel *et al*., 2023) and leads to health deterioration.

Meanwhile, the significantly higher ($p$<0.05) abundance of *Photobacterium* in moribund hindguts is likely correlated with functionally higher degradation of chitin derivatives, though they normally reside in the foregut and midgut to aid the host chitin digestion (Egerton *et al*., 2018). This chitinolytic bacterium is a known opportunistic pathogen in aquaculture (Andreoni & Magnani 2014), and past evidence showed that increased *Photobacterium* may disrupt gut ecological balance, reduce the dominance of beneficial microflora and alter gut-microbiota-mediated functions in groupers (Sun *et al*., 2009; Deng *et al*., 2020; Liu *et al*., 2020). This is reflected in our study, as the moribund hindguts with enriched *Photobacterium* showed functionally enhanced lipid metabolisms, reduced metabolisms of energy, carbohydrate and protein (Figure 7c). To further explore the potential pathogenic role of this genus in moribund fish, the abundance of *Photobacterium* was analyzed at the species level (Figure 5) and revealed that *P. damselae* subsp. *damselae* was significantly enriched in moribund hindguts ($p$<0.05). This strain has previously been known to transmit through water (Fouz *et al*., 2000) and affects a wide range of marine organisms via the skin, leading to skin conditions, including ulcers (Hassanzadeh *et al*., 2015; Gouife *et al*., 2022) and white spots (Shao *et al*., 2019). However, its role as the causative agent of the moribund fish skin conditions (Figure 1b-c) and also the mass mortality episode in Merbok River can only be ascertained by further molecular diagnosis.

Besides *Photobacterium*, MaAsLin2 analysis also flagged the significant abundances of several potentially invasive genera in moribund midguts and hindguts (Figure 6). Despite *Escherichia-Shigella* and *Shewanella* being known bacterial pathogens, information on their harmful roles in marine organisms is limited. Thus, the correlation of their significant abundances on the moribund fish remained unknown.

One big question to be answered was, how did gut microbiomes maintain the well-being of normal fish used in this study? We postulate the supportive role of the enriched *Cyanobacteria* phylum in midguts, and *Eubacterium coprostanoligenes* and *Sphingomonas* genera in hindguts. The presence of gut Cyanobacteria might have played a role in coordinating the microbial community in the normal groupers' gut, promoting the survival of beneficial microbiota and the host immune defences, as reported in Pilotto *et al*. (2019) in Pacific white shrimp. Additionally, *E. coprostanoligenes* is recognized to be beneficial to gut health in humans by positively regulating the host's energy homeostasis and immunity (Mukherjee *et al*., 2020). Furthermore, *Sphingomas* were reported as a core microbiota in Nile tilapia, transferred across generations and presumably supporting the health of offspring (Abdelhafiz *et al*., 2022).

While this study provides novel insights, it is limited by the small number of moribund fish, which may have contributed to data variability across biological replicates in the foregut and midgut (Figure 2), although results were more consistent in the hindgut. In addition, poor documentation and the lack of systematic health assessments in family-run Malaysian fish farms have further hindered the scientific investigation of such mass mortality events. This study lends further support that applying evidence-based scientific approaches is much more effective in identifying and tackling the aquaculture problems, which could facilitate the exponential growth of aquaculture in Malaysia (Ng *et al*., 2023; Yue *et al*., 2023).

## CONCLUSION

In conclusion, our study reveals that, consistent with previous findings in other grouper species (Sun *et al*., 2009; Deng *et al*., 2020; Liu *et al*., 2020), the gut microbiota of farmed orange-spotted grouper (*E. coioides*) undergoes dynamic changes associated with the health status of the host. This is evidenced by the significantly different abundances in health-related microbial taxa between normal and moribund fish. Microbial genera, including *Eubacterium* and *Sphingomonas,* are potentially beneficial gut microbiome that could be developed into probiotics in the aquaculture industry to enhance the general health and pollution tolerance of host fish. On the other hand, the predominant abundance of *Photobacterium* suggests its role in infection and disruption of the gut ecological balance, causing skin conditions. Thus, we strongly recommend the integration of molecular tools into disease management in farm facilities as a whole, and specifically in Malaysia.

## ACKNOWLEDGEMENTS

We thank Universiti Malaysia Terengganu for providing funding support for this project (UMT/SRG/2018/53280) and Pak Su for sharing commercial data collection.

## ETHICAL STATEMENT

The handling of animals complied with the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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