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ARTICLE

Gut dysbiosis induced by florfenicol increases susceptibility to *Aeromonas hydrophila* infection in Zebrafish *Danio rerio* after the recommended withdrawal period

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Abstract

Objective: Florfenicol (FFC) is a broad-spectrum antibiotic approved by the U.S. Food and Drug Administration to treat both systemic and external bacterial infections in food fish. The objective of this study was to evaluate the effect of FFC-medicated feed on the gut microbiota of Zebrafish *Danio danio* to determine (1) if the therapeutic dose of FFC-medicated feed induces dysbiosis and (2) if fish with altered gut microbiota were more susceptible to subsequent infection by the common opportunistic fish pathogen *Aeromonas hydrophila*.

Methods: Zebrafish that were treated with regular and FFC-medicated feeds were artificially challenged with *A. hydrophila* at the end of the recommended 15-day antibiotic withdrawal period. The gut microbiota of the Zebrafish at different stages was analyzed using 16S ribosomal RNA gene sequencing.

Result: Our results found that FFC-medicated feed induced disruption of the gut microbiota. Dysbiosis was observed in all treated groups, with a significant increase in bacterial diversity, and was characterized by a remarkable bloom of Proteobacteria and a drastic decline of *Mycoplasma* and *Cetobacterium* in treated animals but without noticeable clinical signs or mortalities. In addition, the increase of Proteobacteria was not significantly reduced after the recommended 15-day withdrawal period, and the Zebrafish treated with FFC-medicated feed exhibited a significantly higher mortality rate when they were subsequently challenged with *A. hydrophila* compared to the control (regular feed) groups. Interestingly, the most dramatic changes in the gut microbiome composition occurred at the transition time between the late stage of the medicated treatment and the beginning of the withdrawal period instead of the time during the *Aeromonas* infection.

Conclusion: The administration of FFC-medicated feed at the recommended dose induced gut dysbiosis in Zebrafish, and fish did not recover to the baseline after the recommended withdrawal period. Our findings suggest that the use of antibiotics in fish elicits a response similar to those previously described in mammals and possibly makes the host more susceptible to subsequent infections of opportunistic pathogens. This study using a controlled model system suggests that antibiotics in aquaculture may have long-term effects on the general well-being of the fish.

KEYWORDS

Aeromonas hydrophila, florfenicol, gut dysbiosis, gut mucosal epithelium, Zebrafish

[†]Deceased.

INTRODUCTION

The fish gut is colonized by a complex microbial community that is essential in maintaining host homeostasis (Lozupone et al. 2012; Clements et al. 2014; Ghanbari et al. 2015; Tarnecki et al. 2017; Ray et al. 2019). It provides the host with complementary genetic resources, including genes involved in energy harvesting and the production of essential vitamins, bioelements, and other essential metabolites (Caporaso et al. 2011; Faith et al. 2013; Shin et al. 2015). In addition, the gut microbiota supports the development of the host immune system by safeguarding it against pathogen colonization and invasion (Garrett et al. 2010; Carding et al. 2015). Failure to maintain a balanced equilibrium between the host and its gut microbiota results in dysbiosis, and many studies in humans have shown that antibiotic administration induces dysbiosis and causes adverse effects on patient health (Wipperman et al. 2017; Neuman et al. 2018; El Hage et al. 2019).

In the United States and other developed countries, the use of antibiotics in fish intended for human consumption is tightly regulated and the few antibiotics that are approved for use in fish farms are strictly controlled by a veterinary feed directive (Kelly 2013). Florfenicol (FFC) is one of the three antibiotics approved for use in Channel Catfish *Ictalurus punctatus*, the main aquaculture species in the United States. It is commercially available for fish farmers under the trade name Aquaflor (Merck & Co, Inc.) and is typically used in the form of a premix medicated feed (containing 50% FFC; Fukui et al. 1987). In the United States, FFC is only approved to treat columnaris disease (caused by *Flavobacterium columnare*) and edwardsiellosis (caused by *Edwardsiella ictaluri*) in Channel Catfish (Nathan 2004), furunculosis (caused by *Aeromonas salmonicida*) in salmonids, and streptococcal septicemia (caused by *Streptococcus iniae*) in warmwater finfish (U.S. Fish and Wildlife Service 2011). However, as a broad-spectrum antibiotic that inhibits the growth of both gram-positive and gram-negative bacteria (Cannon et al. 1990), FFC is likely to eliminate commensal members of the gut microbiota in fish, resulting in an unbalanced gut microbiota (Pamer 2016).

Florfenicol was the last antibiotic to be approved by the U.S. Food and Drug Administration to treat Channel Catfish, and it rapidly became the drug of choice for producers due to its high efficacy (McGinnis et al. 2003; Bowker et al. 2010; Darwish 2010; Soto et al. 2013). He et al. (2010) investigated the effect of FFC on the intestinal microbiota of tilapia and found a significant reduction in bacterial diversity in fish that received medicated feed. However, their study had a limited scope due to use of a fingerprinting-based method to characterize the bacterial communities. In a recent study, Channel Catfish that were fed FFC-medicated feed exhibited a decreased microbial diversity in the gut, which was accompanied by an increase in the relative abundance of Proteobacteria, suggesting that the fish had become dysbiotic (He et al. 2013). However, that study did not evaluate whether the changes observed in the gut microbiome increased the fish's susceptibility to fish pathogens. Therefore, the comprehensive effect of FFC on the fish gut microbiota, when used at the recommended therapeutic doses, has not yet been investigated. We chose the Zebrafish *Danio rerio* as our animal model, as it has been widely used to study infection, immunity, and inflammation in vertebrates (Pindling et al. 2018; Garcia-Moreno et al. 2019; Renshaw et al. 2019). In addition, our group has used Zebrafish as a laboratory model to study columnaris disease (Olivares-Fuster et al. 2011; Mohammed et al. 2013), one of the diseases that FFC is approved to treat. Studies on chemically induced enterocolitis (Oehlers et al. 2011; He et al. 2013; Bartoskova et al. 2014; Plhalova et al. 2014) and antibiotic administration (Cantas et al. 2012; Johanesen et al. 2015; Patil et al. 2016; Romero et al. 2016; He et al. 2017) in Zebrafish have shown a strong pharmacological effect on the gut microbial communities. *Aeromonas hydrophila* is the causative agent of motile aeromonad septicemia (MAS) and causes significant losses for the U.S. catfish industry (Griffin et al. 2013). In this study, we aimed to (1) test whether antibiotic-induced dysbiosis could predispose the fish to this common opportunistic pathogen and (2) determine the microbiome shaped by both the antibiotic treatment and pathogen infection in the fish host.

Impact statement

Our results suggested that bacterial community in the gut of healthy Zebrafish could be readily disturbed as a result of antibiotic administration. In addition, after medicated feed, Zebrafish were more susceptible to bacterial secondary infection (e.g., *Aeromonas hydrophila*).

METHODS

Zebrafish husbandry

Zebrafish were purchased from Aquatic Bio-Tech (Sun City Center, Florida, USA) as unsexed adults (5 months old; ZDR wild strain; $n = 900$; average weight

[mean ± SD] = 27.0 ± 0.7 g; average length [mean ± SD] = 27.90 ± 2.45 mm). Upon arrival, the fish were quarantined in a stand-alone unit (270 L) for 15 days, and a subsample of 15 fish was inspected for parasites and bacterial pathogens by the Southeastern Cooperative Fish Parasite and Disease Laboratory at Auburn University (Case Identification Number FL16_1). After the quarantine period, fish were stocked into 20 tanks (37 L each) at a stocking rate of 40 fish/tank and were maintained as previously described (Zhang et al. 2016). Fish were fed once daily at approximately 2% of body weight (BW) with commercial catfish feed containing 32% crude protein (Alabama Catfish Feed Mill, LLC, Uniontown, Alabama, USA). Water quality was monitored daily (i.e., alkalinity = 80 mg/L; hardness = 150 mg/L; dissolved oxygen > 8 mg/L; temperature = 27.6 ± 0.50°C; pH = 7.84 ± 0.15; ammonia and nitrites were kept at nondetectable levels), and a 12-h light : 12-h dark photoperiod was maintained throughout the experiment.

Presence of *Aeromonas hydrophila* in the Zebrafish gut

Aeromonas hydrophila has been previously reported as one of the typical opportunistic pathogens in the intestinal tracts of healthy fish. The intestinal tracts (with their contents) of five fish were pooled and weighed to determine the numbers of *A. hydrophila* in the Zebrafish intestine prior to the challenge. The intestinal tracts and contents were then homogenized and resuspended in 1 mL of brain–heart infusion (BHI; BD Difco, Heidelberg, Germany). Plates were incubated at 28°C for 24 h on *Aeromonas*-selective agar (LAB Neugene, Lancashire, UK), and the colony-forming units (CFU) were counted. Putative colonies of *A. hydrophila* were purified and confirmed using the API 20E identification system (BioMérieux, Durham, North Carolina, USA). Susceptibility of the *A. hydrophila* isolates to FFC was carried out. Discs containing 30 µg of FFC (Becton Dickinson, Heidelberg, Germany) were used for the disc diffusion test following the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS). The inhibition zone (32–44 mm) was measured according to the NCCLS manual (Miller et al. 2003). Isolates that grew inside the zone of inhibition were recorded.

Intestinal bacterial resistance to florfenicol

We determined the degree of FFC resistance among culturable bacteria in the Zebrafish gut prior to starting FFC treatment. Briefly, the intestines of five animals were aseptically excised, pooled, homogenized, and resuspended in 1 mL of sterile, 0.9% saline solution. Standard plate counts were conducted on trypticase soy agar (TSA; BD GmbH, Germany) and on TSA supplemented with FFC (Sigma-Aldrich, Darmstadt, Germany) at 25 µg/mL. Plates were incubated at 28°C for 24 h, and colonies were counted.

Experimental design

The experimental design consisted of four treatment groups, with five replicates for each treatment (replicate = tank): system control (regular catfish feed, not challenged), treatment I (regular catfish feed, challenged with *A. hydrophila*), treatment II (FFC-medicated feed, challenged with *A. hydrophila*), and treatment III (FFC-medicated feed, not challenged; Figure 1). Tanks were randomized and assigned blindly to each treatment. During the 10-day acclimation period, all fish were fed a regular 32% protein standard catfish diet at 2% of BW. After acclimation, FFC-medicated catfish feed was administered to fish in treatments II and III at a dosage of 15 mg/kg of BW per day, while regular feed was administered to the control fish (system control and treatment I). After 10 days of FFC treatment, all fish were returned to the normal feed regime for 15 days. Throughout the study, medicated and nonmedicated control diets were coated with fish oil (menhaden fish oil; Jandell Fishing Product, Texas, USA) to increase palatability, and the feed remnants were removed daily.

Bacterial challenge

Aeromonas hydrophila strain ML09-119, which was isolated from an MAS outbreak at a commercial Channel Catfish pond in Alabama during 2009, was used for the experimental infection (Rodriguez et al. 2008). The strain was cultured in BHI broth and incubated at 28°C under shaking for 15 h. Fish in treatments I and II were exposed to *A. hydrophila* by immersion at the end of the 15-day withdrawal period (the final concentration of the pathogen in the immersion bath was 2.65×10^7 CFU/mL) as described previously (Aboyadak et al. 2015; Peatman et al. 2018). Briefly, fish were immersed for 50 min in a challenge tank (25 L) equipped with an air pump before being returned to the rearing tank. Fish from the system control and treatment III were sham-challenged using sterile BHI broth as the inoculum. After the challenge, animals were returned to their respective tanks and maintained under standard husbandry conditions. Fish were observed for clinical signs of disease and mortalities twice daily for 14 days.

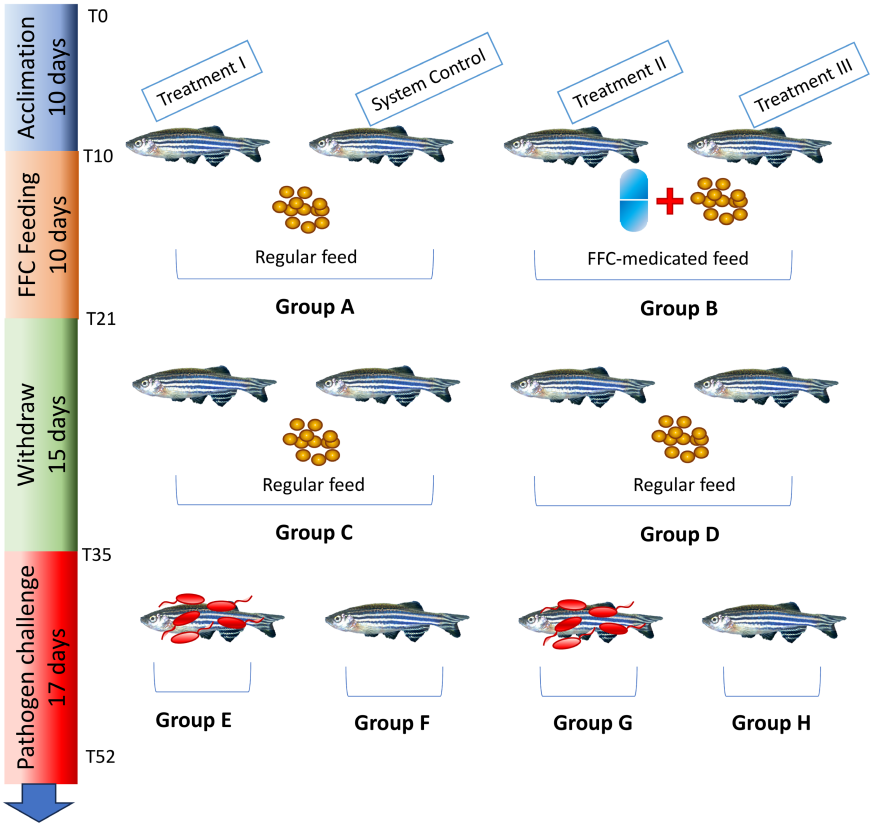

FIGURE 1 Experimental design with the different treatments and time points used in the study of Zebrafish. The four treatments were system control (nonmedicated feed, not challenged), treatment I (nonmedicated feed, challenged with *Aeromonas hydrophila*), treatment II (florfenicol [FFC]-medicated feed, challenged with *A. hydrophila*), and treatment III (FFC-medicated feed, not challenged).

Putative *A. hydrophila* colonies were recovered from the anterior kidneys of moribund and dead fish in BHI agar, and the isolates were confirmed by polymerase chain reaction (PCR) as described by Burgos et al. (2018).

DNA from gut samples

Five fish per tank were randomly sampled for DNA analysis. In general, fish were euthanized with buffered tricaine methanesulfonate (MS 222; 250 mg/L) and the entire intestinal tract was aseptically excised from each fish. Intestines and their contents were pooled, snap-frozen in liquid nitrogen, and stored at −80°C until DNA extractions were carried out. Fish were sampled at day 21 (t21: time after the 10-day medicated feed period), day 35 (t35: time after the 15-day withdrawal period and 3 days before the challenge), and day 52 (t52: the end of the experiment).

The DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA) following the manufacturer's instructions (Spin Column Protocol: Total DNA from Animal Tissues) with modifications, including digestion with proteinase K and pretreatment with lysozyme for lysis of gram-positive bacteria and RNase A treatment. The DNA was eluted with 100 µL of elution buffer. The concentration and purity of the DNA were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA), and the concentration was normalized to 100 ng/µL with double-distilled H_2O.

16S ribosomal RNA sequencing

The DNA samples were submitted to the MR DNA Lab (Molecular Research LP, Shallowater, Texas, USA) for PCR amplification and sequencing using the Illumina MiSeq platform. The V4 region of the 16S ribosomal RNA gene was amplified using 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primers, with a barcode on the forward primer to generate a 300-base-pair (bp) amplicon. The PCR was performed using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) as previously described (Rossello-Mora and Amann 2001). The PCR products were run through a 2% agarose gel to verify successful amplification and relative band intensity of the target DNA. Multiple samples were pooled together and purified using calibrated AMPure XP beads to prepare

the Illumina DNA library. The samples were sequenced as paired-end reads on the Illumina MiSeq platform following the manufacturer's instructions. The sequences were processed using the bTEFAP pipeline (Molecular Research LP). Briefly, after demultiplexing contigs were created, primers, barcodes, and short sequences (<150 bp) were eliminated from the analysis. Ambiguous base calls and homopolymer runs exceeding 6 bp were also removed. Operational taxonomic units (OTUs) were defined at 3% divergence (97% similarity) as per the prokaryotic species concept (Glassing et al. 2015). Final OTUs were taxonomically classified using the nucleotide Basic Local Alignment Search Tool (BLASTn) against a curated database (i.e., Green Genes) and compiled into each taxonomic level (Mohammed and Arias 2015).

Data analysis

For data analysis, samples were clustered based on treatments and sampling time into a total of eight groups (see Figure 1): group A consisted of samples from the system control and treatment I at t21; group B comprised samples from treatments II and III at t21; group C contained samples from the system control and treatment I at t35; group D comprised samples from treatments II and III at t35; group E consisted of samples from treatment I at t52; group F contained samples from the system control at t52; group G comprised samples from treatment II at t52; and group H contained samples from treatment III at t52. Pairwise comparisons between control fish and the fish receiving FFC-medicated feed were conducted at each sampling time since we knew from previous experiments that the microbial communities of fish changed over time when animals were kept under artificial experimental conditions (Schloss et al. 2009).

Mortality data were analyzed using analysis of variance (ANOVA) followed by Tukey's student range (honestly significant difference) test for all pairwise comparisons to determine significant differences ($p < 0.05$) between the mean mortality of the different treatments using JMP version 12.0.1 (SAS Institute Inc., Cary, North Carolina, USA). Alpha diversity was analyzed by generating rarefaction curves, and we calculated the observed OTUs, shared OTUs, and the abundance-based coverage estimation (ACE), Chao1, Good's coverage, inverse Simpson (Invsimpson), and Shannon diversity indices by using Mothur version 1.33.3 (Anderson 2006) after data were rarefied to the lowest number of reads obtained in an individual sample. One-way ANOVA and Tukey's test were used for all pairwise comparisons to determine differences in diversity indices between the groups. Beta diversity was explored by using principal coordinates analysis (PCoA; variables: treatment, time, and group) with optimized positions to visualize the similarities or dissimilarities of the samples. In addition, analysis of similarities (ANOSIM) and permutational multivariate ANOVA (PERMANOVA) were run in PRIMER (Primer E Ltd., Plymouth, UK) to analyze the diversity across groups. Finally, a permutational multivariate analysis of dispersion (PERMDISP) was conducted under the null hypothesis of no differences in within-group multivariate dispersion across sampling time points to determine the within- and between-group dispersion and enable a more accurate interpretation of the PERMANOVA and ANOSIM results (Clarke and Gorley 2006). The PERMANOVA, ANOSIM, and PERMDISP were conducted using 9999 permutations of data following the recommendation of Clarke and Gorley (2006; Sommer et al. 2017). A pairwise test was conducted when statistical tests identified significant effects within the main test ($p \leq 0.05$) to determine the difference between and within groups across sampling events. Similarity percentage (SIMPER) analysis based on a Bray–Curtis similarity matrix assembled from a genus-level table was conducted to determine specific taxonomic differences between communities. The relative abundance at phylum- and genus-level comparisons was examined using a one-way ANOVA.

RESULTS

Zebrafish health status

Routine protocols were followed to examine the health status of five randomly selected Zebrafish before the experiment. The fish did not present with any ectoparasites and were negative for bacterial culture from internal organs. The number of *Aeromonas* sp. in the Zebrafish intestine was 4.5×10^5 CFU/g of tissue. The number of culturable heterotrophic bacteria that were resistant to FFC was 6.25×10^2 CFU/g. The weight and length of fish did not significantly change during the study (data not shown). After the challenge, fish that were infected and colonized by *A. hydrophila* exhibited typical clinical signs of MAS, including external hemorrhages and petechiae in the abdomen and visceral cavity. Anecdotally, fish in group G (survivors sampled at t52 after FFC-medicated feed and after the *A. hydrophila* challenge) contained more feces than fish from the other treatments.

Florfenicol-medicated feed alters bacterial community composition and diversity

A total of 1,402,164 bacterial sequences representing 2046 bacterial OTUs were generated in the study. After sample

normalization to the lowest read ($n = 13{,}408$), a total of 536,320 bacterial sequences and 1866 OTUs remained in the analysis. Sequence coverage was 98% or greater in all sequenced samples (Good's coverage; Table 1).

Rarefaction curves from all groups showed that the fish receiving FFC-medicated feed had higher diversity compared with the system control treatment at t21 and t35. However, diversity decreased over time in all treatments (Figure 2). Group B (FFC-medicated feed) at t21 showed the highest diversity, while group F (system control) at t52 had the least diverse bacterial population. The diversity was very similar between the two groups during the transition from t21 (i.e., group A) to t35 (i.e., group C) under regular feed, while the diversity of bacteria in the fish that were given medicated feed (i.e., group D) decreased considerably compared with samples taken before the medicated feed (i.e., group B) at the same period. Groups E and H showed similar diversity at t52 (Figure 2). Figure 3 shows the unique OTUs for each group as well as those shared between the control and medicated-feed groups at t21 and t35 in addition to the pairwise comparisons between control and challenged groups. The number and abundance of shared OTUs are listed in Supplemental Table 1 available in the online version of this article.

The most significant changes over time were detected in FFC-medicated groups at t21 and t52, as indicated by the observed OTUs and evenness, while the control groups remained relatively constant over time. The microbial community richness (e.g., total number of observed OTUs [Sobs]) in group B was significantly higher compared with that in groups E and F. The total expected richness (as indicated by ACE and Chao1) showed no significant differences among groups. Shannon's evenness index indicated a substantial difference in groups E, F, and H compared to group B. The diversity based on Invsimpson was significantly different in groups A, B, and D compared to the nonchallenged, FFC-medicated animals in group H (Table 1).

These changes in diversity were accompanied by significant changes in the phylum and genus abundances between groups. When bacterial sequences were ascribed at the phylum level, a total of 19 phyla were observed and each group returned a unique bacterial composition (Figure 4). Proteobacteria, Tenericutes, and Firmicutes were the most abundant phyla presented among all groups. Proteobacteria accounted for 53% of the total sequences obtained, whereas Tenericutes and Firmicutes represented 21% and 15%, respectively, of the total sequences. Fusobacteria notably represented only 7%, and other, less common phyla, like Bacteroidetes and Planctomycetes, constituted 1%. The remaining 13 phyla contributed less than 1% of the total sequences. Proteobacteria was the most predominant phylum in six of the eight groups and comprised the majority of all sequences, with a higher proportion in the medicated-feed groups (66% in group B, 64% in group D, 76% in group G, and 71% in group H) than in the regular feed groups (48% in group A, 46% in group C, 31% in group E, and 26% in group F). After the withdrawal

TABLE 1 Diversity indices for the intestinal microbiota of Zebrafish, as calculated by Mothur version 1.33.2. Operational taxonomic units (OTUs) were defined at 97% sequence similarity. Group A received regular feed and was sampled at t21 (see Figure 1) after a 10-day feeding regime; group B received florfenicol (FFC)-medicated feed and was sampled at t21 after a 10-day feeding regime; group C received regular feed and was sampled at t35 after a 15-day withdrawal period; group D received FFC-medicated feed and was sampled at t35 after a 15-day withdrawal period; group E received regular feed and was sampled at t52, after the *Aeromonas hydrophila* challenge; group F received regular feed and was sampled at t52 without an *A. hydrophila* challenge; group G received FFC-medicated feed and was sampled at t52, after the *A. hydrophila* challenge; and group H received FFC-medicated feed and was sampled at t52 without an *A. hydrophila* challenge. Significance among total values for each developmental stage was determined by one-way analysis of variance followed by Tukey's post hoc test. Within a column, values denoted by different letters (z, y, x) are significantly different ($p < 0.05$).

Group	Good's coverage	Sobs[a]	ACE[b]	Chao1	Shannon evenness	Inverse Simpson
A	0.995	886 zy	1711	1442	0.529 zy	7.9 z
B	0.994	1143 z	2102	1678	0.621 z	15.4 z
C	0.995	859 zy	1773	1383	0.504 zy	6.7 zy
D	0.994	990 zy	1734	1409	0.513 zy	8.7 z
E[c]	0.995	807 y	1537	1206	0.453 y	5.2 zy
F	0.995	695 y	1460	1176	0.429 y	3.0 zy
G[c]	0.995	796 zy	1648	1232	0.455 zy	6.1 zy
H	0.995	805 zy	1641	1332	0.418 y	4.2 yx

[a]Sobs is the total number of species observed in the community.

[b]ACE is the abundance-based coverage estimation.

[c]Groups that were challenged with *A. hydrophila*.

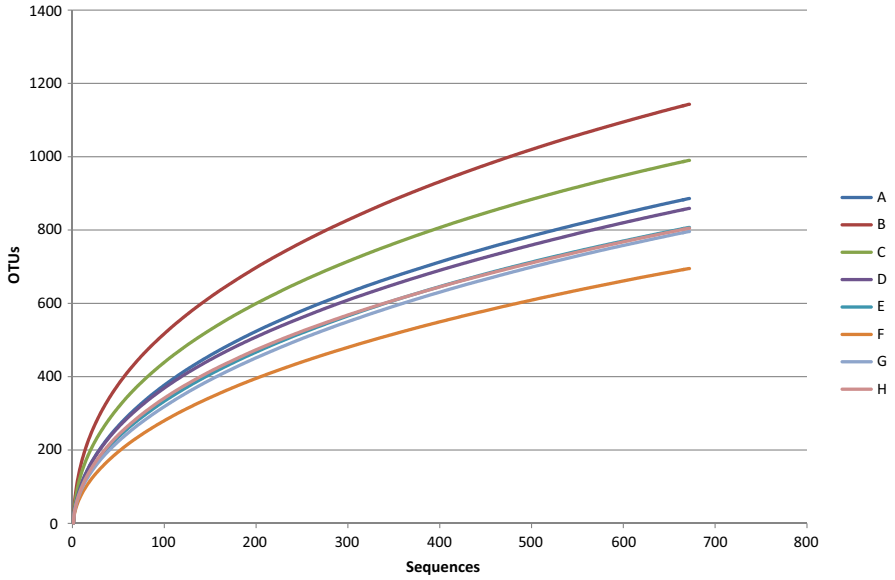

FIGURE 2 Rarefaction curves for Zebrafish belonging to each group; overall, the medicated-feed groups had more diverse intestinal microbiota in terms of operational taxonomic units (OTUs) compared with the nonmedicated control across time in all groups. Group A received regular feed and was sampled at t21 (see Figure 1) after a 10-day feeding regime; group B received florfenicol (FFC)-medicated feed and was sampled at t21 after a 10-day feeding regime; group C received regular feed and was sampled at t35 after a 15-day withdrawal period; group D received FFC-medicated feed and was sampled at t35 after a 15-day withdrawal period; group E received regular feed and was sampled at t52, after the *Aeromonas hydrophila* challenge; group F received regular feed and was sampled at t52 without an *A. hydrophila* challenge; group G received FFC-medicated feed and was sampled at t52, after the *A. hydrophila* challenge; and group H received FFC-medicated feed and was sampled at t52 without an *A. hydrophila* challenge.

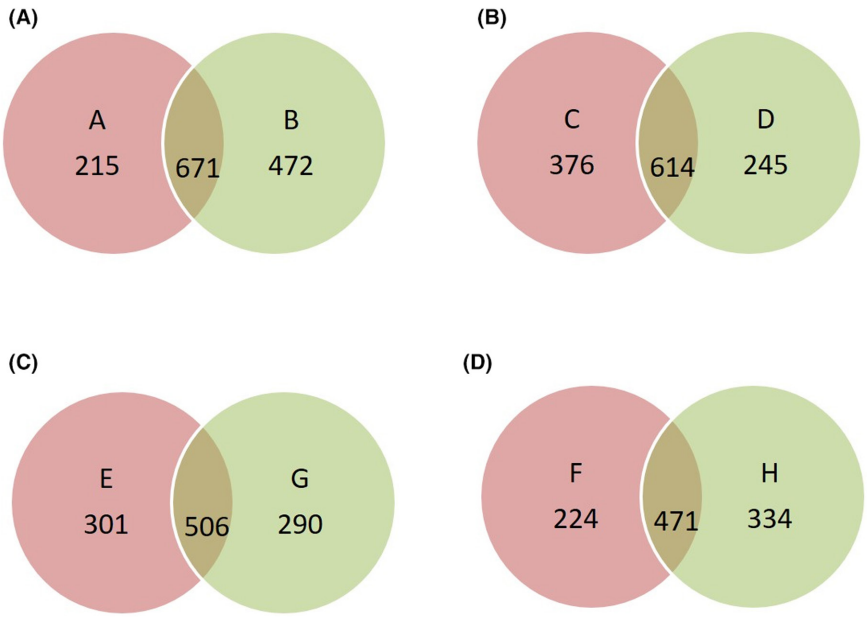

FIGURE 3 Microbial richness shared between nonmedicated and florfenicol-medicated groups of Zebrafish across the study. Groups A–H are depicted in Figure 1 and are also described in Figure 2.

period, Proteobacteria abundance in the medicated-feed group D was significantly higher than in the regular feed group C ($p = 0.0149$). After the bacterial *A. hydrophila* challenge, the Proteobacteria abundance in the medicated-feed group G was still higher than that in the regular feed group E. In contrast, the percentage of Tenericutes was much higher in the regular feed groups than in the medicated-feed groups (group C > group D; $p = 0.0002$). After the bacterial challenge period, the regular feed groups E and F manifested the highest abundance of Tenericutes, at 54%

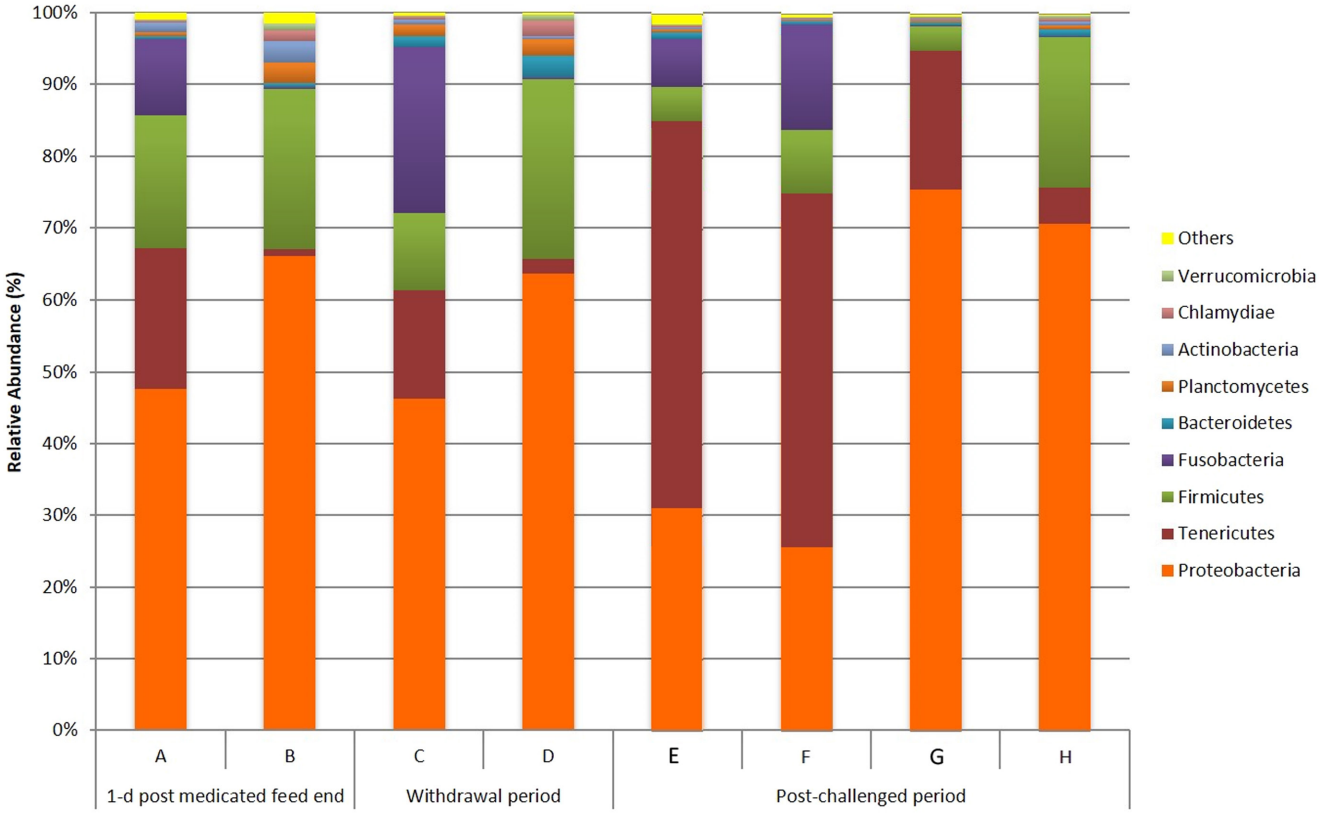

FIGURE 4 Bacterial phylum composition (obtained by Illumina MiSeq) in the intestinal microbiota of each Zebrafish group, representing the average of all replicates. Groups A–H are depicted in Figure 1 and are also described in Figure 2.

and 49%, respectively. Furthermore, Firmicutes showed a reduction in bacterial abundance over time, ranging from 19% to 5% in the regular feed groups (i.e., groups A, C, E, and F), with the lowest value observed in the challenged group E. In the FFC-medicated groups, the abundance of Firmicutes was held relatively constant (~20%) over time, except in challenged group G, which showed a drastic reduction (3%). Fusobacteria was present in all regular feed groups, with group A at 10.7%, group C at 23.2%, group E at 6.7%, and group F at 14.8%, but this phylum was significantly diminished (<1%; $p = 0.0017$) in the medicated-feed groups B, D, G, and H. Other, less common phyla, like Bacteroidetes, were present in groups C and D (>1%), but their abundance was very low (<1%) in the rest of the groups. Planctomycetes was detected in groups A, B, C, D, and H but was limited in the other groups. Actinobacteria was present at approximately 1% in groups A and B, but its abundance was much less (<1%) in other groups. The phylum Chlamydiae showed its highest abundance in group D (2.08%) compared with the rest of the groups (Figure 4). The abundance of the phylum composition is provided in Supplemental Table 2.

At the genus level, the gut microbiota of all groups was composed of a total of 526 genera. Only the most dominant genera present (>0.2%) in all groups are listed in Table 2. *Aeromonas* and *Pseudomonas* (class Gammaproteobacteria, phylum Proteobacteria) were the most abundant bacterial sequences present in all groups, with the highest prevalence occurring in group H (41.7%); *Aeromonas* was significantly higher ($p = 0.0005$) in groups D and H compared with the other groups. Interestingly, *Mycoplasma* (class Mollicutes, phylum Tenericutes) was the most abundant genus in the regular feed groups A, C, and F and in the bacterial-challenged groups E and G and was reduced in the treated groups B, D, and H. Contrarily, *Cetobacterium* (class Fusobacteriia, phylum Fusobacteria) was present in the regular feed groups A, C, E, and F, whereas this genus was reduced in all treated groups (B, D, G, and H). *Mycoplasma* was significantly higher in groups F and E ($p = 0.0016$) compared with the other groups, while *Cetobacterium* was significantly higher ($p = 0.0122$) in group C than in group D. Furthermore, *Ruminiclostridium* (class Clostridia, phylum Firmicutes) was most abundant in groups A, D, and H, whereas it was lowest in groups B, C, E, G, and F.

Bacterial community cluster analysis

Gut microbiota samples were analyzed based on group ascription at the OTU level, and visual representation of the beta diversity using PCoA showed the discrimination of

TABLE 2 Genus identity of sequences in the Zebrafish intestinal microbiome, represented as percentages of the total number of sequences. Only genera accounting for more than 0.2% of the sequences in at least one group are displayed. Groups are depicted in Figure 1 and are also described in Table 1.

	Group							
Genus	**A**	**B**	**C**	**D**	**E**[a]	**F**	**G**[a]	**H**
Aeromonas	11.8	14.2	22.0	36.3	18.2	13.4	35.8	41.7
Mycoplasma	19.5	0.9	15.1	1.9	54.1	49.4	19.4	5.1
Pseudomonas	8.7	18.6	13.3	12.3	6.8	7.0	19.5	19.7
Ruminiclostridium	13.5	2.6	2.0	15.2	3.2	4.4	2.4	19.0
Cetobacterium	10.7	0.3	23.2	0.2	6.7	14.8	0.2	0.2
Geobacillus	2.7	10.2	7.1	7.0	0.7	2.7	0.5	0.6
Phyllobacterium	11.1	0.7	0.3	0.7	0.1	0.5	0.4	0.4
Shinella	0.7	3.2	0.5	0.7	0.7	0.5	5.5	1.4
Bacillus	1.5	1.4	1.1	2.2	0.5	1.0	0.3	1.0
Shewanella	0.6	0.5	0.7	2.1	0.8	0.4	2.0	1.6
Stenotrophomonas	2.8	4.4	0.2	0.5	0.4	0.5	0.2	0.3
Burkholderia	2.2	5.4	0.2	0.5	0.2	0.2	0.3	0.3
Others	14.1	37.5	14.3	20.5	7.6	5.3	13.7	8.7

[a]Challenged group sampled at t52.

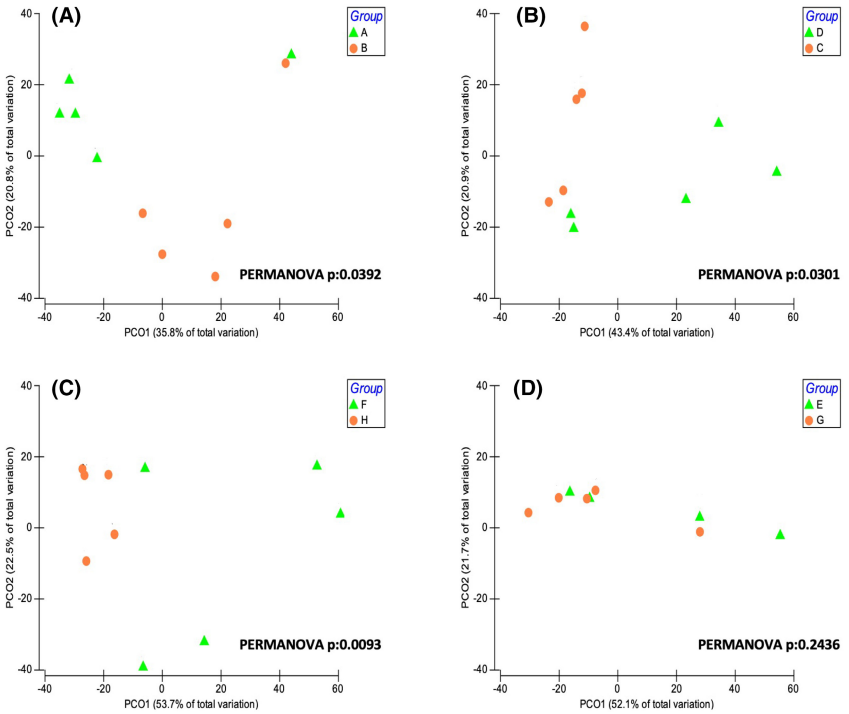

FIGURE 5 Principal coordinates (PC) analysis plots of Bray–Curtis distances based on the intestinal microbiota of Zebrafish belonging to the eight groups. Groups are depicted in Figure 1 and are also described in Figure 2. PERMANOVA, permutational analysis of variance.

samples between regular feed and medicated-feed groups with a small degree of overlap (Figure 5). The ANOSIM and PERMANOVA compared the clusters based on the following variables: treatment (I–III and system control), time (t21, t35, and t52), and group (A–H). Samples clustered significantly ($p \leq 0.05$) by all factors, although some overlap existed. In addition, the separation was most significant when samples were assigned to the cluster based on the group with an *R*-value of 0.22. These global *R*-values indicated that clusters were significantly correlated with all factors, although group (group = treatment + time combined) was the most significant variable and played

the primary role in determining the change in composition of the gut microbiota. The *R*-values for treatment and time were 0.168 and 0.126, respectively.

Furthermore, statistical analysis of beta diversity across sampling events showed a significant divergence of the microbial communities present between gut groups than within the group at the OTU level ($p \leq 0.001$; Table 3). The pairwise PERMANOVA and ANOSIM results were in agreement that significant shifts occurred between microbial communities at every sampling time point ($p \leq 0.05$; Table 3), except for the postchallenge groups E and G (sampled at t52), which failed to show significant differences ($p = 0.243$ and $p = 0.122$, respectively; Table 3). The most noteworthy difference in the microbial composition of nonchallenged groups was detected between groups F and H, as this comparison showed the largest ANOSIM and PERMANOVA test statistics (ANOSIM: *R*-value = 0.416; PERMANOVA: pseudo-value = 2.00). Furthermore, the paired comparison within treatments and across sampling times (i.e., groups A, C, and F) showed nonsignificant changes between all regular feed groups. However, they were significantly different between medicated-feed groups D and G (PERMANOVA; Supplemental Table 3).

Similarity percentage analysis

To further explore microbial differences in groups, SIMPER analysis was used to evaluate within-group similarity and among-group dissimilarity. The SIMPER analysis of bacterial genera within each of the medicated-feed groups showed that similarities increased across sampling time in the FFC-medicated groups B, D, G, and H (39.8, 55.7, 62.9, and 63.8%, respectively), while similarities in the regular feed groups (i.e., A, C, E, and F) were close, ranging from 40.85% to 45.80%. Furthermore, SIMPER analysis showed high pairwise dissimilarities between all groups. The main dissimilarities between groups were mostly due to the different relative abundances of the genera *Mycoplasma*, *Aeromonas*, and *Cetobacterium*. Based on genus composition, SIMPER analysis indicated that groups B and F were the most dissimilar (75.14%), while groups G and H were the least different (37.026%; Supplemental Table 4).

Susceptibility to the opportunistic pathogen *Aeromonas hydrophila*

The mean cumulative percent mortality after the challenge is shown in Figure 6. Mortality was only reported in fish belonging to group G (treatment II; medicated feed, challenged), while other groups, including group F (system control; regular feed, not challenged), group E (treatment I; regular feed, challenged), and group H (treatment III; medicated feed, not challenged), did not show any mortality throughout the challenge. Fish treated with medicated feed and challenged with *A. hydrophila* (group

TABLE 3 Main and pairwise test results of nonparametric, permutation-based multivariate statistical analyses of Bray–Curtis similarity of the intestinal microbiome in Zebrafish by treatment across sampling times (days; t21, t35, and t52). Groups are depicted in Figure 1 and are also described in Table 1. All statistical tests were conducted using a fixed factor of fish treatment across all three sampling times. Test statistics were calculated using up to 9999 permutations, but data structure dictated the number of possible permutations. An asterisk indicates significant difference. ANOSIM, analysis of similarities; PERMANOVA, permutational multivariate analysis of variance; PERMDISP, permutational multivariate analysis of dispersion.

Statistical test	Groups compared	Test statistic	*p*-value	Possible permutations
Main tests across treatments				
PERMDISP		1.9982	0.28	9999
PERMANOVA		2.1698	0.0003*	9875
ANOSIM		0.21	0.0002*	9999
Pairwise tests between treatments over time				
PERMANOVA	A and B	1.4907	0.0392*	126
	C and D	1.6201	0.0301*	126
	E and G	1.1054	0.2436	126
	F and H	2.0045	0.0093*	126
ANOSIM	A and B	0.248	0.05*	126
	C and D	0.336	0.024*	126
	E and G	0.048	0.22	126
	F and H	0.416	0.016*	126

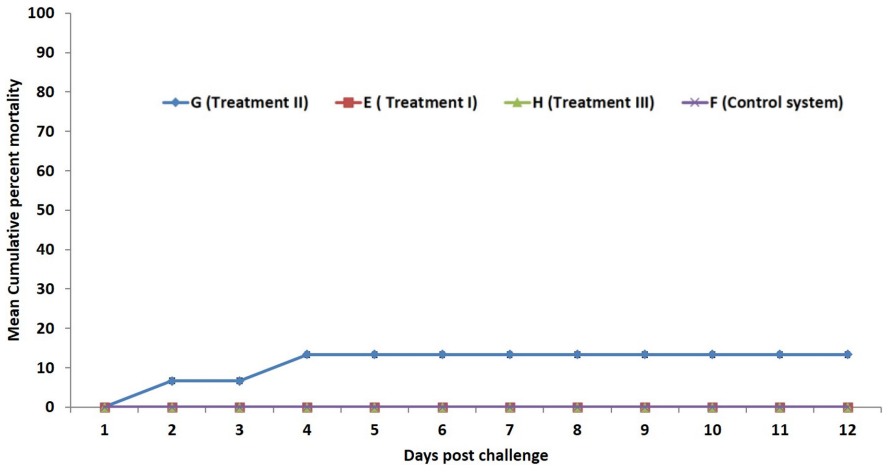

FIGURE 6 Mean cumulative percent mortality of Zebrafish challenged with *Aeromonas hydrophila*. Group E received regular feed and was sampled at t52 (see Figure 1), after the *A. hydrophila* challenge; group F received regular feed and was sampled at t52 without an *A. hydrophila* challenge; group G received florfenicol (FFC)-medicated feed and was sampled at t52, after the *A. hydrophila* challenge; and group H received FFC-medicated feed and was sampled at t52 without an *A. hydrophila* challenge.

G) had a mean mortality of 13.33 ± 0.577% (mean ± SE). *Aeromonas hydrophila* was isolated from the kidneys of dead and moribund fish and confirmed by PCR. Mortality persisted for 4 days, with the majority of fish deaths occurring at 2 and 4 days postchallenge. The study was monitored for a total of 14 days.

DISCUSSION

The gut bacterial community comprises pathogenic and nonpathogenic microorganisms living in a dynamic but balanced equilibrium with their host. Studies show that dysbiosis refers to a disturbed microbial composition (an outgrowth of possible pathogenic species) or a disturbed interaction between bacteria and the host (Hawrelak and Myers 2004). Factors such as antibiotics, physiological and physical stress, and specific dietary components have been found to contribute to intestinal dysbiosis (Gismondo 1998). The use of antibiotics has been proven to be one of the significant drivers for alterations in gut microbiota, and the effect of antibiotics was directly related to their spectrum of activity (Nord 1990), pharmacokinetics dosage (Nord and Edlund 1990), and length of administration (Ryan et al. 2017). Previous studies showed that the use of antibiotics could change the dominant community in the microbiota, such as Proteobacteria (Colistin) and Fusobacteria (Vancomycin; Anderson 2006; Abdelhamed et al. 2019). Similar to previous studies, our results were marked by an overgrowth of Proteobacteria in the microbiota of the FFC-treated groups, while other microbial members of Fusobacteria were diminished. It is known that antibiotics might increase gut permeability, allowing the generation of diverse bacterial species and thus increasing the species richness. This might explain why the microbiome in group B showed the highest diversity at 1 day posttreatment, followed by a continued decrease in diversity in the treated fish until the end of the study.

The microbial diversity was significantly different between the feed treatments (i.e., medicated feed versus regular feed). This study found that the microbial community composition in the regular feed groups was relatively stable and resilient against perturbations. A significant bacterial shift was observed within the medicated-feed groups, but its composition was shaped toward specific bacterial communities, including many species of pathogens. Our results were consistent with previous studies involving treatment with antibiotics, which reported changes in diversity along with the loss of crucial microbial members within the gut microbiota, even after the cessation of the antibiotic treatment (Antonopoulos et al. 2009; Jakobsson et al. 2010; Ubeda et al. 2010; Buffie et al. 2012; Liu et al. 2012; Jernberg et al. 2013; Carlson et al. 2015).

The variability among different groups (i.e., groups A–H) significantly affected the gut microbiota composition. Although each group presented a significantly distinct microbiota, a relatively low sample-to-sample variability within the regular feed or medicated-feed treatment groups was revealed. Furthermore, the microbial composition differed significantly over time during the period of study, including in the control treatment. Furthermore, dysbiosis remained even after the selective pressure induced by the FFC ended. This disruption in microbiota structure was correlated with a significant increase in mortality after *A. hydrophila*

challenge for Zebrafish treated with FFC-medicated feed (13.3%) compared to those that received regular feed (0%). Our mortality results were in accordance with the olaquindox-altered gut microbiota reported by He et al. (2017) for Zebrafish, which showed an increase in susceptibility to *A. hydrophila* infection. It has been suggested that once individuals with pre-existing conditions (e.g., an altered gut microbiota) face an adverse environmental factor, such as viruses, bacterial infection, trauma, or stress, the risk of disease could increase (Scher et al. 2015). Our result indicates that the microbial composition in the Zebrafish gut was changed by antibiotic treatment, accompanied by susceptibility to *Aeromonas*.

Our data suggested that the increase in pathogen susceptibility was possibly due to gut dysbiosis after antibiotic administration in Zebrafish. However, it is worth noting that this study was conducted using a model cyprinid species in a tank system. The result may not sufficiently represent the clinical situation in other common aquaculture species, such as ictalurids or salmonids. Thus, the evidence is suggestive—not conclusive—regarding the effect of antibiotic use on commercial fish species in natural systems. Further studies under field conditions are needed to fully understand the changes in fish gut microbiota after treatment with FFC-medicated feed in aquaculture ponds.

CONCLUSIONS

In conclusion, our results showed that FFC-medicated feed, which is commonly used in aquaculture to treat bacterial diseases, induced a dysbiotic state in healthy adult Zebrafish that was characterized by an increase in diversity, an increase in the abundance of specific phyla containing mostly potential bacterial pathogens, and a reduced cohort of beneficial bacteria, which potentially increased the susceptibility of the fish host to secondary infections. Our results indicated that the gut microbiota in healthy fish could be readily disturbed due to disturbance in the environment of the gastrointestinal tract caused by antibiotic administration. The altered microbiota observed in the FFC-medicated groups was either causative of or consequential to the susceptibility to secondary infection (e.g., *A. hydrophila*), and healthy gut microbiota transplantation followed by a rechallenge experiment might prove this point.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ETHICS STATEMENT

The animal experiment protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Auburn University (IACUC Number 2016-2946).

DATA AVAILABILITY STATEMENT

All data is available from the first author or corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.