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Examining the Effects: Lack of Impact by Endolysin and Phage Treatment on Rotifer and Larvae Microbiota

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Abstract

Background: Bacteriophages and phage-derived lytic enzymes are increasingly considered to be targeted antimicrobial tools in aquaculture; however, their compatibility with non-target microbial communities under hatchery-relevant conditions remains insufficiently characterized. Objectives This study evaluates the impact of a lytic phage (CH20) and a phage-derived lysin (LysVp1), applied under previously validated conditions for rapid *Vibrio* control, on the microbiota associated with seawater, rotifers, and zebrafish larvae challenged with *Vibrio alginolyticus* GV09. Methods: Treatments were independently applied to each biological matrix using short exposure times representative of hatchery practices, intentionally capturing the critical window during which microbial transfer from live feed to larvae occurs. Microbial communities were analyzed using 16S rRNA gene sequencing, with DNA- and RNA-derived datasets evaluated separately. Results: Alpha diversity indices were compared using appropriate statistical tests, while beta diversity was assessed using Aitchison distance, PERMANOVA, and dispersion analyses, and differential abundance was evaluated using ANCOM-BC2. Alpha diversity metrics showed no significant differences among treatments across all matrices, indicating the preservation of microbial richness and diversity. Beta diversity patterns differed according to the nucleic acid source, with RNA-based analyses revealing treatment-associated shifts in rotifer and water microbiota that were not consistently detected at the DNA level. In zebrafish larvae, neither phage nor lysin treatment significantly altered overall community structure, although dispersion effects reflected limitations related to sample size. Conclusions: Overall, these results indicate that phage CH20 and lysin LysVp1 exert minimal impact on alpha diversity and limited, context-dependent effects on microbial community structure, supporting their microbiota-safe potential for aquaculture applications.

Keywords: microbiota; lysin; endolysin; vibrio; lytic phage; rotifer; live feed; targeted antimicrobial tools

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1. Introduction

Microbial communities associated with early life stages of fish play a pivotal role in host development, nutrient assimilation, immune maturation, and protection against opportunistic pathogens [1–4]. In marine hatcheries, the microbiota of rearing water and live feed constitutes the primary source of microbial exposure for fish larvae and strongly influences the establishment of larval-associated microbial assemblages [5–7]. Perturbations of these microbial communities during early development can increase disease susceptibility and compromise larval performance, making microbiota management a central challenge in modern aquaculture.

Among live feeds, rotifers (*Brachionus* spp.) are widely used as the first exogenous food in marine larviculture due to their appropriate size, nutritional flexibility, and ease of large-scale production [8]. However, rotifers harbor dense and metabolically active microbial communities that often exceed those present in the surrounding rearing water [9,10]. These microbiota may include opportunistic and pathogenic bacteria, particularly members of the genus *Vibrio*, which can be efficiently transferred to fish larvae during feeding [11–13]. As a result, rotifers are increasingly recognized as critical microbial vectors in hatchery systems, capable of shaping larval microbiota assembly and influencing the onset of bacterial diseases during sensitive developmental windows.

Vibriosis remains one of the most prevalent and economically damaging diseases in marine aquaculture worldwide [14,15]. While antibiotics have historically been used to control bacterial outbreaks, their extensive application has raised concerns related to antimicrobial resistance, environmental persistence, and unintended disruption of microbial community structure [16–19]. These limitations have accelerated the search for alternative disease control strategies that combine antimicrobial efficacy with microbiota compatibility.

Bacteriophages are frequently considered microbiota-sparing due to their narrow host specificity; however, recent evidence indicates that phage-induced lysis of target bacteria may indirectly alter microbial community structure through nutrient release and ecological interactions [20–23]. Similarly, endolysins—phage-derived enzymes that enzymatically degrade bacterial peptidoglycan—have emerged as promising antibacterial agents, particularly against antibiotic-resistant bacteria [24,25]. Despite their increasing biotechnological relevance, the ecological effects of endolysins on complex microbial communities remain poorly characterized, especially in aquatic environments.

Although phage therapy has been successfully applied in aquaculture to reduce pathogen loads and improve host survival, most studies have focused primarily on disease outcomes rather than microbiota-level responses [26–28]. Only a limited number of investigations have evaluated how phage treatments influence water or host-associated microbiota, often reporting minimal or context-dependent effects [29–31]. In contrast, systematic assessments of endolysin impacts on aquaculture-relevant microbiota are largely absent.

In this study, we examined the effects of a lytic phage and a phage-derived endolysin on the microbiota associated with seawater, rotifers, and fish larvae under controlled, hatchery-relevant conditions. Importantly, the antimicrobial treatments evaluated here were applied under conditions that have been previously validated to achieve rapid and effective reduction in *Vibrio* spp. in live feeds and fish larvae without detectable toxicity, and under exposure times compatible with routine hatchery practices [32]. Rather than comparing these interventions with antibiotics—whose broad-spectrum activity and microbiota-disruptive effects in aquaculture systems are well documented [17]—this work focuses on evaluating phages and endolysins as targeted antimicrobial tools, with particular emphasis on their compatibility with non-target microbial communities. By addressing the critical temporal window during which microbial transfer from live feed to larvae occurs, and by integrating DNA- and RNA-based microbiota profiling to capture both community

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structure and transcriptional activity, this study addresses a key knowledge gap regarding the ecological footprint of targeted antimicrobial tools during early aquaculture stages. By integrating microbiota profiling with targeted antimicrobial exposure, this work addresses a critical knowledge gap regarding the ecological compatibility of phage-based interventions and contributes to the evidence base supporting their rational implementation in sustainable aquaculture.

2. Results

2.1. Effect of Phage and Lysin Application on Rotifer Microbiota

A total of 453 ASVs were retained after applying filters to the dataset of 54 samples. The rarefaction curves of all samples reached the plateau, indicating that the sequencing depth used was sufficient to detect the existing diversity (Supplementary Figure S1).

2.1.1. Alpha and Beta Diversity

Alpha diversity indices (Chao1, Shannon, and Simpson) of the rotifer and water microbiota, calculated from DNA- and RNA-derived datasets, showed no significant differences among control, lysin-, and phage-treated groups, as indicated by the shared superscript letters, confirming the absence of treatment-related effects on microbial diversity (Supplementary Table S1).

At the beta diversity level (Aitchison distance), differences were observed depending on whether DNA- or RNA-derived data were analyzed. In the principal coordinate analysis (PCoA) of rotifer samples (Figure 1A,B), DNA-based profiles did not cluster according to treatment groups, whereas RNA-based profiles showed a distinct separation of the lysin-treated (Lys) samples from the Control and Phage groups (Figure 1B). These differences were supported by PERMANOVA, which confirmed that the Lys group was significantly different from both the Control and Phage groups ($p < 0.05$; Table 1). In contrast, water samples exhibited an opposite pattern, with DNA-based analyses revealing significant differences among groups, while RNA-based analyses did not show significant separation (Table 1).

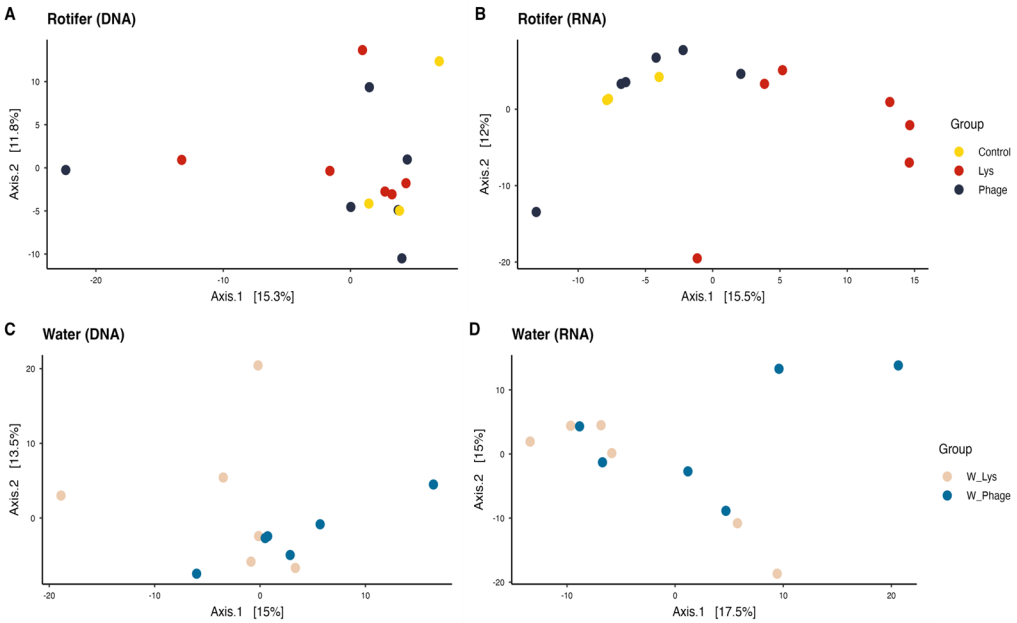


Figure 1. Principal coordinate analysis (PCoA) using clr-transformed data in Aitchison distance. Each point represents a sample corresponding to a group highlighted in different colors. (**A**,**B**) represent the samples of rotifer microbiota, according to DNA and RNA, respectively; (**C**,**D**) similar to the above, but in water samples.

Table 1. Summary of PERMANOVA and Betadisper results comparing microbial community structure (Aitchison distance) in rotifers and water treated with Phage and Lys.

Source	Group	Comparison	PERMANOVA R2	PERMANOVA *p* Value	Betadisper *p* Value
DNA	Rotifer	Lys vs. Phage	0.074	0.875	0.490
		Lys vs. Control	0.121	0.591	0.951
		Phage vs. Control	0.110	0.802	0.505
	Water	Lys vs. Phage	0.109	0.035	0.842
RNA	Rotifer	Lys vs. Phage	0.138	0.004	0.786
		Lys vs. Control	0.181	0.030	0.070
		Phage vs. Control	0.101	0.872	0.086
	Water	Lys vs. Phage	0.099	0.287	0.584

2.1.2. Microbiota Composition of Rotifer

At the phylum level, the rotifer and water microbiota was mainly composed of *Proteobacteria*, *Actinobacteriota*, *Firmicutes* and *Bacteroidota*, in descending order (Figure 2), in both DNA and RNA samples. It is noteworthy that none of these phyla showed significant differences between groups in both rotifers and water samples (Supplementary Table S12).

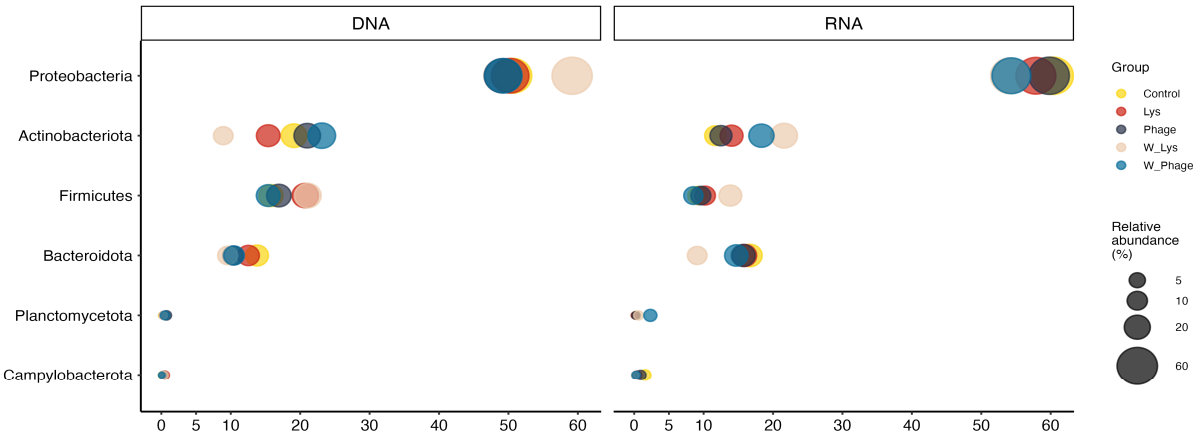


Figure 2. Average relative abundance of major bacterial phyla associated with rotifers and rearing water. Profiles are shown separately for DNA-derived datasets, representing the total bacterial community, and RNA-derived datasets, representing the metabolically active bacterial community. Only phyla with an average relative abundance higher than 1% in at least one experimental group were included.

At the genus level, 18 components were identified with average relative abundance greater than 2% in at least one of the groups (Figure 3). In the rotifer samples, a similar structure was observed in the most abundant components in both DNA and RNA samples, with *Glutamicibacter*, *Escherichia-Shigella*, *Mesoflavibacter* y *Marinomonas* being the most abundant genera, with average relative abundance of at least 4% per group. These results are in agreement with those obtained by ANCOM-BC2 (Supplementary Table S12), where no significant differences were found at the genus level among these groups. These results contrast with the composition of the microbiota in the water samples, where differences in the most abundant components (Figure 3). In both DNA and RNA samples, *Glutamicibacter* was predominantly found in the Phage group, with an average abundance of 20% and 15%, respectively, while *Escherichia-Shigella* was the most abundant in the Lys group, with an average abundance of 23% and 13%, respectively.

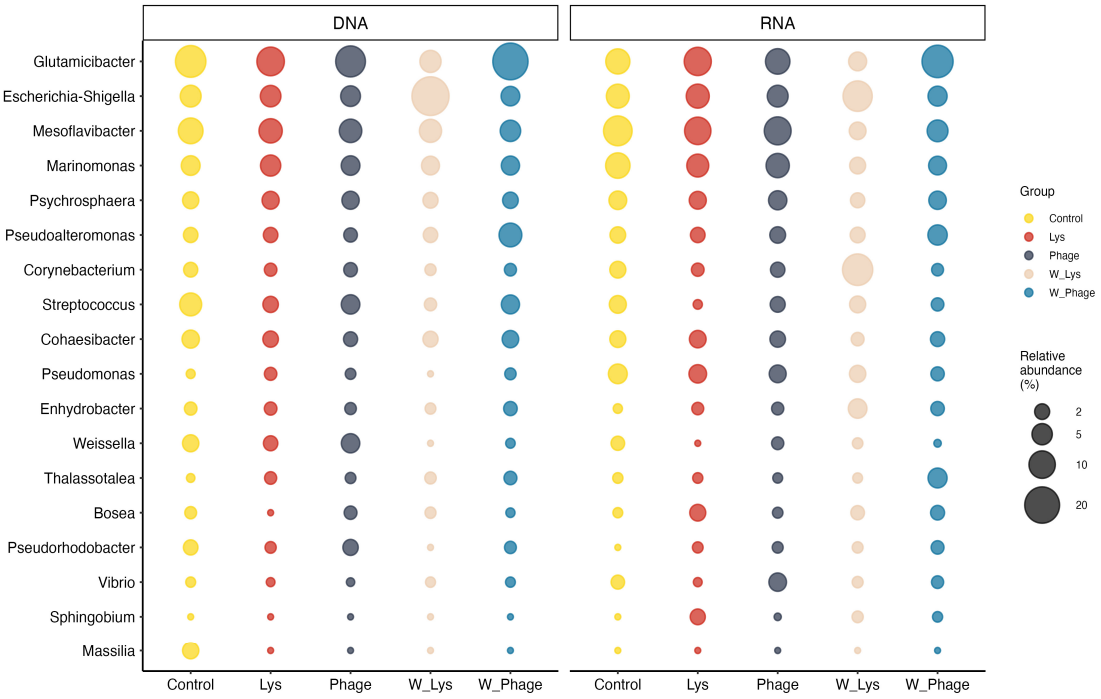


Figure 3. Average relative abundance of major bacterial genera associated with rotifers and rearing water. Profiles are shown separately for DNA-derived datasets, representing the total bacterial community, and RNA-derived datasets, representing the metabolically active bacterial community. Only genera with an average relative abundance higher than 2% in at least one experimental group were included.

Full information on the relative abundance of the components of the microbiota at the different taxonomic levels can be found in the supplementary material (Supplementary Tables S2–S11).

The differences in microbiota composition at the genus level in the water samples described above were statistically significant according to ANCOM-BC2 (Figure 4), adding in the case of DNA samples, two more genera, *Pseudoalteromonas* and *Streptococcus*.

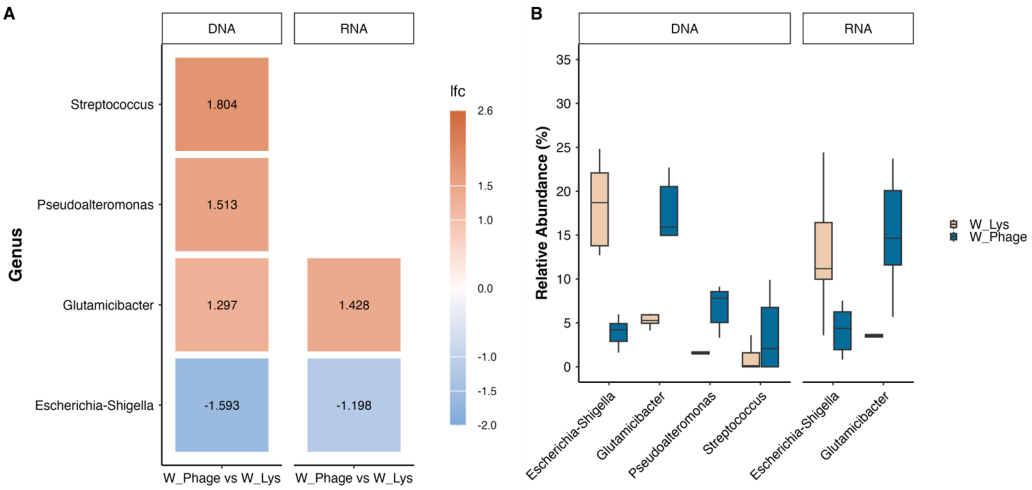


Figure 4. Statistically significant changes at the genus level in water microbiota after exposure to phage and Lys. (**A**) Log fold change (lfc) obtained from pair comparison using ANCOM-BC2; (**B**) relative abundance of genera that showed significant differences according to ANCOM-BC2.

2.2. Effect of Phage and Lysin Application on Zebrafish Microbiota

A total of 302 ASVs were retained after applying filters to the dataset of 18 samples (DNA only). The rarefaccion curves of all samples reached the plateau (Supplementary Figure S2).

2.2.1. Alpha and Beta Diversity

Alpha diversity indices (Chao1, Shannon, and Simpson) of the zebrafish microbiota showed no significant differences among control, phage-, lysin-, and *Vibrio alginolyticus* GV09–challenged groups, as indicated by the shared superscript letters, confirming the absence of treatment-related effects on microbial diversity (Supplementary Table S13).

In addition, visualization of the beta diversity of the zebrafish microbiota by PCoA showed no clustering of the samples according to the different treatment groups (Figure 5). These results were supported by PERMANOVA analysis (Table 2), where no significant differences between groups were reported (p value < 0.05). However, it should be noted that the Betadisper analysis revealed significant differences in some comparison such as, Control vs. GV09 (p value = 0.028), Control vs. GV09_Lys (p value = 0.026), Control vs. GV09_Phage (p value = 0.031) and GV09 vs. GV09_Lys (p value = 0.032), suggesting a difference in dispersion but not in composition, indicating that the PERMANOVA analysis might not be reliable. These results reflect a limitation of the design in which a low number of replicates per group was used.

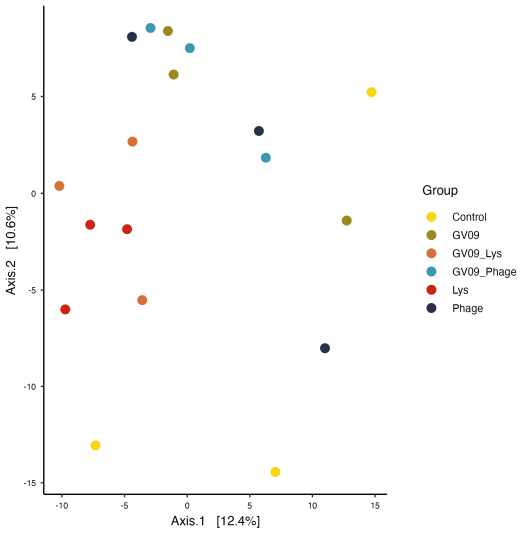


Figure 5. Principal coordinate analysis (PCoA) using clr-transformed data in Aitchison distance. Each point represents a sample corresponding to a group highlighted in different colors.

Table 2. Summary of PERMANOVA and Betadisper results comparing microbial community structure (Aitchison distance) in zebrafish challenged with Vibrio strain GV09 and treated with Phage and Lys.

Comparison	PERMANOVA		Betadisper
	R^2	p Value	p Value
All	0.323	0.058	0.066
Lys vs. Control	0.227	0.1	0.218
Phage vs. Control	0.197	0.6	0.468
GV09 vs. Control	0.220	0.3	0.028
GV09_Lys vs. Control	0.245	0.1	0.026
GV09_Phage vs. Control	0.234	0.1	0.031
Lys vs. GV09_Lys	0.208	0.4	0.474

Table 2. *Cont.*

Comparison	PERMANOVA		Betadisper
	R^2	*p* Value	*p* Value
Phage vs. Lys	0.240	0.1	0.496
Lys vs. GV09	0.263	0.1	0.812
Lys vs. GV09_Phage	0.249	0.1	0.717
GV09_Lys vs. Phage	0.241	0.1	0.064
GV09_Lys vs. GV09	0.223	0.2	0.032
GV09_Lys vs. GV09_Phage	0.212	0.4	0.238
Phage vs. GV09	0.183	0.6	0.174
Phage vs. GV09_Phage	0.196	0.6	0.120
GV09 vs. GV09_Phage	0.189	0.8	0.546

2.2.2. Composition of the Larvae Microbiota

The composition of the zebrafish microbiota was represented by the phylum *Proteobacteria* (ranging from 60 to 83% of average relative abundance), followed by the phyla *Firmicutes*, *Bacteroidota*, *Actinobacteriota* and *Planctomycetota* (Figure 6) regardless of control or treatment group. These phyla, and also those with less than 1% relative abundance, showed no significant differences between groups according to ANCOM-BC2.

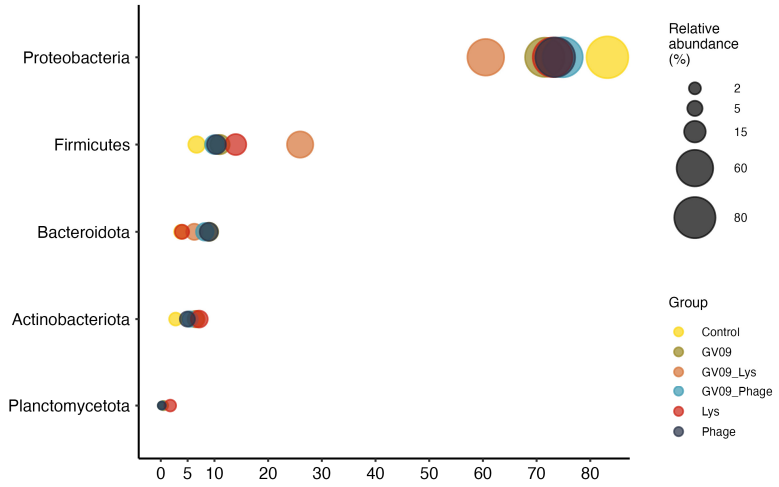


Figure 6. Average relative abundance of major phyla represented in the zebrafish microbiota. In this figure, phyla with an average relative abundance per group higher than 1% in at least one group were included.

Full information on the relative abundance of the components of the microbiota at the different taxonomic levels can be found in the supplementary material (Supplementary Tables S14–S18).

At the genus level, the composition of the microbiota presented different profiles according to the control or treatment groups (Figure 7). In the control group, the most abundant genera were *Pseudomonas*, *Aeromonas*, while the treatment groups containing the *Vibrio* strain GV09 presented *Vibrio* as the most abundant genus, which was lower in the group treated with Lys (GV09_Lys). In the latter, the most abundant genus was *Escherichia-Shigella*, similar to the Lys group. On the other hand, the group containing only the phage addition (Phage group) presented a profile more similar to the control group. However, no significant differences were detected in any genus according to ANCOM-BC2, which could be associated with the dispersion of the samples and the low number of replicates used per group. But differences were found between groups at higher taxonomic levels such as at the family level (Supplementary Table S19), where *Enterobacteriaceae* presented significant

differences between the Lys and Phage groups (lfc = −2.970, p value = 0.038), highlighting that *Escherichia* belongs to this family. Another family that presented differences between the groups was *Pseudomonadaceae*, which was found in the comparison between the Control group and GV09, GV09_Lys and Lys, the genus *Pseudomonas* belonging to this family be highlighted with higher abundance in the Control group.

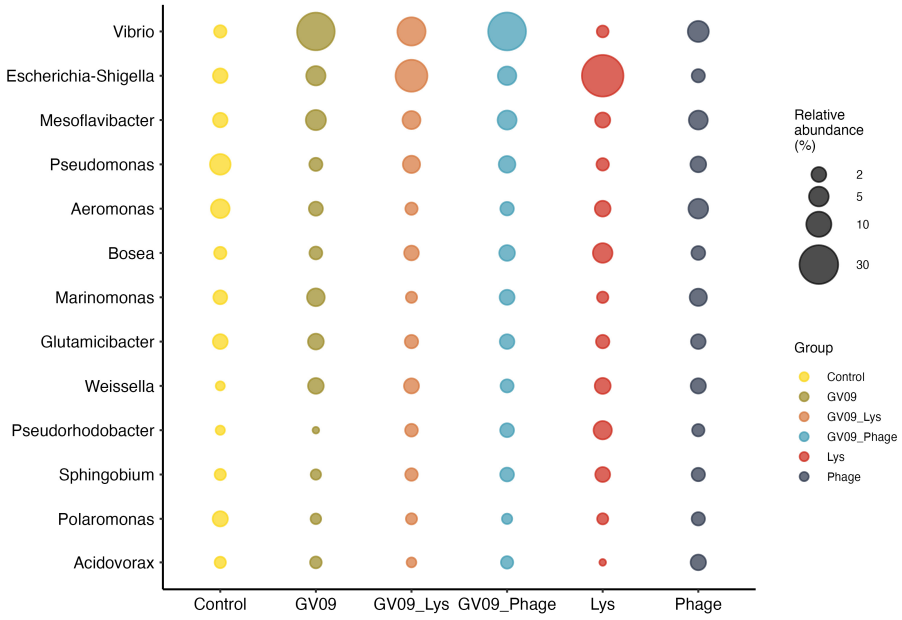


Figure 7. Average relative abundance of major genera represented in zebrafish microbiota. In this figure, genera with an average relative abundance per group higher than 2% in at least one group were included.

Overall, these results suggest that it might be appropriate to include ASVs classified as *Escherichia-Shigella* as a group to be excluded when evaluating the effect on the microbiota of any overexpressed protein in competent bacteria of the *E. coli* species.

3. Discussion

A central challenge in microbial management for aquaculture is achieving pathogen control without destabilizing the microbial communities that support host development, immune maturation, and colonization resistance. Antibiotics remain effective for acute outbreak control, but their broad-spectrum activity frequently produces collateral damage to commensal communities, promotes long-term shifts in community structure, and enriches antimicrobial resistance determinants [16,18]. In fish, antibiotic exposure has repeatedly been associated with reductions in intestinal microbiota diversity and compositional rearrangements, including depletion of putatively beneficial taxa and expansion of opportunistic groups [17]. These microbiota-level consequences are particularly concerning in hatchery settings, where early-life microbial assembly can shape later host performance and disease susceptibility [10,33]. Furthermore, Hurtado [34] highlight live feeds as a critical reservoir of antibiotic-resistant bacteria and resistance genes in larval fish culture systems, particularly under sustained antibiotic pressure. The high prevalence of florfenicol-resistant *Vibrio* spp. and associated resistance determinants underscores the urgent need for alternative, non-antibiotic strategies to control bacterial loads in live feeds and prevent the dissemination of antimicrobial resistance in aquaculture environments.

Phage-based interventions are often positioned as "microbiota-sparing" because of their narrow host range; however, evidence across model systems suggests a more nuanced picture. In murine models, phage administration can modulate both microbiota composi-

tion and metabolite profiles, in part through indirect ecological effects following targeted lysis [20]. Importantly, multiple studies have shown that phage therapy can reduce specific pathogens while preserving overall alpha diversity and avoiding large-scale perturbation of community structure, supporting the idea that phages can provide targeted control with limited collateral impact [21,31]. More recently, work in aquaculture-relevant systems indicates that phage treatment can prevent infection while minimally affecting water microbiota, reinforcing their suitability for aquatic environments where microbial stability is desirable [29]. Taken together, these results indicate that phage-based strategies can achieve pathogen control while maintaining overall microbiota stability, with community-level effects largely shaped by ecological context rather than indiscriminate disturbance. Recent work in marine host-associated microbial communities (sponge) highlights that phage-induced shifts are system specific and reflect structured ecological reorganization rather than community collapse [35]. This perspective aligns with the present findings and supports the positioning of phages—and phage-derived endolysins—as microbiota-compatible alternatives to conventional antibiotics.

Endolysins represent a parallel "precision antimicrobial" strategy, but their ecological footprint on complex microbiomes has been much less studied than that of phages. Foundational work describes endolysins as potent enzymatic antibacterials with potential for high specificity and reduced off-target effects, particularly when engineered or formulated to target defined bacterial groups [24,25]. Recent experimental evidence in aquaculture systems demonstrates that recombinant endolysins can rapidly and significantly reduce pathogenic *Vibrio* loads in both live feed organisms (rotifers) and fish larvae, achieving multi-log reductions within short exposure times without the use of antibiotics [35]. Notably, these effects were observed across several clinically relevant *Vibrio* species, supporting the applicability of endolysins as targeted, non-antibiotic tools for controlling *Vibrio*-associated disease risk during early life stages in marine hatchery settings. In line with this view, recent reviews emphasize that advances in bacteriophage and endolysin engineering have enabled highly targeted antimicrobial strategies that enhance specificity against foodborne pathogens while reducing unintended effects on non-target microbial communities, reinforcing their potential as precision biocontrol tools [36]. Recent reviews also explicitly frame next-generation endolysins as precision antimicrobials and highlight the critical gap in empirical studies assessing their microbiome compatibility [36,37]. Where evaluated, endolysins (phage lysins) are widely framed as microbiome-friendly antimicrobials due to their comparatively high specificity, which can—at least in principle—enable targeted depletion of defined bacterial groups while limiting off-target effects [38]. At the same time, this microbiota-compatibility should not be assumed a priori: recent syntheses stress that specificity does not automatically translate into microbiome-friendliness, and therefore lysins should be assessed case-by-case using complementary in vitro, ex vivo, and in vivo microbiome models [38,39]. In this context, the present study adds aquaculture-relevant evidence that lysin exposure is not associated with broad losses of alpha diversity and that any detectable community-level effects are subtle and matrix-dependent, consistent with a precision-antimicrobial paradigm that prioritizes pathogen control while preserving community-level stability [38,39].

Because ASVs classified as *Escherichia–Shigella* represented a substantial fraction of the microbiota in some rotifer and zebrafish samples, sensitivity analyses were performed to evaluate their influence on beta-diversity patterns (Figures S3 and S4). Reanalysis after excluding these ASVs yielded PERMANOVA and Betadisper results that were highly consistent with those obtained from the full datasets (Tables S20 and S21). In rotifers, DNA-derived profiles remained non-significant across treatments both before and after ASV removal, while RNA-derived profiles preserved the significant separation between lysin-

treated and control groups, with comparisons involving the phage treatment remaining non-significant. In zebrafish larvae, exclusion of *Escherichia–Shigella* ASVs did not generate new treatment-associated clustering, and PERMANOVA results remained non-significant, indicating that observed dispersion effects were not driven by this taxon. Collectively, these sensitivity analyses demonstrate that the treatment-associated patterns reported here—particularly those affecting the metabolically active fraction of the rotifer microbiota—are robust and not driven by the dominance of *Escherichia–Shigella*, supporting the validity of the conclusions regarding microbiota stability under phage and endolysin exposure.

A key interpretive dimension raised by recent studies is that "no change in alpha diversity" does not necessarily imply "no ecological effect". Several phage studies report preserved richness and evenness alongside shifts in beta diversity, reflecting altered relative abundances, niche release, and resource redistribution after lysis [20,40,41]. This framework is particularly useful for interpreting scenarios where compositional separation is detectable without diversity collapse. Moreover, emerging evidence suggests that analytical choice (e.g., profiling total DNA-based communities versus RNA-enriched active fractions) can influence sensitivity to treatment effects. Studies emphasizing functional or activity-linked readouts indicate that active-community shifts can occur without parallel changes in total-community structure, which may explain why RNA-based profiles sometimes reveal treatment-associated separation not evident in DNA-based analyses [42,43]. Consistent with this view, RNA-based analyses have been shown to reveal shifts in the active fraction of microbial communities that remain undetected in DNA-based profiles, despite apparent structural stability [44]. Similar observations in fish-associated microbiota indicate that activity-based approaches can uncover biologically relevant community responses to perturbation without concurrent changes in overall diversity [45]. This concept is especially relevant for live feed and rearing water, where microbial turnover is rapid and activity states can change quickly in response to perturbations. In the present study, this distinction is exemplified by rotifer-associated microbiota, where RNA-based analyses revealed treatment-related clustering not observed in DNA-derived datasets, suggesting modulation of metabolically active fractions rather than restructuring of the total community. In contrast, zebrafish larvae were assessed only at the DNA level, and the absence of detectable beta diversity separation is consistent with either true community stability or limited sensitivity to subtle, activity-level shifts under the experimental conditions used. From a methodological perspective, these results underscore the value of combining DNA- and RNA-based microbiome profiling when assessing targeted antimicrobial interventions. While DNA-based analyses capture overall community structure, RNA-based approaches are more sensitive to shifts in metabolically active fractions, particularly in rapidly turning-over systems such as live feeds and rearing water, thereby improving the detection and interpretation of subtle treatment effects.

An additional point relevant to the interpretation of the zebrafish larval results concerns the distinction between compositional change and dispersion. Although PERMANOVA analyses did not detect significant treatment-associated shifts in overall community composition, Betadisper analyses revealed differences in within-group variability for some comparisons. Importantly, such dispersion effects reflect increased heterogeneity among individual samples rather than coordinated restructuring or destabilization of the microbiome. In early life stages, larval-associated microbial communities are known to exhibit pronounced stochasticity and host-associated variability, driven by factors such as developmental stage, immune maturation, and early colonization dynamics [33,34]. Under these conditions, increased dispersion can occur even when higher-level compositional structure remains conserved. In the present study, the absence of consistent treatment-driven separation in PCoA space, together with stable alpha diversity and non-significant

PERMANOVA results, supports the interpretation that phage and endolysin exposure did not compromise microbiome stability, but instead coincided with natural inter-individual variability characteristic of early larval stages.

Across all matrices, microbial communities were dominated by Proteobacteria, Firmicutes, Bacteroidota, and Actinobacteriota, a taxonomic structure commonly reported in aquatic rearing systems, live feeds, and early fish-associated microbiota [46–48]. Within this conserved phylum-level framework, treatment-associated differences were restricted to a small number of genera and were primarily detected in the rearing water, while rotifer- and larva-associated microbiota remained comparatively stable [49]. Notably, *Glutamicibacter* displayed a higher relative abundance under phage treatment in water samples. Members of this genus are widely distributed environmental bacteria with documented roles in organic matter turnover, nutrient cycling, and stress tolerance in aquatic and terrestrial systems, and are generally regarded as low-virulence or opportunistic taxa rather than primary pathogens [50]. Genomic and ecological studies indicate that *Glutamicibacter* spp. exhibit high metabolic versatility and environmental resilience, without consistent association with virulence in aquatic hosts, supporting their interpretation here as indicators of ecological redistribution rather than dysbiosis or pathogenic selection. In parallel, *Pseudoalteromonas*, a genus frequently associated with marine environments, biofilm formation, and the production of antimicrobial and bioactive compounds, tended to increase under phage treatment, whereas *Streptococcus* showed more variable responses and remained at low relative abundance following lysin exposure. The ecological roles of *Pseudoalteromonas* include competitive interactions with other bacteria and modulation of microbial assemblages in aquatic and aquaculture systems, which may explain its selective enrichment under bacteriophage-driven pressure [51]. Importantly, these genus-level shifts were confined to taxa of low to moderate abundance and did not translate into significant changes in alpha diversity or overall community composition, indicating that phage or lysin treatments induced selective redistribution within an otherwise stable microbial community rather than broad restructuring. Together, these results suggest that the observed genus-level changes reflect context-dependent ecological responses of environmentally relevant bacteria, rather than biologically disruptive effects on the microbial communities supporting live feeds and early larval stages.

A relevant consideration when interpreting these results is that the experiments were conducted under controlled laboratory conditions, whereas commercial hatchery operations involve greater environmental and hydrodynamic complexity. In large-scale live feed production systems, factors such as oxygen distribution, temperature gradients, nutrient availability, and mixing efficiency introduce spatial heterogeneity that may influence antimicrobial–bacteria interactions by modulating physical contact and access of phages and endolysins to *Vibrio* cells. The present study was intentionally designed to isolate the direct microbiota-level effects of phage and endolysin exposure at the critical moment of treatment, independently of large-scale hydrodynamic variability. Consequently, while the observed microbiota responses support the compatibility of these targeted antimicrobial tools, validation under commercial-scale hatchery conditions—where bulk volume and mixing heterogeneity coexist—will be required to fully confirm their robustness and applicability.

4. Materials and Methods

4.1. Bacterial Strain, Phage, and Endolysin

The bacterial strain *Vibrio alginolyticus* GV09, originally isolated from marine aquaculture environments, was used as the target pathogen and has been previously characterized for susceptibility to both lytic phages and phage-derived endolysins [32]. The lytic bacteriophage CH20, specific to *V. alginolyticus*, was propagated using the double-layer agar

method, and high-titer stocks were quantified by plaque assay and stored at 4 °C until use. The recombinant endolysin LysVp1, derived from a *Vibrio*-infecting bacteriophage, was produced by heterologous expression in *Escherichia coli* and purified by affinity chromatography as previously described [32].

4.2. Experimental Design

The experimental design was conducted under controlled laboratory conditions and included three biological matrices: rotifer rearing water, rotifers (*Brachionus plicatilis*), and zebrafish larvae (*Danio rerio*), following procedures previously validated and described by Romero et al. [32] (Figure 8). Importantly, the targeted antimicrobial treatments evaluated in this study were applied under conditions that had been empirically optimized in prior work to achieve rapid, reproducible, and multi-log reductions in *Vibrio* spp. in live feeds and fish larvae, without detectable toxicity to rotifers or larvae, and using exposure times compatible with routine hatchery practices [32]. Each biological matrix was independently subjected to three experimental conditions: untreated control, phage treatment (CH20), and endolysin treatment (LysVp1). The present study does not aim to reassess antimicrobial efficacy or dose–response relationships, as these aspects have already been established, but rather to evaluate microbiota compatibility under previously validated treatment conditions. All assays were performed using independent biological replicates. For rotifer assays, cultures were concentrated by filtration through a 30 µm nylon mesh and resuspended in 1 µm-filtered seawater. Rotifer suspensions were inoculated with *Vibrio alginolyticus* GV09 to a final concentration of 5×10^6 CFU mL^{-1} and incubated for 15 min at 22 °C to allow bacterial association. Subsequently, aliquots of 9 mL, containing approximately 350 rotifers per experimental unit, were distributed into sterile plates. Treatments were applied by adding 1 mL of the corresponding solution: sterile seawater for controls, phage CH20, or endolysin LysVp1, using the same concentrations and short-contact exposure times previously validated for effective *Vibrio* control [35]. Following treatment, samples were incubated for 20 min at 22 °C. Rotifers were then recovered by filtration, washed, and processed for downstream microbiota analyses. For zebrafish larval assays, larvae were maintained in E3 medium and distributed into sterile plates at a density of six larvae per 9 mL well. Larval cultures were challenged with *V. alginolyticus* GV09 at 5×10^6 CFU mL^{-1} and incubated for 15 min at 22 °C prior to antimicrobial treatment. Control groups received 1 mL of E3 medium, while treated groups received phage CH20 or LysVp1 under the same previously validated application conditions [32]. Incubation was continued for 20 min, after which larvae and surrounding water were collected for microbiota characterization.

The selected exposure times, antimicrobial concentrations, and organism densities were designed to reflect short-contact bioassays compatible with hatchery practices, as previously validated for phage and endolysin activity in live feed and fish larvae systems. Sample sizes were constrained by larval availability and ethical considerations, and these constraints were accounted for during statistical analysis.

4.3. Nucleic Acid Extraction

For simultaneous DNA and RNA extraction, rotifer and water samples were processed following the protocol described by [44] using a single co-extraction workflow. Rotifer samples were recovered by filtration, thoroughly washed with phosphate-buffered saline to remove loosely associated microorganisms, and transferred to extraction tubes. Water samples were processed by filtration through 0.45 µm membrane filters, and the filters were directly used as input material for nucleic acid extraction. Both rotifer biomass and water filters were subjected to enzymatic pretreatment with lysozyme and proteinase K, followed by mechanical disruption by bead beating [44]. Total DNA and RNA were simultane-

ously extracted using the AllPrep PowerFecal DNA/RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Nucleic acid concentrations were quantified fluorometrically, and RNA extracts were reverse-transcribed using random primers to generate cDNA. DNA- and cDNA-derived templates were subsequently processed independently for downstream sequencing analyses, as previously described [44].

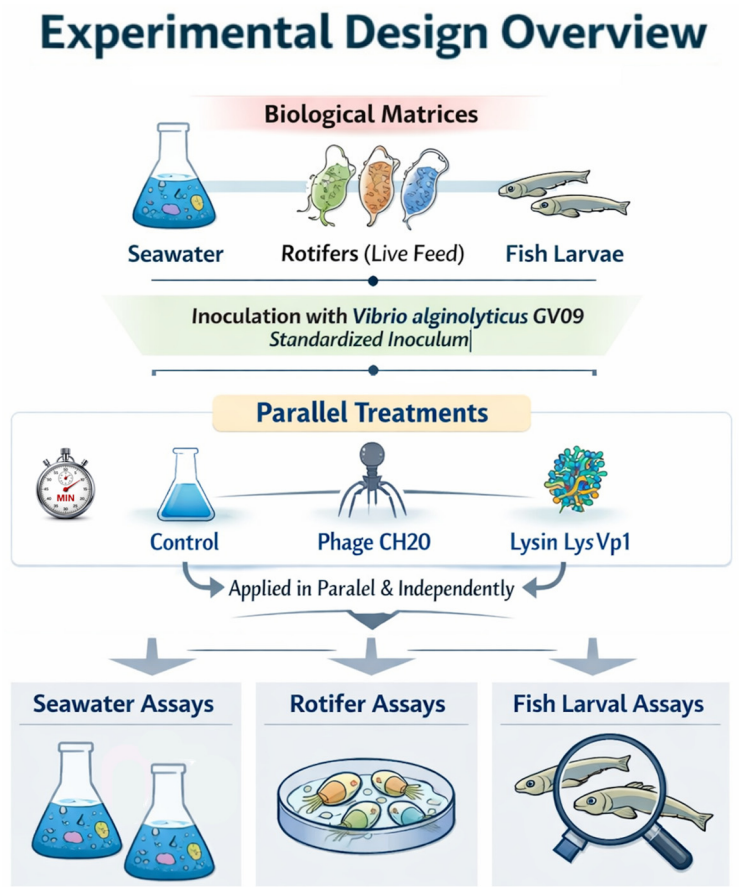


Figure 8. Overview of the experimental design used to evaluate the effects of targeted antimicrobial tools on microbiota associated with seawater, rotifers (live feed), and fish larvae.

Genomic DNA from zebrafish larvae was extracted following a washing step analogous to the procedure applied to rotifer samples. Briefly, pooled larvae were rinsed 2 times with sterile phosphate-buffered saline and transferred to 2 mL bead-beating tubes. Samples were subjected to enzymatic pretreatment with lysozyme and proteinase K as above. DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with minor modifications to enhance inhibitor removal.

DNA concentration and purity were assessed using a NanoDrop spectrophotometer and a Qubit dsDNA High Sensitivity assay (Thermo Fisher Scientific, Waltham, MA, USA), and only high-quality extracts were used for downstream analyses. For bacterial community profiling, the V4 region of the 16S rRNA gene was amplified using the universal primers 515F and 806R. PCR reactions were performed in a final volume of 30 µL containing 1.5 U of GoTaq® G2 Flexi DNA Polymerase (Promega, Madison, WI, USA), 6 µL of 5× reaction buffer, 1.5 mM $MgCl_2$, 0.25 µM of each primer, 0.5 mM of each dNTP, and 20 ng of template DNA. Negative controls without DNA template were included in all PCR runs. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced using paired-end chemistry (2 × 300 bp) on an Illumina HiSeq PE250 platform at CD Genomics [52].

4.4. Metagenomic Analysis

Raw paired-end 16S rRNA gene sequences were processed in R (v4.4.0) using the DADA2 pipeline, following the workflow described by [52]. Primer sequences were removed, and reads were quality filtered and truncated based on quality score profiles. Error rates were learned from the data, and amplicon sequence variants (ASVs) were inferred through denoising, merging of paired-end reads, and removal of chimeric sequences. Taxonomic assignment was performed using a naïve Bayesian classifier against the SILVA v138.1 reference database [53]. ASV count tables, taxonomic assignments, and sample metadata were integrated into phyloseq objects for downstream analyses. Alpha diversity indices (Chao1, Shannon, and Simpson) were calculated from untransformed ASV count data. For beta diversity analyses, ASV tables were subjected to a centered log-ratio (clr) transformation after zero handling, and Aitchison distances were calculated as Euclidean distances on clr-transformed data [54–56]. Community dissimilarities were visualized using principal coordinate analysis (PCoA). Differences in bacterial community composition among experimental groups were tested using PERMANOVA with 999 permutations [57]. Homogeneity of multivariate dispersions was assessed using betadisper followed by permutation testing [57]. Differential abundance analyses were conducted using ANCOM-BC2, which corrects for compositional and sampling biases [58]. All analyses were performed in R using the packages phyloseq, vegan, microbiome, and ANCOMBC.

5. Conclusions

This study demonstrates that exposure to a lytic phage (CH20) or a phage-derived endolysin (LysVp1) does not induce broad disruptions of microbial diversity in seawater, rotifers, or zebrafish larvae, supporting their microbiota-compatible profile in aquaculture settings. Across all matrices, alpha diversity remained stable, while detected compositional changes were limited, context-dependent, and largely restricted to low-abundance taxa, with clearer treatment signals emerging only when RNA-based analyses were applied. Together, these findings indicate that phages and endolysins can achieve targeted antimicrobial effects without compromising overall microbial community structure, reinforcing their potential as precision, non-antibiotic tools for sustainable disease management in early-life aquaculture systems.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/antibiotics15020204/s1. Doc file: Figure S1: Rarefaction curves of rotifer and water microbiota; Figure S2: Rarefaction curves of zebrafish microbiota; Figure S3: Principal coordinate analysis (PCoA) using clr-transformed data in Aitchison distance excluding ASVs classified as *Escherichia–Shigella*; Figure S4: Principal coordinate analysis (PCoA) using clr-transformed data in Aitchison distance excluding ASVs classified as *Escherichia–Shigella*. Table S1: alpha diversity indices of rotifer and water microbiota; Table S13: Alpha diversity indices of the zebrafish microbiota; Table S20: Summary of PERMANOVA and Betadisper results comparing microbial community structure excluding ASVs classified as *Escherichia–Shigella* (Aitchison distance) in rotifers and water treated with Phage and Lys; Table S21: Summary of PERMANOVA and Betadisper results comparing microbial community structure excluding ASVs classified as *Escherichia–Shigella* (Aitchison distance) in zebrafish challenged with Vibrio strain GV09 and treated with Phage and Lys. Excel file: Table S2: Relative abundance (%) at phylum level from DNA samples of microbiota; Table S3: Relative abundance (%) at phylum level from RNA samples of microbiota; Table S4: Relative abundance (%) at class level from DNA samples of microbiota; Table S5: Relative abundance (%) at class level from RNA samples of microbiota; Table S6: Relative abundance (%) at order level from DNA samples of microbiota; Table S7: Relative abundance (%) at order level from RNA samples of microbiota; Table S8: Relative abundance (%) at family level from DNA samples of microbiota; Table S9: Relative abundance (%) at family level from RNA samples of microbiota;

Table S10: Relative abundance (%) at genus level from RNA samples of microbiota; Table S11: Relative abundance (%) at genus level from DNA samples of microbiota; Table S12: Summary of results of pairwise comparison using ANCOM-BC2 in microbiota samples from rotifers and water exposed to Phage and Lys; Table S14: Relative abundance (%) at phylum level of zebrafish microbiota; Table S15: Relative abundance (%) at class level of zebrafish microbiota; Table S16: Relative abundance (%) at order level of zebrafish microbiota; Table S17: Relative abundance (%) at family level of zebrafish microbiota; Table S18: Relative abundance (%) at genus level of zebrafish microbiota; Table S19: Summary of results of pairwise comparison using ANCOM-BC2 in zebrafish microbiota simples.

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