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RESEARCH PAPER

Effect of Silver Nanoparticles on Gut Microbiota Composition of Striped Dwarf Catfish (*Mystus vittatus*): A Metagenomic Analysis

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Abstract

This study was initiated to investigate the impact of dietary silver nanoparticles (AgNPs) on the gut microbiota of *Mystus vittatus* using metagenomic analysis. Fish were fed a regular diet and an AgNPs-supplemented diet (50 mg/kg) for 75 days, and the gut content was collected for microbial profiling using short-read sequencing of the hypervariable V3-V4 region of the *16S rRNA* gene. The sequencing results identified 18 phyla, 25 classes, 67 orders, 92 families, and 107 genera, clustering into 361 amplicon sequence variants (ASVs). Fish in the AgNPs-fed group showed higher microbial richness, diversity, and evenness, as indicated by increased Chao1 and Shannon index values, whereas Simpson's dominance index was lower. However, both alpha and beta diversity showed no significant difference ($P>0.05$). Taxonomic analysis revealed Proteobacteria, Fusobacteriota, and Firmicutes as the dominant phyla in both groups. Particularly, the AgNPs-fed fish showed an increased abundance of *Bacillus*, suggesting a potential probiotic-promoting effect of AgNPs. Moreover, the exclusive presence of *Ralstonia* and *Candidatus Arthromitus* in the AgNPs group was identified as potential genus-based biomarkers. These results indicate that dietary AgNPs influence gut microbiota in *M. vittatus*, with potential benefits for fish health and aquaculture nutrition.

Introduction

Nanoparticles are minute substances with a size ranging from 1 to 100 nm, and exhibit a high surface-to-volume ratio, enabling enhanced biochemical activity, stability, and biocompatibility (De Silva et al., 2021). Due to these properties, nanoparticles are increasingly explored in aquaculture nutrition and health management. Among them, biosynthesized gold nanoparticles (AuNPs) have been shown to improve growth, survival, and hematological parameters in *Mystus vittatus* (Shahariar et al., 2024). Our earlier experimental trials suggested possible positive responses of *M. vittatus* to nanoparticle-enriched diets. Furthermore, chitosan nanoparticle-enriched diets have been reported to enhance disease resistance in rainbow trout (*Oncorhynchus mykiss*) against *Yersinia ruckeri* (Ahmed et al., 2021).

Conventional strategies to enhance fish growth, immunity, and disease resistance have relied on probiotics, plant extracts, and antibiotics (Harikrishnan et al., 2019). While, probiotics are widely applied in aquaculture, their effects may vary among species (Hossain et al., 2023; Shelby et al., 2006). Excessive antibiotics use can contribute to antimicrobial resistance, disrupts beneficial gut microbiota, and poses

environmental risks (Journal, 2005). These challenges highlight the need to explore additional dietary approaches, such as nanoparticles, that may support fish health while maintaining gut microbiota balance.

Silver nanoparticles (AgNPs) are known for their bactericidal properties, enabling them to attach to and penetrate bacterial cell walls (Sondi and Salopek-Sondi, 2004). AgNPs have also been investigated in livestock and aquaculture for their antibmicrobial microbiota-modulating effects (De Silva et al., 2021; Udayangani et al., 2017; Kumar et al., 2020).

The gut microbiota is a diverse community of microorganisms that plays a crucial role in nutrient metabolism, immune modulation, and overall fish health (Feng et al., 2018). It contributes to nutrient digest and immune defense, for example through short-chain fatty acids (SCFA) produced by *Clostridia* (Eichmiller et al., 2016). In aquaculture and livestock, AgNPs supplementation has also been linked with reduced pathogenic infections, such as *Escherichia coli* in poultry leading to improve growth and survival (Kumar et al., 2020). Examples of other functional groups such as, *Verrucomicrobia* in carp aiding β-glycan breakdown (van Kessel et al., 2011) are discussed later in this section.

Metagenomics is a culture-independent sequencing approach that enables comprehensive profiling of microbial communities without the need for cultivation (Staley & Konopka, 1985). This technique provides detailed taxonomic and functional insights and analyzes gut microbiota composition, microbial diversity, and metabolic potential (Farber & Lusis, 2008). Unlike traditional microbial studies, metagenomics can identify novel or uncultivable microorganisms, which helps to understand host-microbiota interactions (Didelot et al., 2012). Metagenomic approaches have been increasingly applied in aquaculture to explore gut microbial diversity and function. For instance, a higher abundance of some bacteria found in farmed *Labeo rohita* (*Bacillus*, *Clostridium*) compared to wild fish (*Vagococcus*, *Carnobacterium*), highlighting their roles in metabolism and immunity (Ashok Pingle & John Khandagle, 2022). Similarly, chitosan silver nanocomposites altered gut microbiota composition and improved immune responses in *Danio rerio* (Udayangani et al., 2017). However, the effects of AgNPs on gut microbiota diversity and function in fish remain largely unexplored, necessitating further research.

The striped dwarf catfish (*M. vittatus*), a member of the Bagridae family, is a small and valuable indigenous species with high market demand (Mondal et al., 2017). Despite its aquaculture potential, limited research exists on how dietary silver nanoparticles (AgNPs) influence its gut microbiota and physiological responses. *Mystus vittatus* was selected as the experimental species due to its small body size, high adaptability to captive conditions, and economic importance as an indigenous catfish in South Asia. Under laboratory conditions, this species exhibits a high survival rate (approximately 98%), making it suitable for controlled feeding and microbiome studies (Rahman et al., 2022).

To address these knowledge gaps, this study investigates the impact of dietary AgNPs supplementation on the gut microbiota of *M. vittatus* using *16S rRNA* high-throughput sequencing. This study aims to identify key bacterial taxa influenced by AgNPs and evaluate how these nanoparticles shape microbial communities within the fish gut ecosystem. Furthermore, it explores functional alterations in the gut microbiota, particularly in nutrient metabolism and immune-related pathways, which could have physiological implications for fish growth and overall health. The findings will contribute to the development of nanoparticle-based dietary strategies for sustainable aquaculture practices aimed at enhancing fish health, disease resistance, and production efficiency.

Materials and Methods

Experimental Setup, Feeding Trial, and Sample Collection

Fish were obtained from a commercial hatchery and acclimated under controlled laboratory conditions before the experiment. The feeding trial was conducted in six rectangular glass aquariums (60 cm×45 cm×45 cm), each containing 70 L of water and arranged according to the completely randomized design. Each dietary group was assigned to three replicate aquariums, with a total of 360 striped dwarf catfish (60 fish per aquarium) randomly distributed across six aquariums. which were evenly divided into two groups based on diet composition. Water temperature, pH, dissolved oxygen, and ammonia were monitored regularly to maintain optimal conditions, with partial water exchange performed every 2–3 days. Afrin et al. (2023) is cited only as a methodological reference, and the full design details are now provided here for clarity.

The control group (T0), received a commercial feed (0.6 mm, Mega Feed, Spectra Hexa Feeds Limited) without AgNPs (0 mg/kg) supplementation, while the experimental group (T1) was fed the same commercial feed supplemented with 50 mg/kg silver nanoparticles. The selected AgNPs concentration was based on previous findings showing improved microbial modulation, growth and hematological performance at this level. To ensure uniform distribution, AgNPs were ultrasonically dispersed in distilled water using an Elma ultrasonic bath (35 kHz for 10 minutes; Banaee et al., 2016) and thoroughly mixed with the ground feed. The mixture was pelleted, sun-dried for six hours, and stored at -20°C until use. Fish were fed to apparent satiation three times daily, which lasted for 75 days, and the feeding trial was conducted by an assistant to reduce the biasness. The water quality parameters, including DO, pH, and NH_3, were regularly monitored to ensure optimal conditions.

For gut microbiome analysis, six biological replicates were collected, with one fish randomly selected from each of the three replicated aquariums per dietary group. Although limited, this sample size is consistent with previously published metagenomic fish gut studies (Udayangani et al., 2017; Ashok Pingle & Khandagle, 2022) and provides adequate sequencing coverage while maintaining ethical use of experimental animals. Before dissection, all surgical tools, including tweezers, scalpels, and scissors, were sterilized with 70% ethanol (Thermo Scientific, USA) to prevent contamination. The fish were initially rinsed with distilled water, followed by disinfection with 70% ethanol, and rinsed again to remove residual ethanol. Under sterile conditions, the entire gut was extracted while carefully separating the liver and bile ducts to prevent cross-contamination. The gut samples were immediately transferred to sterile Eppendorf tubes (Thermo Scientific, USA), flash-frozen in liquid nitrogen, and stored at -80°C for further analysis.

DNA Extraction and Amplicon Generation

Genomic DNA was extracted from the gut samples using the phenol-chloroform isoamyl alcohol (PCI) method. The purity and concentration of the extracted DNA were evaluated by 1% agarose gel electrophoresis, and the DNA was diluted to 1 μg/μL using sterile water.

For amplicon sequencing, the hypervariable V3-V4 region of the *16S rRNA* gene was amplified using the universal primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2012). The PCR reactions were conducted using 15 μL Phusion® High-Fidelity PCR Master Mix (New England Biolabs), with 0.2 μM of each primer and 10 ng of template DNA. The thermal cycling conditions included an initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s, with a final extension step at 72°C for 5 min.

Following amplification, the PCR products were mixed with an equal volume of 1X loading buffer containing SYBR Green and subjected to 2% agarose gel electrophoresis for quality verification. Equimolar amounts of the PCR products were pooled and purified using the Qiagen Gel Extraction Kit (Qiagen, Germany) to prepare for sequencing.

Library Preparation, Sequencing, and Quality Control

Amplicon sequencing was conducted at Novogene (China). The sequencing libraries were prepared using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA), and index codes were incorporated to allow for sample identification. Library quality assessment was performed using a Qubit® 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system.

Sequencing was performed using an Illumina NovaSeq 6000 platform, generating 250 × 2 bp paired-end reads targeting the V3-V4 hypervariable region of the *16S rRNA* gene. The raw sequencing reads were demultiplexed based on barcode sequences, followed by the removal of barcode and primer sequences. High-speed merging of paired-end reads was conducted using FLASH (v1.2.7) to produce full-length sequences. The QIIME2 (v1.9.1) pipeline was used for quality filtering, following the stringent criteria described by (Bokulich et al. 2013). Chimera sequences were detected and removed using the UCHIME algorithm, with reference-based filtering performed using the Silva database to retain only high-quality sequences.

Data Processing and Bioinformatics Analysis

The raw sequence data underwent denoising through the DADA2 algorithm, which filtered out low-abundance sequences and generated high-resolution amplicon sequence variants (ASVs). Taxonomic annotation was found performing a pre-trained Naive Bayes classifier within QIIME2 and compared with silva database(Quast et al., 2013) .

A Venn diagram was established to show the shared and unique part of the observed ASVs. To assess microbial diversity, alpha diversity indices such as Chao1, Shannon index, and Simpson's dominance index were calculated to evaluate species richness and evenness. Beta diversity analysis was conducted using principal coordinate analysis (PCoA) to compare microbial community structures between treatment groups. The R statistical package was used for data visualization.

For taxonomic characterization, species abundance was analyzed at multiple levels, including phylum, genus, and species. Relative species composition was visualized using Qiime2 plugins, and bar plots were generated to compare microbial distributions between experimental groups.

Functional Prediction and Statistical Analysis

To predict microbial functional potential, the PICRUSt2 bioinformatics tool was used to infer gene pathways from *16S rRNA* data. Some figures related to PICRUSt2 functional annotation have been moved to Supplementary Materials, as recommended by the reviewers, to optimize readability and maintain the primary focus on taxonomic findings.

Statistical analyses were performed using multiple methods. The T-test and MetaStat approaches were applied to determine differences in microbial composition between groups. Additionally, LEfSe (Linear Discriminant Analysis Effect Size) analysis was used to identify significantly enriched taxa, with a cutoff threshold of LDA score >2.0 and $P<0.05$(Segata et al., 2011).

Results

Sequencing Outcomes

The sequencing data from this study were submitted to NCBI under BioProject ID PRJNA1193237, with BioSample accession numbers SAMN45134591 to SAMN45134593 and SAMN45134597 to SAMN45134599. High-throughput sequencing of the *16S rRNA* gene was performed to analyze the gut microbial communities of *M. vittatus* fed either a control diet or a diet supplemented with AgNPs. At the domain level, bacterial sequences accounted for 98.35% of the total reads, while Archaea contributed 1.65%.

A total of 716,968 raw paired-end reads were generated. After quality filtering and chimera removal, the final dataset contained 694,540 high-quality reads, comprising 297,373,141 base pairs (bp), with an average sequence length of 428.15 nucleotides (nt). The sequencing reads identified 18 phyla, 25 classes, 67 orders, 92 families, and 107 genera, which were clustered into 361 amplicon sequence variants (ASVs).

The sequencing depth was assessed using Good's coverage index, which was 0.99 for both groups, indicating that further sequencing would not significantly increase the detection of novel microbial taxa. The Phred quality score (Q30) was 95.03%, ensuring high sequencing accuracy with minimal errors.

Gut Microbiome Diversity Analysis

A comparison of unique and shared ASVs between the control and AgNPs-fed groups revealed that 219 ASVs were unique to the AgNPs-fed group, while 97 ASVs were unique to the control group. Both groups shared 45 ASVs (Figure 1).

Alpha diversity indices, including Chao1, Shannon, Simpson, and Dominance indices, were calculated to assess microbial richness and diversity (Table 1). The average Chao1 index was significantly higher in the AgNPs-fed group (105) compared to the control (58.22), suggesting an increased number of microbial species. Similarly, the Shannon diversity index was slightly higher in the AgNPs-fed group (2.58) compared to the control (2.47), indicating greater microbial diversity and evenness. In contrast, the Simpson's dominance index was higher in the control group (0.739) than in the AgNPs-fed group (0.677), suggesting that a few dominant species were more prevalent in the control group.

While no statistically significant differences were observed (P>0.05), the trends in microbial richness and evenness indicate a potential microbial modulation effect of dietary AgNPs. Beta diversity was analyzed using Principal Coordinates Analysis (PCoA) based on the unweighted UniFrac method (Figure 2). The PCoA plot demonstrated no distinct clustering between the

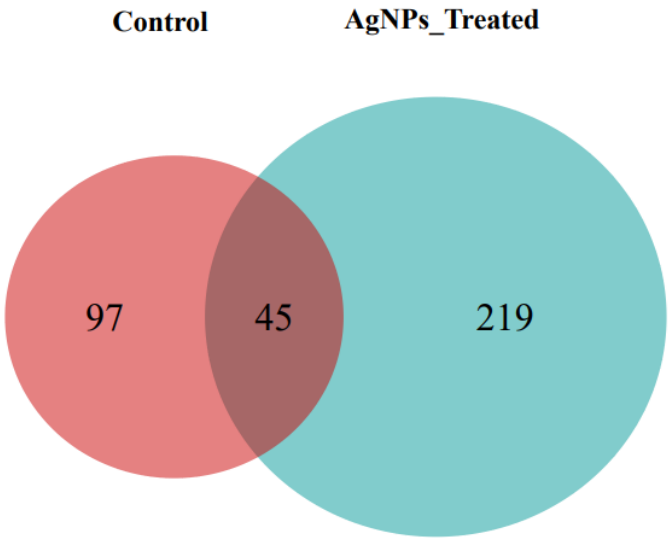

Figure 1. Venn diagram showing shared and unique ASVs of *M. vittatus* gut microbiota communities from each group.

Table 1. Alpha diversity index of control and AgNPs_treated groups

Sample Name		Chao1	Goods Coverage	Shannon	Simpson
Control	R1	72.66667	0.999964	2.674439	0.755883
	R2	73	0.999994	2.793187	0.804096
	R3	29	0.999994	1.933708	0.659885
AgNPs	R1	44	1	2.69112	0.735887
	R2	114	0.999994	1.508212	0.470903
	R3	157	1	3.536545	0.828134

control and AgNPs-fed groups, indicating that the overall microbial community structure remained similar between the two groups. The greatest dissimilarity was observed between Control_R1 and AgNPs_Treated_R1, but the differences were statistically insignificant (P>0.05).

Taxonomic Profiling of Gut Microbiota

The taxonomic composition of the gut microbiota in both groups was analyzed at different levels. At the phylum level, the gut microbiota was dominated by Proteobacteria, Fusobacteriota, and Firmicutes, which together accounted for more than 98% of the total microbial community. Proteobacteria was the most abundant phylum in both groups, comprising 98.20% in the control and 93.49% in the AgNPs-fed group (Figure 3). Fusobacteriota were the second dominant phylum in the control group (1.26%), and Firmicutes were the second dominant phylum in AgNPs_treated group (2.53%) indicating a microbial shift.

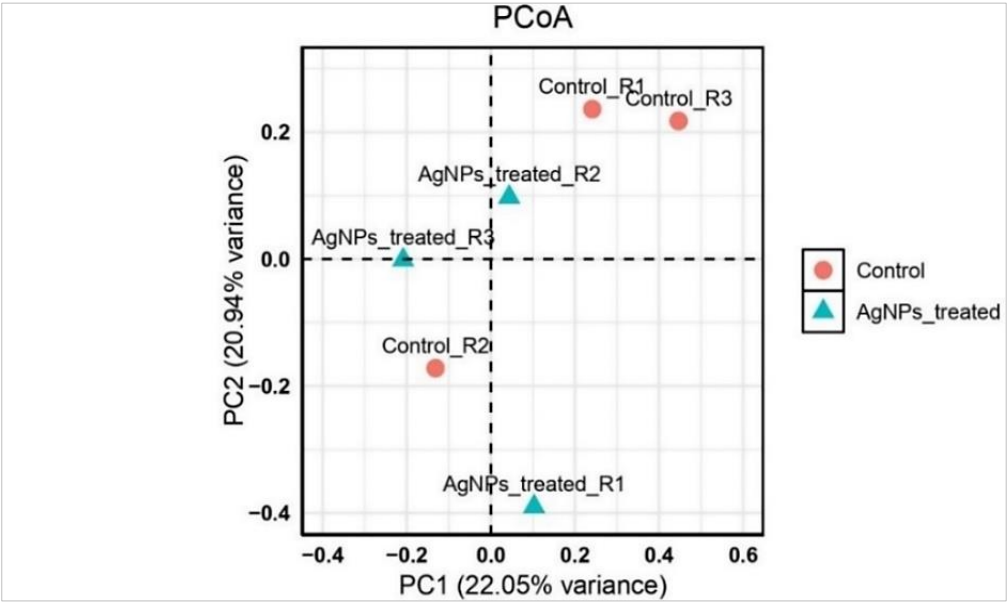

Figure 2. Beta diversity visualization through a PCoA plot.

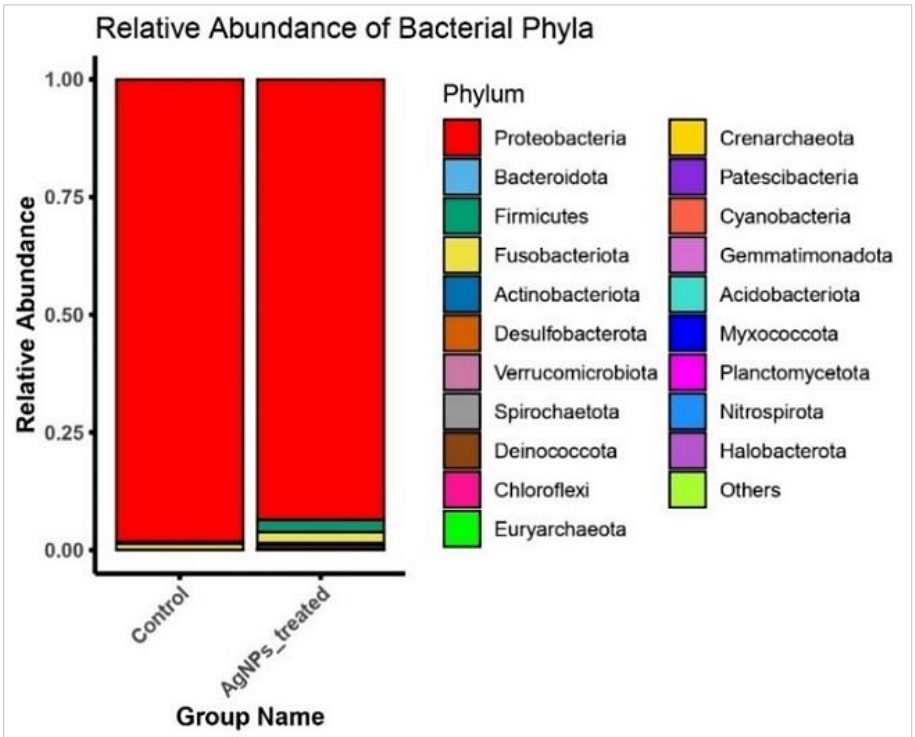

Figure 3. *M. vittatus* gut microbiota composition at the phylum level. Multi-colored bar plots showing the relative abundance of microbial phyla.

At the genus level, a substantial proportion of bacterial taxa in the control group remained unclassified, accounting for 97.29% of the total bacterial community. In contrast, the AgNPs-fed group exhibited a higher proportion of classified genera, with *Enterobacter* (48.75%), *Plesiomonas* (22.24%), and *Cetobacterium* (2.27%) being the dominant genera (Figure 4). Interestingly, *Ralstonia* and *Candidatus Arthromitus* were exclusively identified in the AgNPs-fed group. T-test analysis identified *Ralstonia*, *Candidatus Arthromitus*, and *Bacillus* as genus-level biomarkers associated with dietary AgNPs supplementation ($P<0.05$). Additionally, 23.35% of bacterial genera in the AgNPs-fed group remained unidentified, suggesting the presence of potentially novel or understudied microbial taxa.

Predictive Functional Analysis of Gut Microbiota

Predictive functional analysis was performed using PICRUSt2, revealing functional differences between the microbial communities of the control and AgNPs-fed groups. Functional pathways were classified according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) at three hierarchical levels. At Level 1, functional pathways were primarily associated with metabolism, environmental information processing, genetic information processing, cellular processes, human diseases, and organismal systems. Metabolism was the most dominant functional category in both groups, with a slight increase in the AgNPs-fed group, suggesting that dietary AgNPs may enhance metabolic activity in *M. vittatus* (Figure 5). At Level 2, a total of 41 sub-pathways were identified, with membrane transport, carbohydrate metabolism, amino acid metabolism, and replication and repair being the most prominent pathways (Figure 6). Notably, except for carbohydrate metabolism, which was slightly higher in the control group, all other metabolic sub-pathways were more prevalent in the AgNPs-fed group. Membrane transport-related pathways exhibited a marked increase in the AgNPs-fed group, indicating potential alterations in nutrient absorption and microbial interactions. At Level 3, specific metabolic sub-pathways related to amino acid metabolism, lipid metabolism, and vitamin biosynthesis were significantly enriched in the AgNPs-fed group (Figure 7). These included pathways for glycine, serine, and threonine metabolism, valine, leucine, and isoleucine biosynthesis, histidine metabolism, and phenylalanine, tyrosine, and tryptophan biosynthesis. Additionally, pathways associated with fatty acid metabolism, glycerophospholipid metabolism, and lipopolysaccharide biosynthesis were also more abundant in the AgNPs-fed group. Conversely, pathways associated with carbohydrate metabolism, including glycolysis, fructose and mannose metabolism, and starch and sucrose metabolism, were more prevalent in the control group.

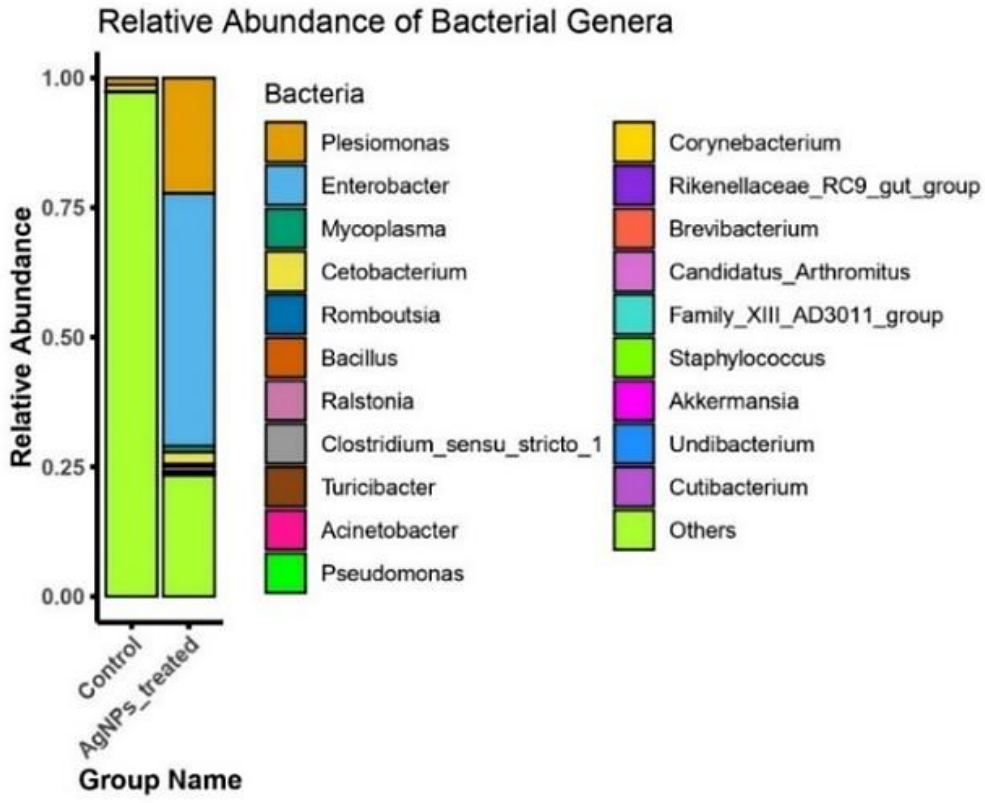

Figure 4. *M. vittatus* gut microbiota composition at the genus level. Multi-colored bar plots showing the relative abundance of microbial genera.

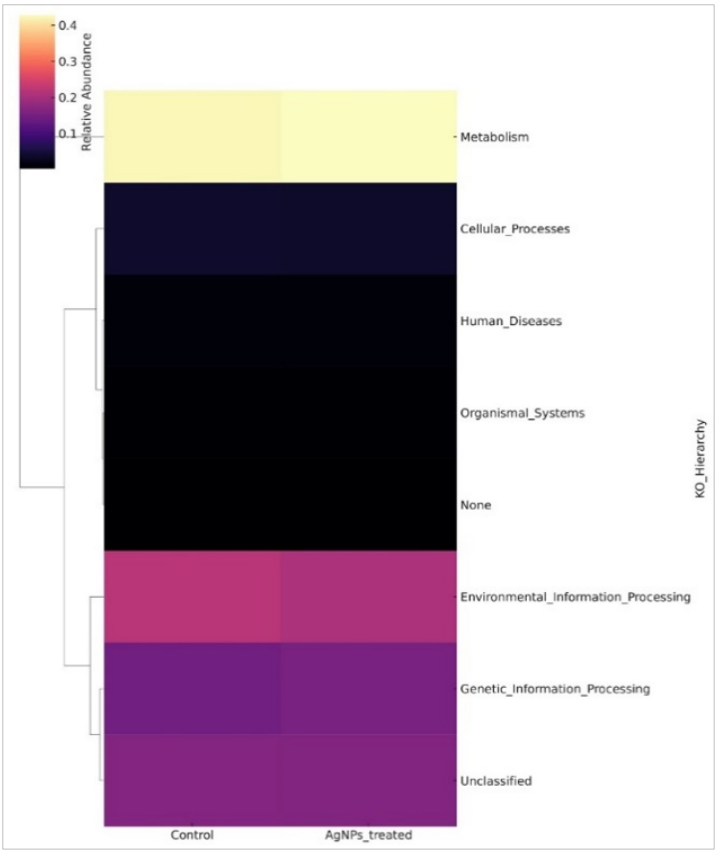

Figure 5. Illustration of KEGG pathway at level 1 through heatmap. Multiple colors showing the relative abundance of microorganisms associated with the function.

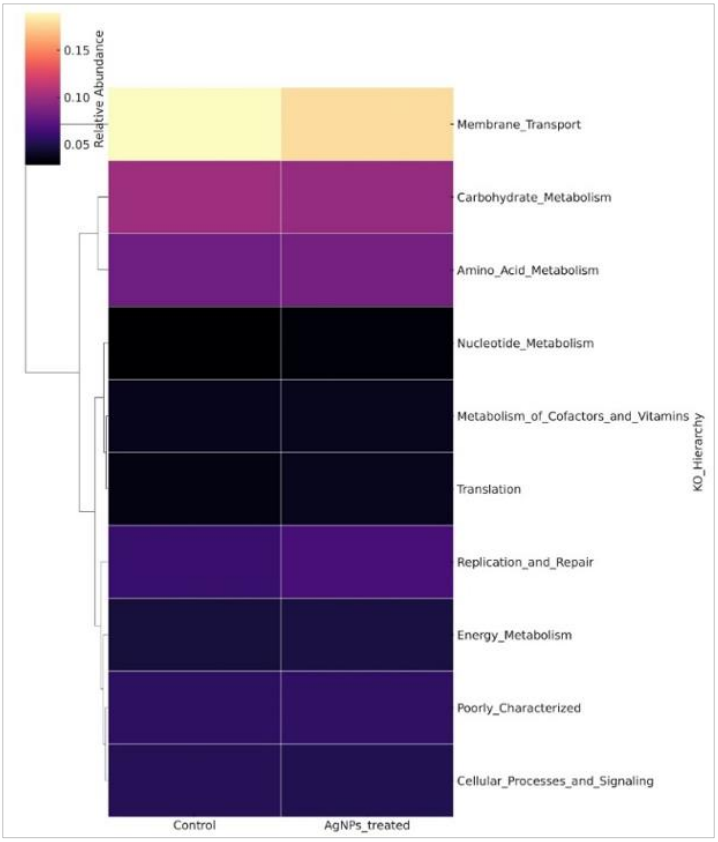

Figure 6. Illustration of KEGG pathway at level 2 through heatmap. Multiple colors show the relative abundance of microorganisms associated with the function.

Discussion

Influence of AgNPs on Gut Microbiota Diversity

This study investigated the effects of dietary silver nanoparticles (AgNPs) on gut microbiota composition in *M. vittatus* using metagenomic sequencing. Although no statistically significant differences were detected in overall microbial diversity, trends in richness and evenness suggest that AgNPs may help establish a more balanced gut microbial community. The lower Simpson's dominance index suggests a reduction in dominance by specific taxa, consistent with previous findings in zebrafish fed chitosan–silver nanocomposites (Udayangani et al., 2017). Similar diversity-enhancing effects have been reported in carp (*Cyprinus carpio*), where balanced microbial communities promote cellulose digestion and nutrient assimilation (van Kessel et al., 2011), and in invasive carp microbiomes shaped by environmental exposure (Eichmiller et al., 2016). Although beta diversity analysis revealed no significant clustering between control and AgNPs-fed groups, subtle taxonomic shifts at the genus level indicate that AgNPs may selectively modulate specific microbial populations rather than cause broad compositional restructuring.

Selective Modulation of Gut Microbiota by AgNPs

The metagenomic analysis revealed that AgNPs selectively influence specific bacterial communities in the gut. Notably, *Ralstonia* and *Candidatus Arthromitus* were found exclusively in the AgNPs-fed group, suggesting that these genera may serve as biomarkers for AgNPs exposure. *Ralstonia* species play a role in microbial metabolism and enzymatic activity, contributing to nutrient cycling within the gut (Fishman et al., 2004). *Candidatus Arthromitus* is associated with immune system development, suggesting that AgNPs supplementation may foster beneficial bacterial populations that support host immunity (Didelot et al., 2012). A noticeable increase in *Bacillus* abundance was also observed in the AgNPs-treated group. *Bacillus* species are widely recognized for their probiotic

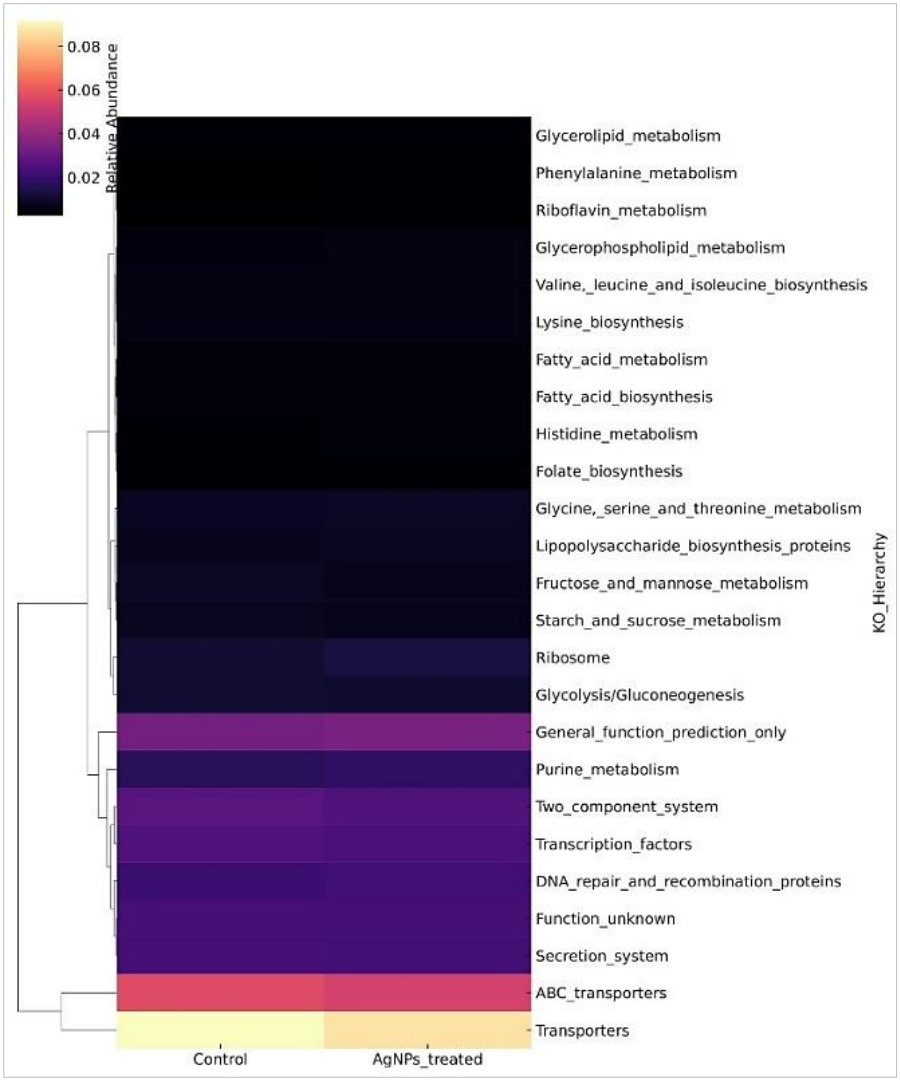

Figure 7. Illustration of KEGG pathway at level 3 through heatmap. Multiple colors showing the relative abundance of microorganisms associated with the function.

properties—such as antimicrobial compound production and digestive enzyme secretion—that contribute to maintaining gut homeostasis (Abriouel et al., 2011; Rawls et al., 2006). This aligns with our Introduction, where probiotics were described as beneficial yet species-specific; our findings suggest that AgNPs may indirectly promote probiotic-associated taxa like *Bacillus*, potentially enhancing intestinal health.

Functional Implications of AgNPs on Gut Microbial Metabolism

Predictive functional analysis using PICRUSt2 showed that AgNPs supplementation altered microbial metabolic pathways. Specifically, pathways related to amino acid metabolism, lipid metabolism, and vitamin biosynthesis were more prevalent in the AgNPs-fed group, suggesting that AgNPs may enhance microbial contributions to nutrient absorption, potentially benefiting fish growth and metabolism (Kolodziejczyk et al., 2019). In contrast, carbohydrate metabolism pathways, such as glycolysis and starch metabolism, were more active in the control group. This suggests that AgNPs supplementation may shift gut microbial activity toward protein and lipid metabolism instead of carbohydrate utilization (Liu et al., 2016). These metabolic changes highlight the need for further research to assess their long-term impact on gut microbiota stability and host metabolism.

Conclusions

This study provides preliminary insights into the effects of dietary silver nanoparticles (AgNPs) on gut microbiota of *M. vittatus*. Although overall microbial diversity did not differ significantly, certain bacterial taxa—including *Bacillus*, *Ralstonia*, and *Candidatus Arthromitus*—appeared to respond to AgNPs supplementation. These trends suggest that AgNPs may influence microbial composition and metabolic pathways related to amino acid, lipid, and vitamin metabolism. However, as these observations were based on a single dose and non-significant diversity changes, further multi-dose and long-term studies are needed to confirm these effects. Future research should also assess potential nanoparticle accumulation in tissues and ecological risks to ensure safe and sustainable use. A cautious, evidence-driven approach will be essential before considering AgNPs as dietary additives in aquaculture.

Ethical Statement

The Institutional Animal Research Ethics Committee (AREC) of the Gazipur Agricultural University declares that the experiments were carried out according to the committee guidelines, and performed with their knowledge and permission (Ref. No. FVMAS/AREC/2023/31).

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Author Contribution

MSN: Methodology, Analysis, Investigation, Original draft preparation. **AK:** Methodology, Analysis, and Draft Preparation. **MAS:** Methodology, Analysis, and Draft Preparation. **MSH:** Analysis and Draft Paper Reviewing. **AQMRK:** Review, and Editing. **MSA:** Conceptualization, Methodology, Investigation, Writing - Review and Editing, Supervision, Project administration, Funding acquisition.

Conflict of Interest

The authors declare no conflicts of interest.

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