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Research Article

Aquaculture Phage AS-A: Effects on Zebrafish Conventionalised with Human Microbiota

Nerea Arias-Jayo[1,5]*, Laura Alonso-Sáez[2], Miguel A Pardo[1], Amaia Lasagabaster[1], Andoni Ramirez-Garcia[3] and Igor Hernández[1,4]

[1]Food Research, Azti, Parque Tecnológico de Bizkaia, Spain

[2]Marine Research, Azti, Txatxarramendi Ugartea Z/G, Spain

[3]Fungal and Bacterial Biomics Research Group, Department of Immunology, Microbiology and Parasitology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Spain

[4]Food Technology Department, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Spain

[5]UCLA School of Medicine, Department of Digestive Diseases, Los Angeles, Charles E. Young, Los Angeles, California, United States

***Corresponding Author:** Nerea Arias-Jayo, UCLA School of Medicine, Department of Digestive Diseases, Los Angeles, Charles E. Young, Los Angeles, California, United States.

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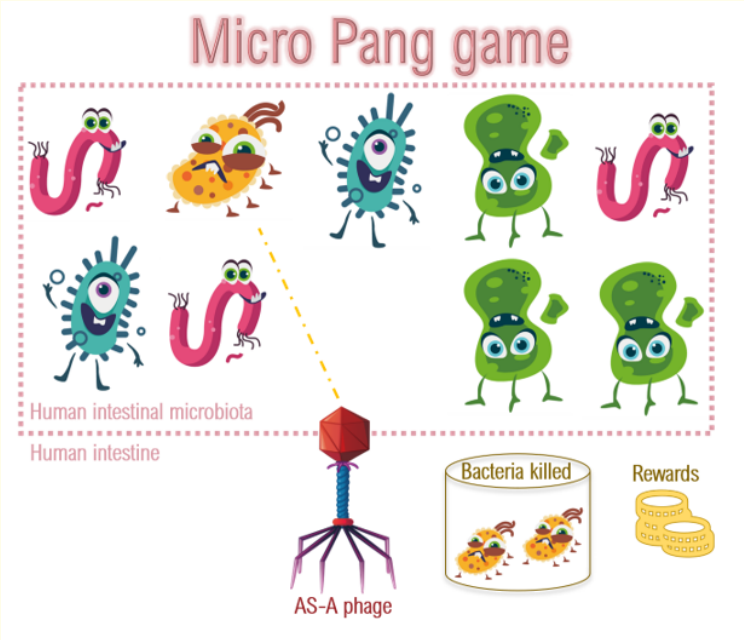
Graphical Abstract



Abstract

Phage therapy has been proposed as a new strategy in aquaculture against pathogen bacteria, especially against antimicrobial resistant strains. It is target specific, and promises to be fast, flexible, and inexpensive. However, it requires a deep characterisation of experimental phages before use. For that reason, many researches have focused on the effect of different phages on aquaculture fish. In contrast, to date, the effect of phage in the human bacteriome has not been studied. Zebrafish (Danio rerio) shares homologies with human in terms of organs and cell types. For that reason, the use of this animal model has increased in recent years. The aim of this report was to study the effect of the AS-A phage, highly active against fish pathogens, on the immunity and intestinal microbiota of a human-conventionalised zebrafish model. The expression level of several immune system associated genes was evaluated by real-time qPCR to achieve this aim. In addition, the microbial population was characterised by 16S sequencing after an over-therapeutic phage exposure for 24 hours. Results showed that AS-A phage did not stimulate zebrafish immune response. However, the bacterial population structure was affected. This research provides evidences of the impact of AS-A from fish to human wellbeing.

Keywords: Phage Safety; Conventionalised Zebrafish; Aquaculture; Animal Model

Introduction

Phages are estimated to be the most abundant and the most diverse entities in the biosphere, with a large impact on bacterial biomass in all environments [1]. As they are highly specific to a single species or even a strain of bacteria, they usually do not infect other bacteria [2].

In the last years, the use of phages has gained popularity on different research field, due to the increasing problem of multidrug-resistant bacteria. They are currently being used in pharmaceutical initiatives and agricultural uses, to eliminate contamination from food and water supplies [3]. Another interesting application for phage therapy is in aquaculture, involving phages active against many fish pathogens, like *Vibrio*, *Flavobacterium, Pseudomonas, Aeromonas* or *Edwardsiella* [4]. For example, protective effects of phages against fish diseases have been tested in Yellowtail (*Seliora quinqueradiata*) infected by *Lactococcus garviea*, Ayu (*Plecoglossus altivelis*) infected by *Pseudomonas plecoglossicida* or in Brook trout (*Salvelinus fontinalis*) infected with *Aeromonas salmonicida* [5,6].

However, there still are many unconclude premises that might be elucidated to estimate the value for the phage therapy in the future. The most important one, is the safety issue [7]. Phages are self-replicating as well as self-limiting. Thus, they replicate exponentially, as bacteria do, and decline when bacterial numbers decrease; representing a limited environmental impact. It is assumed that phages are selective and they will have no effect on non-target microorganisms. However, if a phage is able to switch to consume other bacteria is yet unknown. The potential effects of massive phage utilisation on the environmental bacterial communities in the surrounding ecosystems is of critical importance to safety evaluation, as a result, in aquaculture plants. Additionally, as the human will be the consumer of these aquaculture species, discarding the deleterious effects of aquaculture phages on human gut microbiota would be critical in order to convince producers to use this technology to help legislative and regulation agencies to allow bacteriophage utilisation as zoosanitary agents [8].

In this work, we have used a human microbiota-conventionalized zebrafish model (Danio rerio) to test the selectivity and innocuousness of the phage AS-A for the non-target bacterial community. Zebrafish has been developed with an increasing recognition as a model organism for biomedical and toxicity research in recent years. It represents a rapid and cost-effective model. Here all the experiment was performed in 7 days; and even though axenic animals were used non-specific facilities were needed. Furthermore, zebrafish is physiologically and genetically similar to mammals, and its genome is completely sequenced and available [9,10]. AS-A phage was isolated from sewage water in Aveiro (Portugal) by Pereira., *et al.* [11]. It was described that *Aeromonas salmonicida* is the specific target of this phage. However, it also inactivates other fish pathogenic bacteria, such as *Vibrio angillarum* and *Vibrio parahemoliticum* [11].

In a previous research, the efficacy and safety of AS-A phage in *Senegalese sole* (*Solea senegalensis*) and the surrounding natural bacterial community of aquaculture water were evidenced [12]. Here, the effect of AS-A phage on zebrafish immunity and human microbiota, transplanted in the zebrafish model [13], was analysed for the first time.

Materials and Methods

Zebrafish husbandry

Zebrafish (D. rerio, Hamilton 1822) embryos were obtained from wild-type adult zebrafish bred in the AZTI Zebrafish Facility (REGA number ES489010006105; Derio, Spain). Adult zebrafish were reared on a 12 h light/12 h dark cycle, fed with a pellet-formulated diet (Gemma Micro 300; Skretting) and maintained at 27°C in 60 L tanks, with aerated freshwater; according to standard protocols [14]. All experimental procedures were approved by the regional animal welfare body (NEIKER-OEBA-2015-004).

Procedure for obtaining germ-free (GF) larvae

Zebrafish embryos were collected directly from the breeding tanks immediately after fertilisation and germ-free larvae were obtained following a well-established protocol [15]. Briefly, embryos were washed with a sterilised embryo wash buffer (EWB) solution (embryo water (EW): $CaCl_2$ 294 mg/ml, $MgSO_4$ $7H_2O$ at 123.3 mg/ml, $NaHCO_3$ at 63 mg/ml, KCl at 5.5 mg/ml and supplemented with methylene blue 0.01% (w/v)), antibiotic solution (AB) (kanamycin 15 μg/ml, ampicillin 300 μg/ml and amphotericin B 1.25 μg/ml), 0.02% (w/v) polyvinylpyrrolidone (PVP) solution for 2 min, 0.003% (v/v) bleach solution for 1 h and finally with EWB solution. Afterwards, the embryos were incubated overnight in AB solution. The following day, 50 embryos were collected and transferred to a Petri plate (5.5 cm diameter×1.0 cm) to be immersed in 5 ml EWB solution and treated with two UV light pulses of 1.6 kV (Pulsed UV System XeMatica 1:2L-SA, Steri Beam Systems, GmbH) to inactivate any bacteria present in the sample. The entire procedure was carried out inside a laminar flow cabinet to maintain sterile conditions. Sterile solutions and materials were also used.

Sterility was routinely tested after 96 hpf (hours post fertilization), by culturing on general aerobic and yeast and mould culture media (Petrifilm aerobic count plates and Petrifilm yeast and mould count plates, 3M) and by PCR amplification using primers targeting the 16S ribosomal RNA gene (63f: CAGGCCTAACACATGCAAGTC and 1387r: GGGCGGWGTGTACAAGGC) .

Phage inoculum preparation

We used phage AS-A, isolated from sewage water from the sewage network of Aveiro, Portugal [11]. Phage stocks were stored in 1% chloroform at 4°C. Fresh phage suspension with a titre of approximately 108 plaque forming units per millilitre PFU/ml was prepared in tryptone soy agar (TSB; Oxoid) with $CaCl_2$ (10 mM) and $MgSO_4$ (5mM) using *A. salmonicida* (CECT894) as described before [12]. The phage suspension titre was determined in duplicates by a two-layer method using Tryptic Soy Agar medium (TSA; Oxoid) as base agar and soft TSA (0.4% agar) as soft agar using CECT894 as host after cultivation at 72ºC overnight. Results were expressed as PFU/mL.

Phage effect on zebrafish larvae immunity by qPCR

A transcriptomic analysis was carried out to further characterise the immune response of axenic embryos to phage challenge test at 24 and 48 h. Six pools of twenty 5 dpf zebrafish embryos (n = 120) were exposed to 106 UFP/ml as previously described [17]. Six pools of the same axenic individuals were used as control group (n = 120). After 24 and 48 hour of exposure 3 pools of each condition were euthanised and homogenised in 500 µL of dd H_2O, at each time point. Total RNA was isolated with TRIzol® following manufacturer instructions (Invitrogen Life Technology, Merelbeke, Belgium). The RNA concentration and integrity were assessed with an Agilent 2100 Bioanalyzer (Agilent Technology, Santa Clara, Ca). RNA samples with a RIN (RNA Integrity Number) lower than 8.5 were discarded. The reverse transcription was carried out with TaqMan® Reverse Transcription following the instructions of the manufacturer. cDNAs were synthesised from the RNA samples in a reverse transcription reaction (RT) containing 20 ng of RNA per assay. RT was performed in a mix containing 1x TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 µM dNTPs, 2.5 µM oligo-dT, RNase inhibitor (0.4 U/µL), and 1.25 U/µL MultiScribe reverse transcriptase (Applied Biosystems). The mixture was incubated at 25°C for 10 min and at 48°C for 30 min, and the enzyme was inactivated at 95°C for 10 min. Quantitative PCR was performed with SYBR Green PCR master mix (Roche Diagnostic Rotkreuz, Switzerland.) on a Roche Light Cycler 480. Reaction conditions were as follows: 50ºC for 2 min and 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 1min. Beta actin and elongation factor 1 were used as reference genes. Each sample was tested in triplicate. The mean Ct of each of each gene, normalised against the reference gene and the corresponding control, was calculated by the 2-ΔΔCt method, using the REST 2009 software (Qiagen, Hilden Germany). The transcript levels of six genes related to innate immunity were monitored after exposure to the inflammation inductor agents to select the most appropriate markers. The evaluated genes are listed in table 1.

Abbr.	Gene	Description	NCBI ID	Sequence
ACT	Beta actin	Reference gene	NM_131031.1	TGCTGTTTTCCCCTCCATTG TTCTGTCCCATGCCAACCA
EF1	Elongation factor 1 alpha	Reference gene	ENSDART00000023156	CTGGAGGCCAGCTCAAACAT ATCAAGAAGAGTAGTACCGCTAG CATTAC
IL1β	Interleukin 1β	Pro-inflammatory cytokine	NM_212844.2	CATTTGCAGGCCGTCACA GGACATGCTGAAGCGCACTT
IL10	Interleukin 10	Anti-inflammatory cytokine	NM_001020785	ATATTTCAGGAACTCAAGCGGG ACTTCAAAGGGATTTTGGCAAG
IL22	Interleukin 22	Pro-inflammatory cytokine	NM_001020792.1	TGAGGGAGGGTCTGCACAG CACAAGCGGATGGCTGG
MYD88	Myeloid differentiation primary response gene 88	Inflammation mediator	NM_212814.2	CACAGGAGAGAGAAGGAGTCACG ACTCTGACAGTAGCAGATGAAAGCAT
NFKB	Nuclear factor kappa beta	Immune response regulator	NM_001003414	AGAGAGCGCTTGCGTCCTT TTGCCTTTGGTTTTTCGGTAA
MPO	Myeloid-specific peroxidase	Immune response regulator	NM_212779	CAATGGCCCGCATAATCTG GCGAAAAGGATCTCTGGGAACT

Table 1: Primers for transcriptomic analysis.

Zebrafish axenic larvae conventionalization with human microbiota

Germ-free zebrafish larvae were colonised at 5 days-post-fertilization (dpf) with the human intestinal microbiota (HCONV), previously extracted from a human donor, by static immersion following an infection protocol previously published with some modifications [13]. Briefly, one aliquot of the human fecal slurry was thawed at 4ºC and inoculated into rPBS+ medium at a final density of 105 CFU/mL inside the anaerobic chamber. Then, outside in aerobic conditions, it was poured into a petri dish containing zebrafish larvae inside a laminar flow cabinet. After 24 h incubating at 27ºC shaking at 50 rpm (Heidolph unimax 1010), the whole medium was removed, and larvae were washed with clean medium twice. Immediately, zebrafish larvae were divided into 6 pools (3 replicates per condition) containing 20 larvae per pool. After that, AS-A phage culture was added to 3 of the larvae-pools at a final density of 106 UFP/ml (HCONV_P). Larvae were further incubated for 24 h with the fresh medium and the phage inoculum (HCONV_P) and in fresh medium (HCONV). Afterwards, pools were washed with fresh sterile rPBS+, passed into a new petri dish and maintained for 6 h in fresh rPBS+ medium. All larvae from the same pool were washed twice in a Tween 20 0.1% (Merck) baths and then washed twice in fresh sterile PBS (1X) to remove any bacteria attached on the skin [18]. Finally, larvae were collected and total gDNA was extracted. Each pool was considered as an independent sample, resulting in 3 samples for phage treated fishes (HCONV_P, N = 3) and 3 samples for untreated fishes (HCONV, n = 3). HCONV samples were considered as controls for non-phage samples. Axenic larvae conventionalization and infection is schematized in figure 1.

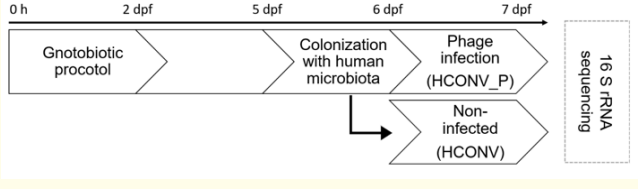


Figure 1: Schema of the conventionalization and infection processes of HCONV and HCONV_P samples for the microbial analysis, and normally raised larvae for gene expression analysis. Time is represented from 0 h, when eggs are fertilized and the gnotobiotic protocol starts, to 7 dpf where samples are taken for the posterior microbiota analysis by 16 S rRNA sequencing.

Analysis of microbial community composition by meta-barcoding

Total gDNA was extracted from 3 pools of 20 zebrafish larvae per condition, HCONV as a control and HCONV_P as conventionalised larvae exposed to phage (QIAamp DNA mini kit, Qiagen). DNA quantity and purity were quantified using a Nanodrop 1000 spectrophotometer (Thermo). The microbial community composition was characterized by sequencing the V3-V4 16S rRNA region using the primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 [19] in an Illumina Miseq Platform as previously described [13]. Briefly, Paired reads were trimmed and merged using Trimmomatic and flash [20]. The rest of the bioinformatics analysis was performed using the mothur platform [21] (1.37.2). Reads were aligned against a reference SILVA alignment. For operational taxonomic unit (OTU) construction, reads were clustered at the genetic distance cut-offs 0.01 and 0.03 substitution per nucleotide, using the average linkage method. Taxonomic assignment of the OTUs was obtained by classification with SILVA taxonomy (version 128) using the Wang approach [22]. Alpha diversity indexes were calculated in Mothur; Beta diversity was calculated with Bray-Curtis algorithm in Mothur. Other statistics and figures were performed in R (3.4.0 version).

Results and Discussion

AS-A phage effect on zebrafish larvae immunity

The expression of several cytokines associated with the inflammatory cascade (IL1B), cellular mediators involved in cellular signaling and triggering inflammation (NFKB, IL10, IL22 and MYD88) and antimicrobial peptides secreted by the host (MPO) were monitored by quantitative qPCR [15]. None of the genes included in this experiment significantly changed in phage-treated samples at 24 or 48 hours of treatments (Figure 2), compared with a control group (larvae without phage solution).

AS-A phage effect on zebrafish larvae microbiome

Considering samples treated and non-treated with the phage, 1962 OTUs were identified at the 0.03 distance cut-off level. The bacterial communities were dominated by members of Proteobacteria and Firmicutes, and also included Actinobacteria and Bacteroidetes. In both cases, the dominant families were *Enterobacteriaceae* (more than 90% of the identified sequences), *Bacillaceae* (up to 2.51%), *Lactobacillaceae* (1.13 - 1.64%), *Streptococcaceae* (1.24 - 1.55%) and *Enterococcaceae* (0.31 - 1.11%) (Figure 3). Aeromonas, the target genera described for this phage, was not detected in any sample as it was not previously identified in the human inoculum used to conventionalized the larvae [13] (Figure 3).

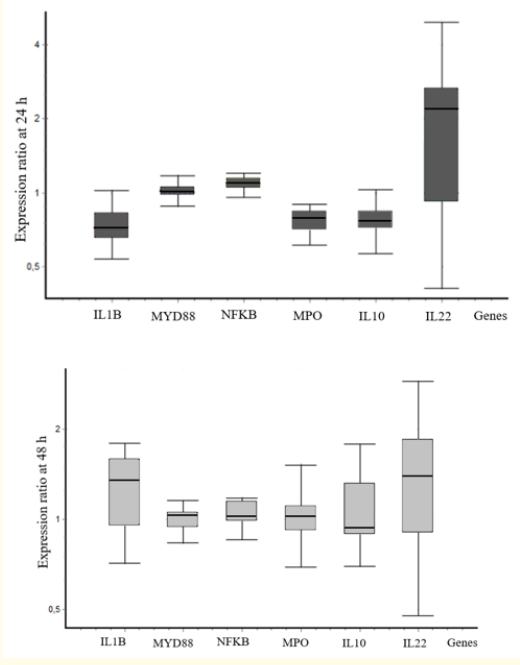


Figure 2: Gene expression fold change analysis after 24 and 48 hours of phage treatment exposure. Each box represents the mean of three replicates, and error bars indicate the standard error of the mean. The expression of the control group is 1 for all genes. Differences were considered statistically significant at $p < 0.05$ (*).

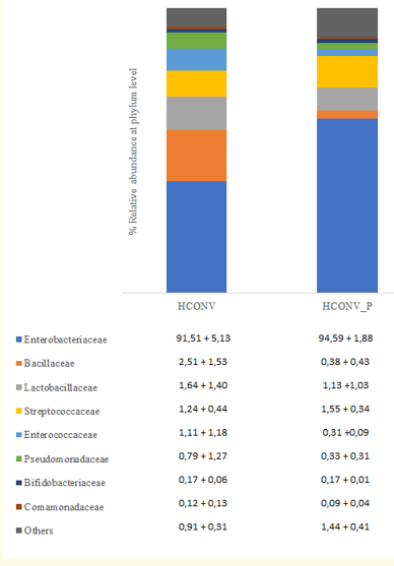


Figure 3: Relative abundances at family level in HCONV and HCONV_P samples represented in stacked bar charts. The relative abundances of each family and the standard deviation was also represented.

The community diversity significantly decreased with the phage treatment calculated by Shannon and Inverse Simpson indices (Figure 4). Non-metric multidimensional scaling (NMDS) and a phylogenetic tree based both on Bray-Curtis algorithm was used to visualise dissimilarities between groups. As shown in Figure 5, different sample clustering was observed between groups, suggesting that the phage treatment changed the community structure (p = 0.095, AMOVA test) (Figure 5).

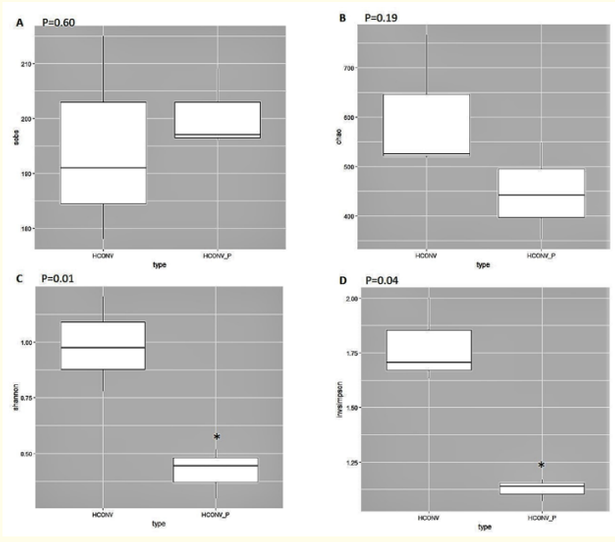


Figure 4: Alpha diversity. (A) Observed richness of bacterial OTUs, (B) Chao estimates of total OTU richness, (C) Shannon index, and (D) Inverse of Simpson diversity estimator are presented for HCONV and HCONV_P samples. *p < 0.05.

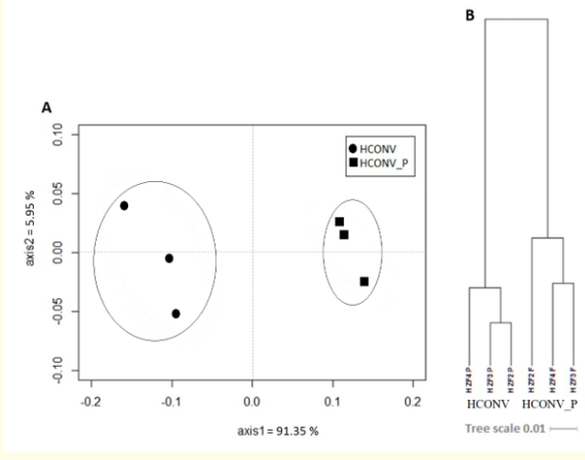


Figure 5: (A) Non-metric multidimensional scaling (NMDS) based on Bray Curtis algorithm for HCONV and HCONV_P samples (p = 0.095). (B) Bray Curtis dendrogram (p = 0.0329) HCONV clusters away from HCONV_P samples. (R1 = 0.95, R2 = 0.99, R3 = 0.99).

In order to further analyse the observed differences in the microbial community, a LEfSe analysis was performed. Results (Figure 6) showed that the most affected OTU by the phage treatment was OTU 2, affiliated to *Enterobacter*. The relative abundance of this OTU was reduced from 18% in the control group to 0.01% in samples treated with the phage. In contrast, OTU 1, identified as Escherichia-Shigella, increased its abundance in phage-treated samples (Results are summarised in Table 2). Changes in those OTUs could explain the differences in the community diversity presented above, as the major alterations were related to the reduction in the OTU 2 and the increase of the OTU 1.

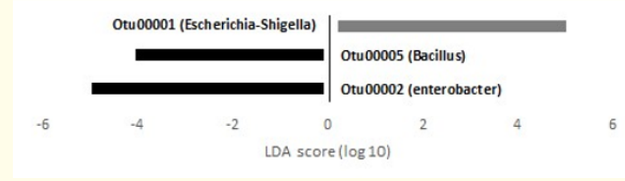


Figure 6: Lineal discriminant analysis effect size (LEfSe) analysis. LDA score for samples is represented in bars. OTUs enriched in phage treated samples are represented in grey, whereas reduced OTUs are in black.

OTUs	Associated taxonomy	HCONV	HCONV_P
OTU 1	Escherichia-shigella	72.7589	94.2373
OTU 2	Enterobacter	18.4016	0.0135
OTU 5	Bacillus	2.3708	0.0489

Table 2: OTU relative abundance.

Discussion

This paper describes for the first time the effect of phage treatment on an animal model conventionalized with human microbiota. Since our aim was to evaluate the effects of phage therapy under a high dose scenario, we used a phage concentration of 106 PFU/ml (i.e., 100 times higher than the effective concentration), and contact time of 24 hours, 4 times the period require for fish protection against *Aeromonas salmonicida* [17].

As the zebrafish immune system activation and signalling molecules of inflammatory response are greatly conserved in mammals [23], these results may be extrapolated to humans. Zebrafish has both innate and adaptive immune systems equivalent to mammals. This species also has an active complement system with three activation routes: the classical, alternative, and lectin pathways [24]. Microbial-associated molecular pattern (MAMP) receptors, which detect conserved molecular patterns in bacteria, viruses and the associated signal-transduction pathways are present in zebrafish

[23]. The genes selected for this experiment are cytokines and other inflammatory mediators that are typically activated in common inflammatory processes not only in zebrafish [25], but also in human. In previous experiments, T-4 like phages demonstrated non-adverse health effects on humans, even under high oral doses administration [26]. Similarly, our results indicate that AS-A phage may not affect the host immune system, as no significant differences in expression levels of the selected genes (Figure 2) were found after 24 and 48 hours exposure to the phage. These results support the hypothesis of the innocuousness of the phage for the host immunity.

The effect of the AS-A phage on the human microbiota was also tested in this experiment, using a zebrafish model conventionalized with human-derived microbiota previously described [13]. Despite the fact that the microbial community of zebrafish and mammals is different in terms of taxonomic composition, dominated by Firmicutes and Bacteroidetes in mice and humans [27] and by Proteobacteria in zebrafish, the responses to microbial colonisation are conserved [28]. Furthermore, the importance of the gut microbiota in the normal intestinal development and modulating host metabolism in zebrafish has been also highlighted in several studies [29,30].

The concentration of the phage used in this experiment was much higher than that needed to reduce the population of its target, *A. salmonicida.* Indeed, in the microbial community of conventionalized zebrafish larvae, *A. salmonicida* was not detected. Even though phages are thought to be highly specific to a single bacteria or strain, in previous studies AS-A phage was strongly active not only against *A. salmonicida* but also against *V. anguillarum* and *V. parahemoliticum*. Those bacteria are common inhabitants in aquaculture ecosystems [11]. In addition, it was described that *Solea senegalensis* intestinal microbial community was moderately affected by this phage [17]. However, if this phage could be active against other bacterial species from other ecosystems it not yet reported.

According to our results, AS-A phage might reduce the population of *Enterobacter* present in the human microbiota. This genus is affiliated to the class gamma-proteobacteria as well as *vibrio*. Furthermore, in our study the lack of *Enterobacter* is related to an increase of *Escherichia-Shigella*.

Due to the lack of the AS-A phage specific target in the human microbiota, this phage might have infected *Enterobacter*, reducing its population and allowing the growth of other non-sensitive strains, such as *Escherichia-Shigella*. Accordingly, it was previously demonstrated that AS-A phage is not active against *Escherichia coli* [12].

Conclusion

Taking all together, it might be speculated that even the zebrafish immune system is not affected by AS-A phage in terms of the expression levels of a battery of marker genes related to the innate immune system that are conserved between zebrafish and mammals, this phage has a broader host range than expected and could have a lytic activity against more targets, such as bacteria from *Enterobacter genera*. Additional studies are required to confirm this speculation; however, the preliminary results presented in this work open a window to further research.

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Conflict of Interest

The authors declare no conflicts of interest.

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