

## ORIGINAL ARTICLE

# The effect of sulfamonomethoxine treatment on the gut microbiota of Nile tilapia (*Oreochromis niloticus*)

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## Abstract

To investigate the possible effects of sulfamonomethoxine (SMM) on Nile tilapia (*Oreochromis niloticus*), we quantitatively evaluated the microbial shifts in the intestines of Nile tilapia in response to different doses of SMM (200 and 300 mg/kg) using 16S rRNA gene sequencing. At the phylum level, the control group (0 mg kg<sup>-1</sup> SMM) was dominated by Actinobacteria, Proteobacteria, and Firmicutes. In the treatment groups, Firmicutes, Proteobacteria, and Chloroflexi were the dominant phyla. Cluster analysis indicated that the two groups treated with SMM clustered together. Similarly, the bacterial families that dominated the control group differed from those dominating the treatment groups. The changes in intestinal microbial composition over time were similar between the two SMM treatment groups. In both groups, the abundances of some families, including the Bacillaceae, Streptococcaceae, and Pseudomonadaceae, increased first and then decreased. Overall, the addition of SMM to the feed changed the structure of the intestinal microbiota in Nile tilapia. This study improves our understanding of the impact of SMM on the intestinal microenvironment of Nile tilapia. Our results provide guidelines for the feasibility of SMM use in aquaculture production.

## KEYWORDS

community abundance, community composition, community diversity, intestinal microbiota, Nile tilapia, sulfamonomethoxine

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## 1 | INTRODUCTION

The microbial community in the fish body is diverse and includes protozoa, fungi, yeast, viruses, bacteria, and Archaea (Merrifield & Rodiles, 2015). Bacteria are the most abundant microbes in fish intestines and have been intensively studied over the past several decades (Rombout, Abelli, Picchiatti, Scapigliati, & Kiron, 2011). Compared to the large body of work covering the intestinal microbiota of humans and other mammals, there are few studies of fish intestinal microbiota. However, the fish intestinal microbiota represents a research area of historic significance, which can be traced back to the first half of the 20th century (Gibbons, 1933; Reed & Spence, 1929). Interest in this field has recently increased due to the growth of the aquaculture industry worldwide. The relationship between a host fish and its microbes can be mutually beneficial or pathogenic (Derome, Gauthier, Boutin, & Llewellyn, 2016). The gastrointestinal microbiome is critical to the growth and survival of the host (Fouhy, Ross, Fitzgerald, Stanton, & Cotter, 2012). For instance, the microbiome may benefit the host by aiding digestion, helping to maintain energy balance, preventing colonization by pathogens, and improving mucosal immunity (Merrifield & Ringo, 2014; Nicholson et al., 2012). Conversely, intestinal pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella flexneri*, and *Vibrio cholerae* can cause diseases in fish (China et al., 2012). To prevent disease outbreaks and to avoid economic loss, antibiotics have been frequently used in traditional aquaculture (Alderman & Hastings, 1998; Defoirdt, Sorgeloos, & Bossier, 2011).

Antibiotics are natural or synthetic drugs that have been developed to kill microorganisms or to inhibit their growth. Antibiotic mechanisms range from the destruction of cell membranes to the inhibition of various metabolic pathways (Defoirdt et al., 2011; Serrano, 2005). In hosts, antibiotics may increase intestinal absorption, improve the digestibility of dietary proteins, and stimulate other metabolic processes (Serrano, 2005). Despite the potential benefits of antibiotics used in aquaculture, antibiotics may also affect hosts negatively. Besides, microbial resistance to various widely used antibiotics is common due to the rapid reproduction of various pathogens and because microbes acquire resistance through interspecific and intraspecific plasmid exchange (Tuan, Duc, & Hatai, 2013). Nonetheless, antibiotics play an important role in the treatment and prevention of animal diseases in modern animal husbandry and aquaculture (Armstrong, Hargrave, & Haya, 2005; Kemper, 2008). Antibiotics are usually given to animals through the feed. However, this practice often results in the release of antibiotic residues from residual feed or animal feces; antibiotics from feed may even contaminate the surrounding surface water (Nikolaou, Meric, & Fatta, 2007). These residues may have direct toxic effects on microorganisms (Lai, Hou, Su, & Chen, 2009), and promote the development of antibiotic-resistant bacteria (Cabello, 2006). Also, the residues may transfer antibiotic resistance to pathogenic bacteria that affect humans, causing widespread antibiotic resistance and further adverse effects (Baran, Adamek, Ziemiańska, & Sobczak, 2011; Kümmerer, 2009).

Sulfonamides (SAs) are widely used in the medical treatment of livestock and aquatic animals, accounting for a large proportion of the antibiotics in use globally (Baran et al., 2011). Sulfamonomethoxine [4-amino-N-(2-methoxypyrimidinyl) benzene-sulfonamide] is the most common SA (Huang, Hou, Kuo, & Lai, 2014). Sulfamonomethoxine (SMM) is a broad-spectrum antibiotic that affects both gram-positive and gram-negative bacteria; sulfamonomethoxine effectively treats various aquaculture diseases, such as *Vibrio* disease, *Salmonella* disease, and redfin disease (Duijkeren, Vulro, & Miert, 1994; Ueno, 1999). Because sulfamonomethoxine is structurally similar to para-aminobenzoic acid (PABA), this SA can compete with PABA to act on bacterial dihydrofolate synthase, preventing bacteria from using PABA to synthesize necessary folic acid, and reducing the metabolic activity of tetrahydrofolate (Achari et al., 1997). Bacteria require tetrahydrofolate to synthesize purines, thymine nucleotides, and DNA; thus, decreases in tetrahydrofolate inhibit bacterial growth and reproduction (Connor, 1998). Several studies have investigated SMM pharmacokinetics, bioavailability, aquatic animal toxicity, and tissue residues, but few studies have investigated the effects of SMM on the intestinal microecology of aquatic animals (Huang et al., 2014; Ismail et al., 2012; Ueno, 1998; Ueno & Aoki, 1996). In this study, Nile tilapia (*Oreochromis niloticus*) were used as the research object. First, we classified the intestinal microbiota of this fish using high-throughput sequencing analysis, and we then investigated the effects of SMM on the species richness and composition of the microbiota over time. The purpose of this study was to evaluate the use of SMM in the prevention of fish diseases from the perspective of intestinal microecology and to discuss the advantages and disadvantages of SMM application. Our work will provide a reference for SMM administration in the aquaculture industry.

## 2 | MATERIALS AND METHODS

### 2.1 | Experiment design and system

Nile tilapia individuals (bodyweight  $137.36 \pm 22.61$  g) were raised at Nanquan Experimental Base, Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, Wuxi, China. The indoor experimental tank simulated the outdoor Nile tilapia culture pond, with a sediment layer that was 5 cm thick. The sediment and water were both obtained from the outdoor Nile tilapia culture pond, and they were not treated with antibiotics. We randomly divided 180 fish among nine tanks (400 L each, 20 fish per tank). Fish were allowed to acclimate for 7 days, and they were only fed commercial complete feed during this period. Upon completion of the acclimation period, experimentation began and lasted 4 weeks. During the experimentation period, fish were fed twice daily at 08:00 and 17:00. Fish were fed 2% of their initial body weight. The control group (C) was fed commercial complete feed with no added SMM. The first treatment group ( $T_1$ ) was fed commercial complete feed supplemented with SMM (200 mg kg<sup>-1</sup> body weight), while the second treatment group ( $T_2$ ) was fed commercial

complete feed supplemented with SMM (300 mg kg<sup>-1</sup> body weight). For each group, three replicates were used. During the experimentation period, there was no water exchange in any tank. The water parameters were measured daily and were maintained at pH 7.8, dissolved oxygen >7.0 mg/L, and water temperature 25.0 ± 1.0°C.

## 2.2 | Sample collection

Initial samples were taken before the experiment began, and additional samples were taken every 7 days during the experiment. Three fish were randomly collected from each tank and were anesthetized with 200 mg/L MS-222 before sampling. The intestine of each fish was removed using sterilized scissors and tweezers. The external intestinal wall was immersed in 75% ethanol for 3 min and then rinsed with sterile 0.85% (w/v) saline solution three times. Next, the contents of the entire intestine were removed by scraping with a sterile scalpel. The intestinal contents from the three fish were mixed, transferred to a sterile freezing tube, snap-frozen in liquid nitrogen, and stored at -80°C for DNA extraction.

## 2.3 | DNA extraction

Total DNA was extracted from the intestinal contents using TIANamp Stool DNA Kits (Tiangen), following the manufacturer's instructions. A NanoDrop 2000 (Thermo Scientific) and agarose gel electrophoresis were used to determine DNA quantity and quality.

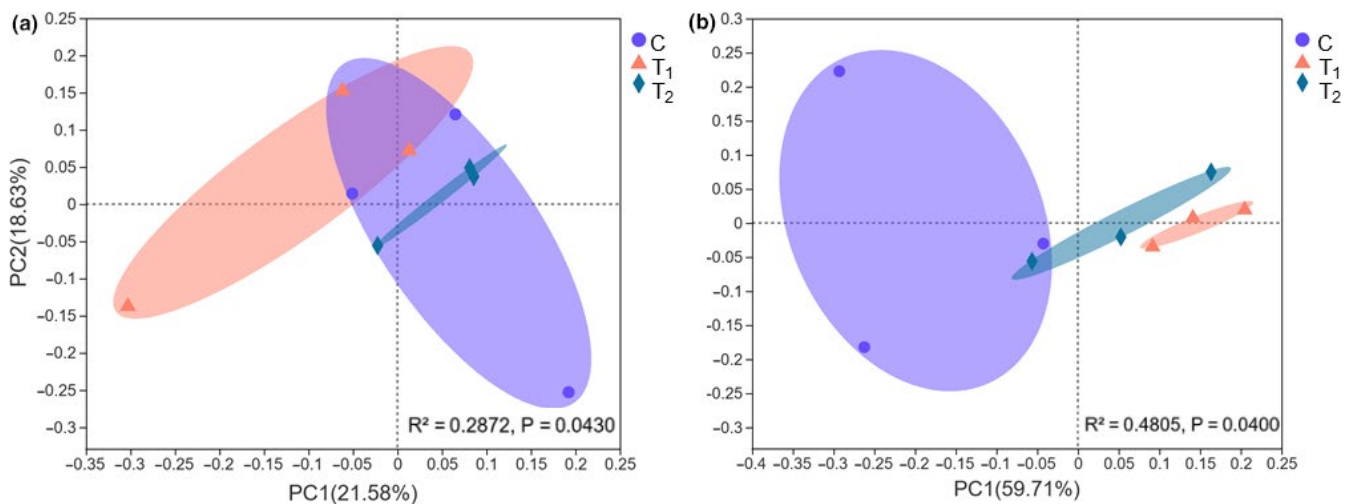
## 2.4 | PCR amplification and 16S rRNA gene library construction

The V3-V4 hypervariable region of the bacterial 16S rRNA gene was PCR-amplified using universal primers

**TABLE 1** Sequence statistics and community coverage for each treatment.

	Sequences number	Mean length (bp)	Good's coverage
C-28d	36374	433	0.99
T <sub>1</sub> -0d	29560	445	0.99
T <sub>1</sub> -7d	29132	437	0.99
T <sub>1</sub> -14d	24797	436	0.99
T <sub>1</sub> -21d	33907	441	0.99
T <sub>1</sub> -28d	31932	440	0.99
T <sub>2</sub> -0d	32501	441	0.99
T <sub>2</sub> -7d	28726	436	0.99
T <sub>2</sub> -14d	24467	436	0.99
T <sub>2</sub> -21d	35893	440	0.99
T <sub>2</sub> -28d	34852	442	0.99

(338F: 5'-ACTCCTACGGGAGGCAGCAG-3', 806R: 5'-GGACTACH VGGGTWCTAAT-3'). Indexed adapters were added to the ends of the 16S rRNA gene amplicons to generate indexed libraries for downstream NGS sequencing on the Illumina MiSeq platform. Sequencing adapters were also added to the termini of the PCR products to facilitate MiSeq sequencing. All of the PCR amplifications were performed in triplicate using TransStart Fastpfu DNA Polymerase Kits (TransGen). Each 20 µl PCR mixture contained 4 µl of 5×FastPfu Buffer, 2.5 µl of dNTPs, 0.8 µl of each primer, 0.4 µl of FastPfu Polymerase, 0.2 µl of BSA, 10 ng of template DNA, and ddH<sub>2</sub>O to make 20 µl. The thermal cycling conditions were as follows: initial denaturation at 95°C for 3 min; 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s; and a final extension at 72°C for 10 min. All of the PCR products were visualized on agarose gels (2% in TAE buffer) containing ethidium bromide and purified using DNA gel extraction kits (Axygen).



**FIGURE 1** The principal coordinate analysis (PCoA) plots were based on unweighted/weighted UniFrac dissimilarity of samples. The significant effect of SMM dose and time on OTUs was detected by PERMANOVA (A: unweighted UniFrac; B: weighted UniFrac)

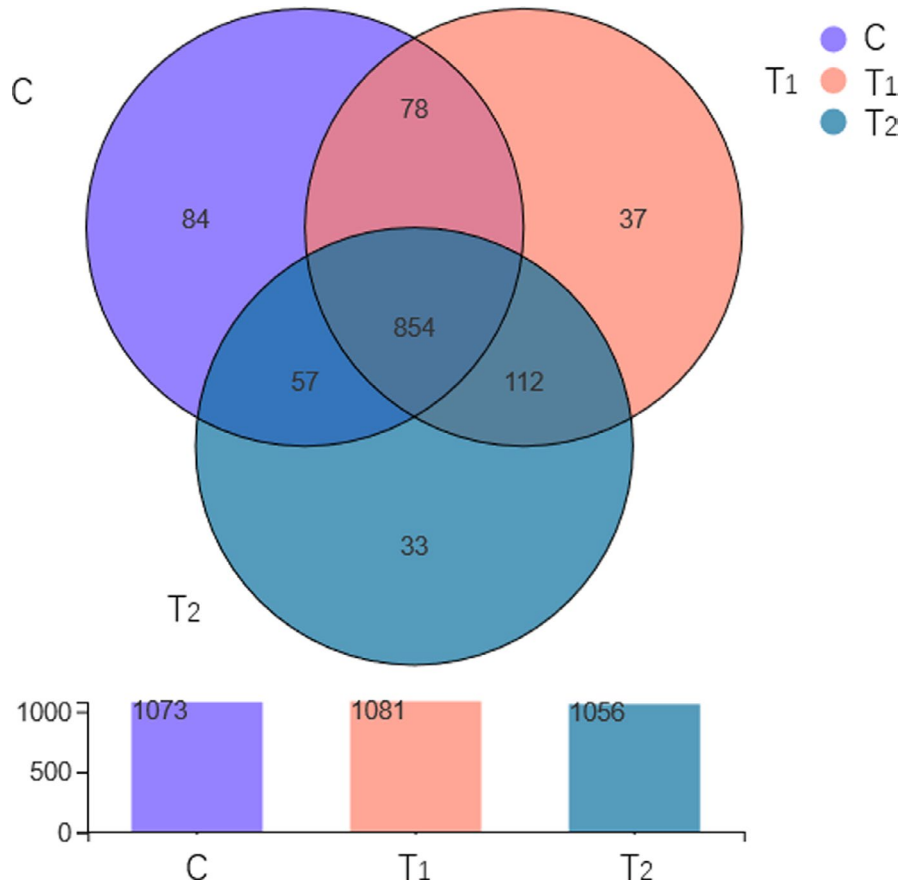


FIGURE 2 Venn diagram representing shared OTUs between treatments. Bar plots representing the total number of OTUs in treatments

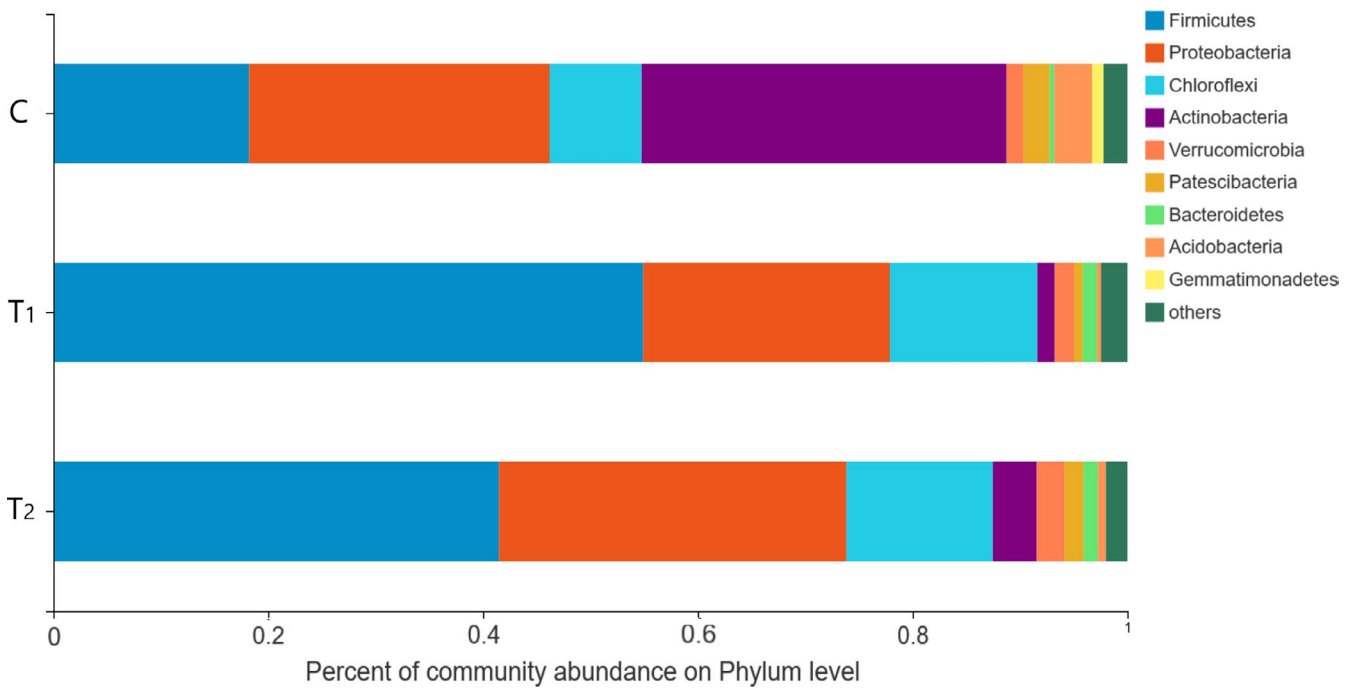


FIGURE 3 Intestinal microbiota composition analysis bar plots on the phylum level of different treatments (A: C; B: T<sub>1</sub>; C: T<sub>2</sub>)

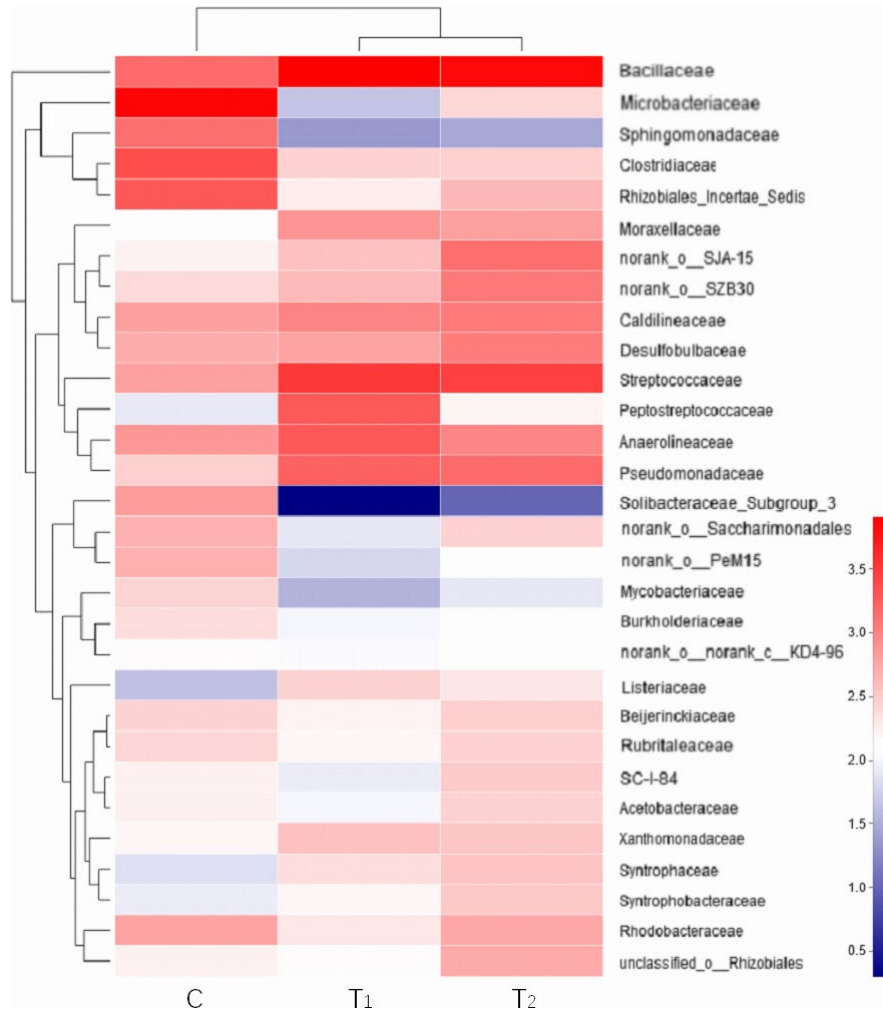


FIGURE 4 Hierarchical clustering heat map showing the abundances of various families among treatments

## 2.5 | Bacterial 16s rRNA gene sequencing and analyses

The PCR products were quantified using the QuantiFluor-ST fluorescent quantitative system (Promega), based on the preliminary quantitative electrophoresis results. PCR products were then mixed in proportion to the sequencing volume of each sample. The 16s rRNA library was multiplexed and loaded on Illumina MiSeq instruments according to the manufacturer's instructions (Illumina). Paired-end (PE) reads obtained using MiSeq sequencing were spliced based on the overlap, quality controlled, and quality filtered. Raw data were merged using Flash (version v1.2.11; <https://ccb.jhu.edu/software/FLASH/index.shtml>) and filtered using QIIME (version v1.9.1; <http://qiime.org/install/index.html>). Effective data were clustered at a 97% sequence identity into operational taxonomic units (OTUs) using UPARSE (version v7.0.1090; <http://www.drive5.com/uparse/>), and OTUs were taxonomically identified using the Ribosomal Database Project (RDP) Classifier (version v2.11; <https://sourceforge.net/projects/rdp-classifier/>) against the SILVA database (version v132; <https://www.arb-silva.de/>).

OTU with <0.01% abundance was filtered out to reduce spurious OTU. Mothur (version v1.30.2; [https://www.mothur.org/wiki/Download\\_mothur](https://www.mothur.org/wiki/Download_mothur)) was used for rarefaction analysis, and graphs were constructed in R (<http://www.r-project.org/>). Alpha diversity indexes and beta diversity distance were calculated in QIIME from rarefied samples; we used the Shannon index to measure species diversity and the Ace index to measure species richness. Principal coordinate analysis (PCoA) was used to visualize differences in the bacterial community based on a weighted/unweighted UniFrac similarity matrix of the square-root-transformed relative abundance of the different OTUs per sample.

## 2.6 | Statistical analysis

Data were analyzed using the SPSS 19.0 statistical software package (SPSS Inc.). All of the values are presented as means  $\pm$  standard deviation (mean  $\pm$  SD). Significant differences in the data were identified using one-way analyses of variance (ANOVAs). We considered  $p < 0.05$  statistically significant.

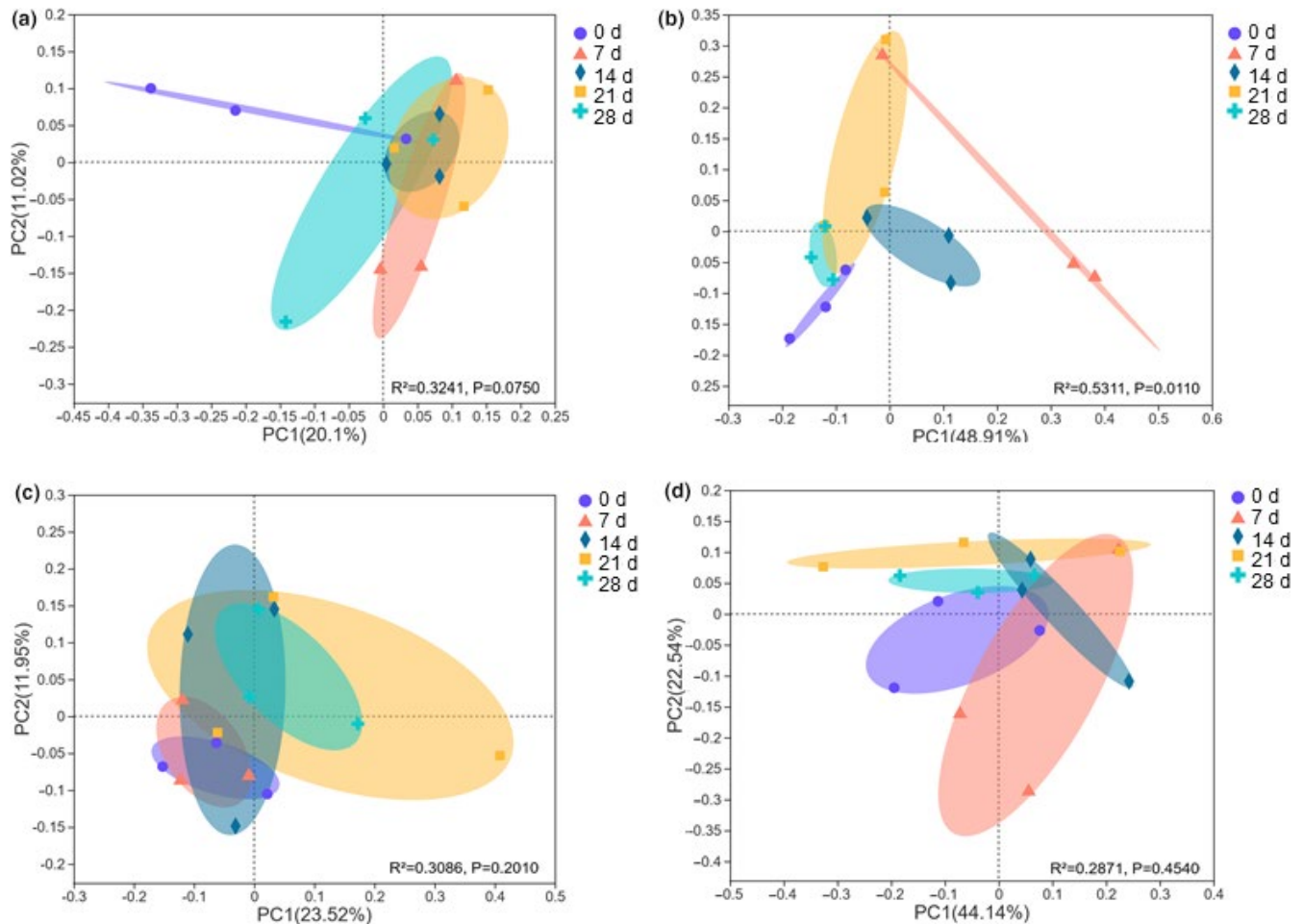


FIGURE 5 The principal coordinate analysis (PCoA) plots were based on unweighted/weighted UniFrac dissimilarity of samples. The significant effect of SMM dose and time on OTUs was detected by PERMANOVA (A:  $T_1$ , unweighted UniFrac; B:  $T_1$ , weighted UniFrac; C:  $T_2$ , unweighted UniFrac; D:  $T_2$ , weighted UniFrac)

### 3 | RESULTS

#### 3.1 | Distribution of the 16S rRNA gene library

We obtained 1,026,423 effective sequences in total, with an average number of sequences per treatment of 24,467–36,374. The average length of the effective sequences in each treatment was 433–442 (Table 1). As sequencing depth increased, the rarefaction curves flattened (Figure A1). The coverage reached 99% (Table 1).

#### 3.2 | Intestinal microbiota community diversity and composition among treatments

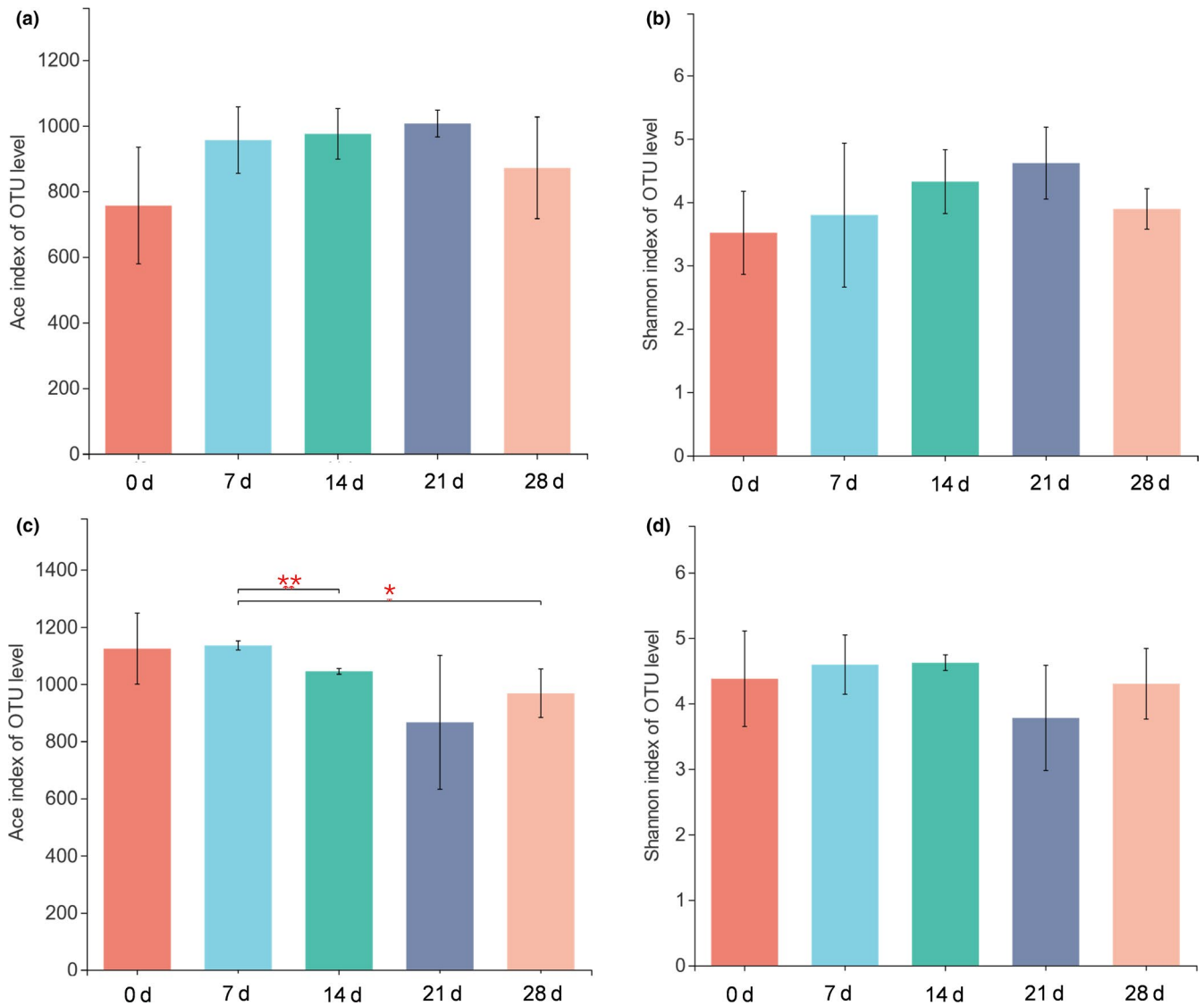
Visual representation of beta diversity using PCoA showed partial overlapping of individual Nile tilapia intestinal microbiota samples among treatments after a four-week experiment based on unweighted UniFrac distance metric and showed moderate grouping based on weighted UniFrac distance metric (Figure 1). Permutational multivariate analysis of variance (PERMANOVA) corroborated

weighted UniFrac distance metric could better explain the changes of microbiota community composition of samples among treatments after 28 days ( $R^2 = .4805$ , Figure 1B), but the difference of bacterial composition between SMM doses was significant in both unweighted UniFrac distance metric and weighted UniFrac ( $p < 0.05$ ).

The Ace index reflects community richness, while the Shannon index reflects community diversity. There was no significant difference in the richness or diversity of the intestinal microbial community among treatments after the four-week experiment ( $p > 0.05$ , Figure A2).

To evaluate the distributions of OTUs among treatments after the four-week experiment, Venn diagrams were drawn (Figure 2). There were 1,073, 1,081, and 1,056 OTUs in C,  $T_1$ , and  $T_2$ , respectively, which were in line with the results of the Ace index and Shannon index. Venn diagrams show common and unique OTUs. The common OTUs reflect the similarities among the microbial communities, while the unique OTUs reflect the differences. After the four-week experiment, 78 OTUs were shared between C and  $T_1$ ; 112 OTUs were shared between  $T_1$  and  $T_2$ ; and only 57 OTUs were shared between  $T_2$  and C. There were 84 unique OTUs in C, 37 unique OTUs





**FIGURE 6** Intestinal microbiota community richness and diversity indexes over time in T<sub>1</sub> (A, B) and T<sub>2</sub> (C, D). Significant differences between connected groups are indicated with asterisks: \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$

in T<sub>1</sub>, and 33 unique OTUs in T<sub>2</sub>. The results showed that microbial community structure changed after adding SMM to the feed, but community patterns remained similar.

The phylum-level composition of the intestinal microbiota differed after the four-week treatment period (Figure 3). Group C was dominated by the Actinobacteria (34.10%), Proteobacteria (27.98%), and Firmicutes (18.25%). The dominant phyla in T<sub>1</sub> and T<sub>2</sub> were the same: Firmicutes, Proteobacteria, and Chloroflexi. The proportions of Firmicutes, Proteobacteria, and Chloroflexi in T<sub>1</sub> were 55.14%, 22.97%, and 13.77%, respectively, while the proportions of Firmicutes, Proteobacteria, and Chloroflexi in T<sub>2</sub> were 41.62%, 32.38%, and 13.67%, respectively.

A hierarchical clustering heat map analysis was performed at the family level, including the 30 most abundant microbial families across the three treatments (Figure 4). This analysis indicated that group C was distinct from groups T<sub>1</sub> and T<sub>2</sub>. The dominant

families in the SMM-supplemented groups T<sub>1</sub> and T<sub>2</sub> primarily fell into the phyla Firmicutes (Bacillaceae, Streptococcaceae, and Peptostreptococcaceae), Proteobacteria (Moraxellaceae, Desulfobulbaceae, and Pseudomonadaceae), and Chloroflexi (Caldilineaceae and Anaerolineaceae). The dominant families in the unsupplemented group C were Bacillaceae (in the Firmicutes), Sphingomonadaceae, and an indeterminate family of Rhizobiales (in the Proteobacteria), and Microbacteriaceae (in the Actinobacteria).

### 3.3 | Intestinal microbiota community diversity and composition varied over time among SMM treatment groups

In T<sub>1</sub>, visual representation of beta diversity using PCoA showed partial overlapping of individual Nile tilapia intestinal microbiota

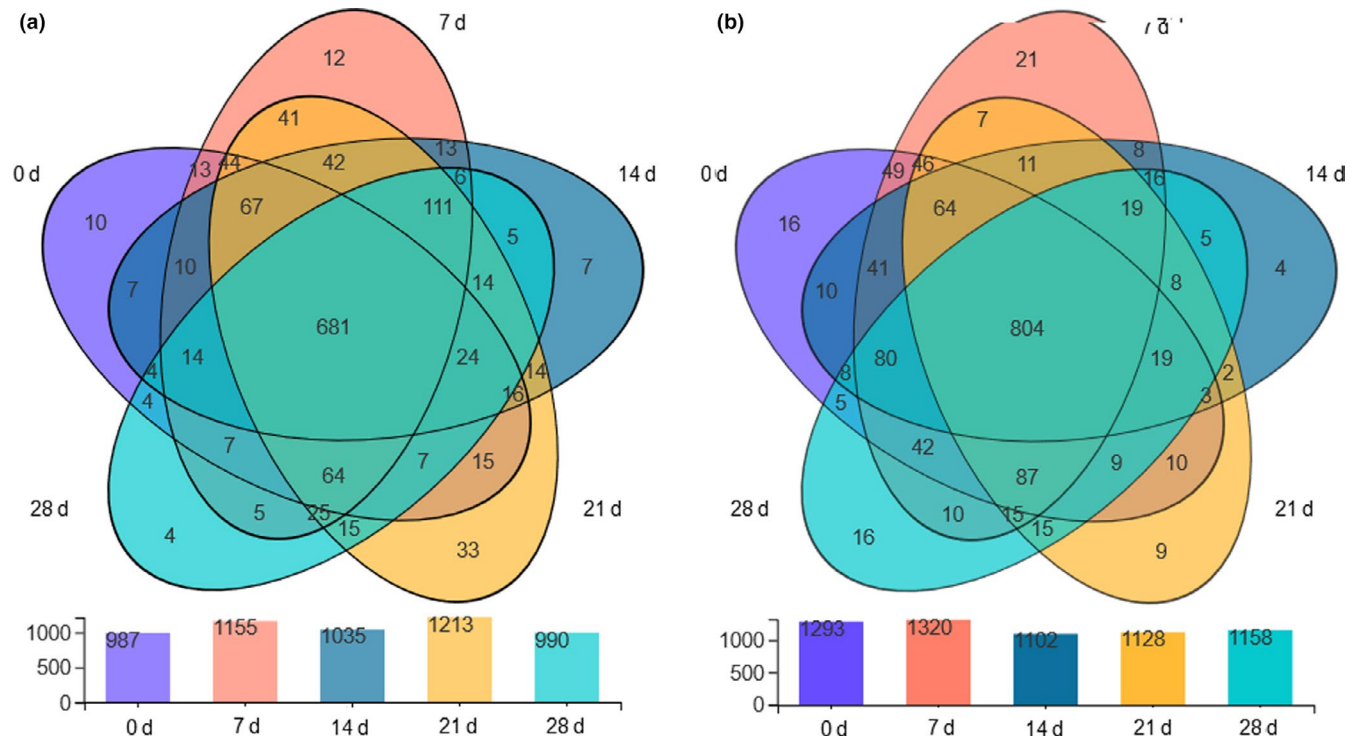


FIGURE 7 Venn diagram showing shared OTUs over time. Bar plots representing the total number of OTUs in treatments (A: T<sub>1</sub>; B: T<sub>2</sub>)

samples within time points based on unweighted UniFrac distance metric and showed moderate grouping based on weighted UniFrac distance metric. PERMANOVA with weighted UniFrac distance metric indicated that time contributed 53.1% and had significantly different bacterial compositions over time ( $p < 0.05$ , Figure 5B). In T<sub>2</sub>, visual representation of beta diversity using PCoA shows partial overlapping of individual Nile tilapia intestinal microbiota samples within time points based on weighted UniFrac and unweighted distance metric. PERMANOVA indicated no significant difference in bacterial compositions over time in both unweighted UniFrac distance metric and weighted UniFrac ( $p > 0.05$ , Figure 5C,D).

In T<sub>1</sub>, there were no significant differences in the Ace and Shannon indexes of intestinal microbiota over time ( $p > 0.05$ , Figure 6A,B). In T<sub>2</sub>, the Ace index of intestinal microbiota changed significantly ( $p < 0.05$ ), decreasing significantly on day 14, day 21, and day 28 compared to day 7 (Figure 6C,D). Among them, there were extremely significant differences between day 7 and day 14 ( $p < 0.01$ ).

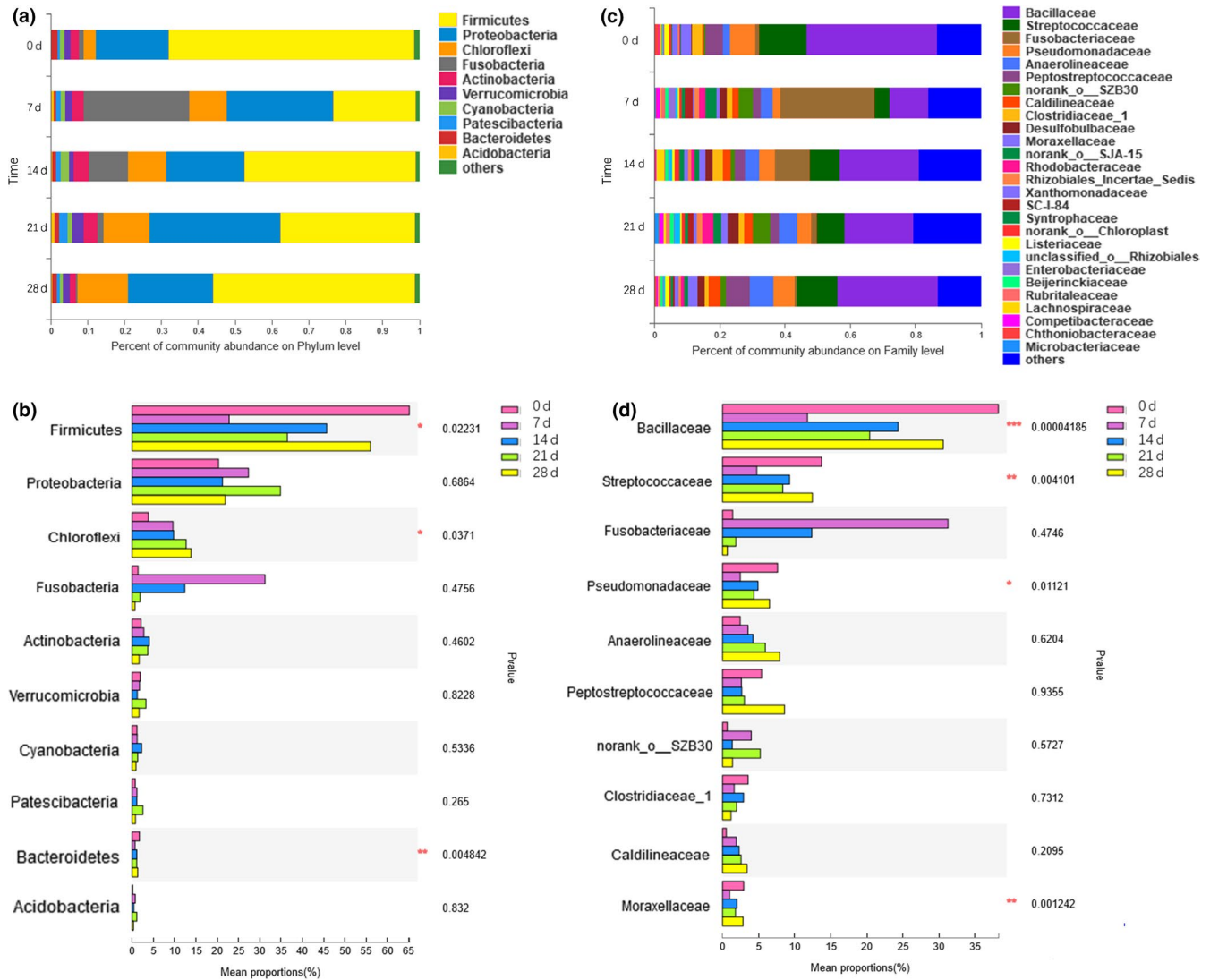
In T<sub>1</sub>, there were 987, 1,155, 1,035, 1,213, and 990 OTUs on day 0, day 7, day 14, day 21, and day 28, respectively. 13 OTUs were shared between the samples from day 0 and day 7; 13 OTUs were shared between the samples from day 7 and day 14; 14 OTUs were shared between the samples from day 14 and day 21; and 15 OTUs were shared between the samples from day 21 and day 28. 7 OTUs were shared between the samples on day 28 and day 0. For the samples taken on days 1, 7, 14, 21, and 28, the numbers of unique OTUs were 10, 12, 7, 33, and 4, respectively (Figure 7A). In T<sub>2</sub>, there were 1,293, 1,320, 1,102, 1,128, and 1,158 OTUs on day 0, day 7, day 14, day 21, and day 28, respectively. 49 OTUs were shared between the

samples from day 0 and day 7; 8 OTUs were shared between the samples from day 7 and day 14; 2 OTUs were shared between the samples from day 14 and day 21; and 15 OTUs were shared between the samples from day 21 and day 28. 5 OTUs were shared between the samples on day 28 and day 0. For the samples taken on days 0, 7, 14, 21, and 28, the numbers of unique OTUs were 16, 21, 4, 9, and 16, respectively (Figure 7B).

In T<sub>1</sub>, Firmicutes and Proteobacteria were the dominant phyla in the intestinal microbiota at different times (Figure 8A). From day 7, the proportion of Chloroflexi in the intestinal microbiota began to increase significantly ( $p < 0.05$ ). At this point, Fusobacteria also increased sharply to account for a large proportion of the intestinal microbiota (28.8%); eventually, this phylum decreased in abundance to initial levels. Firmicutes and Bacteroidetes differed significantly at different times ( $p < 0.05$ ). The abundances of both phyla first decreased and then increased almost to initial levels (Figure 8B). At the family level, similar to the phylum Fusobacteria, Fusobacteriaceae abundance increased sharply on day 7, to account for a large proportion of the intestinal microbiota. The abundance of this family eventually decreased to initial levels (Figure 8C). There were significant differences at different times in the relative abundances of Bacillaceae, Streptococcaceae, Pseudomonadaceae, and Moraxellaceae ( $p < 0.05$ ). The abundances of these families also first decreased and then increased almost to initial levels (Figure 8D).

Throughout the experiment, changes in the intestinal microbiota of T<sub>2</sub> were less obvious than those of T<sub>1</sub>. Although the abundance of Fusobacteria increased significantly on day 7 ( $p < 0.05$ ), this phylum remained less abundant than the dominant phyla (Firmicutes, Proteobacteria, and Chloroflexi) (Figure 9A). At the family level,





**FIGURE 8** Phylum- and family-level composition of the intestinal microbiota composition over time in group  $T_1$ . Significant differences between selected groups are indicated with asterisks: \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$

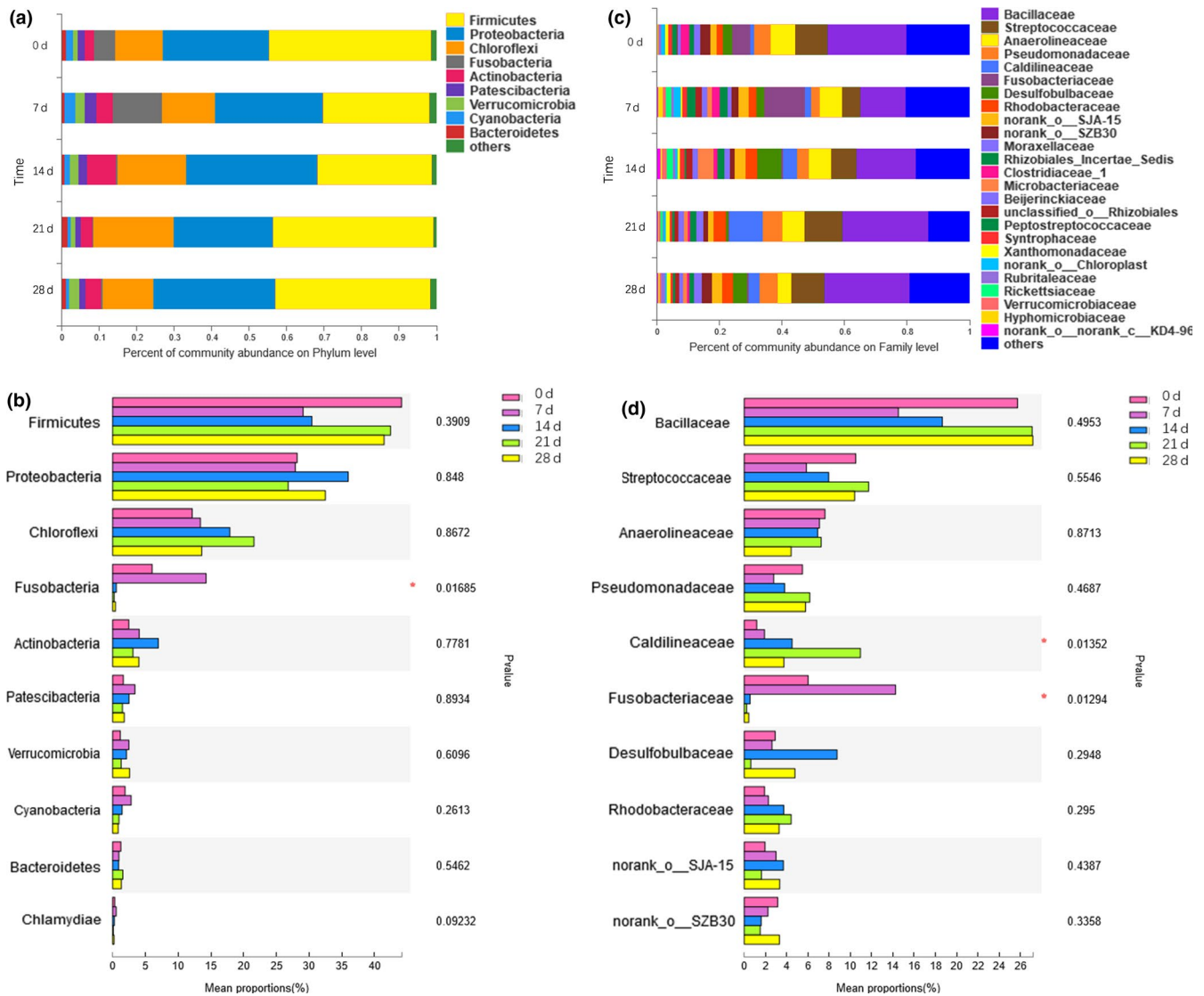
Caldilineaceae and Fusobacteriaceae differed significantly during the experimental period ( $p < 0.05$ ), first increasing and then decreasing (Figure 9D). In the most abundant families (Bacillaceae, Streptococcaceae, and Pseudomonadaceae), abundance tended to decrease and then recover to initial levels, but these changes were not significant ( $p < 0.05$ ).

## 4 | DISCUSSION

The microbiota of the animal intestinal tract is complex and diverse, and together, they constitute an important functional unit that plays an important role in the growth, metabolism, and immunity of the host (Daugbjerg, Cesaroni, Ottesen, Diderichsen, & Osler, 2014; Nayak, 2010). Antibiotics, which are one of the main treatments for aquatic animal diseases, have been shown to affect the intestinal microbiota (Hansen, Strøm, & Olafsen, 1992; Jernberg, Löfmark,

Edlund, & Jansson, 2010). Here, we investigated how sulfonamide concentration affected the intestinal microenvironment of Nile tilapia over time by using an artificial environment to exclude external influences on fish microecology. Specifically, we used 16S rRNA gene sequencing to explore the possible correspondence between the intestinal microbiota of Nile tilapia and SMM.

After the four-week feeding experiment, the composition of the microbiota between SMM feeding groups and the control group had a significant difference. In the Nile tilapia fed with SMM, the proportion of Actinobacteria in the intestinal microbiota was significantly reduced, and the microbiota became dominated by the Microbacteriaceae. The actinobacteria are arguably the richest source of small-molecule diversity on earth (Ventura et al., 2007). However, actinomycetes are also opportunistic pathogens (Miao & Davies, 2010). Some saprophytic bacteria participate in the natural nitrogen cycle by secreting extracellular enzymes, secondary metabolites, and other



**FIGURE 9** Phylum- and family-level composition of the intestinal microbiota composition over time in group T<sub>2</sub>. Significant differences between selected groups are indicated with asterisks: \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$

substances; other actinomycetes are pathogenic (notably, species of *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Propionibacterium*, and *Tropheryma*) and infect the host when immune capacity is low, resulting in chronic or subacute diseases (Barka et al., 2016; Evtushenko & Takeuchi, 2006). The addition of SMM to feed reduced the risk of disease in the Nile tilapia, indicating that SMM plays a role in disease prevention and control. In the Nile tilapia fed with SMM, the relative abundance of Firmicutes in the intestinal microbiota increased significantly, primarily due to the substantial increase in the abundance of Bacillaceae. Firmicutes are the dominant phylum in the intestinal microbiota of most vertebrates (Cantas, Sørby, Aleström, & Sørum, 2012; Scott, Gratz, Sheridan, Flint, & Duncan, 2013; Wang, Ran, Ringø, & Zhou, 2018). *Bacillus* is found in probiotic formulations that are added to the feed or live bait for many aquatic animals such as grouper (*Epinephelus coioides*), pompano (*Trachinotus ovatus*), and grass carp (*Ctenopharyngodon idellus*); in these fish, *Bacillus* balance

the intestinal microbiota, promoting digestion and improving immunity (Guo et al., 2016; Sun, Yang, Huang, Ye, & Zhang, 2013; Zhang et al., 2014). In the guts of Nile tilapia fed with SMM, Proteobacteria were no longer the dominant phylum. Instead, the composition of the intestinal community became more complex. The abundance of the Sphingomonadaceae decreased, while the abundances of the Moraxellaceae, Desulfobulbaceae, and Pseudomonadaceae increased. Proteobacteria, the largest of the bacteria, are ubiquitous in the intestinal tract of aquatic organisms (Egerton, Culloty, Whooley, Stanton, & Ross, 2018; Wang et al., 2018). Excessive proportions of Proteobacteria damage the intestinal microenvironment and increase the possibility of disease in the host (Estruch et al., 2015; Shin, Whon, & Bae, 2015).

By observing changes in the intestinal microbiota of Nile tilapia fed different amounts of SMM over time, we identified some specific phenomena and rules. When Nile tilapia were fed 300 mg kg<sup>-1</sup> SMM, the population richness of the intestinal microorganisms decreased

significantly. Similar results were observed in zebrafish (*Danio rerio*) and channel catfish (*Ictalurus punctatus*) after antibiotics were used (Wang et al., 2019; Zhou et al., 2018). The addition of SMM disrupted the stability of the intestinal microbiota, causing population richness to decline. In the SMM-treated groups, many bacterial families, especially those with higher abundances (e.g., Bacillaceae, Streptococcaceae, and Pseudomonadaceae), became first less abundant and then more abundant over time. This may suggest that 4 weeks of SMM treatment significantly increased the antibiotic resistance in the intestinal microbiota of the Nile tilapia. Antibiotic residues in animal products, if ingested in low doses for long periods, can repeatedly stimulate pathogenic bacteria and induce antibiotic resistance (He et al., 2017). Bacteria become resistant by reducing the permeability of the cell membrane to antibiotics (Delcour, 2009). Bacteria also develop resistance by changing the target of antibiotic action and producing enzymes that decompose or modify antibiotics (Hooper, 1999). Genetic variation in bacteria may also lead to antibiotic resistance (Cabello, 2006). SMM has been shown to lead to antibiotic resistance in the Firmicutes (e.g., Bacillaceae and Clostridiaceae) and the Actinobacteria (Lin et al., 2016). However, whether and how antibiotic resistance developed under the experimental conditions remain relatively poorly understood. Importantly, the abundance of Fusobacteriaceae in both SMM-treated groups increased significantly on day 7 and then decreased to a very low level. This might have been due to the decreases in the relative abundances of other dominant families. Competition among bacteria offsets the instability caused by the decrease in intestinal microbiota diversity via a negative feedback loop; this feedback loop maintains the stability of the intestinal microbial ecosystem (Coyte, Schluter, & Foster, 2015).

## 5 | CONCLUSIONS

Dietary supplementation with SMM had a significant effect on the structure of the intestinal microbiota in Nile tilapia. SMM treatment did lead to abnormally abundant (or rare) microbiota, decrease community richness or diversity, or suddenly increase the abundance of pathogenic bacteria. However, in the group treated with 300 mg kg<sup>-1</sup> SMM, community richness decreased with time. Therefore, we recommend preventing Nile tilapia disease using mixed feeding with a low dose of SMM (200 mg/kg), and shortening the treatment time to the extent possible. Our study provides preliminary guidance for the use of SMM in Nile tilapia, and it will support the development of appropriate treatment regimens that account for the effects on the intestinal microbiota. Finally, our results may help to reduce the spread of resistant bacterial strains associated with antibiotic overuse.

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## AUTHOR CONTRIBUTIONS

**Junchao Ming:** Conceptualization (lead); data curation (supporting); formal analysis (supporting); funding acquisition (equal); investigation (lead); methodology (lead); project administration (lead); resources (equal); supervision (lead); validation (lead); visualization (equal); writing – original draft (lead); writing – review and editing (lead). **Zhengyi Fu:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); software (equal); validation (equal); visualization (equal); writing – original draft (equal). **Zhenhua Ma:** Conceptualization (equal); data curation (equal); formal analysis (equal); project administration (equal); resources (equal); supervision (equal); validation (equal); writing – original draft (equal). **Lijun Zhou:** Conceptualization (equal); formal analysis (equal); investigation (equal); methodology (equal); project administration (equal); validation (equal). **Zongli Zhang:** Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); validation (equal). **Chao Song:** Investigation (equal); methodology (equal); project administration (equal); resources (equal); validation (equal). **Xinhua Yuan:** Conceptualization (equal); funding acquisition (equal); project administration (equal); resources (equal); supervision (equal); writing – review and editing (equal). **Qinglong Wu:** Conceptualization (equal); funding acquisition (equal); project administration (equal); resources (equal); supervision (equal); writing – review and editing (equal).

## ETHICS STATEMENT

The study complied with the ethical animal use protocol approved by the Animal Welfare Committee (E437-16).

## DATA AVAILABILITY STATEMENT

Raw sequence data have been deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive database (BioProject ID: PRJNA650039). <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA650039>.

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## APPENDIX

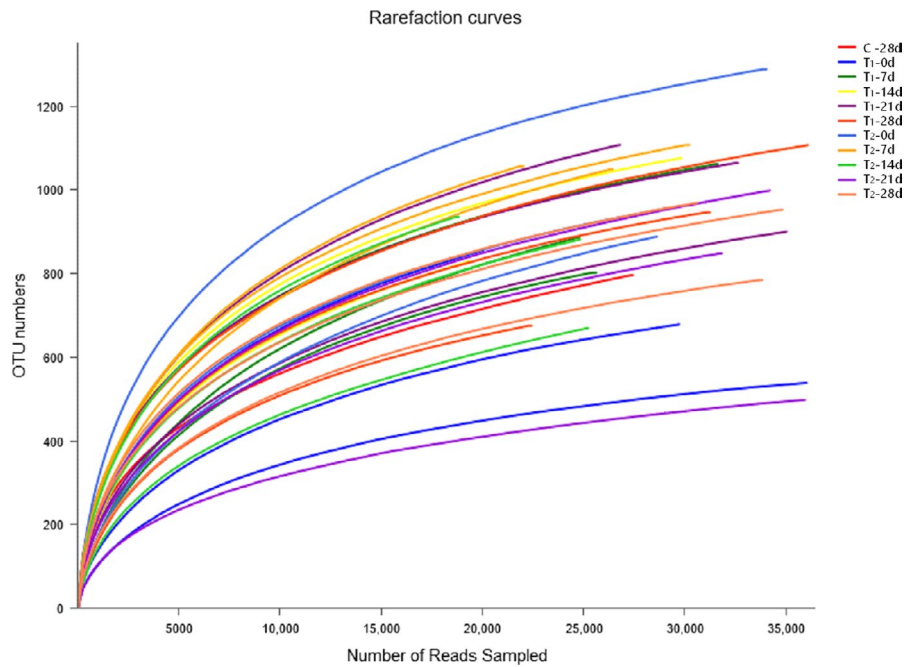


FIGURE A1 Rarefaction curves for each sample. Curves represent the number of operational taxonomic units (OTUs) at 97% sequence identity as a function of sequencing effort. The number of species can be inferred based on OTUs.

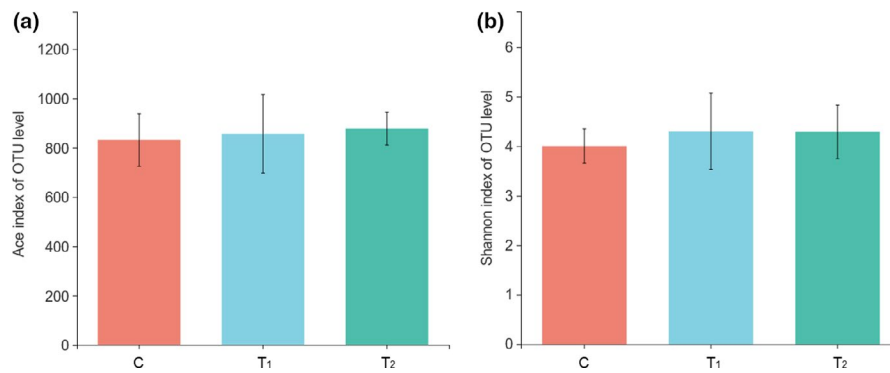


FIGURE A2 Richness and diversity indexes for the intestinal microbiota communities of different treatments.