



OPEN Conventional diversity metrics underestimate gut microbiome disruption under florfenicol exposure in Nile tilapia (*Oreochromis niloticus*)

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The gut microbiome is an integral component of fish health and growth performance and therefore represents an important production factor in aquaculture. Antibiotics are a major stressor, not only by disturbing microbial diversity, but also by altering microbial interactions involved in digestion, colonization resistance and functional redundancy. Current risk assessments, however, rely largely on diversity-based evaluations and rarely consider changes in network topology. Here, we investigated the impact of florfenicol on the gut microbiome of Nile tilapia (*Oreochromis niloticus*) using 16S rRNA sequencing, combining conventional diversity metrics with microbial co-occurrence network modelling. While the microbial community remained overall stable at the diversity level, with treatment explaining only a minor proportion of the observed variation, network analysis revealed substantial fragmentation and a less robust network structure. Network connectivity was significantly reduced, with a near-complete turnover of central hub taxa in the florfenicol network. Our findings demonstrate that diversity-based assessments alone may substantially underestimate the extent of antibiotic-induced microbiome disturbance, with network analysis capturing changes within the community that are highly relevant for microbiome stability and would otherwise remain undetected when evaluations are restricted to diversity metrics.

Keywords Gut microbiome, Florfenicol, Co-occurrence networks, Antibiotics, Aquaculture, *Oreochromis niloticus*

Abbreviations

SCFA	Short-chain fatty acids
ARG	Antibiotic resistance gene
ASV	Amplicon sequence variant
C	Control
FFC	Florfenicol
IVI	Integrated value of influence
LCC	Largest connected component
AUC	Area under the curve
RAS	Recirculating aquaculture system
CSS	Cumulative sum scaling

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Commercial aquaculture relies on optimal growth performance of farmed fish under intensive rearing conditions to meet the rising global demand for aquatic products¹. The dependence of fish on the symbiotic relationship with their gut microbiome for optimal development and resilience under stressful conditions is increasingly recognized as a key component for sustainable farming practices and veterinary health management in aquaculture. Bacteria support host physiology through their involvement in nutrient digestion, including short-chain fatty acid (SCFA) production and synthesis of essential vitamins such as cobalamin (vitamin B12), immune modulation and colonization resistance against pathogens. Gut microbiome composition differs with host species and the associated feeding ecology^{2,3}, habitat⁴ and changes throughout ontogenetic development^{5,6}. Environmental conditions such as temperature⁷, salinity⁸, water pollution⁹, as well as the type of rearing system used¹⁰ impose further selective pressures on gut microbiome assembly. Across freshwater species, the intestinal microbiota is dominated by *Proteobacteria*, *Bacteroidota*, *Firmicutes* and *Fusobacteriota*¹¹. However, the functional repertoire of microbial communities, both free-living and host-associated, is not only defined by the overall taxonomic composition, but also by how microbiome members interact with one another. Microbial interactions range from cooperative behaviors that benefit all interacting partners, such as metabolic cross-feeding or antibiotic resistance conferral, to competitive and antagonistic interactions that negatively affect other community members, for example through niche exclusion or antimicrobial activity¹². The balance between positive and negative interactions is integral for maintaining core microbiome functions relevant for host health and determines the capacity of the microbial community to adapt to abiotic and biotic disturbances. As the gut microbiome of farmed fish is both a determinant of host performance and a direct target of multiple production-related stressors, microbiome disruption, either at the compositional or network level, can directly affect production outcomes in commercial aquaculture. Gut microbiome dysbiosis is frequently linked to extensive antibiotic use, driven by the increasing infection pressure within aquaculture systems as a consequence of rising water temperatures, inadequate hygiene and high stocking densities¹³. Florfenicol (FFC) is one of the most widely used veterinary antibiotics and is highly effective in treating bacterial diseases of major economic concern in freshwater aquaculture, such as streptococcosis and vibriosis¹⁴. Beyond the risk of promoting the transmission of antibiotic resistance genes (ARGs)¹⁵, several studies have reported histopathological effects on the host and the associated microbiome^{16–18}, which may in turn increase disease susceptibility due to the impairment of intestinal barrier integrity and immunosuppression, thereby reinforcing the need for repeated antibiotic intervention. However, growing evidence suggests that antibiotic-induced microbiome disruption cannot be summarized by a single universal marker of dysbiosis. Instead, both the type and magnitude of disturbance depend strongly on the investigated species, the environmental context, the type of antimicrobial compound used, co-application with antiparasitics and host age^{19,20}. For example, metabolism and persistence of antimicrobial compounds can vary with temperature²¹, whereas the susceptibility to adverse microbiome responses differs between juvenile and adult fish²⁰. To date, most studies investigating antibiotic effects on fish gut microbiota primarily rely on standard diversity metrics (e.g. α -, β -diversity, differential abundance testing) derived from 16S rRNA amplicon sequencing or sometimes shotgun metagenomics¹⁹. While highly informative for compositional turnover, these approaches fail to capture structural shifts at the level of microbe-microbe interactions. As abundance and network influence are not strictly coupled, rare taxa may act as keystone taxa with a disproportionately large impact on ecosystem stability relative to their abundance²². Consequently, focusing on compositional analysis alone may underestimate the extent of microbiome disruption following antibiotic treatment. Resolving these response patterns in a holistic manner is therefore not only important for a broader ecological understanding of gut microbiome dysbiosis, but also for the refinement of husbandry practices and veterinary intervention strategies in aquaculture. Because of its relatively low dietary and husbandry requirements and its rapid growth, tilapia is one of the most widely farmed fish species in the world¹. However, despite its global relevance, tilapia has received comparatively little attention in microbiome research. To address this gap, we exposed adult Nile tilapia (*Oreochromis niloticus*) to a 10-day florfenicol treatment at a daily dose of 10 mg/kg bw followed by a 20-day recovery without further intervention and characterized gut microbiome dynamics using both conventional diversity metrics and microbial co-occurrence network analysis based on 16S rRNA sequencing of the V1–V2 region.

Results

Limited impact of florfenicol on gut microbiome diversity

A total of 10,028 ASVs were detected in the dataset, representing 31 phyla and 854 genera. Control and FFC-treated tilapia shared a similar set of dominant genera, including *Cetobacterium*, *Romboutsia*, *Streptococcus*, *Diaminobutyricimonas* and *Bacteroides* (Supplementary Fig. S2a). As shown in Fig. 1a, b, time was the primary determinant of observed changes in alpha diversity and overall community composition. Shannon diversity varied significantly across sampling time points (ANOVA: time, $F = 7.05$, $p < 0.001$); the treatment main effect was not significant ($F = 2.83$, $p = 0.099$), whereas the time $\times$ treatment interaction was significant ($F = 4.69$, $p < 0.001$). Time was also the primary determinant of beta diversity (PERMANOVA: time, $R^2 = 0.258$, $p = 0.001$). Although florfenicol had a significant but small main effect on the gut microbiome (PERMANOVA: treatment, $R^2 = 0.027$, $p = 0.001$), its influence was mainly reflected in the temporal trajectory of community turnover (time $\times$ treatment, $R^2 = 0.139$, $p = 0.001$). This pattern was most distinct in the post-treatment phase, where between-group Bray–Curtis dissimilarities tended to be higher than during the exposure phase (Supplementary Fig. S3). To identify taxa driving the temporal and between-group differentiation, we first identified within-group responders relative to the common pretreatment baseline and then used the union of these responders to test whether FFC altered the magnitude or direction of their temporal trajectories. Of the 854 genera tested, 31 showed temporal changes relative to the pretreatment baseline in the control group, compared to 37 genera shifting under FFC exposure, with 25 genera shared between the two sets (Supplementary Table S5). 43 differed significantly at selected time points between groups, although no consistent changes across the experimental

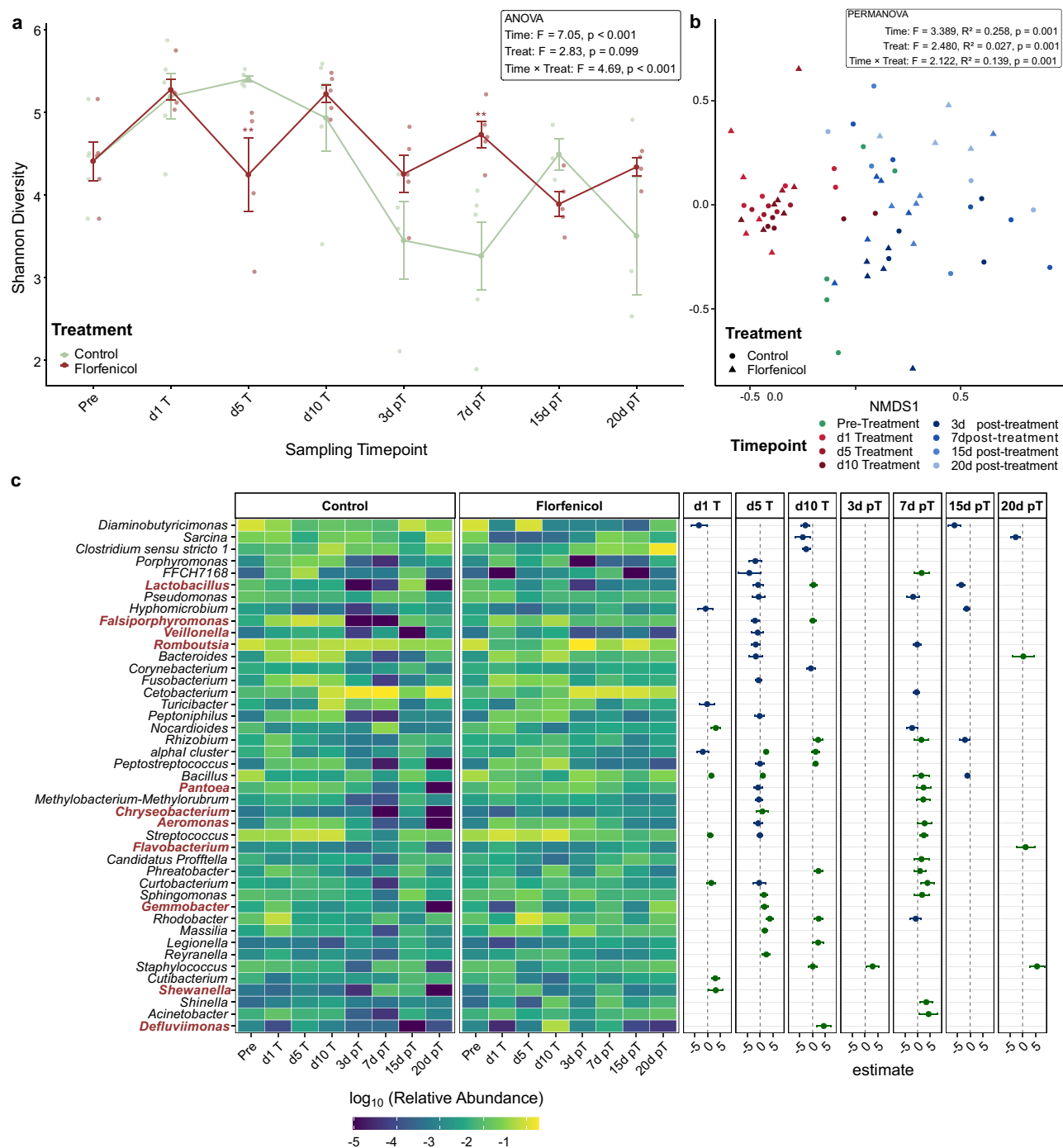


Fig. 1. (a) Comparison of Shannon diversity between pre-treatment baseline ($n = 5$), control ($n = 30$) and FFC-treated fish ($n = 33$). Differences between treatments at each time point were tested using Wilcoxon rank-sum tests and are indicated by asterisks ($p < 0.05$). (b) NMDS plot of Bray–Curtis dissimilarities between treatment groups over the experimental period. (c) Heatmap showing log₁₀ relative abundance of 43 within-group temporal responders with significant between-group differences per time point identified with beta-binomial regression modelling ($p < 0.05$). Red labels indicate temporal responders that were only identified in the FFC group. p -values were adjusted for multiple testing using the Benjamini–Hochberg procedure.

period could be detected. In line with the diversity analysis, the largest numbers of responders were observed at day 5 of treatment and 7 days post-treatment (Fig. 1c). Florfenicol treatment was associated with a stronger depletion of *Diaminobutyricimonas*, *Sarcina*, *Lactobacillus*, *Romboutsia* and *Pseudomonas* over time, among others. After an initial decrease during the early treatment phase, *Aeromonas*, *Pantoea*, *Methylobacterium-Methylorubrum* and *Bacteroides* rebounded to control levels or even increased in abundance following florfenicol withdrawal. A further characteristic of exposed individuals was the temporarily higher abundance of *Bacillus*,

Streptococcus, *Phreatobacter*, *Sphingomonas*, *Rhodobacter* and *Staphylococcus* in the gut microbiome. Consistent with the limited effects of florfenicol on gut microbiome diversity, the core community (aggregated over all time points) remained stable in both groups, with differences mainly restricted to low-abundance and lower-prevalence taxa (Supplementary Fig. S2b,c).

Reduced network stability and interactions under florfenicol exposure

Because the resilience of microbial communities to external stressors is determined not only by their overall diversity and composition, but also by the type of interactions between microbiome members, we next analyzed how florfenicol affected microbial co-occurrence patterns as a secondary layer of microbiome restructuring under stress. Because the primary focus of this analysis was on the disruptive potential of florfenicol as the main factor, samples from all time points were pooled within each treatment, and two genus-level networks were constructed to characterize overall treatment-associated differences while retaining sufficient sample size for network inference. An additional sensitivity analysis using phase-stratified networks suggested stronger fragmentation of the FFC networks, particularly during recovery, but did not support a robust phase-wise comparison between treatments. As shown in Supplementary Tables S3 and S4, the phase-specific networks were based on smaller and unequal sample sets, showed lower edge-selection stability during exposure, and differed substantially in network size and sparsity. These technical differences precluded reliable separation of biological phase effects from variation introduced by network inference. The networks contained similar numbers of nodes (261 in the control and 268 in the FFC network) and similar proportions of positive associations (~74%), but the FFC network contained fewer edges (409 vs. 516). A full list of network topology metrics is provided in Supplementary Table S1. While overall modularity based on fast-greedy clustering only showed minor differences between treatments (0.57 vs. 0.64), FFC affected several parameters related to network connectivity and information flow between nodes. Degree (C: 9.91 ± 3.42 ; FFC: 8.1 ± 3.96) and neighbour centrality (C: 4.43 ± 0.97 ; FFC: 3.55 ± 1.47) were significantly reduced under antibiotic treatment, resulting in an overall sparser and more fragmented network compared to the control (Fig. 2a). Betweenness centrality did not differ significantly between groups (C: 450.77 ± 378.24 ; FFC: 557.94 ± 673.62), however, the broader distribution of node-level values in the FFC network suggests a more heterogeneous, taxon-specific response. In addition, network-wide connectivity was reduced under FFC, as indicated by an increase in average path length between nodes from 4.49 to 5.54 and a higher proportion of within-module edges (65.9% in the control vs. 73.3% in the FFC network; Fig. 2b). To estimate network robustness under repeated secondary node-loss events, we conducted in silico attack simulations by sequentially removing either random nodes or hub nodes (highest to lowest degree). The faster decline in relative LCC size and lower AUC values of the FFC network indicate reduced structural robustness and increased vulnerability to node loss under both scenarios, with the strongest difference observed for the targeted attack (random AUC: 0.405 vs. 0.338; targeted AUC: 0.226 vs. 0.135; Fig. 2c).

Central hub nodes shift under florfenicol treatment

We initially hypothesized that network influence would be at least partially tied to core membership and high abundance. Based on hubness scores, with IVI used as a secondary ranking criterion, we identified 23 (C) and 22 (FFC) nodes as central hubs in the two networks. However, no correlation between relative abundance and hubness scores across all nodes could be detected in either network (Spearman's rho (C)=0.12, $p=0.06$; rho (FFC)=0.05, $p=0.42$). In the control group, hubs were mainly affiliated with *Firmicutes* (e.g. *Clostridium sensu stricto*, *Lactobacillus*, *Proteiniclasticum*), *Proteobacteria* (e.g. *Dechloromonas*, *Hyphomicrobium*) and *Bacteroidota* (e.g. *Macellibacteroides*, *Aureispira*) (Fig. 3a). Following FFC treatment, all control hubs except *Clostridium sensu stricto* 2 and *Proteiniclasticum* lost their central network positions, as shown by a drastic decrease in degree, betweenness and neighbour centrality values relative to their corresponding positions in the FFC network (Fig. 3b). However, while *Dechloromonas* and *Actinobacillus* no longer met the hub criteria in the FFC network, both taxa shifted from a more peripheral position in the control to positions as bridge nodes connecting different submodules in the FFC network. Accordingly, FFC hubs were mainly distributed across *Firmicutes* (e.g. *Streptococcus*, *Erysipelothrix*, *Clostridium sensu stricto* 2, *Peptostreptococcus*), *Actinobacteriota* (e.g. *Propioniceella*, *Herbiconiux*) and *Proteobacteria* (e.g. *Pasteurella*, *Aggregatibacter*). A complete list of all hubs and their respective network metrics is supplied in Supplementary Table S2. Despite the stringent filtering prior to network construction, only four control hubs overlapped with core genera, namely *Cetobacterium*, *Clostridium sensu stricto* 1, *Rhizobium* and *Lactobacillus*, whereas in the FFC network only *Streptococcus* was identified as both a hub and a core genus (Supplementary Fig. S2b,c). Likewise, the abundance changes reported above did not consistently translate into gains or losses in network importance among hub genera. For *Streptococcus*, increased abundance coincided with increased network importance under FFC treatment. By contrast, *Lactobacillus* and *Hyphomicrobium* showed temporal reductions in relative abundance together with a drastic loss of network influence.

Florfenicol induces strong turnover in association partners

The structural changes in network architecture described above were accompanied by both a reduction in total edge count from 516 to 409 and the extensive reorganization of association partners. Based on the subset of nodes shared between both networks, 79 associations were lost under FFC treatment, whereas 54 new associations were formed in the treated group (Supplementary Fig. S4a). Edge loss largely affected highly connected hubs such as *Aggregatibacter*, *Lactobacillus*, *Macellibacteroides* and *Hyphomicrobium*, but also non-hub taxa like *Murdochella* and *Bacteroides* (see Supplementary Fig. S4b). Conversely, taxa with the strongest gain in associations were mostly newly established hubs in the FFC network, like *Erysipelothrix*, *Erysipelatoclostridium*, *Frisingicoccus* and *Porphyromonas*, although non-hub taxa such as *Nocardioides* showed comparable shifts in connections. Hubs that retained their central position under treatment, namely *Clostridium sensu stricto* 2 and *Proteiniclasticum*,

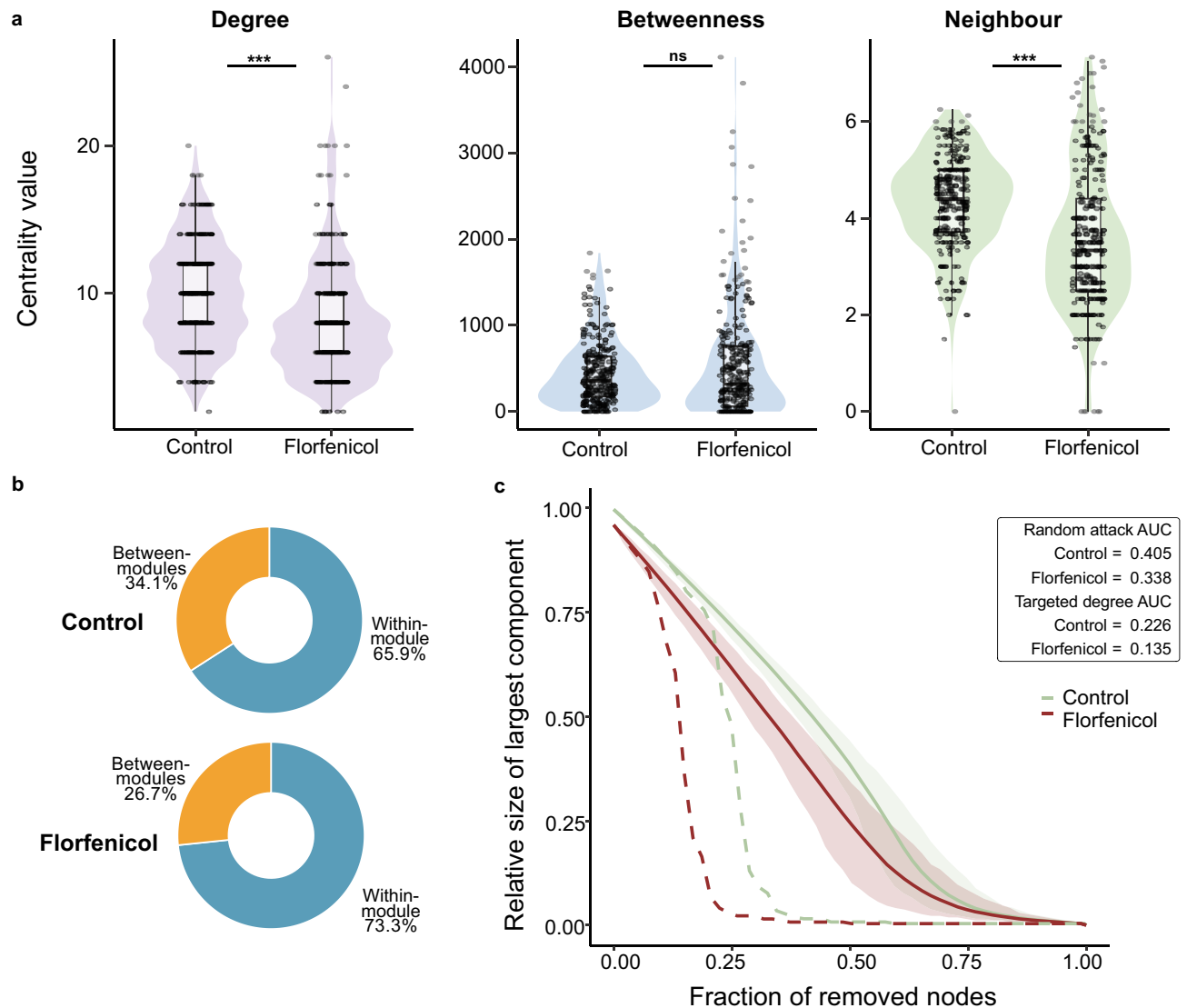


Fig. 2. (a) Node-level degree, betweenness and neighbour centrality in control ($n = 30$) and FFC networks ($n = 33$). Dots represent nodes ($n_C = 261$, $n_{FFC} = 268$); group differences were tested with Wilcoxon rank-sum tests (** $p < 0.001$, BH-adjusted). (b) Relative distribution of edges connecting nodes within or between network modules. (c) Network robustness analysis under random (solid lines) and targeted (dashed lines) node removal, quantified by relative LCC size and AUC. Random removal was repeated 500 times; shading shows the corresponding 95% simulation intervals.

showed complete replacement of direct association partners in the exposed network. The observed edge turnover particularly affected connections among well-documented gut commensals and fermenters, replacing previously functionally coherent clusters with associations that may have lower functional complementarity in the FFC network, as shown in Fig. 4. Under control conditions, *Clostridium sensu stricto* 2 formed a cluster with *Succiniblasticum* and *Alloprevotella*, whereas in the FFC network these connections were replaced by links to *Proteus* and *Eubacterium nodatum* group. Similarly, *Lactobacillus* lost its associations with *Limosilactobacillus* and *Aurantimicrobium* and instead was associated with *Porphyromonas* in the FFC network, connected to the newly formed *Clostridium sensu stricto* 2 cluster (Supplementary Fig. S5). Central links of *Bacteroides* to *Fusobacterium*, *Aeromonas* and *Sutterella* were absent in the FFC network, while *Cetobacterium* as a key fish gut commensal shifted from an association with *Macellibacteroides* to one with the Firmicutes Family XIII AD3011 group. Altered connections following florfenicol treatment also included links between commensals and genera containing potential pathogens, such as the negative association between *Streptococcus* and *Macellibacteroides*. Newly formed associations included *Erysipelothrix* with *Succiniblasticum*, *Pasteurella* and *Alloprevotella* and *Proteiniclasticum* with *Butyrivibrio* and *Prevotella* 7.

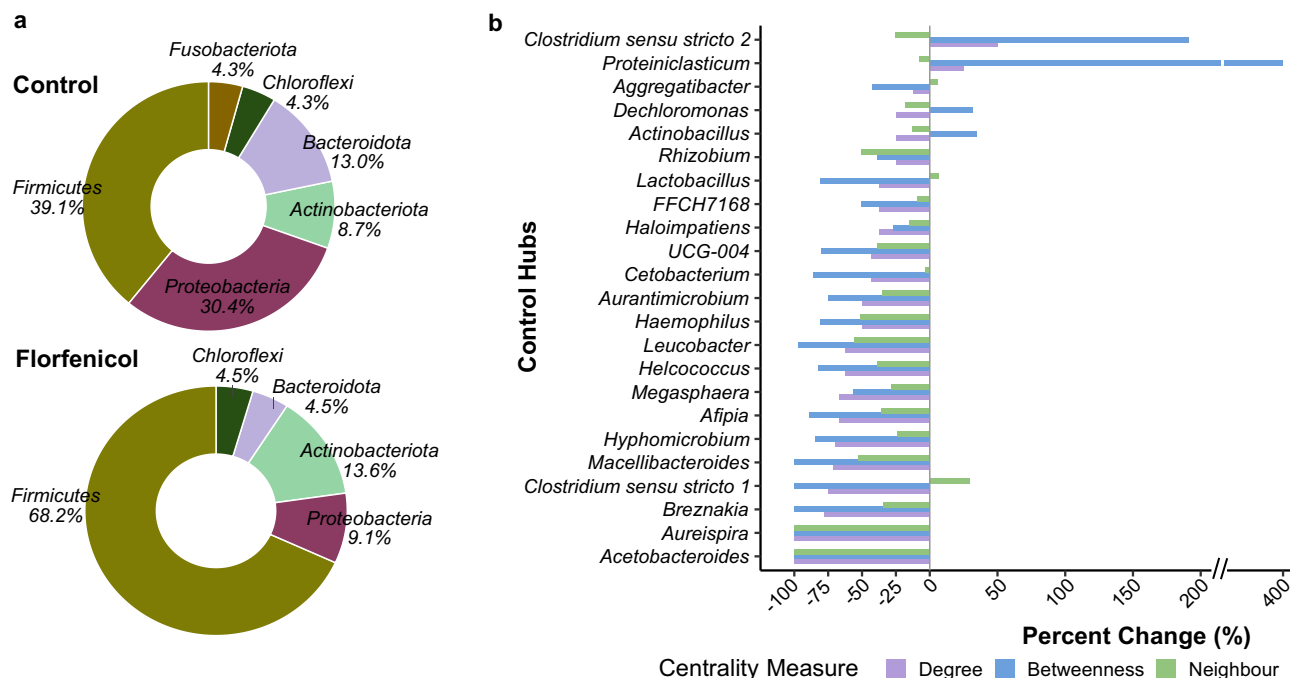


Fig. 3. (a) Phylum-level composition of hubs identified in the control (23 hubs; network $n = 30$ fish) and FFC networks (22 hubs; network $n = 33$ fish). (b) Relative changes in the network positions of control hubs following FFC treatment. Bars are colored according to the corresponding centrality value (degree, betweenness, neighbour).

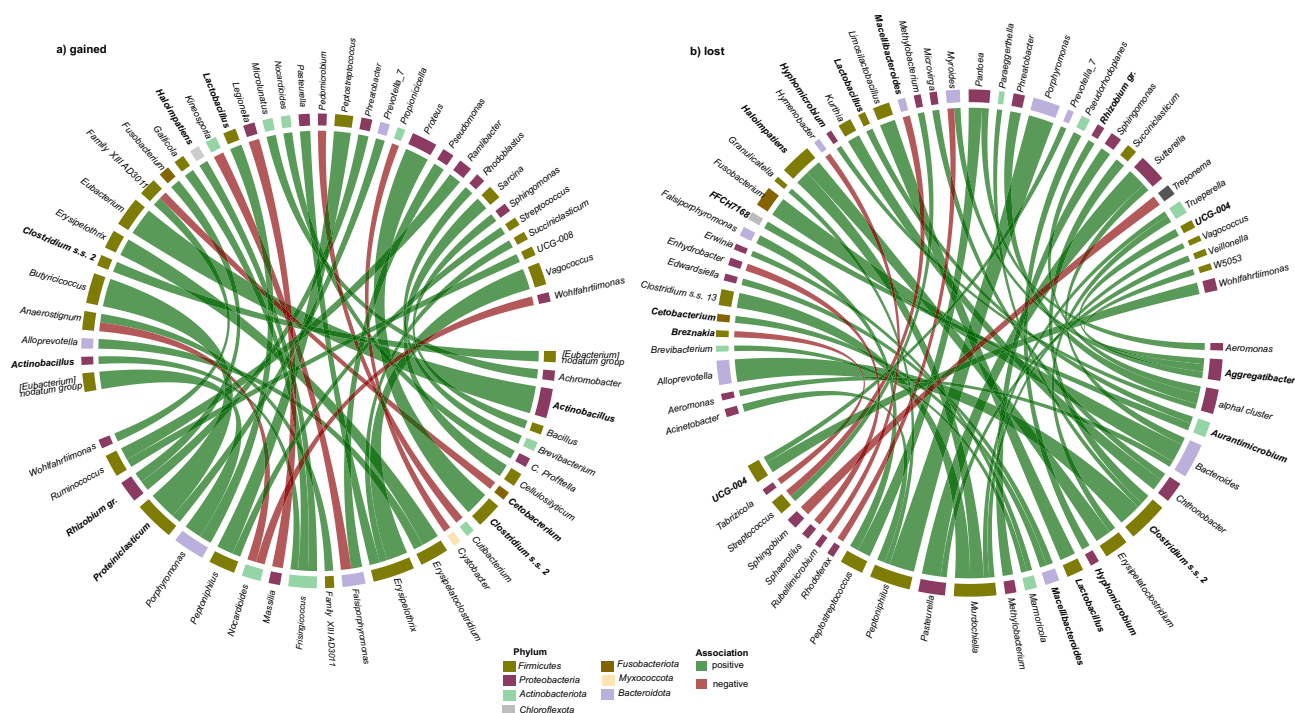


Fig. 4. Top 20 taxa, ranked by the number of gained (a) or lost (b) associations in the FFC network relative to the control network, and their respective association partners. The analysis was restricted to the 209 nodes shared between networks, among which 54 associations were gained and 79 were lost; only associations with an absolute weight ≥ 0.1 are shown. Chord color indicates association sign (green, positive; red, negative), and chord width represents association weight. Outer-ring colors indicate phylum affiliation; hub genera are shown in bold.

Discussion

Potential impairment of growth and increased disease susceptibility associated with antibiotic-induced gut microbiome dysbiosis represent major challenges to sustaining high production yields in commercial fish farming. However, as alternatives for controlling infectious diseases, such as prebiotics or vaccines, are currently only available for a limited number of fish species and pathogens, antibiotics remain a critical bottleneck in current husbandry practices despite their associated risks^{23,24}. Available microbial risk assessment studies provide limited resolution of system-specific risks, often focus on a restricted range of model species, mainly zebrafish (*Danio rerio*), and rarely incorporate analyses at the network level¹⁹. In this study, the overall effect of FFC on the gut microbiota of adult Nile tilapia was moderate when assessed by conventional diversity metrics. Time had the strongest influence on the observed taxonomic changes in our study, whereas treatment-dependent modulation of natural temporal dynamics was less pronounced. Differences in β -diversity over time between control and FFC-treated fish at a dose of 10 mg/kg bw for 10 consecutive days were mainly attributed to the transient depletion of abundant microbiome members like *Diaminobutyricimonas*, *Lactobacillus* and *Romboutsia*. These genera, together with *Bacteroides* and *Pseudomonas*, are commonly identified members of the healthy gut microbiota of various freshwater fish and are likely to provide important functions to the host, including degradation of complex carbohydrates, lipid digestion and SCFA production (e.g. acetate and propionate)^{2,25–28}. Probiotic feed supplementation with *Lactobacillus plantarum* is frequently linked to improved immune function, feed utilization and growth performance^{29,30}. Furthermore, *Lactobacillus* spp. contribute to community stability by limiting pathogen overgrowth through bacteriocin production and acidification by lactic acid, with documented evidence for increased host resilience to *Aeromonas hydrophila* infection in Nile tilapia²⁹. While these commensal taxa had recovered their initial abundances by the end of the experiment, this temporary imbalance may still impose additional stress on the host by impairing intestinal barrier integrity and metabolism, thereby making it more vulnerable to subsequent stressors within the production system. This hypothesis is supported by a recent study by Han et al.²⁷, which reported persistent intestinal damage in koi carp following FFC treatment despite recovered microbiome diversity. A common signature of antibiotic-induced dysbiosis is the selective enrichment of opportunistic pathogens. Increased pathogen burden is a concern for aquaculture for two main reasons: (i) it may cause secondary infections during the vulnerable post-treatment phase; and (ii) multidrug-resistant pathogens may promote the dissemination of antibiotic resistance genes (ARGs) within the population, thereby reducing the effectiveness of future interventions, especially given the limited range of approved therapeutics. Due to the spread of ARGs and pathogens via water and food chains, this may also pose a public health risk³¹. In the present study, transient increases were observed only for *Streptococcus* sp. and *Aeromonas* sp., although these changes were not consistent across the experiment. While frequently detected as part of the normal gut microbiota, both genera contain fish pathogens of high economic concern, such as *S. iniae*³², *S. agalactiae*³³ and *A. hydrophila*³⁴. Their resilience to FFC may be related to the high prevalence of multidrug resistance determinants in subpopulations of these taxa, alongside R-strategy traits that enable them to outcompete other taxa for vacated ecological niches and resources^{33,35,36}. Other commonly reported genera containing potential pathogens, including *Citrobacter* and *Plesiomonas*^{19,37}, were not detected in our study. Although full-length 16S rRNA sequencing could improve species-level resolution, confident identification of pathogenic strains would require higher-resolution approaches such as shotgun metagenomics or targeted cultivation. This represents a major methodological constraint of the present study. Overall, the patterns observed in this study are consistent with similar studies in Nile tilapia^{37–39}, channel catfish¹⁷, salmon⁴⁰, and European seabass⁴¹. Differences in the magnitude of microbiome shifts, such as stronger and more persistent pathogen enrichment or sustained depletion of beneficial commensals, may be partly explained by the use of substantially younger fish rather than fully grown tilapia at market age. As shown in our previous study, juvenile brown trout exhibited stronger microbiome shifts and higher pathogen loads than adult fish under otherwise identical rearing conditions²⁰. Further variation between studies may stem from the antibiotic tested, temperature-dependent differences in pharmacokinetics and metabolism of the compounds by the fish²¹, differences between gut compartments⁴¹ and the type of rearing system used. Florfenicol and its metabolites are excreted via feces and disseminated into the rearing water from uneaten feed. In fully closed or recirculating systems, prolonged exposure to (sub-)therapeutic concentrations may effectively increase the selective pressure on the microbiome compared to flow-through systems with continuous water exchange as used here. The substantial variation among aquaculture systems and husbandry practices is one of the reasons why defining a common marker of antibiotic-induced microbiome damage and subsequent identification of reliable microbial biomarkers for monitoring purposes remains challenging. Compared to other relevant aquaculture species, tilapia is frequently described as being relatively resilient to antibiotic treatment and tolerant of broad temperature and salinity gradients^{42,43} as well as hypoxia⁴⁴. The detection of resistance determinants in treatment-naïve tilapia by Payne et al.³⁷ further suggests a pre-existing resistome within the baseline microbiome. Taken together, these factors may explain the observed apparent resilience at the diversity level, especially given the persistence of the core community throughout the experiment. Nevertheless, these findings should be interpreted within the context of a controlled laboratory setting, in which FFC represented the only major stressor. Commercial production systems expose fish to multiple pressures, including different therapeutic compounds, repeated treatments, changing physicochemical conditions and high stocking densities, all of which may intensify microbiome changes following antibiotic treatment. Because the experiment lacked independent replication at the tank level, treatment effects cannot be fully separated from potential tank effects; some tank-associated bias therefore cannot be excluded. Another important caveat is that persistence at the abundance level does not provide information on whether the ecological relationships between individual microbiome members are retained under pressure. Especially in the host context, microbial ecosystem services are often the product of interspecies cooperation within trophic networks⁴⁵. Regarding optimal growth, one of the most important processes in this context is metabolic cross-feeding between primary carbohydrate degraders and secondary

fermenters, which convert intermediates like succinate and lactate into SCFAs (e.g. propionate, butyrate)⁴⁶. Even when restricted to taxonomic data, shifts in network associations can provide indications that functional relationships within the microbiome may have changed. Despite the minor effect on microbial diversity and community composition, FFC treatment resulted in a more fragmented and compartmentalized network, with reduced network-wide connectivity between nodes. Comparable effects on microbial networks have been shown for murine models and humans^{47,48} but, to the best of our knowledge, not yet at this level of detail for fish. Empirically, reduced network complexity is linked to a lower capacity to maintain homeostasis under stress and to reduced efficacy of microbiome functions if established synergistic relationships or bridge species are lost. Even if a taxon remains stable at the abundance level, changes in its position within the network or its association partners may still alter its functionality because of metabolic interdependencies and weaker coupling between modules. Among other things, associations lost under FFC affected taxa with documented complementary substrate utilization within trophic networks. One example is the coupling of succinate-producing *Alloprevotella*⁴⁹ with *Succinivibrio*⁵⁰ (converting succinate to propionate) and *Clostridium* sensu stricto as a butyrate producer, which occupies a similar metabolic niche. In the rumen, synergistic interactions between these taxa have been associated with epithelial barrier integrity and gut homeostasis^{51,52}. Their co-detection in fish gut microbiomes warrants testing whether comparable relationships occur in fish. A similar syntrophic relationship between *Lactobacillus* and *Limosilactibacillus* is well established in vaginal microbiomes⁵³. The disappearance of negative links, commonly attributed to antagonistic relationships through resource competition or niche exclusion⁵⁴, between *Streptococcus* and *Macellibacteroides*, may indicate weakened pathogen suppression following FFC treatment. This interpretation is further supported by the emergence of *Streptococcus* as a new hub in the FFC network with the highest connectivity, as also observed for other opportunistic pathobionts such as *Erysipelothrix*⁵⁵. Under FFC, the network structure was reorganized, potentially also in response to changes in the intestinal milieu. Newly formed associations included those linking the proteolytic hub *Proteiniclasticum* to the major butyrate producer *Butyrivibrio* and to *Prevotella*, as well as *Clostridium* sensu stricto 2 to *Proteus*, all of which are low-abundance taxa and have so far been considered of limited relevance to fish health. These newly established edges may not provide functionally equivalent compensation for the lost metabolic synergies adapted to host and dietary conditions. While the interpretation of interactions inferred from statistical network modelling is rooted in ecological theory, co-occurrences may equally result from shared niches rather than direct ecological interactions (e.g. mutualism, predation, competition). Direct synergism between taxa requires validation through co-culture experiments, which are still unavailable for most gut microbiome members, especially in fish⁵⁶. Nevertheless, these observations may provide a possible ecological explanation for the reduced feed utilization, lipid digestion and reduced growth previously reported in antibiotic-treated tilapia and seabass^{57–59}. Further evidence for network re-organization is provided by the extensive turnover of hub taxa between control and FFC networks. Hubs are highly interconnected nodes with potentially disproportionate influence on community structure; however, network centrality alone identifies candidate keystone taxa rather than demonstrating keystone function. Members of several genera represented among control hubs, such as *Cetobacterium*, *Clostridium* and *Lactobacillus*, have documented roles in supporting host physiology^{2,28}. The availability of cobalamin (vitamin B12), an essential cofactor for several physiological processes such as the formation of red blood cells, DNA synthesis and nervous system functioning, depends on the de novo synthesis by specialized bacterial and archaeal clades, including *Cetobacterium ceti* and *C. somerae*⁶⁰. Likewise, *Cetobacterium* supplementation has been shown to increase colonization resistance against *A. hydrophila* and strengthen intestinal barrier integrity through acetate production and a cobalamin-dependent increase in intestinal levels of essential SCFAs^{61,62}. The role of bacterial predation in modulating microbiome dynamics is currently an understudied mechanism. *Aureispira* could contribute to the suppression of pathogen overgrowth through targeted ixotrophic predation, which so far has been demonstrated against *Vibrio* spp.⁶³. Increased resilience through predator–prey dynamics has already been described in coral microbiomes^{64,65}. The detection of *Aureispira* in different freshwater and marine hosts, including Japanese eel⁶⁶ and swan mussels⁶⁷, together with its high connectivity despite low abundance and its ecological characteristics, makes this genus a candidate keystone taxon that should be investigated in further studies. Other identified hubs, like *Hyphomicrobium*⁶⁸ or *Dechloromonas*⁶⁹, have been described in other studies as transient, low-abundance taxa that are primarily associated with the environment. Because hubs may provide important functions and connect otherwise separated modules, their loss or replacement may result in network collapse if localized failures can no longer be adequately compensated⁷⁰. In our study, in silico node-loss simulations indicated that the more fragmented FFC network lost connectivity substantially faster than the control network, especially when removing highly connected hubs. In a realistic aquaculture setting, comparable secondary node-loss events may result from repeated antibiotic interventions, particularly shortly after an initial treatment, co-exposure to other therapeutics, secondary infections, or additional environmental stressors. Another important finding for distinguishing eubiotic from altered microbiome states is that neither hub identity nor changes in network position are strictly correlated with taxon abundance. Consequently, changes in abundance do not allow direct conclusions about topological changes within the community and vice versa. Ecologically important taxa may lose central positions without corresponding changes in abundance and would therefore remain undetected as key responders to network restructuring in diversity-based analyses. For example, for *Cetobacterium*, no uniform response to antibiotic treatment can be derived from abundance data: some studies reported increased abundance after oxytetracycline and sulfamonomethoxine^{37,38}, others decreased abundance following FFC^{17,20} or no changes, as observed here. Consistent with the results presented here, we previously showed in a study on FFC treatment in brown trout that the centrality of key responders, including *Cetobacterium*, remained significantly reduced in post-treatment networks despite full recovery in abundance²⁰. This discrepancy between compositional stability and structural disruption at the interaction level raises the question of whether current operational definitions of gut dysbiosis can adequately capture microbiome responses to antibiotic treatment or other stressors to allow

for an accurate assessment of the associated risks. The concept of gut dysbiosis is widely used to distinguish healthy microbiome states from altered states that are frequently associated with disease phenotypes and reduced fitness. The exact criteria used for classification remain a subject of debate, but commonly applied definitions include: (i) deviation of microbiome composition from a defined normal state, reflected by shifts in β -diversity, reductions in α -diversity, or changes in biomarker ratios, e.g. *Proteobacteria:Bacteroidota* or *Proteobacteria:Firmicutes*; (ii) enrichment of pathobionts; and (iii) simultaneous depletion of keystone taxa or commensals with known or potential benefits for the host⁷¹. A major limitation of these definitions is their predominant reliance on diversity metrics, while network topology is rarely included in the assessment. This partly reflects the absence of a methodological gold standard for network construction, the higher computational demands, and the substantially larger sample sizes required for reliable network inference. Here, the limited statistical power resulting from insufficient replication per timepoint prevented phase- or even timepoint-stratified network inference, which would have allowed us to quantify the underlying temporal dynamics in addition to the overarching treatment effect. When considering diversity alone, our results suggest that FFC treatment did not induce clear patterns of gut microbiome dysbiosis or persistent microbiome shifts in adult Nile tilapia. Analysis of the microbial co-occurrence structure, however, showed that FFC shifted the microbiome towards a destabilized and more vulnerable community state that may also be functionally distinct from the initial state, despite similar diversity. From an industry perspective, impaired nutrient utilization and reduced resilience due to the loss of synergistic interactions as detailed above may lead to reduced growth and increased mortality, ultimately resulting in lower production yields⁷². Diversity metrics and network topology capture different dimensions of microbiome stability; resilience in one cannot be directly translated to the other. Evaluating diversity alone may consequently underestimate the extent of microbiome disruption following antibiotic exposure. Rather than treating compositional and network stability as separate concepts, they should be considered as complementary levels that are both required for a comprehensive understanding of microbiome responses and for reliable risk assessment. Integrating both levels in future research may also contribute to a more robust baseline definition of what constitutes a healthy fish microbiome, facilitate the identification of suitable biomarkers for the discrimination of eubiotic and dysbiotic states in aquaculture and support the development of new probiotic candidates as alternatives to repeated chemical interventions.

Methods

Experimental design

To assess the impact of florfenicol (FFC) on the gut microbiome of Nile tilapia (*Oreochromis niloticus*), 255 healthy adult fish (mean body weight $\pm$ SD: 370 ± 62 g) were randomly distributed into seventeen 450-L water tanks (15 fish per tank) with continuous aeration and open water flow at the Aquaculture Center of the University of São Paulo (Jaboticabal, SP, Brazil). All experimental procedures complied with national guidelines for animal care and were approved by the Ethics Committee for Animal Experimentation of UNESP, Brazil (Protocol 1115/2023). Fish were sourced from a commercial fish farm (André Luiz Scarano Camargo e Outro; Santa Bárbara, Zacarias, São Paulo, Brazil) and had not previously been exposed to antimicrobial treatment. Prior to the study, fish presenting clinical signs of disease (e.g., skin lesions, body spots, or fin erosion) were excluded. Fish were acclimated together for 12 days and, during both the acclimation phase and post-treatment period, were fed a commercial non-medicated diet (Aqua Pirá®, Guabi, Brazil) ad libitum. During the 10-day treatment period, fish received a laboratory-prepared FFC-medicated diet once daily (9:00–11:00 a.m.) at a feeding rate of 1.0% of body biomass, providing a target dose of 10 mg of FFC per kg body weight per day. The medicated feed was prepared at the University of Campinas by top-coating the commercial feed with a mixture of vegetable oil and a premix containing 50% (w/w) FFC, as previously described by Valim et al.⁷³. The FFC concentration in the medicated feed (1280 ± 13 mg/kg) was confirmed using a validated liquid chromatography–tandem mass spectrometry (LC–MS/MS) method⁷⁴. Control fish received identical top-coated feed without the FFC premix to ensure comparability between groups. Fish were maintained at ambient temperature (28.1 ± 1.4 °C). Water quality parameters, including dissolved oxygen (6.6 ± 1.9 mg/L) and pH (7.6 ± 0.4), were monitored twice daily⁷⁴. Samples were collected at seven time points to evaluate both treatment effects and microbiome recovery: days 1, 5, and 10 during FFC administration and days 13, 17, 25, and 30 (post-treatment phase). Five fish were randomly sampled from the common acclimation tank prior to allocation to the experimental tanks before treatment (day 0) to establish baseline microbiome composition. Following allocation, fish from the control and FFC groups were sampled according to the same schedule. Prior to sampling, fish were deeply anesthetized with benzocaine (100 mg/L), followed by brain perforation to ensure death before dissection. The intestine was aseptically dissected, and fecal material was collected by gentle compression. Five fish were sampled per treatment group and time point, immediately snap-frozen, and stored at -20 °C until further processing. Each fish was sampled only once. However, because each treatment-by-time-point combination was represented by a single tank, the five fish sampled from that tank constitute biological specimens but not independent tank-level replicates.

DNA extraction and 16S rRNA amplicon sequencing

Genomic DNA from 75 samples was extracted using the QIAamp FAST DNA Stool Mini Kit (QIAGEN, Netherlands) following the supplier's "pathogen detection protocol". DNA concentration was measured using the Quant-iT PicoGreen dsDNA assay (Thermo Scientific, USA). Bacterial community profiling was performed by 16S rRNA gene amplicon sequencing targeting the V1–V2 region (008F 5'-AGAGTTTGATCMTGGC-3', 343R 5'-CTGCTGCCTYCCGTA-3'⁷⁵), as described previously²⁰. The primary PCR comprised 35 cycles, followed by 10 cycles of indexing PCR using Nextera XT Index Kit v2 (Illumina Inc., USA). No-template controls and extraction controls were included as blanks to monitor potential contamination during library preparation. Library yield and quality were evaluated on a Fragment Analyzer device (Agilent Technology, USA; DNF-43 Standard Sensitivity NGS Fragment Kit). Samples were re-purified in the case of primer dimers. Finally, high-

quality samples were pooled in an equimolar ratio of 4 nM and sequenced on an Illumina MiSeq (2 × 300 bp) using the MiSeq Reagent Kit v3 (600 cycles). Raw sequence data are available in the NCBI Sequence Read Archive under BioProject PRJNA1474536.

Raw data processing

Processing of the obtained 11,146,440 raw sequences and downstream microbiome analyses were conducted in R (version 4.5.1) using RStudio. After adapter removal with *cutadapt* v4.5 and initial quality check with FastQC v0.12, an ASV (amplicon sequence variant) abundance table was built with DADA2 v1.30.0 using the following trimming settings: *truncLen* = 240/200; *maxEE* = 2/4; *trimLeft* = 20. Taxonomic annotation of inferred ASVs was performed using the SILVA v138 database. ASVs were classified as contaminants based on a 25% prevalence threshold in extraction and PCR negative controls, corresponding to a presence in ≥ 2 of 8 controls. ASVs associated with negative controls (41), as well as those assigned to eukaryotes (460), chloroplasts (1), or mitochondria (356) were removed. In addition, seven low-coverage samples were excluded following evaluation of adequate sequencing depth based on rarefaction curves (vegan v2.7.1; see Supplementary Fig. S1). The final dataset contained 4,147,654 reads (mean sequencing depth: 60,994.10 ± 30,734.86), distributed across 10,028 ASVs and 68 samples.

Microbial diversity analysis and statistics

Unless specified otherwise, R packages phyloseq v1.52, microbiome v1.31.3, tidyverse v2.0, ggplot2 v3.5.2 and patchwork v1.3.2 were used for data analysis and visualization. Cumulative sum scaling (CSS) was used for normalization to mitigate potential sequencing depth bias between samples. Data distribution and homoscedasticity were checked prior to statistical testing using Levene's test and visually via Q-Q plots. Microbial alpha diversity was estimated using the Shannon index. Differences between treatment groups were assessed using a two-way ANOVA (Shannon ~ Treatment * Timepoint) and additionally by non-parametric Wilcoxon rank-sum test per time point after adjusting for multiple testing with the Benjamini-Hochberg (BH) method. Beta diversity was based on Bray-Curtis distances and visualized by NMDS (non-metric multidimensional scaling). The effects of treatment, sampling time point, and their interaction on ASV-level community composition were tested with PERMANOVA (9999 permutations). Tank could not be included as an independent random effect because tank identity was confounded with treatment and sampling time point; consequently, treatment effects cannot be separated completely from potential tank effects. Multivariate dispersion was assessed using the *betadisper* function (vegan) to verify that PERMANOVA results were not primarily driven by differences in within-group variability. To reduce noise from sparse, low-prevalence ASVs, the non-normalized dataset was aggregated to genus level prior to differential abundance testing. Time-dependent key responders to florfenicol were identified using corncob v0.4.2 via the *differentialTest* option fitting taxon-wise beta-binomial regression models. As both treatments shared a common baseline and time explained the majority of observed variation in microbiome composition independent of treatment, a dual-modelling approach was applied: first, within-group responders relative to pre-treatment were identified with ~ *Timepoint* as the key variable. Then, the subset of time responders was used to test whether treatment changed the magnitude or direction of the temporal trajectory. For all tests, an FDR threshold of 0.05 was chosen. The core microbiome (genus level) per treatment group was defined based on a 60% prevalence threshold with a minimum detection limit of 0.05%.

Microbial co-occurrence network analysis

For the construction of genus-level co-occurrence networks per treatment, we used the SPIEC-EASI method implemented in NetCoMi v1.2 with identical settings for each subset (Meinshausen-Bühlmann neighborhood selection; *repnum* = 20, *nlambda* = 20). As the primary objective was to quantify global shifts in microbial association patterns following florfenicol treatment rather than time-point-specific dynamics, samples were pooled across time points within each treatment, resulting in two distinct networks (control: n = 30; florfenicol: n = 33). Pre-treatment samples were excluded. To evaluate whether phase-stratified network construction could provide a robust alternative despite the reduced sample size below the recommended threshold for stable network inference (n < 25⁷⁶), exposure- and recovery networks were constructed using identical SPIEC-EASI parameters. The suitability of the phase-stratified approach was evaluated based on StARS instability, edge-selection stability, network size, and sparsity (Supplementary Tables S3 and S4). For the pooled analysis, the abundance table was filtered to retain genera with a relative abundance > 0.005% that were present in ≥ 5 samples, preserving ≥ 95% of total read counts for network construction. For the sensitivity analysis, prevalence was scaled proportionally to the respective sample size. Data were normalized using the default centered log-ratio (CLR) transformation to account for compositionality. The resulting network adjacency matrices were then transformed into an igraph object (v2.1.4) for the calculation of global network characteristics and node-level centrality measures. Modularity, defined as clusters of highly interconnected nodes, was determined by the fast-greedy clustering algorithm. Wilcoxon rank-sum tests with BH-correction were used to evaluate differences in degree, betweenness and neighbour centrality between untreated and treated communities. For the identification of nodes with high network influence ("hubs"), we used the influential package (v2.2.9), with the hubness score (a synergistic index integrating both the local H index and degree centrality) as the main selection parameter⁷⁷. Nodes with hubness scores exceeding the empirical 90th percentile of the hubness-score distribution (~ 1.45) within the respective network were classified as hubs. To test whether the selected cutoff value for hub classification may affect the observed hub turnover between control and FFC networks, an additional sensitivity analysis was performed using the 85th and 95th percentile as more permissive and more stringent cutoffs (Supplementary Table S6). The IVI score (Integrated Value of Influence) was used as a secondary ranking criterion, as absolute IVI values are not directly comparable across networks with differing global topology. The correlation between abundance and hubness score was tested using Spearman's rank correlation. To characterize shifts in association patterns

under florfenicol exposure, edge turnover was quantified on the subset of nodes shared between the control and florfenicol networks ($n = 209$). Associations were classified as lost if they were present exclusively in the control network, whereas associations were classified as gained if they were present only in the florfenicol network. Subnetworks containing hubs and their direct interaction partners were visualized with ggraph (v2.2.2). Chord diagrams of altered network associations were prepared using the circlize package (v0.4.16). For Fig. 4, associations were restricted to an absolute weight ≥ 0.1 ; taxa were then ranked by the number of qualifying gained or lost associations, and the top 20 taxa in each category were displayed. For the evaluation of network robustness under stress, we simulated sequential node-loss events and quantified network vulnerability based on the relative size of the largest connected component (LCC) and the area under the corresponding decay curve (AUC). The relative LCC was calculated as the number of nodes within the current LCC divided by the total number of nodes in the original network. Two attack scenarios were tested: (i) random node removal and (ii) targeted removal of highly connected nodes. For the random attack, 500 simulations were run per network, whereas the targeted attack was performed deterministically by sequentially removing the node with the highest degree and recalculating node degree after each removal step. In both scenarios, network decay was recorded at node-removal increments corresponding to 2% of the original network size.

Data availability

Raw sequencing data from demultiplexed samples analyzed in this study have been made available in the NCBI Sequence Read Archive (SRA) under BioProject accession PRJNA1474536.

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Author contributions

IMA, FP, GFPS and APQ conducted the animal experiment and sampling. SR, GFPS, MS and LMS designed the experiment. LMS conducted the laboratory work, data analysis and drafted the manuscript. SW conducted the sequencing. SR and MS critically reviewed the manuscript and provided overall guidance. MS and SR secured funding. All authors read and approved the final version of the manuscript.

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Declarations

Competing interests

The authors declare that they have no competing interests.

Ethical approval and consent to participate

All experimental procedures complied with national guidelines for animal care and were approved by the Ethics Committee for Animal Experimentation of UNESP, Brazil (Protocol 1115/2023).

Additional information

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