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# Gut Microbiome Dynamics in Farmed Tilapia: A Comparative Analysis of Fingerlings and Adults Infected with Tilapia Lake Virus

**Asha Gopan and K. Riji John***
*Department of Aquatic Animal Health Management, Faculty of Fisheries, Kerala University of Fisheries and Ocean Studies, Panangad, Kochi 682 506*

## Abstract

Tilapia lake virus (TiLV) poses a significant threat to global tilapia aquaculture, yet its impact on the gut microbiota across developmental stages remains poorly understood. This study aimed to compare the gut microbial composition and functional potential of TiLV-infected adult and fingerling tilapia (*Oreochromis niloticus*) from aquaculture systems, using 16S rRNA gene sequencing and PICRUSt2-based prediction. Phylum-level analysis revealed that Proteobacteria dominated in adults, whereas fingerlings exhibited higher levels of Verrucomicrobiota, followed by Proteobacteria and Actinobacteriota. Genus-level differences were prominent: *Acinetobacter, Novosphingobium,* and *Methylobacterium* dominated in adult samples, while fingerlings showed an increased abundance of *Akkermansia*. Alpha diversity was slightly higher in adults, though not statistically significant. Beta diversity analysis revealed greater inter-individual variation among adults, while fingerlings exhibited tighter clustering, indicating a more conserved microbiota. Functional prediction showed enrichment of the membrane transport pathway in fingerlings. In contrast, adults showed higher activity in pathways related to replication and repair, nucleotide metabolism, metabolism of cofactors and vitamins, energy metabolism, and folding, sorting, and degradation. These findings demonstrate apparent life-stage-dependent differences in gut microbiota structure and function in TiLV-infected tilapia. Understanding these microbial patterns may inform age-specific strategies for disease management and microbiome modulation in aquaculture.

**Keywords:** Tilapia lake virus (TiLV), gut microbiota, 16S rRNA gene sequencing, tilapia fingerlings, microbial diversity, functional prediction

## Introduction

The vertebrate gut microbiome is a complex, dynamic community of microorganisms inhabiting the gastrointestinal tract. It contributes to host physiology by modulating nutrient metabolism, immune system development, and defense against pathogens (Sender, Fuchs, & Milo, 2016). In recent years, the gut microbiota of aquatic species has received increasing interest, not only for its effects on host metabolism and growth, but also for its involvement in disease processes and immune modulation (Llewellyn, Boutin, Hoseinifar, & Derome, 2014; Wang, Ran, Ringø, & Zhou, 2018; Luan et al., 2023; Kanika et al., 2025). In aquaculture, microbial dysbiosis has been associated with higher vulnerability to pathogenic infections, reduced growth rates, and increased mortality (Gómez & Balcázar, 2008). The fish gut microbiome is known to undergo significant developmental changes, influenced by diet, immune system maturation, and environmental stressors (Ghanbari, Kneifel, & Domig, 2015). In aquaculture settings, cultured fish are continuously exposed to fluctuating microbial loads, antibiotic residues, and pathogen reservoirs—all of which can influence microbial homeostasis. Life stage is a key determinant of microbial community structure: fingerlings possess an immature immune system and a gut microbiome still undergoing colonization, while adults typically harbour more

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**Email: rijijohn@gmail.com*

stable and resilient microbial communities (Esteban, 2012; Stephens et al., 2016).

In aquaculture, particularly in economically important species like tilapia (*Oreochromis niloticus*), a healthy and diverse gut microbiome is crucial for optimal growth, disease resistance, and overall productivity (Wu et al., 2020). Therefore, understanding the factors that can disrupt this delicate microbial ecosystem is paramount for sustainable aquaculture practices. Tilapia aquaculture, however, is increasingly threatened by infectious diseases, one of the most notable being Tilapia Lake Virus (TiLV), an emerging RNA virus causing significant mortalities in both cultured and wild populations (Eyngor et al., 2014; Ferguson et al., 2014; Behera et al., 2018; Rao et al., 2021; Rajendran et al., 2023). It has been found in more than 16 countries and can result in the mortality of up to 90% of infected tilapia stocks (Surachetpong et al., 2017). The virus primarily affects the liver, kidney, brain, spleen, gills, eyes, and connective tissue of muscle, leading to clinical signs such as lethargy, anorexia, exophthalmia, and scale erosion, often resulting in high mortality rates, particularly in younger fish (Dong et al., 2017). While the primary focus of TiLV research has been on its pathology, epidemiology, and diagnostics, the potential impact of viral infections on the host gut microbiome is increasingly being recognized as a critical aspect of disease progression and overall host health (Thaiss, Zmora, Levy, & Elinav, 2016). Despite extensive work on TiLV pathology and diagnosis, there is still limited understanding of how the virus impacts host-associated microbial communities, particularly at different developmental stages. Viral infections can induce significant alterations in the gut microbial community structure and function, leading to dysbiosis, which may further compromise the host's immune response and increase susceptibility to secondary infections (Belkaid & Hand, 2014). These virus-induced changes in the gut microbiome can vary depending on the host species, the type of virus, and the stage of infection (Mizutani, Ishizaka, Koga, Tsutsumi, & Yotsuyanagi, 2022). In the context of farmed tilapia, limited research has explored the interplay between TiLV infection and the gut microbiome, other than the study by Paimeeka et al. (2024). Considering the varying susceptibility and physiological status of tilapia at different life stages commonly encountered in aquaculture, particularly between juvenile fingerlings and those approaching market size, it is plausible that the TiLV infection may differentially impact the gut microbiome. Fingerlings, often experiencing higher stress in intensive culture systems and possessing developing immune systems and gut microbiota, might exhibit a different response than mature adult fish with a more established microbial community within the controlled environment of culture ponds (Zhou et al., 2018).

Given the increasing incidence of TiLV and growing evidence that gut microbial communities influence disease outcomes, this study investigates and compares the gut microbiome of farmed Nile tilapia (*O. niloticus*) infected with Tilapia Lake Virus at fingerling and adult stages, aiming to identify stage-specific shifts in microbial diversity, composition, and function, and to explore microbial signatures linked to susceptibility or resilience in aquaculture systems. This research will enhance our understanding of host–microbiome–virus interactions in fish and provide valuable insights for developing microbiome-informed disease management strategies in aquaculture.

## Materials and Methods

Moribund Nile tilapia (*O. niloticus*) exhibiting clinical signs of TiLV were collected from two different culture ponds located at 9°54′40.4" N 76°18′57.1" E and 9°59′20.7" N 76°15′23.5" E in Ernakulam district, Kerala, India, during October 2022, where mortality events had been reported. Fish were captured using cast nets and immediately transferred to aerated containers for transport. Individuals were categorized into two developmental stages based on weight and morphology: fingerlings (≤ 5 g) and adults (≥ 100 g). A total of 30 fish per group were sampled. Upon arrival at the laboratory, fish were euthanized using Tricaine Methane Sulfonate (MS-222, 100 mg $L^{-1}$), in compliance with institutional ethical guidelines. Affected fish displayed external clinical signs including lethargy, loss of equilibrium, exophthalmia, body darkening, abdominal distension, and frayed fins. Internally, pale or swollen liver, splenomegaly, and ascites were frequently noted during necropsy. All dissections and sample processing were conducted aseptically for further viral and microbiome analysis.

Tissues (brain, liver, and spleen) were excised and preserved in RNA later at −80 °C until RNA extraction. Total RNA was isolated using the Trizol reagent following the protocol described by Vineetha,

Asha, and Devika (2021). RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and 1% agarose gel electrophoresis.

The diagnosis of TiLV infection was performed primarily by RT-PCR using the sequence of the third segment of TiLV (Behera et al., 2018). RT-PCR was conducted using the detection kit (DSS Takara Bio India) following the manufacturer's instructions. The primers used for the first step RT-PCR were nested ext-1 5′ -TATGCAGTACTTTCCCTGCC-3′ and ME1 5′ -GTTTGGGCACAAGGCATCCTA-3′ with an amplicon size of 415 bp (Eyngor et al., 2014; Tsofack et al., 2017). For semi-nested PCR, primers 7450/150R/ME2 5′ -TATCACGTGCGTACTCGTTCAGT-3′ and ME1 5′ -GTTGGGCACAAGGCATCCTA-3′ with an amplicon size of 250 bp (Eyngor et al., 2014; Tsofack et al., 2017) were used. PCR products were electrophoresed on a 2% agarose gel and visualized in a gel documentation system (Bio-Rad, USA). TiLV-positive individuals were selected for gut microbiome analysis.

Gut microbiome analysis included 48 TiLV-positive Nile tilapia across six groups (three fingerling and three adult groups; n = 8 per group), with samples pooled within each group for analysis. The gastrointestinal tract of each TiLV-positive fish was dissected aseptically. Midgut to hindgut contents were carefully collected into sterile microcentrifuge tubes and stored at −80 °C until further use. DNA was extracted using the CTAB method as described by McMurtrie et al. (2022), with slight modifications. Specifically, the samples were incubated in the dark at room temperature for 2 h after the addition of 0.7 volumes of isopropanol, followed by centrifugation at 10,000 rpm for 30 min at room temperature. DNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer and 1% agarose gel electrophoresis.

Sequencing was performed on a total of 48 TiLV-positive Nile tilapia, comprising three fingerling groups (n = 8 per group) and three adult groups (n = 8 per group), by BioXplore Labs in Tamil Nadu, following the sequencing company's guidelines. The V3–V4 region of the 16S rRNA gene was amplified using the primers: 16sF: 5′ -AGAGTTTGATGMTGGCTCAG-3′ and 16sR: 5′ -TTACCGAGGAMGCSGGCAC-3′. PCR reactions were conducted in triplicate per sample using high-fidelity DNA polymerase (TAQ Master mix). Amplicons were pooled, purified using AMPure XP beads (Beckman Coulter), and quantified with Qubit. Libraries were further purified with AMPure beads and quantified using the Qubit dsDNA High Sensitivity assay kit. Sequencing was carried out on Illumina MiSeq with a 2×300PE V3 sequencing kit.

Raw sequencing reads were demultiplexed and quality-checked using FastQC. Sequence processing, denoising, chimera removal, and Amplicon Sequence Variant (ASV) inference were performed using DADA2 within the QIIME2 environment (v2023.2). Taxonomic classification was assigned using a pre-trained Naïve Bayes classifier trained on the SILVA 138 database. Alpha diversity indices (Shannon, Simpson, Chao1) and beta diversity measures (Bray-Curtis) were calculated. Statistical significance was evaluated using PERMANOVA in QIIME2 and R (v4.2.2) using the phyloseq packages.

PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2) was employed to infer the metabolic potential of the gut microbiota, which estimates functional gene content from 16S rRNA gene sequences (Langille et al., 2013). The predicted gene family abundances were mapped to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) Orthology (KO) database to identify potential functional pathways associated with the microbiota.

## Results and Discussion

The relative abundance of the top bacterial phyla differed markedly between TiLV-infected adult (T) and fingerling (TF) tilapia from culture ponds (Fig. 1). At the phylum level, Proteobacteria was the most dominant group in adult Nile tilapia, accounting for approximately 70–75% of the gut microbiota, followed by Actinobacteriota (5–10%) and Bacteroidota (8–10%). In contrast, Verrucomicrobiota emerged as the predominant phylum in the fingerling stage, comprising 60–65%, while Proteobacteria and Actinobacteriota represented 30–35% and 20–25%, respectively. These stage-specific shifts in dominant phyla indicate substantial variation in gut microbial composition associated with host developmental stage and viral infection.

The gut microbiota at the genus level revealed distinct compositional differences between TiLV-infected adult and fingerling tilapia from culture ponds (Fig. 2). In the adult group, the microbiota

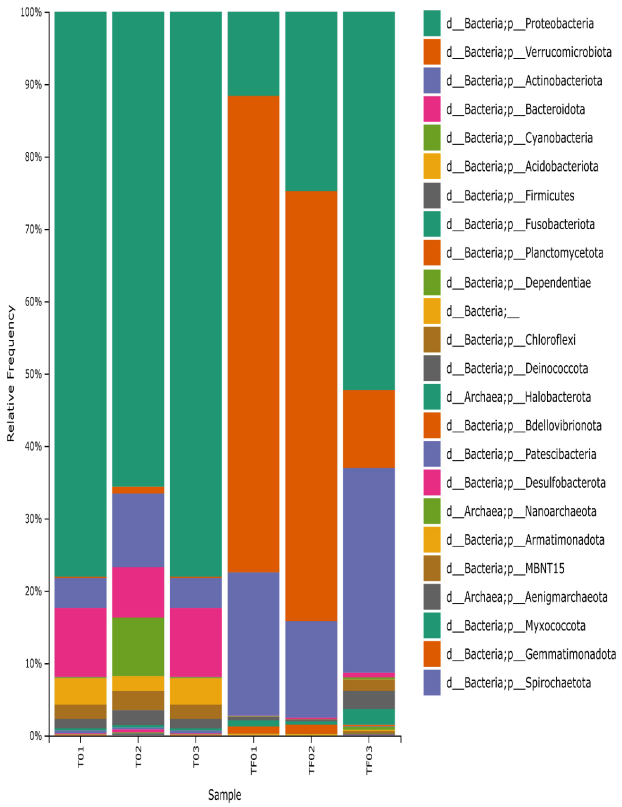


Fig. 1. The abundance of dominant phyla in the adult (T) and fingerling (TF) Nile tilapia infected by TiLV.

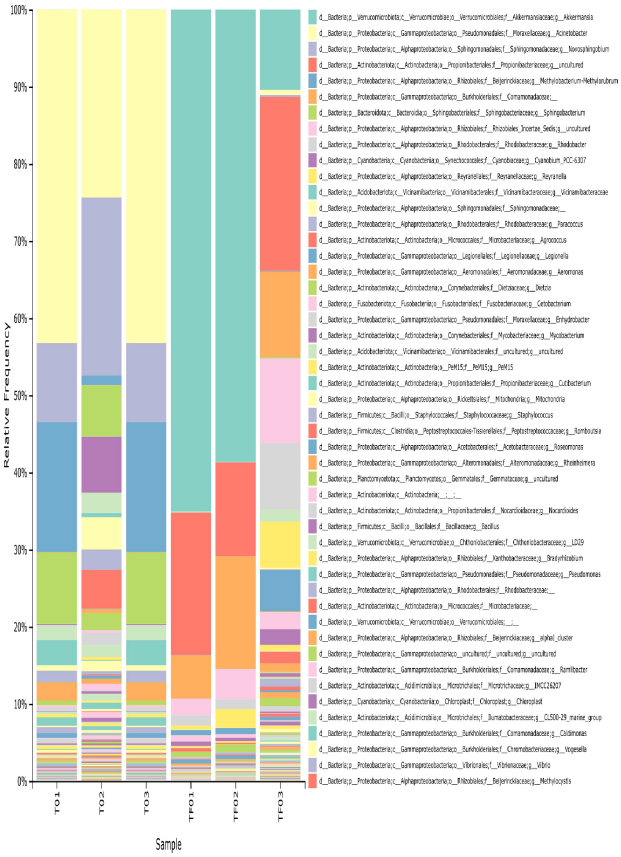


Fig. 2. The abundance of dominant genera in the adult (T) and fingerling (TF) Nile tilapia infected by TiLV.

was predominantly composed of the genera *Acinetobacter, Novosphingobium,* and *Methylobacterium,* belonging to the phylum Proteobacteria, accounting for most of the observed relative abundance. Moderate levels of *Sphingobacterium*, of phylum Bacteroidota, were also detected, consistent with a typical aquatic gut microbiome profile in mature fish. In contrast, the microbiota of fingerlings was markedly different, with a substantial enrichment of *Akkermansia*, a genus associated with Verrucomicrobiota. Additionally, genera belonging to Actinobacteriota and Proteobacteria were also prevalent.

Alpha diversity indices were used to evaluate within-sample microbial diversity in the gut microbiota of TiLV-infected fingerling and adult tilapia (Fig. 3). Although the Chao1 richness index appeared higher in adults, suggesting greater microbial richness, statistical analysis using the Mann–Whitney U test revealed that this difference was not statistically significant ($U$ = 8.0, $p$ = 0.184). Similarly, Shannon and Inverse Simpson diversity indices, which account for richness and evenness, did not differ significantly between the groups ($p$ = 0.400 and $p$ = 0.700, respectively). These findings suggest that, although adult tilapia tend to harbour richer microbial diversity, the observed differences in alpha diversity between fingerlings and adults were not statistically significant.

Beta diversity analysis was performed using Bray-Curtis dissimilarity metrics to assess gut microbial community composition differences between TiLV-infected fingerling and adult *O. niloticus*. PERMANOVA (Permutational Multivariate Analysis of Variance) revealed a statistically significant difference between the two groups ($F$ = 4.27, $p$ = 0.012), indicating that host developmental stage significantly influences microbial community structure in the presence of TiLV infection. A beta diversity heatmap based on pairwise Bray–Curtis dissimilarities further supported this finding. Fingerling samples (TF01, TF02, TF03) exhibited high similarity, as shown by lower dissimilarity values and lighter shading in the heatmap. In contrast, adult samples (T01, T02, T03) displayed greater

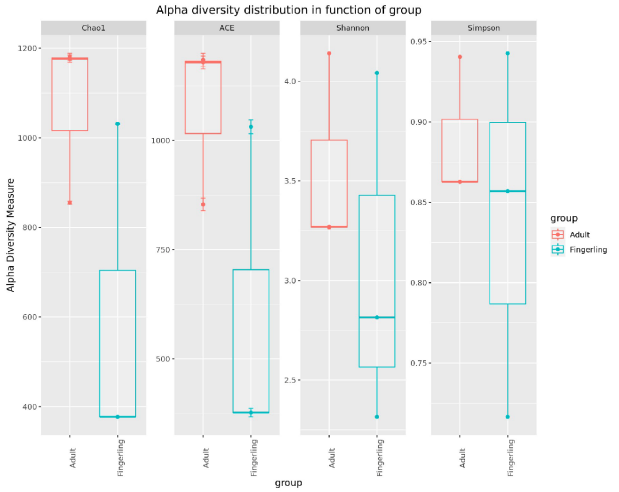


Fig. 3. Alpha diversity indices in the gut microbiota of adult and fingerling Nile tilapia infected by TiLV.

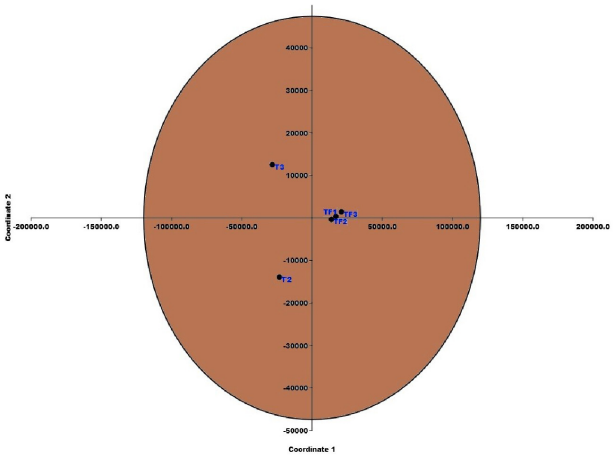


Fig. 4. Principal Coordinates Analysis (PCoA) plot based on Bray-Curtis dissimilarity representing the beta diversity of gut microbiota in TiLV-infected adult (T) and fingerling (TF) Nile tilapia.

inter-individual variability, reflected by a broader range of pairwise distances (Fig. 4). These results suggest that while fingerlings harbour a more conserved gut microbiota, adult fish possess a more individualized and diverse microbial composition.

The functional potential of gut microbiota in TiLV-infected tilapia fingerlings and adults was predicted using PICRUSt2 based on 16S rRNA gene sequences. The KEGG Level 2 pathway analysis revealed distinct functional profiles between the two groups (Fig. 5). In tilapia fingerlings infected with TiLV, metabolic pathways related to membrane transport exhibited higher relative abundances and were significantly more enriched than those in adults. On the other hand, the gut microbiota of adult tilapia with TiLV infection showed increased abundance in pathways associated with replication and repair, nucleotide metabolism, cofactor and vitamin metabolism, energy metabolism, and protein folding, sorting, and degradation. Several pathways, such as amino acid metabolism, carbohydrate metabolism, lipid metabolism, and glycan biosynthesis and metabolism, displayed comparable levels between the two groups, indicating conserved metabolic functions.

This study investigated the compositional and functional shifts in gut microbiota between TiLV-infected adult and fingerling tilapia (*O. niloticus*) from aquaculture systems, revealing clear life stage-dependent patterns in microbial colonization and functional profiles. At the phylum level, distinct differences were evident between fingerling and adult, with Proteobacteria dominating the adult gut microbiota, while Verrucomicrobiota was predominant in fingerlings. Proteobacteria are widely reported as a dominant phylum in freshwater fish gut environments due to their metabolic versatility and ability to respond to environmental shifts (Llewellyn et al., 2014; Ghanbari et al., 2015). Their enrichment in adults may reflect a more complex gut environment and immune landscape in mature hosts. Conversely, the prominence of Verrucomicrobiota in fingerlings, particularly the genus *Akkermansia*, suggests a developing microbiota structure possibly shaped by early host-microbe interactions and immune immaturity. *Akkermansia* spp. are mucin-degrading bacteria often associated with gut barrier function and mucosal health (Cheng & Xie, 2021), and their enrichment in fingerlings may indicate early-stage mucosal development or compensatory responses to TiLV infection. At the genus level, the adult tilapia microbiota was characterized by genera such as *Acinetobacter*, *Novosphingobium*, and *Methylobacterium*, previously linked with aquatic environments and opportunistic pathogenicity (Austin, 2006). The predominance of *Acinetobacter* is of particular interest, as this genus has been implicated in disease states in aquaculture systems (Dong et al., 2019), raising questions about its potential opportunistic role in TiLV-infected adults. In contrast, the microbiota of fingerlings was more homogenized, dominated by *Akkermansia*, and also included Actinobacteriota-related genera, commonly found in early fish gut colonizers and known

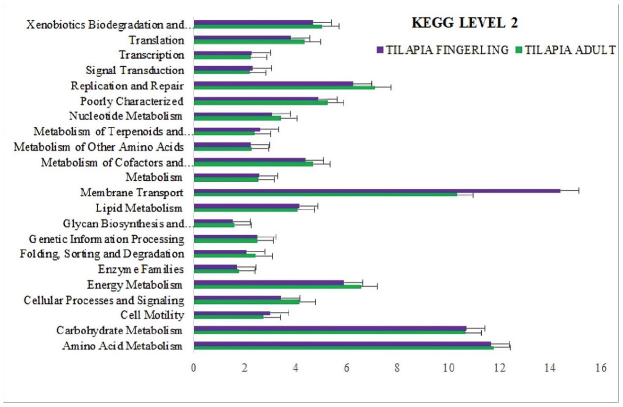


Fig. 5. Predicted functions of gut microbiota of adult and fingerling Nile tilapia infected by TiLV representing the differences in KEGG pathways in level 2.

for their immunomodulatory properties (Ringø et al., 2018).

Alpha diversity indices suggested slightly higher richness in adults. However, the lack of statistical significance aligns with prior studies showing that diversity metrics alone may not fully capture microbial compositional or functional disruptions during infection (Egerton, Culloty, Whooley, Stanton, & Ross, 2018). In contrast, beta diversity analysis revealed statistically significant separation between adult and fingerling microbial communities, supporting the idea that developmental stage contributes to inter-individual variation in microbial assemblages (Stephens et al., 2016). The tighter clustering of fingerling samples in the Bray–Curtis heatmap further supports the hypothesis of a less individualized and more conserved microbiota in early developmental stages, possibly due to limited environmental exposure or immune filtering mechanisms (Giatsis et al., 2015).

The functional prediction analysis offers novel insights into the metabolic capacities of the microbiota under TiLV infection. Fingerlings showed significant enrichment in membrane transport pathways, indicating a potential adaptation toward efficient nutrient acquisition or stress response during early development (Zhou et al., 2018). In contrast, adults exhibited greater enrichment in diverse metabolic functions, including replication and repair, and nucleotide and vitamin metabolism. These functions may reflect a more mature and metabolically versatile microbiota, better equipped to support host maintenance and respond to immunological challenges (Butt & Volkoff, 2019). Interestingly, pathways related to core metabolic functions such as amino acid, carbohydrate, lipid, and glycan metabolism were comparable between groups, suggesting a degree of functional stability despite taxonomic shifts, in line with the concept of functional laying-off in microbial ecosystems (Moya & Ferrer, 2016).

These results, derived from PICRUSt2-based functional prediction, offer insights into the potential metabolic capacities of the microbiota under TiLV infection. However, functional prediction methods, such as PICRUSt2, rely on marker gene inference and reference genome databases, and do not provide direct evidence of gene presence or expression. Limitations include the assumption that closely related organisms share similar functional repertoires, limited database coverage of aquaculture-associated microbes, and the inability to capture strain-level variation or real-time gene expression (Douglas et al., 2020). Therefore, future validation using metagenomic or meta-transcriptomic approaches is recommended. However, the findings from this study highlight the complex interplay between host developmental stage and gut microbiota under viral infection, offering implications for disease management in aquaculture. The age-specific microbiome responses suggest that fingerlings may be particularly susceptible to microbial dysbiosis during TiLV infection, potentially impacting immune system development and nutrient assimilation. Advanced bioinformatics approaches will be essential to further unravel how these metabolic shifts influence TiLV pathogenesis and guide aquaculture management practices. Understanding these shifts could inform the development of life stage-specific targeted probiotic or prebiotic interventions, ultimately improving resilience against viral infections like TiLV.

This study highlights the significant influence of host developmental stage on gut microbiota composition and function in Nile tilapia infected with TiLV. Fingerlings exhibited a more conserved microbial community dominated by *Akkermansia* and enriched membrane transport functions, reflecting a microbiota in early developmental adaptation. In contrast, adults harboured a more individualized and metabolically diverse microbiota, including genera with potential pathogenic roles. Despite similarities in core metabolic pathways, the observed taxonomic and functional divergences underscore the dynamic host–microbe interactions, shaped by both age and infection status. These findings emphasize the need

for stage-specific microbiome management strategies in aquaculture to enhance disease resilience and maintain host health.

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