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Metagenomic analysis of gut microbiome in Nile tilapia, *Oreochromis niloticus*: Insights from cultured and lake populations

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ABSTRACT

The application of high-throughput sequencing to investigate the gut microbiome has become increasingly frequent in aquaculture due to its role in monitoring the fish immune system and nutrient absorption. This study examines the gut microbiome of Nile tilapia (*Oreochromis niloticus*) from two distinct environments: a cultured pond and a natural lake. Using 16S rRNA Next-Generation Sequencing (NGS), the microbial communities within the gastrointestinal tract were characterized, and the impact of habitat on microbial diversity and composition was assessed. This study represents the first NGS-based study of the Nile tilapia microbiome in Malaysia. The findings showed that the lake population had significantly higher microbial diversity than the cultured pond population, likely attributable to the more diverse and natural diet in the lake. The dominant bacterial phyla differed by habitat, with *Fusobacteriota* (41.4 %) prevailing in lake samples and *Firmicutes* (71.1 %) in pond samples. Principal Coordinate Analysis (PCoA) revealed significant differences in gut microbiota between habitats. Despite these differences in microbial composition, most phyla were shared across both populations. Overall, these findings provide valuable insights into the gut microbiome of tilapia reared under Malaysian conditions, offering data to support the optimization of tilapia feed and management practices.

Introduction

The global demand for seafood is rising rapidly, increasing pressure on wild fish stocks and the aquatic environment. To address this, aquaculture has become a crucial industry, offering a sustainable solution to meet the growing need for seafood (Theuerkauf et al., 2019). According to the FAO (2020), aquaculture accounted for 46 % of total fish production and 52 % of fish for human consumption in 2018, and this figure is expected to grow to about 59 % by 2030. This trend highlights the importance of implementing efficient and sustainable aquaculture practices to meet future demands. Reflecting this global trend, Malaysia recorded notable growth in aquaculture production, with brackish water output rising by 47.1 % and freshwater output by 9.4 % in 2022 compared to 2021. Species such as grass carp, silver carp, and tilapia are among the major species farmed in global aquaculture (FAO, 2020). Although these species are not native to Malaysia, they have become integral to the aquaculture industry. Over the past decade, fish such as tuna, freshwater catfish (*keli*), river catfish (*patin*), and tilapia have been among the most produced in both freshwater and brackish water environments (Department of Statistics Malaysia, 2023).

As the third most produced species in global aquaculture (FAO, 2020), Nile tilapia (*Oreochromis niloticus*) is particularly important in tropical and subtropical regions, supporting both commercial aquaculture and small-scale farming (Serdiati et al., 2021). Native to the Nile

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River basin in Africa, Nile tilapia has been introduced worldwide and became one of the most widely farmed fish globally. In Malaysia, Nile tilapia was introduced in 1979 due to its fast-growing characteristics and suitability for aquaculture, making it an ideal species to enhance local fish farming efforts (Ang et al., 1989; Lim et al., 2016). Its success in aquaculture can be attributed not only to its hardiness, rapid growth, and adaptability to diverse environmental conditions, but also to its simple dietary requirements and tolerance of varying water quality parameters (Abd El-Hack et al., 2022; Bereded et al., 2022; Da-oh et al., 2024; Mirera & Okemwa, 2023).

However, like other aquaculture species, the sustainability and productivity of tilapia farming are closely linked to fish health, which is significantly influenced by the gut microbiome. This microbiome, comprising diverse bacterial communities, plays a crucial role in various physiological processes including digestion, nutrient absorption, immune system development, and disease resistance (Goh et al., 2023; Talwar et al., 2018). Factors such as diet, environmental conditions, water quality, and habitat strongly influence the composition and diversity of the gut microbiota (Goh et al., 2023). Understanding these influences is essential for optimizing aquaculture practices, as the gut microbiome directly impacts fish growth, feed efficiency, and resilience to disease. Previous studies have shown that the gut microbiota of fish can vary significantly depending on environmental conditions (Bereded et al., 2021; Goh et al., 2023; Talwar et al., 2018; Younes et al., 2023). Wild fish populations, for example, are exposed to a more complex and diverse array of dietary sources and environmental microbes, leading to a richer and more diverse gut microbiome than fish raised in controlled or cultured environments. Wild fish typically consume a variety of natural food sources such as algae, zooplankton, and detritus, which contribute to this diversity (Talwar et al., 2018). In contrast, cultured fish are generally fed formulated pellets that may not support the same level of microbial diversity, potentially resulting in a less complex gut microbiome (Luan et al., 2023).

The significance of the gut microbiome in aquaculture has become increasingly recognized, with research showing that manipulating the microbial community can enhance fish health and productivity (Younes et al., 2023). For instance, the use of probiotics and prebiotics has been shown to modulate the gut microbiome, leading to enhanced growth performance and disease resistance (Luan et al., 2023; Schmidt et al., 2017). Additionally, the composition of the gut microbiome has been linked to a fish ability to metabolize different dietary components, with specific bacterial taxa being associated with the digestion of proteins, lipids, and carbohydrates (Talwar et al., 2018).

In the context of Nile tilapia farming in Malaysia, it is important to understand the influence of different environmental settings, such as natural lakes and cultured ponds, on the gut microbiome of this species. Malaysia's diverse aquatic environments provide a valuable opportunity to study these effects, as tilapia are farmed in a range of settings, from highly controlled aquaculture systems to more natural, extensive systems. The limitation of the present study is that only one site was examined for each population type. Nonetheless, the sequencing depth was sufficient to capture the dominant microbial populations, and the results provide meaningful insights into the differences between natural lake and cultured pond populations. This study aims to address this knowledge gap by employing advanced Next-Generation Sequencing (NGS) techniques to characterize and compare the gut microbiomes of Nile tilapia from a natural lake and a cultured pond in Malaysia. By analysing the microbial diversity and community composition in these different environments, the study seeks to identify the key factors driving these differences and explore their potential implications for tilapia aquaculture. The findings of this research could provide valuable insights into how environmental management and habitat selection influence microbiota in Nile tilapia, offering critical data for optimizing management practices in Malaysian aquaculture.

Material and methods

Specimen handling and processing

A total of eight Nile tilapia (*Oreochromis niloticus*) samples were collected from two different habitats: a cultured pond at an aquaculture extension centre in Machang, Kelantan (5.8007° N, 102.2264° E), and a natural lake in the Universiti Sultan Zainal Abidin (UniSZA) campus in Besut, Terengganu (5.7575° N, 102.6287° E). The cultured pond was supplied with water pumped and filtered from the nearby river, supplemented by the district water supply. These sites were selected to represent contrasting aquaculture and natural environmental conditions. The cultured pond in Machang is a semi-intensive aquaculture site, enclosed with a KOI-net and maintained with a regular feeding schedule of formulated pellets. In contrast, UniSZA's lake is a natural habitat that offers a diverse range of dietary resources, including algae, zooplankton, and detritus. Each fish sample weighed approximately 250–300 g, with a standard length of 18.4–20.2 cm. Water quality parameters (pH, dissolved oxygen, temperature, and salinity) were measured on-site using a YSI multiparameter device and are presented in Table S1.

The gut content was then collected from the midgut to the hindgut (Liu et al., 2016). The gut was sliced open and transferred into a tube containing 30 mL of 99 % ethanol, followed by vigorous vortexing for 5 min to dislodge the gut content from the tissue. The gut tissues were then removed with sterile forceps, leaving only the suspended contents in the tube.

Microbial DNA extraction and 16S rRNA gene sequencing

Microbial DNA was extracted from the intestinal contents of Nile tilapia (*Oreochromis niloticus*) using a DNeasy PowerSoil Kit (Qiagen, UK) with specific modifications. The ethanol tube was vigorously mixed to fully disperse the gut contents. Then, 1.5 mL of the mixture was transferred into a 1.5 mL tube and centrifuged at maximum speed for 10 min to collect the solid gut content at the bottom. After removing the ethanol, the gut content was resuspended in lysis buffer and transferred into a bead-beating tube. Bead-beating was performed by shaking the tube horizontally at approximately 3000 rpm for 30 min. The subsequent steps followed the manufacturer's protocol. The DNA concentration and purity were assessed using a Nanodrop N60 spectrophotometer (IMPLEN, Germany), with the A260/280 ratio used to measure protein content, the 260/230 ratio for organic pollutants, and A260 for DNA concentration.

The 16S rRNA V3 hypervariable region of bacteria was amplified from the genomic DNA template using primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 518R (5′-ATTACCGCGGCTGCTGG-3′) (Klindworth et al., 2013; García-López et al., 2020). To facilitate inline barcoding, the primers were modified by adding an extra 5 bases of inline barcode and a partial Illumina adaptor at their 5′ end (Glenn et al., 2019). Different samples were amplified using different combinations of the forward and reverse inline primers. The polymerase chain reaction (PCR) amplification was conducted using REDiant 2X PCR Master Mix (Axil Scientific Pte Ltd, Singapore). Each PCR reaction was conducted in a 20 μL volume, consisting of 5 μL of REDiant Master Mix, 1 μL of each forward and reverse primers (10 μM), and 4 μL of template DNA (~25 ng/μL). The PCR protocol consisted of an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 20 s, and extension at 72 °C for 10 s. The PCR products were subsequently purified and subjected to a second PCR to incorporate the standard Illumina index. The barcoded amplicons were pooled, purified, and sequenced on the Illumina NovaSeq 6000 (Illumina, San Diego) using the 2 × 150 bp paired-end sequencing configuration.

Data analysis

Demultiplexing and primer trimming of the raw paired-end reads were performed using Cutadapt v1.18 (Martin, 2011) to remove adapter sequences and primers, ensuring high-quality reads for subsequent analysis. The trimmed reads were then merged using Fastp v0.21 (Chen et al., 2018), which also incorporated additional quality control steps, such as filtering low-quality bases and removing short reads, to produce high-quality merged reads. The processed reads were imported into QIIME2 v2023.9 (Bolyen et al., 2019) and denoised using DADA2 v.1.26.0 (Callahan et al., 2016), generating the Amplicon Sequence Variants (ASVs). This denoising step included the removal of chimeric sequences by comparing them with a reference database, thereby ensuring that the resulting ASVs accurately represented unique biological sequences.

For taxonomic classification, the ASVs were assigned to taxa using the q2-feature-classifier (Bokulich et al., 2018), trained with the latest GreenGenes2 database (McDonald et al., 2024). Only ASVs classified at the phylum level were selected for further analysis. The ASV and taxonomic classification tables were then exported from QIIME2 as tab-separated values (TSV format) and manually formatted to ensure compatibility with MicrobiomeAnalyst (Chong et al., 2020) for subsequent analyses.

Alpha diversity, beta diversity, and core microbiome analyses were performed using the Marker DNA Profiling implemented in the user-friendly web-based platform, MicrobiomeAnalyst (Chong et al., 2020). Prior to community profiling analyses, the data were rarefied to the sample with the lowest number of reads to ensure uniform sequencing depth across all samples. Within-sample diversity (alpha diversity) was assessed using indices including Observed, Chao1, Shannon, Simpson, ACE, and Fisher, with Welch's *t*-test applied for statistical comparisons. Beta diversity was evaluated by constructing a dissimilarity matrix, followed by principal coordinates analysis (PCoA) and analysis of group similarities (ANOSIM) based on both Unweighted and Weighted UniFrac distance methods. Core microbiome analysis was carried out in MicrobiomeAnalyst (Chong et al., 2020), applying a prevalence threshold of 20 % and a minimum relative abundance cutoff of 0.01 % to identify core microbial taxa. To assess significantly different abundances of bacterial taxa across samples, the Linear Discriminant Analysis Effect Size (LEfSe) method was employed. Additionally, phylogenetic tree analysis was conducted using MicrobiomeAnalyst to infer evolutionary relationships between the identified taxa and their abundance across habitat.

To explore potential functional pathways across groups, PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Douglas et al., 2020) was used, utilising the KEGG (Kyoto Encyclopaedia of Genes and Genomes) database. The resulting data were then imported into STAMP (Statistical Analysis of Metagenomic Profiles) (Parks et al., 2014) to generate graphical representations of the relative protein abundance.

Results

Aquatic physicochemical factors in different habitats

The primary water quality parameters of two different habitats of the sampled adult *O. niloticus* are presented in Table S1. Temperature and pH levels were similar across both habitats. However, dissolved oxygen and salinity levels were higher in the natural lake (4.90 mg/L and 0.07 ppt, respectively) compared to the cultured pond (4.70 mg/L and 0.02 ppt).

Sequence profiles

The Illumina NovaSeq 6000 16S rRNA sequencing data of eight samples from two different populations were examined for gut microbiota composition. After a series of quality filtration procedures, a total of 3,032,818 read counts were recovered, with an average of 379,102 reads per sample. Of these, 1,478,435 reads originated from the cultivated pond samples, while 1,554,383 reads were obtained from the natural lake samples. The number of ASVs was higher in the natural lake samples (325) compared to the cultured pond samples (249). The rarefaction curves reached a plateau, suggesting that the specific microbial diversity within each sample had been accurately identified (Fig. S1). The sequence integrity was further evaluated using Good's coverage. The coverage estimators for all samples in this study, as determined by Good's method, yielded a value of one (Table S2), confirming that sequencing depth was sufficient and that the majority of bacterial populations present in the samples were captured.

Alpha diversity of gut bacterial communities

The alpha diversity indices (Observed, Chao1, Shannon, Simpson, ACE, and Fisher) were compared to assess the bacterial diversity and richness in the intestines of both cultured and lake Nile tilapia (Fig. 1). The Observed, Chao1, ACE, and Fisher indices indicated that the natural lake population had higher species richness, with p values of 0.044, 0.052, 0.048 and 0.047, respectively. This suggests that the gut microbiome of fish from the natural lake population has more diverse species. The Shannon index ($p = 0.693$) did not differ significantly between habitats. Although the Simpson index was numerically lower in the natural lake population, the difference was not statistically significant ($p = 0.297$). Overall, these findings suggest that the natural lake supports a richer gut microbiome compared to the cultured pond population.

Beta diversity of gut microbiota

Beta diversity analysis was conducted to compare the gut bacterial communities between the two populations. Principal Coordinate Analysis (PCoA) was used to visualize differences, with both unweighted and weighted UniFrac distances were applied to capture variations in species presence and abundance (Fig. 2). For unweighted UniFrac distance, Axis 1 accounted for 46.5 % of the overall variation, while Axis 2 accounted for 28.4 %. For the weighted UniFrac distance, Axis 1 accounted for 75.4 % of the variation, with Axis 2 accounting for 12.9 %. Statistical significance for the differences visualized in PCoA was assessed using ANOSIM and PERMANOVA. The results for the unweighted UniFrac revealed significant separation between the two populations (ANOSIM: $R = 0.77083$, $p < 0.04$; PERMANOVA: R-squared = 0.43604, $p < 0.04$). In contrast, the weighted UniFrac did not yield significant results (ANOSIM: $R = 0.41667$, $p = 0.082$; PERMANOVA: R-squared = 0.37813, $p = 0.127$). These findings suggest that the significant differences in beta diversity detected by unweighted UniFrac, but not weighted UniFrac, are driven primarily by the influence of rare taxa in shaping the gut microbiomes of natural and cultured populations.

Core microbiota composition and taxa abundance

The core gut microbiota composition at the phylum level revealed a similar bacterial structure between the two environments, except that *Planctomycetota* was not detected from the natural lake, and *Actinobacteriota* was absent from the cultured pond population (Fig. 3). The natural lake samples were dominated by *Fusobacteriota* (41.4 %), while cultured pond samples were dominated by *Firmicutes* (71.1 %).

The gut microbial abundance at the phylum level was recovered by specimens (Fig. 4). In the cultured pond specimens, *Firmicutes A* (71.1 %) was the dominant phylum, followed by *Fusobacteriota* (19.1 %) and *Firmicutes D* (4.1 %). In contrast, within the natural lake specimens, *Firmicutes A* dominated only in specimen A8. The remaining three natural lake specimens were primarily dominated by *Fusobacteriota*, with *Firmicutes A* as the secondary phylum. Overall, in the natural lake

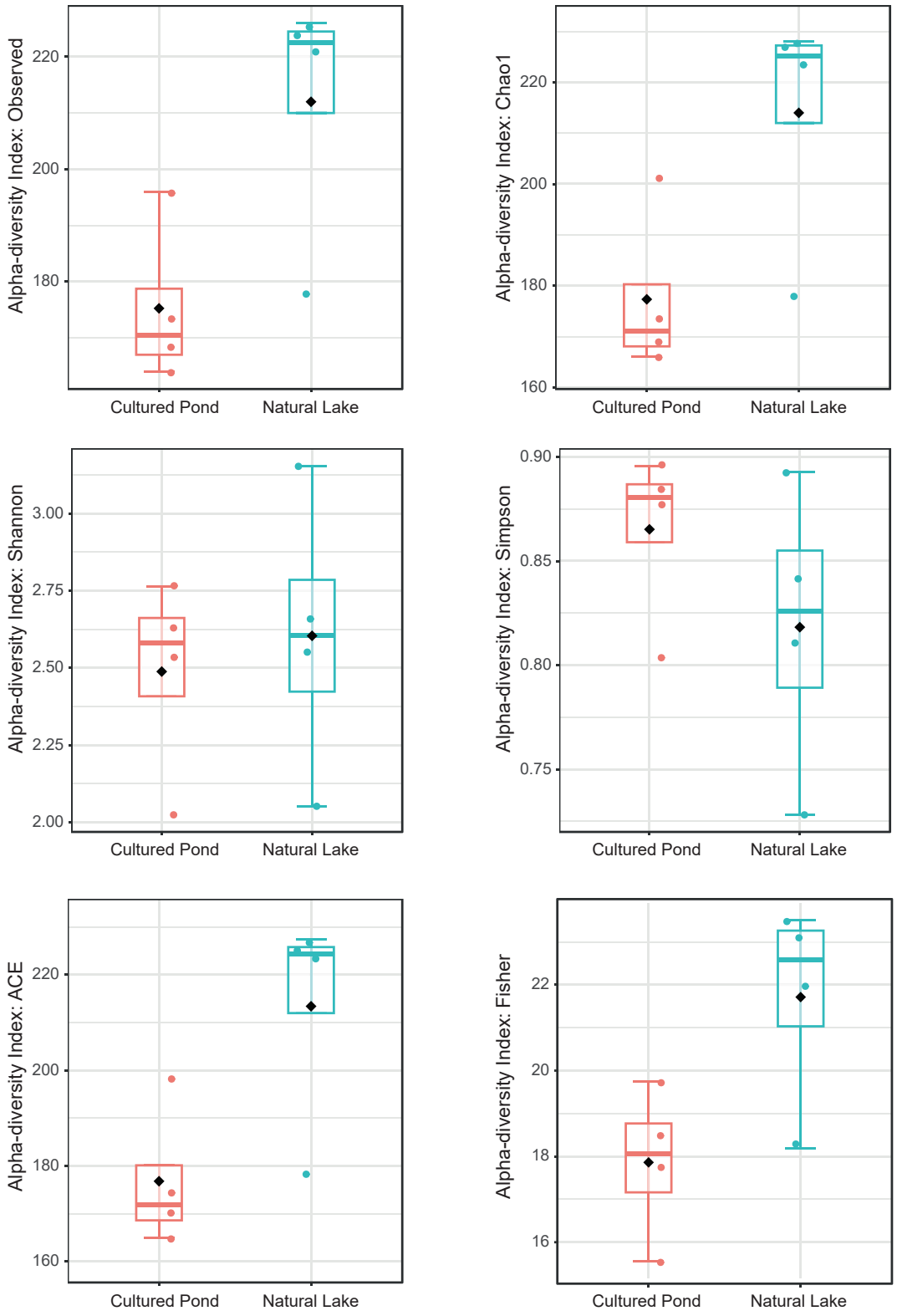

Fig. 1. Alpha diversity indices between the cultured pond and natural lakes population.

population, *Fusobacteriota* (41.4 %) was the dominant phylum, followed by *Firmicutes A* (30.5 %) and *Cyanobacteria* (7 %) across all samples. The phylum *Cyanobacteria* was notably abundant in the natural lake specimens.

Bacterial signatures in different samples

At the lowest taxonomic level, the natural lake samples were predominantly characterized by ASVs from the genera *Cetobacterium*, *Clostridium*, and the family *Peptostreptococcaceae*. In contrast, the cultured pond samples were mainly dominated by ASVs from the family *Peptostreptococcaceae*, with *Clostridium* and *Cetobacterium* following in

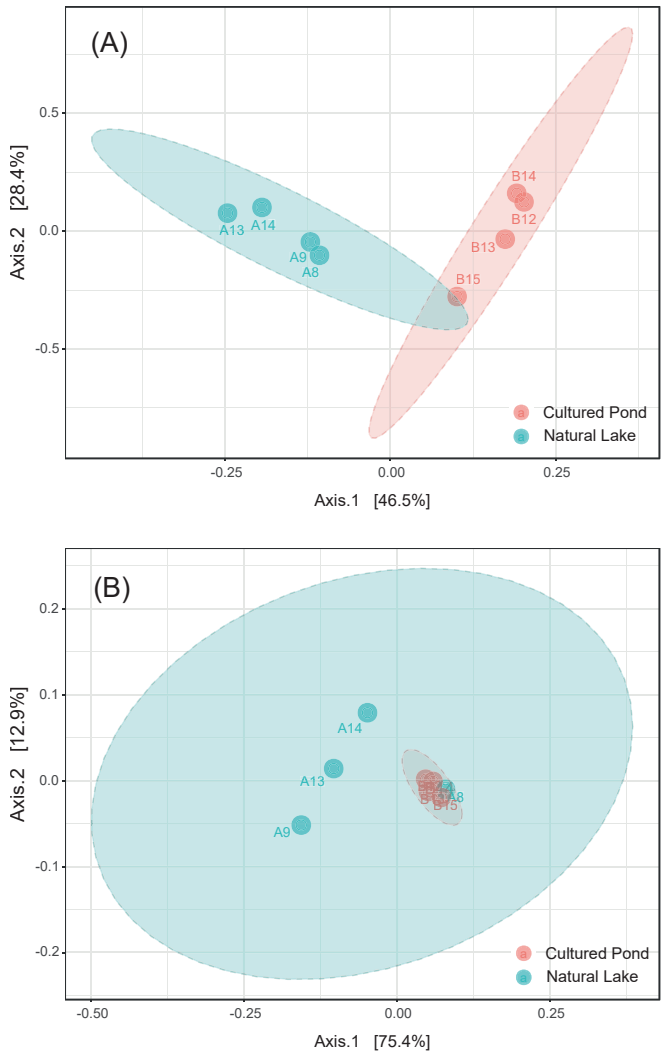

Fig. 2. Principal coordinate analysis based on (A) unweighted UniFrac distance and (B) weighted UniFrac distance between populations.

abundance. The top 50 ASVs heatmap provides a detailed comparison of the gut microbiome composition between the two distinct populations of *Oreochromis niloticus* (Fig. 5). It displays the relative abundance of the most dominant ASVs across all samples, allowing for a clear visualization of microbial community differences influenced by environmental conditions. In the natural lake population, certain ASVs, such as the genera *Cetobacterium* and *Clostridium* were observed to be more abundant compared to the cultured pond population. These ASVs may be associated with the unique environmental conditions of the lake, such as the availability of natural food sources, water quality, and the presence of distinct microbial communities within the lake ecosystem. The diversity in ASV profiles within the natural lake samples suggests a more heterogeneous gut microbiome, potentially reflecting the varied diet and natural habitat of the fish in this environment.

The microbial signatures from the two different populations were detected using Linear Discriminant Analysis Effect Size (LEfSe) to identify microbial communities at both the phylum and ASV levels (to the lowest taxonomic level). Among the identified phyla, *Proteobacteria*, *Actinobacteriota*, and *Bdellovibrionota* were more abundant in the cultured pond population (Fig. 6B). At the ASV level, 20 ASVs showed significant variation between the two populations. LEfSe analysis revealed that *Firmicutes A* (genus *Clostridium P*), *Firmicutes A* (family Peptostreptococcaceae), *Firmicutes A* (order Lachnospirales), *Planctomycetota* (genus *UBA1268*), and *Firmicutes D* (class *Bacilli*) were most

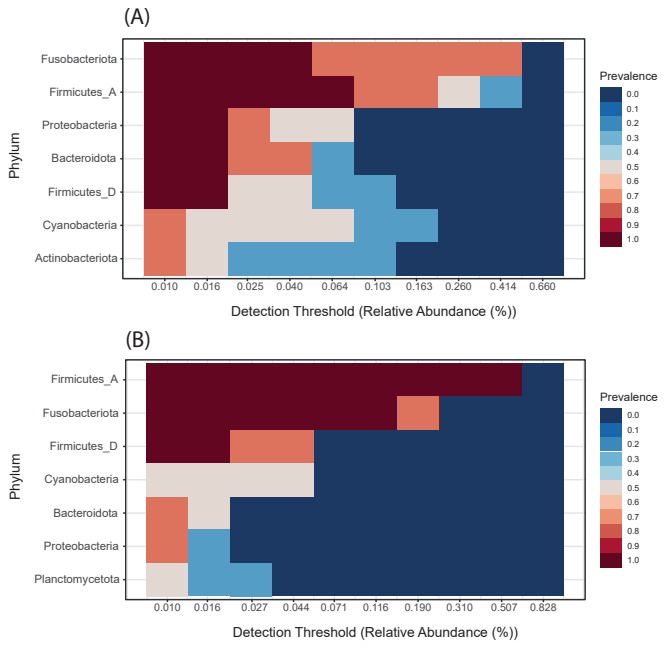

Fig. 3. Core microbiome from (A) natural lake and (B) cultured pond.

abundant in the cultured pond population. In contrast, *Firmicutes D* (genus *Turicibacter*), *Cyanobacteria* (family *Elainellaceae*), *Proteobacteria* (genus *Stenotrophomonas A*), *Bacteroidota* (genus *Paludibacter*), and *Proteobacteria* (genus *Vibrio*) were most abundant in the natural lake population (Fig. 6A).

Bacterial KEGG pathways

KEGG pathway analysis revealed that the gut microbiota was enriched with a total of 398 pathways, of which 55 were significantly different functional pathways related to metabolism ($p < 0.05$, FDR corrected). Based on KEGG level 2 pathways, the overall pathways included amino acid metabolism (29.1 %), carbohydrate metabolism (20 %), energy metabolism (1.8 %), glycan biosynthesis and metabolism (9.1 %), lipid metabolism (21.8 %), cofactor and vitamin metabolism (9.1 %), nucleotide metabolism (7.3 %), and xenobiotics biodegradation (1.8 %). At KEGG level 3 ($p \leq 0.01$, FDR corrected), a total of 10 pathways were statistically significant (Fig. 7). Enriched functional categories such as Urate Biosynthesis/Inosine 5′-Phosphate Degradation, Pyridoxal 5′-Phosphate Biosynthesis I, Superpathway of Pyridoxal 5′-Phosphate Biosynthesis, and Glucose and Glucose-1-Phosphate Degradation were found to be enriched in the natural lake. In contrast, pathways including GDP-Mannose Biosynthesis, Glycogen Degradation I (Bacterial), L-Arginine Biosynthesis IV (Archaebacteria), L-Arginine Biosynthesis I (via L-ornithine), Superpathway of UDP-N-acetylglucosamine-derived O-antigen biosynthesis, and Sucrose Degradation IV (sucrose phosphorylase) were enriched in the cultured farm population.

Discussion

The concept of a fundamental gut microbiome has been suggested for some fish species (Baldo et al., 2015). The core gut microbiota in fish is largely shaped by trophic level, environment, and host phylogeny (Sullam et al., 2012). In this study, significant differences in gut microbiota diversity were observed between Nile tilapia populations from a natural lake and a cultured pond, as indicated by Observed, Chao1, Simpson, ACE and Fisher indices (Fig. 1). Beta diversity analysis based on unweighted UniFrac distance revealed a significant separation between these populations (Fig. 2A), underscoring habitat-driven

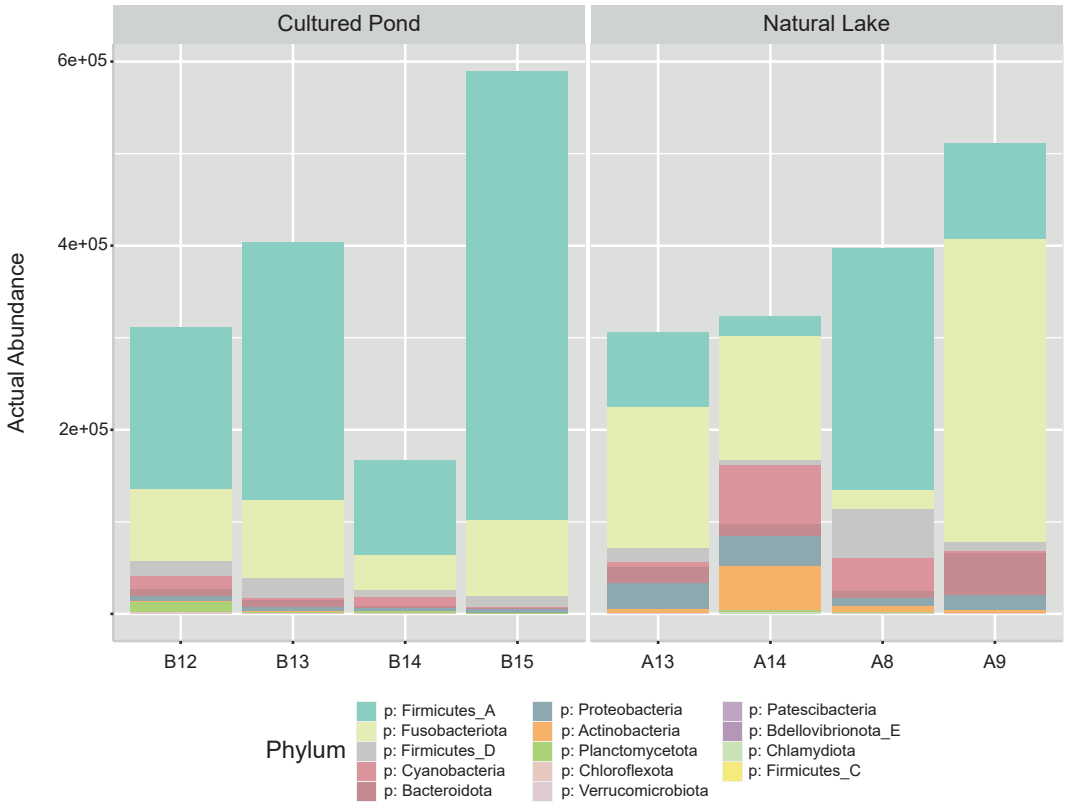

Fig. 4. Abundance bar plot representing the core gut microbiome for all specimens at the phylum level.

differences in microbiome composition.

The dominant phyla identified in this study—*Firmicutes*, *Fusobacteriota*, and *Cyanobacteria*—are consistent with previous findings on the gut microbiota of Nile tilapia. These findings align with previous research on the gut microbiome of Nile tilapia, although variations were observed depending on environmental and dietary factors. For instance, Younes et al. (2023) reported that *Fusobacteriota*, *Proteobacteria*, and *Firmicutes* were dominant in their samples collected from five Japanese prefectures. Their study emphasized the influence of ecological background and source of water (groundwater vs. river) on microbial composition. Zheng et al. (2018) found *Proteobacteria*, *Firmicutes*, and *Cyanobacteria* to be predominant in juvenile genetically improved farmed tilapia reared in aquaculture systems supplemented with resveratrol, highlighting the impact of dietary supplementation on gut microbiota. Similarly, Bereded et al. (2020) identified a core microbiome dominated by *Proteobacteria*, *Firmicutes*, and *Cyanobacteria* in tilapia from Ethiopian lakes, representing wild populations feeding on natural diets. Serag et al. (2022) found *Proteobacteria*, *Firmicutes*, and *Actinobacteria* were the most dominant phyla in tilapia gut microbiota from Egyptian aquaculture. These variations underscore the role of environmental conditions, dietary inputs, and aquaculture practices in shaping the gut microbiome, aligning with the habitat-specific findings observed in this study.

In this study, most phyla were present in both populations, except that *Planctomycetota* was not detected in the natural lake samples, and *Actinobacteriota* was absent from the cultured pond sample. The natural lake samples were primarily composed of *Fusobacteriota*, while the cultured pond samples were dominated by *Firmicutes* (Fig. 3). The variation in bacterial dominance between these habitats can be attributed to differences in food source utilization. This finding is consistent with Bereded et al. (2021), who also found that *Firmicutes* and *Fusobacteria* were the most dominant phyla in natural lake samples, further corroborating the patterns observed in this study. However, unlike the present findings, they reported *Proteobacteria* as the most dominant phylum in their aquaculture samples.

Firmicutes possess numerous enzymes that facilitate the breakdown of dietary components, aiding the host in nutrient digestion and absorption. The predominance of *Firmicutes* in the gut microbiota of Nile tilapia from cultured ponds suggests that these fish are efficient at extracting energy from their food. The primary diet for cultured fish consists mainly of carbohydrate-rich pellets, which are more abundant than natural food sources. *Firmicutes* are known to convert carbohydrates and fibres into short-chain fatty acids (SCFAs) (Bereded et al., 2021). Additionally, they play a key role in fermenting dietary fibres and regulating fat absorption in the intestines. In contrast, the lake environment offers a greater variety of zooplankton and small invertebrates, which are richer in protein and serve as the primary food sources for Nile tilapia in the wild. *Fusobacteriota*, which dominate the gut microbiota in the lake samples, are known to convert proteins and amino acids into butyrate. These bacteria may contribute to modulating the immune system and reducing inflammation in fish. *Fusobacteria*, known for their anaerobic metabolism, ferment amino acids and carbohydrates to produce butyrate, a compound that exhibits immunomodulatory and anti-inflammatory properties (Bereded et al., 2021).

Proteobacteria, another significant bacterial group, play a key role in maintaining a stable gut environment by tolerating toxic conditions and supporting homeostasis under anaerobic environments (Bereded et al., 2021; Moon et al., 2018). In contrast to the present findings, Zheng et al. (2018) and Bereded et al. (2021) found that *Proteobacteria* were the most dominant phylum in their farmed tilapia samples. The higher abundance of *Proteobacteria* in farmed environments may be linked to their prevalence in aquaculture settings, where they are involved in organic matter degradation and nitrogen fixation. These differences highlight the uniqueness of cultured farming in Malaysia and offer new insights into the gut microbiome dynamics of Nile tilapia.

Cyanobacteria, present in both environments, are known to be important food sources for Nile tilapia (Mohamed et al., 2019; Salazar Torres et al., 2016). They possess the ability to fix atmospheric nitrogen into bioavailable forms, enhancing nutrient availability in the gut. They

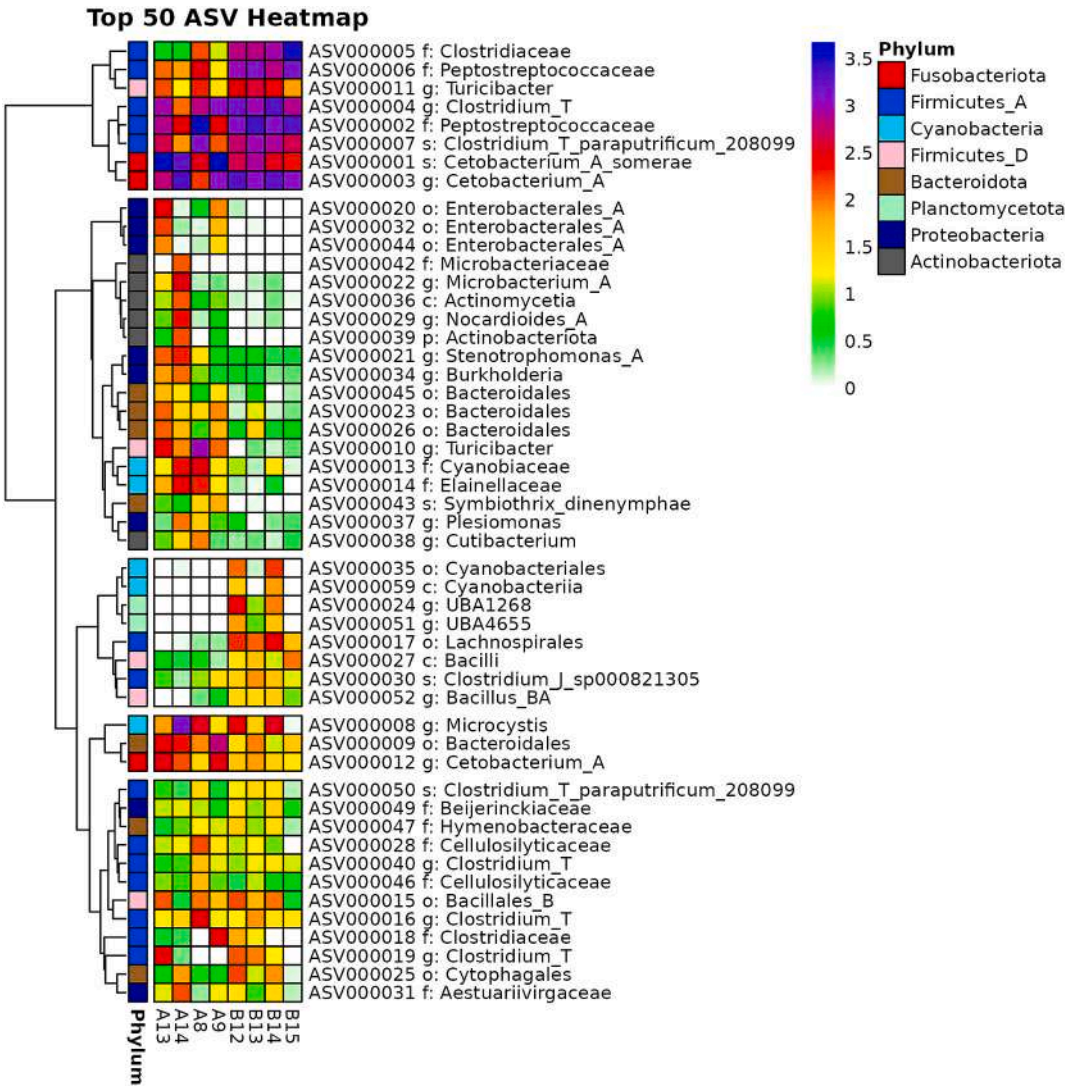

Fig. 5. Heatmap plot of the most abundant ASV identified across samples. The y-axis shows ASVs that were clustered based on Ward2 hierarchical clustering method. ASVs were annotated and coloured based on their taxonomic affiliation at the phylum level. The heatmap scale indicates the relative abundance of ASV (normalized to 10,000 reads/sample) in a 10-based logarithmic scale.

also produce bioactive compounds with detoxifying properties that help neutralise harmful chemicals in the gastrointestinal tract (Paerl & Otten, 2013). Notably, the lake samples showed a higher abundance of *Cyanobacteria* (Fig. 4), which is particularly beneficial given Malaysia's climate, with year-round sunlight provides optimal conditions for *Cyanobacteria* growth. In contrast, farm-reared fish are kept under covered conditions (e.g., with KOI-net cover), limiting sunlight exposure and consequently reducing the presence of algae and green bacteria. This difference in *Cyanobacteria* abundance may contribute to the distinct nutritional profiles of tilapias from natural versus cultured environments.

At the lowest taxonomic level, the most abundant ASVs in the natural lake samples belonged to the genera *Cetobacterium*, *Clostridium*, and the family *Peptostreptococcaceae*. The genus *Cetobacterium*, belonging to the family *Fusobacteriaceae*, was strongly associated with the lake habitat. *Cetobacterium* is commonly found in the gastrointestinal tract of tilapia, as confirmed by previous studies (Bereded et al., 2021; Wu et al., 2021). A strain of *Cetobacterium* isolated from freshwater fish has been shown to synthesize vitamin B-12 and improve glucose metabolism (Tsuchiya et al., 2008). Additionally, *Cetobacterium* can break down organic substances such as ingested organic waste, phytoplankton, and zooplankton (Borsodi et al., 2017).

In contrast, the cultured pond samples were dominated by the family *Peptostreptococcaceae*, followed by the genus *Clostridium* and *Cetobacterium*. The dominance of *Peptostreptococcaceae* in cultured pond samples suggests a microbiome adapted to a carbohydrate-rich, formulated diet. In cultivated ponds, *Peptostreptococcaceae* play a crucial role in nutrient cycling, enhancing water quality by diminishing nitrogenous chemicals and preventing disease through competitive interactions with pathogens. This result was consistent with LEfSe analysis. At the ASV level, numerous ASVs showed significant differences in abundance between the two populations (Fig. 6A). LEfSe analysis identified the genus *Clostridium*, a known cellulose degrader, as a key contributor to the dissimilarity between the populations. This finding aligns with the results of Clements et al. (2007) and Bereded et al. (2020).

Functional analysis of the gut microbiota revealed that pathways related to nucleotide metabolism (Urate Biosynthesis/Inosine 5′-Phosphate Degradation), metabolism of cofactors and vitamins (Pyridoxal 5′-Phosphate Biosynthesis I and the Superpathway of Pyridoxal 5′-Phosphate Biosynthesis), and carbohydrate metabolism (Glucose and Glucose-1-Phosphate Degradation) were significantly enriched in the natural lake populations (Fig. 7). This enrichment may be attributed to the presence of diverse microbial communities and a natural diet rich in organic matter, algae, and *Cyanobacteria*. Several factors could explain

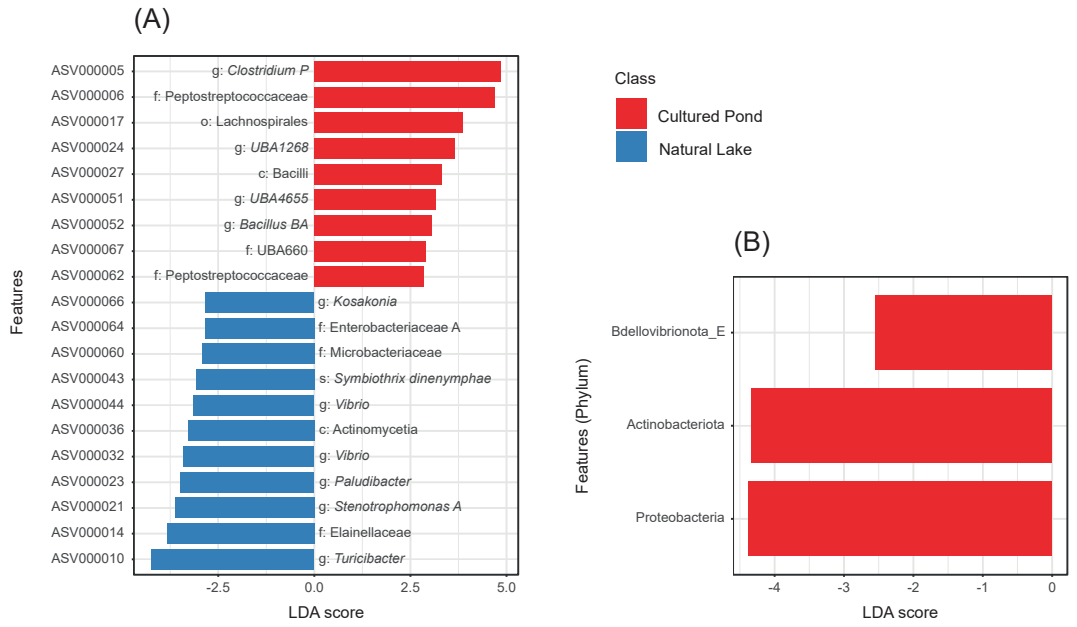

Fig. 6. Linear Discriminant Analysis Effect Size (LEfSe), highlighting the differences in microbial community abundance between the populations, comparing the cultured pond (red) and the natural lake (blue). The bar lengths represent the Linear Discriminant Analysis (LDA) scores. The data presented at (A) the ASV level (lowest taxonomic level) and (B) the phylum level.

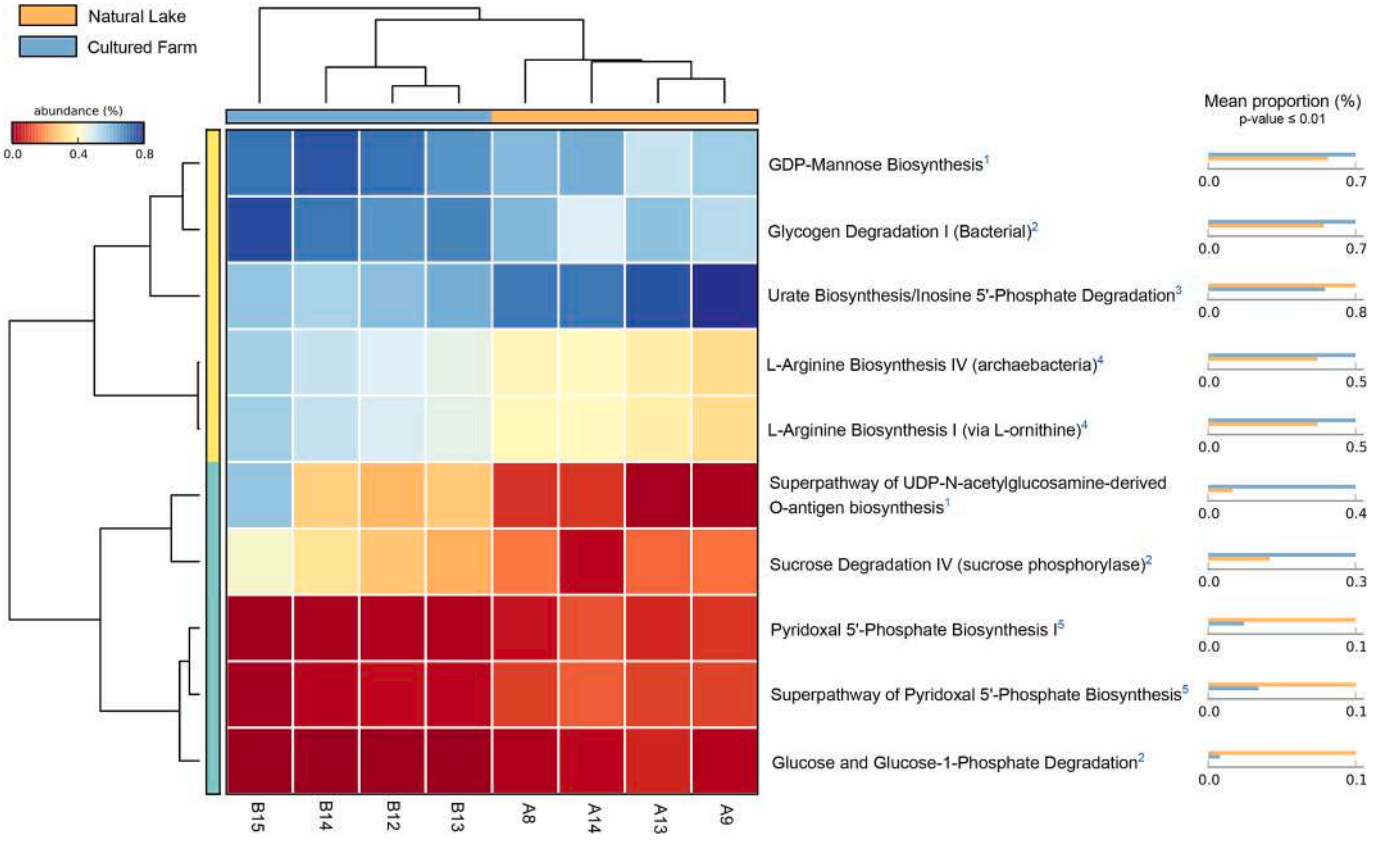

Fig. 7. Functional prediction of level 3 KEGG pathways for two populations using PICRUSt2. A total of 10 KEGG pathways were statistically significant ($p \leq 0.01$, FDR corrected). The numbers at the end of each pathway correspond to KEGG level 2 pathways: 1- Glycan Biosynthesis and Metabolism, 2- Carbohydrate Metabolism, 3- Nucleotide Metabolism, 4- Amino Acid Metabolism and 5- Metabolism of Cofactors and Vitamins.

the differences in these pathways between the natural lake and cultured farm populations. In the natural lake, fish have access to a broader range of food sources, including algae, zooplankton, and microorganisms, all of which contribute to a more diverse gut microbiota (Talwar et al., 2018). This diversity likely enhances metabolic activity, particularly in pathways related to nutrient absorption, such as nucleotide and vitamin

metabolism. The increased availability of essential cofactors like vitamin B6 (Pyridoxal 5′-Phosphate), driven by enhanced vitamin metabolism in the natural lake diverse microbial communities, plays a crucial role in supporting fish growth and overall health in this population (Javanmardi et al., 2020; Teixeira et al., 2012).

In contrast, pathways associated with glycan biosynthesis and metabolism (GDP-Mannose Biosynthesis and the Superpathway of UDP-N-acetylglucosamine-derived O-antigen biosynthesis), carbohydrate metabolism (Glycogen Degradation I [Bacterial] and Sucrose Degradation IV [sucrose phosphorylase]), and amino acid metabolism (L-Arginine Biosynthesis I and IV) were significantly enriched in the cultured pond populations (Fig. 7). Farm-reared fish are typically fed controlled diets, often consisting of processed feed that lacks the diversity of natural nutrients found in the wild (FAO, 2011, 2024). Amino acid pathways were higher in the cultured farm populations may attributed to the reliance on formulated feed, which often includes byproducts such as trash fish or other inexpensive protein sources (Wangkahart et al., 2023).

Conclusion

This study utilized 16S rDNA Next-Generation Sequencing to analyse the gut microbiome of Nile tilapia from two different populations. Significant differences in gut bacterial composition were observed between habitats. Samples from natural lakes exhibited a richer microbiome, with *Fusobacteriota* as the predominant phylum, while cultured pond samples were mainly dominated by *Firmicutes*. This study demonstrates significant differences in microbial diversity and composition between natural and cultured environments, primarily driven by variations in low-abundance taxa. However, the presence of shared core phyla, including *Firmicutes* and *Fusobacteriota*, indicates a conserved microbiome structure across habitats. Future research should expand sampling to multiple sites to explore the functional roles of key bacterial groups and their implications for sustainable aquaculture.

Data availability

Sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra) under BioProject ID PRJNA1184845, with accession numbers SAMN44677341 to SAMN44677348.

Ethical statement

This research was conducted in accordance with the ethical standards of the UniSZA Animal and Plant Research Ethics Committee (UAPREC), Universiti Sultan Zainal Abidin (UniSZA), Malaysia, under approval number UAPREC/008/021.

CRediT authorship contribution statement

Siti Zafirah Ghazali: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Nur Ainin Sofiya Md Gani:** Writing – review & editing, Methodology, Investigation, Data curation. **Nor Ainsyafikah Madiran:** Writing – review & editing, Methodology. **Norshida Ismail:** Writing – review & editing, Validation, Resources, Funding acquisition, Conceptualization. **Veryl Hasan:** Writing – review & editing, Visualization, Methodology. **Romi Novriadi:** Writing – review & editing, Visualization, Methodology. **Han Ming Gan:** Writing – review & editing, Validation, Methodology, Formal analysis. **Victor Feizal Knight:** Writing – review & editing. **Mohd Nor Faiz Norrrahim:** Writing – review & editing, Funding acquisition. **Ahmad Syazni Kamarudin:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejar.2025.10.005.

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