Biological control of *Aeromonas salmonicida* infection in juvenile Senegalese sole (*Solea senegalensis*) with Phage AS-A.

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Abstract:

One of the major sources of financial loss for the fish farming industry is the occurrence of infections by pathogenic bacteria, especially multidrug-resistant variants. This problem is most prominent during the early stages of fish development and is difficult to address with traditional antibiotic treatment or vaccination. In this way, alternative environmentally-friendly biological strategies to control bacterial infections need to be implemented. Under this scenario, phage therapy appears as a useful and flexible tool for the inactivation of bacterial pathogens in aquaculture. The aim of this study was to test the efficacy of phage therapy to inactivate *Aeromonas salmonicida*, the causative agent of furunculosis, a fish disease characterized by high mortality and morbidity. In order to achieve this goal, a new phage was isolated, characterized and tested in artificially-infected *Solea senegalensis* juveniles and in batch bacterial cultures. Results showed that after 6 h of treatment the phage inhibited the growth of *A. salmonicida* both in batch cultures and seawater in the presence of fish juveniles (≈ 4 and $2.5 \text{ Log PFU mL}^{-1}$, respectively). After 72 h, fish juveniles treated with phages after exposure to *A. salmonicida* showed no mortality, contrarily to fishes that were only exposed to the bacterium, which presented a mortality of 36%. This result indicated that phage treatment was effective. In general, it was observed a limited regrowth of resistant cells and absence of lysogeny conversion. No significant impact of phage inoculation on natural bacterial communities of aquaculture water was detected. However, the bacterial community associated with the fish intestinal tract was moderately affected by the addition of the phage. Interestingly, the differences were not significant when the phage was added in the presence of the host bacteria. Taking this into account, this study provides evidences that the tested phage can be effective and safe against furunculosis during the production of juvenile fish.

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41 **Keywords:** Disease; fish pathogenic bacteria; bacterial control; phage therapy;

42 *Aeromonas salmonicida*; furunculosis; *Solea* juveniles.

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1. Introduction

Aquaculture is an increasingly important seafood source worldwide, supplying around 30% of all seafood presently consumed worldwide (FAO, 2014). In general, aquaculture fish are subjected to a greater stress than wild conspecifics, which contributes to a reduction of their natural immune system and often favours bacterial infections, namely during their early life stages. This scenario can favour opportunistic infections and cause significant economic losses to producers.

Aeromonas salmonicida, the causative agent of furunculosis, is a relevant fish pathogen in aquaculture, responsible for causing significant economic losses worldwide (Dallaire-Dufresne and Tanaka, 2014; Diamanka et al., 2013; Gonzalez et al., 2004; Janda and Abbott, 2010; Noga, 2011) due to high mortality and morbidity in a variety of fish species (e.g., salmon (Ringø et al., 2004; Verner-Jeffreys et al., 2007), trout (Imbeault et al., 2006; Nikoskelainen et al., 2001), turbot (Coscelli et al., 2014; Farto et al., 2011; Lago et al., 2012), Atlantic cod (Arnesen et al., 2010), rockfish (Kim et al., 2013), seabream (Zorrilla et al., 2003) and wolffish (Grøntvedt and Espelid, 2004)).

The consequences of furunculosis are specially severe in farmed fish in the absence of vaccination (Bergh, 2008). Although efficient vaccines are available, they are still expensive and often associated with undesirable side effects (Björnsdóttir et al., 2005; Cipriano and Austin, 2011; Midtlyng, 1997). On the other hand, the recent emergence of antibiotic resistant *A. salmonicida* strains in aquaculture has been observed (Kim et al., 2011; Reith et al., 2008), making alternative control methods to treat furunculosis urgently needed.

Phage therapy is based on the use of bacteriophages (viruses that infect bacteria) to inactivate pathogenic bacteria. Compared to conventional methods this approach presents several advantages: (a) phages are target specific; (b) nearly no serious or

irreversible side effects of phage addition have been described to date, although phages may carry virulence or toxin genes; (c) phages can be chosen to have no environmental impact, infecting only pathogenic bacteria; (d) phages have high resistance to different conditions; (e) while phage-resistant cells regrow after treatment, these mutants can recover phage sensibility after being grown in culture media; and (f) phage therapy is a highly flexible, fast and inexpensive technology (Almeida et al., 2009; Silva et al., 2015; Skurnik and Strauch, 2006; Vieira et al., 2012).

A number of phages infecting *Aeromonadaceae* isolated from different environments around the world have already been characterized (Ackermann et al., 1985; Beilstein and Dreiseikelmann, 2008; Chow and Rouf, 1983; Ishiguro et al., 1984; Kim et al., 2012a, 2012b; Popoff, 1971; Rodgers et al., 1981), and, in recent years, phages of *A. salmonicida* have been successfully used to treat furunculosis in Atlantic salmon and rainbow trout (Imbeault et al., 2006; Kim et al., 2015). However, less promising results have also been reported, showing that furunculosis in brook trout was not readily controllable by phage therapy (Verner–Jeffreys et al., 2007). Consequently, additional studies targeting new phages and different fish species are needed to evaluate the effectiveness of phage therapy to fight infections promoted by *A. salmonicida*.

In the present study our main goals were to isolate, characterize and evaluate the safety and effectiveness of an *A. salmonicida* phage to control furunculosis infection in Senegalese sole (*Solea senegalensis*) juveniles. Sole species are economically important for aquaculture and fisheries, being frequently used as biological models to study different aspects of fish nutritional requirements, hormonal mechanisms and genomics (Benzekri et al., 2014).

2. Materials and methods

2.1 Ethics Statement

Sewage water was collected in the EEIS9 lift station of the Aveiro Network Sewage (Portugal) with the permission of *Sistema Multimunicipal de Saneamento da Ria de Aveiro* (SIMRIA). (<http://www.simria.pt/index.php>). Aquaculture water was collected with the permission of Corte das Freiras - Aquicultura, Lda. (<http://codigopostal.ciberforma.pt/dir/509467393/corte-das-freiras-aquicultura-lda/>).

All *in-vivo* studies were carried out in accordance with the recommendations of European Commission (2003/65/EC, 2003; 2007/526/EC, 2007). Protocols were designed to comply with the European policy of the “3 Rs” (Reduce, Refine, and Replace) in aquatic animal experimentation. Fish juveniles were collected directly from grow-out tanks and kept in optimal culture conditions until they were used for experimentation. No fish were intentionally sacrificed during the experiment, with all specimens being euthanized following standard good practice procedures (namely cold treatment) at the end of the experimental period.

2.2 Bacterial strain and growth conditions

The bacterial strains used in this study are listed in Table 1. *A. salmonicida* (CECT 894), *Aeromonas hydrophila* (ATCC 7966), *Vibrio fischeri* (ATCC 49387), *Salmonella enterica* (ATCC 13311 and ATCC14028), *Escherichia coli* (ATCC 25922 and 13706), *Vibrio parahaemolyticus* (DSM 27657), *Vibrio anguillarum* (DSM 215 97), *Photobacterium damsela* subsp. *damsela* (DSM 7482) were purchased from CECT, ATCC and DSM collection, respectively. All other bacterial strains used in the present study were isolated in previous works from water samples collected in Ria de Aveiro (Louvado et al., 2012; Silva et al., 2014). Fresh plate bacterial cultures were maintained in solid Tryptic Soy Agar medium (TSA; Liofilchem, Italy) at 4 °C. Before each assay,

one isolated colony was aseptically transferred to 10 mL of Tryptic Soy Broth medium (TSB; Liofilchem, Italy) and was grown overnight at 37 °C. An aliquot of this culture (100 µL) was aseptically transferred to 10 mL of fresh TSB medium (Liofilchem, Italy) and grown overnight at 37 °C to reach an optical density (O.D. 600) of 0.8, corresponding to about 10^9 cells mL⁻¹.

2.3 Bacteriophage isolation and purification

Phage AS-A was isolated from sewage water from a lift station of the sewage network of Aveiro, Portugal (station EEIS9 of SIMRIA Multi Sanitation System of Ria de Aveiro) using *A. salmonicida* as host, according to Pereira et al. (2011). Phage stocks were stored at 4 °C and 1% chloroform (final volume) (Scharlau, Spain) was added. The phage suspension titre was determined by the double-layer agar method using TSA (Liofilchem, Italy) as culture medium (Silva et al., 2014). The plates were incubated at 25 °C for 12 h and the number of lysis plaques was counted. The results were expressed as plaque forming units per millilitre (PFU mL⁻¹).

2.4 Electron microscope examination

Phage particles of a highly concentrated suspension (10^9 PFU mL⁻¹) were negatively stained with 2% uranyl acetate (Electron Microscopy Sciences, Hatfield, UK). Electron micrographs were taken using a JEOL 1011 transmission electron microscope (JEOL USA Inc, Peabody, MA, USA) operating at 100 kV and images were acquired with a Gatan CCD-Erlangshen ES100W.

2.5 Phage host range determination and efficiency of plating (EOP) analysis

Bacterial strains used in the present work are listed in Table 1. As a first approach, phage host range was determined by spot testing according to Vieira et al., 2012. The plates were incubated at 25 °C and examined for plaques after 8 - 12 h. Bacterial

sensitivity to a bacteriophage was established by a lysis cleared zone at the spot. According to the clarity of the spot, bacteria were differentiated into three categories: large clear lysis (+), no lysis (-). The EOP was determined for bacteria with positive spot tests (occurrence of lysis plaques), using the double-layer agar method. For each host, EOP was calculated by comparison with an efficacy of 100% for the AS-A phage on *A. salmonicida*. Three independent experiments were performed for each phage and results presented are averages of the three assays.

2.6 Bacterial kill curves

Bacterial inactivation was determined using the bacterium *A. salmonicida* as host, at a Multiplicity of infection (MOI) of 100. For each assay, two control samples were included: the bacterial control (BC) and the phage control (PC). While the bacterial control was not inoculated with phages, the phage controls were inoculated with phages but not with bacteria. Controls and test samples were incubated exactly in the same conditions, with 3 replicates being performed per sample. Aliquots of test samples and controls were collected after 0, 2, 4, 6, 8, 10, 12 and 24 h of incubation. In all assays the phage titre was determined in duplicate through the double agar layer method after an incubation period of 18 h at 25 °C. Bacterial concentration was determined in duplicate in TSA medium (Liofilchem, Italy) after an incubation period of 24 h at 25 °C. Three independent experiments were performed for each condition.

2.7 Prophage detection in host bacteria after phage therapy.

In order to evaluate the occurrence of lysogenic conversion (evaluate if the tested phage has the ability to incorporate its own genome in the bacterial genome), the mitomycin C test was applied, according to Silva et al. (2014). The absence of a clear zone after the exposure to mytomycin C was interpreted as an indicator of the absence of prophages in the bacterial genomes. Three independent assays were performed.

2.8 Determination of frequencies of spontaneous phage-bacterial mutants

Spontaneous *A. salmonicida* mutants resistant to bacteriophages were isolated by plating on double-layer agar with the phage (10^9 PFU/mL per plate), according to Filippov et al. (2011) To calculate the mutation frequencies, mean numbers of mutants in 1 mL of culture (prepared from the culture with phages) were divided by mean total numbers of live bacterial cells (prepared from the culture without phages) (Filippov et al., 2011).

2.9 In vivo experiments with *S. senegalensis* juveniles

S. senegalensis juveniles (~30 mm total length) were selected as biological models to test the efficacy of phage therapy against *A. salmonicida* infections. All juveniles used in this experiment were purchased at Safiestela Sustainable Aquafarming Investments, Lda. (Group Sea8), a Senegalese sole hatchery in Póvoa do Varzim (Portugal). This hatchery enforces a bio-security protocol to prevent disease in its facilities, with emphasis to furunculosis. All fishes were transported to the laboratory by the research team in a controlled temperature container, with an oxygen saturated atmosphere, and quarantined to ensure that solely healthy fishes were selected for the experimental trials (fish were surveyed daily and no abnormal behaviour or disease signal was recorded during this period).

2.9.1 Determination of LD50 of *A. salmonicida* infection on *S. senegalensis*

Bacteria were grown overnight in TSB supplemented with 1.5% NaCL, at 25 °C. Bacterial cells were centrifuged and washed twice with PBS. The bacterial suspension was adjusted to about 10^9 CFU mL⁻¹ with PBS.

Determination of Lethal Dose (LD50) of *A. salmonicida* infection on *S. senegalensis* was performed in order to establish the concentration of *A. salmonicida* able to cause death in about 50% of juvenile fish in 72 h. A fresh culture of *A. salmonicida* was produced and then resuspended in PBS in order to reduce the introduction of organic matter in the tanks. The control tank was inoculated with PBS without any addition of bacteria. Bacterial suspension was added to the water and the juvenile fish of the four tanks employed were maintained under optimal growth conditions. Fish mortality was determined by visual inspection every 12 h. Three independent assays were performed in different dates.

2.9.2 Phage therapy in *S. senegalensis* juveniles

In these experiments 4 groups of juvenile fish were used. Each group included 3 samples of 10 juveniles each (a total $4 \times 3 \times 10 = 120$ juveniles per experiment). Samples of the first group were infected with *A. salmonicida* and treated with AS-A phage (test group: fish + bacterium + phage-), samples of the second group were infected with the bacteria but not treated with the phage (bacterium control: fish + bacterium), samples of the third group were not infected with bacteria but phages were added (phage control: fish + phage) and samples of the fourth group received neither bacteria nor phages (fish control: fish). All 3 samples of each group were maintained in an independent tank, each one with an aerator and sterile synthetic seawater (4 L)

(prepared by mixing Tropic Marin ProReef salt (Tropic Marine, Germany) with freshwater purified by a reverse osmosis unit), temperature was maintained at 22 ± 1.5 °C, pH 8.0 ± 0.2 , salinity 35, and dissolved oxygen above 5.5 mg L^{-1} , during the entire trial. A fresh culture of *A. salmonicida* was added to the test group and bacterium control to obtain a final concentration of 10^8 CFU mL^{-1} . Immediately after bacterial addition, the phage suspension was added to the test group and phage control to attain a final concentration of $10^{10} \text{ CFU mL}^{-1}$ (MOI 100).

In each experiment, *A. salmonicida* was incubated in sterile synthetic seawater at a concentration of 10^8 CFU mL^{-1} , under the same conditions as the phage therapy assays described above, in order to determine the number of autochthonous bacteria present in juvenile fish at the beginning of the experiences and also to verify the behaviour of the bacteria in the absence of fish and phages (control bacteria: saline water + bacteria). Water samples were collected at 1, 6, 12, 24, 48, and 72 h, for bacterial and phage quantification. Serial dilutions were performed and plated in TSA in duplicate and plates were incubated at 30 °C for 24 h. Fish mortality was determined by visual inspection every 12 h. This procedure was repeated three times (three independent assays) in different days.

2.10 Impact of AS-A phage treatment on bacterial community structure

2.10.1 Aquaculture water

Water was collected in July 2014 (summer) from culture tanks in the aquaculture system Corte das Freiras (Aveiro, Portugal) two hours before low tide at 0.2 m from the water surface. Samples of 150 mL of water were aseptically distributed in twelve 600 mL acid-washed and sterilized glass flasks. AS-A phage was added to three of the glass flasks at a final concentration of $8 \times 10^7 \text{ PFU} \cdot \text{mL}^{-1}$ (T8-Phage). Three types of negative

controls were also included: water without incubation (T0), water incubated without phage for 8 h (T8) and the phage preservative solution (TSB with 1 % chloroform; T8-TSBCL). T8-Phage, T8 and tT8-TSBCL samples were incubated at 25 °C and 80 - 100 rpm for 8 h. After incubation, each sample was filtered through 0.22 µm pore-size filters (Poretics). T0 samples were not incubated, being immediately filtered. The bacterial cells retained on the membranes were resuspended in 2 mL of TE buffer (10 mM Tris HCl, 1 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0 and centrifuged, pellet was resuspended in 100 µL of TE and stored at 4 °C until extraction. Three independent assays were done for each condition.

2.10.2 Fish gastrointestinal tract

The impact of phage addition on the bacterial communities of fish gastrointestinal tract was assessed in *S. senegalensis* juveniles used in the phage therapy experiments. After 8 h of incubation, five juveniles of each group (a total $4 \times 3 \times 5 = 60$ juveniles) were randomly selected in order to collect their intestine. Tissues were homogenized mechanically in Buffer RLT Plus (lysis buffer of the RNeasy Plus Mini kit, Qiagen), using a sterile rotor/stator homogenizer. All samples were immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

2.10.3 RNA extraction

Bacterial community analysis in aquaculture water and in fish intestinal tissues were based on RNA pure extracts with the objective to provide insights into changes of bacterial community structure and activity (Urich et al., 2008). RNA is usually more concentrated in active bacteria and is commonly used as molecular marker for activity (living microorganisms). Total RNA was extracted using the RNeasy Plus Mini Kit

(Qiagen, Valencia, CA, USA) according to the protocol supplied by the manufacturer. The presence of RNA was detected using Qubit (Invitrogen, Carlsbad, CA, USA), a fluorometric quantitation device.

2.10.4 cDNA synthesis

Reverse transcription was performed using NZY First-Strand cDNA Synthesis kit (NZYtech, Lisbon, Portugal) as indicated by the instructions provided by the manufacturer. Reverse transcriptase products were kept frozen at -80 °C until use.

2.10.5 PCR amplification of 16S rcDNA

The reverse transcriptase products were used to amplify 16S rRNA (rcDNA) fragments, using a nested PCR approach. Reaction was performed in a MyCycler™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). In the first PCR, the universal bacterial primers F-27 and R-1492 were used to amplify c. 1450 bp of the 16S rcDNA gene (Weisburg et al., 1991). The PCR reaction mixture and the PCR conditions were the same used by Gomes et al. (2008). The amplicons obtained from the first PCR were used as a template for a second PCR with bacterial DGGE primers F984-GC and R1378 (c. 473 bp) according to Heuer et al. (1997). Positive (DNA from a known bacterial species) and negative controls were included in every PCR reaction. PCR products were checked after electrophoresis in a 0.8% (w/v) agarose gel.

2.10.6. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed with the DCode System (Universal Mutation Detection System, Bio-Rad). PCR amplification products were loaded onto 6.5% acrylamide gels using a denaturing gradient ranging from 40% to 58%, where 100% denaturant corresponded to 7 M urea (VWR International, LLC, Radnor, PA) and 40% (v/v) formamide (Sigma–

Aldrich, Co., St. Louis, MO). The electrophoresis was performed at 58 °C for 160 min at 160 V in 1 x Tris–acetate-EDTA buffer (5 Prime, Hamburg, Deutschland). After electrophoresis, the gels were stained and fixed for 20 min with 0.3 g silver nitrate (Sigma–Aldrich, Co., St. Louis, MO) in 0.1% (v/v) ethanol 96% (Aga S. A., Loures, Lisboa) and 0.005% acetic acid (Merck KGaA, Darmstadt, Germany), rinsed, and then submerged in a developing solution of 0.003% (v/v) formaldehyde 37% (Sigma–Aldrich, Co., St. Louis, MO) and 0.33% sodium hydroxide (9%) (Merck KGaA, Darmstadt, Germany). Finally, 0.75% Na₂CO₃ was added to stop the staining process.

2.10.7 Analysis of DGGE patterns

Stained gels were digitalized with an Epson perfection V700 Photo Scanner and analysed with the software package Gelcompar 4.0 program (Applied Maths) as previously described (Smalla et al., 2001). After automatic band search, the bands detected were carefully checked and artefacts removed. The band positions and their corresponding intensities (surface) from each treatment were exported to Excel (Microsoft). Band position and intensity data were converted into a relative abundance matrix.

2.11. Statistical analysis

The statistical analysis was performed using the IBM SPSS Statistics 22.0 software. The significance of bacterial and viral concentrations between treatments and along the experiments was tested using a one-way ANOVA and Bonferroni post-hoc test. The significance of fish mortality between treatments was also evaluated using a one-way ANOVA. Normal distributions of data were checked by Kolmorov-Smirnov test and homogeneity of variances by Levene's test. A value of $p < 0.05$ was considered to be statistically significant.

The relative abundance matrix obtained from DGGE profiles was used to investigate the effect of each treatment on the bacterial community structure. Bray–Curtis similarity indices, multidimensional scaling (MDS) ordination and analysis of similarities (ANOSIM) were calculated with the PRIMER 5 software package (Primer-E Ltd., Plymouth, UK). ANOSIM was used to assess significant differences in DGGE profiles (microbial communities) within and between treatments (R). The R statistic in ANOSIM ranges from 0 to 1, with higher values indicating greater variation in composition among samples; $R > 0.75$, well separated; $0.50 \leq R \leq 0.75$, moderately separated but overlapping; $0.25 < R < 0.50$, separated but strongly overlapping groups; $R \leq 0.25$, barely separated (Ramette, 2007).

3. Results

3.1 Phage isolation and enrichment

AS-A, isolated from Aveiro municipal sewage using *A. salmonicida* as host, formed clear plaques on the host strain with a diameter of 0.5 - 2 mm (Fig. 1). High titre suspensions (10^9 PFU mL⁻¹) were obtained.

3.2. Virion morphology

Based on the morphological analysis by Transmission Electron Microscopy (TEM) (Fig.1). AS-A phage was identified as order Caudovirales and family Myoviridae of double-stranded DNA phages (Fig. 1). The phage has an elongated icosahedral head and a rigid tail 220 nm long. The head is 90 nm width (85-95 nm) and 115 nm long.

Fig. 1. Phage plaque morphology and electron micrograph of *Aeromonas salmonicida* phage AS-A. The bars represent 50 nm.

3.3 Phage host range determination and efficiency of plating (EOP) analysis

The AS-A phage infected only *A. salmonicida* and could not infect *A. caviae*, *A. hydrophila*, *V. parahaemolyticus*, *V. anguillarum*, *V. fischeri*, *P. damsela damsela*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas gingeri*, *Pseudomonas segetis*, two strains of *S. enterica* and eleven strains of *E. coli* (Table 1).

Table 1. Lytic spectra and efficiency of plating of *A. salmonicida* bacteriophages determined on 25 bacterial strains included in eight genera. Large clear lysis (+), no lysis (-).

Species	Infectivity of phage	Efficacy of plating (%)
	AS-A	AS-A
<i>A. salmonicida</i> CECT 894	+	100
<i>A. caviae</i>	-	0
<i>A. hydrophila</i> ATCC 7966	-	0
<i>V. parahaemolyticus</i> DSM 27657	-	0
<i>V. anguillarum</i> DSM 21597	-	0
<i>V. fischeri</i> ATCC 49387	-	0
<i>P. damsela damsela</i> DSM 7482	-	0
<i>P. aeruginosa</i>	-	0
<i>P. fluorescens</i>	-	0
<i>P. putida</i>	-	0
<i>P. segetis</i>	-	0
<i>P. gingeri</i>	-	0
<i>S. enterica</i> serovar Typhimurium ATCC 13311	-	0
<i>S. enterica</i> serovar Typhimurium ATCC 14028	-	0

<i>E. coli</i> ATCC 25922	-	0
<i>E. coli</i> ATCC 13706	-	0
<i>E. coli</i> BC30	-	0
<i>E. coli</i> AE11	-	0
<i>E. coli</i> AD6	-	0
<i>E. coli</i> AF15	-	0
<i>E. coli</i> AN19	-	0
<i>E. coli</i> AC5	-	0
<i>E. coli</i> AJ23	-	0
<i>E. coli</i> BN65	-	0
<i>E. coli</i> BM62	-	0

3.4 Killing curves

At a MOI of 100, the maximum of bacterial inactivation recorded was 4 Log CFU mL⁻¹ achieved after 8 h of phage therapy (Fig. 2).

No decrease on phage survival was observed during the 24 h of the experiments, for both AS-A phage alone and for the phage in the presence of the bacterial host (ANOVA, $p > 0.05$) (Fig. 2A). While the concentration of phage incubated alone remained constant during the experiment, a significant increase of Log PFU mL⁻¹ was observed, after 8 h (ANOVA, $p < 0.05$), when the phage was incubated in the presence of its host (Figure 2B).

Fig. 2. Inactivation of *A. salmonicida* by the AS-A phage at MOI 100, during 24 h. Bacteria and phage were inoculated in TSB and incubated at 25 °C. (A) Bacterial concentration (Log CFU mL⁻¹): BC – Bacterial control; BP – Bacteria plus phage. (B) Phage concentration (PFU mL⁻¹): PC – Phage control, and PB – Phage plus bacteria. Values represent the mean of three independent experiments; error bars represent the standard deviation.

3.5. Prophage detection in host bacteria after phage therapy

No lysis plaques were observed after the addition of mitomycin C to the mixture of bacteria and phages, indicating that lysogeny did not occur during the experiment.

3.6 Frequency of phage-resistant mutants

The mean frequency of emergence of resistant mutants was 2.24×10^{-4} (Table 2). The bacterial colonies were smaller than those of the control without added phage and were visible only after 3 days of incubation. In the control colonies were visible after 24 h of incubation in the same conditions.

Table 2. Frequencies of *A. salmonicida* spontaneous AS-A phage-resistant mutants (average of 10 colonies).

Control sample (CFU mL ⁻¹)	Sample treated with phages	Frequency of mutants
$1.72 \pm 0.12 \times 10^9$	$2.81 \pm 0.15 \times 10^5$	2.24×10^{-4}

3.7 Phage Therapy in *S. senegalensis* Juveniles

LD50 determination

The results indicate that 10^8 CFU mL⁻¹ of *A. salmonicida* caused 40% mortality of fish juveniles after a 72 h infection, while a concentration of 10^7 promoted 30 % mortality. Therefore the initial concentration of *A. salmonicida* used in the phage treatment was 10^8 CFU mL⁻¹. As at 96 h of incubation some juvenile fish were dead in the controls (maybe due to toxic compounds accumulation), the phage therapy experiments were done only over 72 h.

3.8 Phage Therapy *in vivo*

After 72 h, the group with juvenile fish infected with *A. salmonicida* (Fish+AS) presented the highest mortality (36 %) (ANOVA, $p < 0.05$). The control group displayed a similar (ANOVA, $p > 0.05$) mortality to that of groups Fish+AS+Phage AS-A and Fish+Phage AS-A (3 %, 0% and 0%, respectively) (Table 3).

Table 3. Fish mortality after 72 h of treatment at 25 °C. Control Fish- fish sample without phage and bacteria; Fish+Phage AS-A - fish sample after incubation with phage; Fish + AS - sample fish after incubation with bacteria; Fish + AS + Phage – fish sample after incubation with bacteria and phage.

Sample	Mortality				
	Assay 1 (n = 30)	Assay 2 (n = 30)	Assay 3 (n = 30)	Sum of all assays (n = 90)	Average percentage of all assays (%)
Control Fish	0	2	1	3	3.3
Fish+Phage AS-A	0	0	0	0	0
Fish+AS	11	10	11	32	35.66
Fish+AS+Phage AS-A	0	0	1	1	1.1

After 6 h of incubation, the bacterial concentration in the seawater of the group Fish+AS+Phage AS-A decreased by approximately 2.2 Log CFU mL⁻¹ (ANOVA, $P < 0.05$) (Fig. 4). After 12 h, bacterial concentration stabilized until the end of the experiment (concentration around 5 x 10⁵ CFU mL⁻¹ after 72 h of incubation). In the groups control (non-challenged and non-treated fish) and Fish+Phage AS-A, without the addition of pathogenic bacteria, the bacterial concentration was relatively stable (± 0.37 Log CFU mL⁻¹ oscillation) throughout the experiment (ANOVA, $p > 0.05$), but at the beginning of the experiment, there were approximately 2.5 x 10⁵ CFU mL⁻¹ in the seawater of these groups, corresponding to other bacteria already present in juvenile

fish. Both groups presented a similar pattern of bacterial concentration. In the seawater of the group containing only fish and *A. salmonicida* (Fish+AS) there was a progressive decrease ($\pm 1.5 \text{ Log CFU mL}^{-1}$) in the concentration of bacteria until 48 h (ANOVA, $p < 0.05$) (Figure 3). A similar pattern was observed when *A. salmonicida* was incubated in sterile seawater, with bacteria surviving at high concentrations ($\pm 6.5 \text{ Log CFU mL}^{-1}$) during the 72 h experiment (ANOVA, $p > 0.05$).

Fig.3. Concentration of viable bacteria (CFU mL^{-1}) during the 72 h experiment. Control Fish- fish sample without phage and bacteria; Fish+Phage AS-A - fish sample after incubation with phage; Fish + AS - sample fish after incubation with bacteria; Fish + AS + Phage - fish sample after incubation with bacteria and phage. Values represent the Mean $\pm$ Standard Deviation of three independent assays.

3.9 Effect of bacteriophage AS-A on active bacterial community structure

3.9.1 Aquaculture water

The effect of the addition of AS-A phage on the structural diversity of active bacterial communities in aquaculture water was assessed by DGGE profiles of 16S rDNA (Fig.

428 4). The addition of phages did not promote any significant shift in bacterial ribotype
 429 diversity. The MDS analysis of the band patterns obtained from DGGE analysis of the
 430 experiment with AS-A phage revealed the occurrence of two groups (Fig. 5), one that
 431 included water samples with AS-A phage (T8- Phage AS-A) and negative control with
 432 the preservative solution (TSB with 1% chloroform, T8-TSBCL) and the other group
 433 including control without incubation (T0) and water incubated without phage AS-A
 434 (T8).

435
 436 **Fig. 4.** DGGE profile rcDNA fragments of the 16S gene amplified by PCR following
 437 addition of phage of AS-A to the natural bacterial community of aquaculture water. (T0)
 438 Control without incubation, (T8) water incubated without phage AS-A added, (T8-
 439 TSBCL) phage preservative solution, TSB with 1 % chloroform and (T8-Phage AS-A)
 440 water incubated with phage AS-A (T8-Phage AS-A).

Fig. 5 Multidimensional scaling (MDS) ordination comparison of DGGE fingerprints of 16S rcDNA fragments after the addition of AS-A phage to the natural bacterial community of aquaculture water (Fig. 5). (T0) Control without incubation, (T8) water incubated without phage AS-A added, (T8- TSBCL) phage preservative solution, TSB with 1 % chloroform and (T8-Phage AS-A) water incubated with phage AS-A (T8-Phage AS-A).

Analysis of similarities (ANOSIM) suggested a moderated separation of bacterial communities between water incubated with phage AS-A and water incubated without phage AS-A (T8 vs T8- Phage) ($R=0.633$). A separated but strongly overlapping groups was observed for water incubated without phage in phage preservative solution (T8-TSBCL) and water incubated with phage AS-A in phage preservative solution (T8-Phage AS-A) (Table 4). Water samples without incubation (T0) displayed a higher similarity, comparatively with water incubation during 8 hours (T8) ($R=0.148$) (Table 4).

Table 4. ANOSIM pairwise comparison of DGGE fingerprints of 16S rcDNA gene fragments after the addition of AS-A phage to the natural bacterial community of aquaculture water. Negative control without incubation (T0), water incubated for 8 h without phage (T8), phage preservative solution in aquaculture water containing TSB with 1 % chloroform (T8- TSBCL) and water incubated for 8 h with phage AS-A (T8- Phage AS-A).

Groups	<i>R</i>
T0, T8	0.148
T0, T8-TSBCL	0.536
T0, T8-Phage	0.602
T8, T8-TSBCL	0.426
T8, T8-Phage	0.633
T8-TSBCL, T8-Phage	0.356

3.9.2. Fish Gastrointestinal Tract

The DGGE analysis of bacterial communities present in fish gastrointestinal tract (Fig. 6) showed more variable community profiles in samples obtained from fishes exposed to AS-A phage.

Fig. 6. DGGE profile of 16S rcDNA fragments. Control Fish - fish sample (gastrointestinal tract) without phage; Fish+Phage AS-A - fish sample (gastrointestinal tract) after incubation with phage (72 h); Fish + AS - sample fish (gastrointestinal tract)

473 after incubation with bacteria (72 hours); Fish+AS+Phage – fish sample
474 (gastrointestinal tract) after incubation with bacteria and phage (72 h).

475 The MDS analysis of the band patterns obtained from DGGE analysis of fish intestinal
476 tracts revealed the occurrence of three groups (Figure 7). The first one included fish
477 infected with *A. salmonicida* but not treated with the phage (Fish+AS) and fish infected
478 with the bacterium and treated with AS-A phage (Fish+AS+Phage). The other two
479 groups, included Control Fish (neither bacteria nor phages were added) and fish with
480 phages (Fish+Phage AS-A).

481
482 **Fig. 7** Multidimensional scaling (MDS) ordination comparison of DGGE fingerprints of
483 16S rcDNA fragments from fish gastrointestinal tissues. Control Fish - fish without
484 phage; Fish+Phage AS-A - fish after incubation with phage (72 h); Fish + AS - fish
485 after incubation with bacteria (72 hours); Fish + AS + Phage – fish after incubation with
486 bacteria and phage (72 h).

487 Analysis of similarities (ANOSIM) (Table 5) indicate that communities in Control Fish
488 were statistically different from those recorded in specimens that were exposed to AS-A
489 phage (Fish+Phage AS-A) (R=0.792). The same trend was observed for fish juveniles

exposed to of the AS and AS-A (Fish+AS+Phage) (R=0.825). Bacterial communities in the group Fish+AS was statically similar to the group exposed to bacteria AS and phage AS-A (R=0.269). The ANOSIM analysis also suggested moderate separation of bacterial communities between Fish+Phage AS-A and group Control Fish (R=0.669), Fish+AS (R=0.569) and Fish+AS+Phage (R=0.666).

Table 5. ANOSIM pairwise comparison of DGGE fingerprints of 16S rcDNA fragments from fish gastrointestinal tissues. Control Fish - fish without phage; Fish+Phage AS-A - fish after incubation with phage (72 h); Fish + AS - fish after incubation with bacteria (72 hours); Fish + AS + Phage – fish after incubation with bacteria and phage (72 h).

Groups	R
Control Fish, Fish+Phage AS-A	0.669
Control Fish, Fish+AS	0.792
Control Fish, Fish+AS+Phage AS-A	0.825
Fish+Phage AS-A, Fish+AS	0.569
Fish+Phage AS-A, Fish+AS+Phage AS-A	0.666
Fish+AS, Fish+AS+Phage AS-A	0.269

4. Discussion

The current challenges to phage therapy studies are: (i) to demonstrate feasibility *in vivo*, (ii) to provide new insights at the *in vitro* level, or (iii) to address the generality of principles that define the forefront of the field.

The results of the present work, the first reporting the use of phages to control bacterial infections in *S. senegalensis* juveniles, show that the agent of furunculosis was efficiently inactivated by the new isolated phage. *In vitro* studies indicated that some

bacteria survived to the infection by the AS-A phage, probably due to the development of phage-resistance. However, *in vivo* studies showed that the presence of these phage-resistant mutants did not negatively affected the control of the infection.

4.1. Phage control of *A. salmonicida* in *S. senegalensis* juvenile fish

Our results strongly suggest that the AS-A phage can protect *S. senegalensis* juveniles against infection by *A. salmonicida*, as in the absence of the phage the mortality of infected fish was significantly higher (up to 40%) The results of our study are in line with those from previous experiments on other fish species (Imbeault et al., 2006; Kim et al., 2015), Atlantic salmon and rainbow trout, respectively, in which the addition of phage controlled infections by *A. salmonicida*. Imbeault et al. (2006) reported a mortality of up to 70% in phage-treated fish against 100% in non-treated fish. In the study by Kim et al. (2015) the mortality recorded was solely of 10% in the treated fish, contrasting with 100% mortality in non-treated fish. It must be highlighted that distinct MOI were employed on our study and these different works: 100 in our study, 10000 in Imbeault et al. (2006) and 1 in Kim et al. (2015). As already stated by several authors (Mateus et al., 2014; Nakai et al., 2010; Y. Silva et al., 2014) and contrarily to the case of chemicals and other substances, precise initial doses of phages may not be essential in aquaculture due to their self-perpetuating nature. This feature was evidenced by an increasing trend of phage titers along with bacteria in infected fish or contaminated waters. However, contrarily to that observed in *in vitro* assays, an increase in MOI does not seems to promote a significant increase in the efficiency of phage therapy *in vivo*. In the study of Verner–Jeffreys et al. (2007) a high MOI of around 100000 was used and the control *A. salmonicida* infection was not readily achieved through phage therapy. In this way, further studies are still needed in order to better understand the *in vitro* and *in vivo* effects of low or high MOI.

4.2. Implications of the use of phages in *A. salmonicida* control due to the development of phage resistance.

A major concern of bacterial inactivation by phages is the emergence of phage-resistant bacteria (Filippov et al., 2011; Levin and Bull, 2004; Y. Silva et al., 2014; Vieira et al., 2012). In fact, the results of the present study indicate that phage-resistant mutants did emerge in the presence of the AS-A phage, even though the phage was capable to control *A. salmonicida* infection in juvenile fish. The regrowth of resistant cells was however limited (2.24×10^{-4}) and reached values already reported in previous studies (Filippov et al., 2011; Levin and Bull, 2004; Y. Silva et al., 2014). It has been stated in the literature that resistance to phages can be overcome by the phage itself, because it evolves along with the host. This feature is one of the major advantages of a phage-derived therapeutic approach over that of “static” antibiotics. Moreover, after 6 h of treatment, only one log of *A. salmonicida* was detected, with this value likely being low enough to allow the immune system of infected fish to control the infection. In fact, this is what happens in the case of antibiotics, which also do not completely remove bacteria but rather lower their numbers so that the immune system of the infected organism can control the remaining bacteria. Although, in challenged and treated fish, around 5 log of bacteria were found after the treatment, in the fish control, not challenged and not treated, around 4 log of bacteria were also detected, corresponding to other bacteria already present in the juveniles at the beginning of the experiment, meaning that only 1 log of *A. salmonicida* resisted to the treatment. However, in the *in vitro* studies, as the remaining viable bacteria are not controlled by a host and the culture conditions are suitable for bacterial growth, bacteria can reach concentrations similar to those of the non-treated control as already reported in other studies (Carey-Smith et al., 2006; Lu and Breidt, 2015; Mateus et al., 2014; Park et al., 2000; Y. Silva et al., 2014; Y. J. Silva

et al., 2014; Vieira et al., 2012). Additionally, previous studies suggest that phage-resistant bacteria tend to be less fit or lose their virulence properties (Castillo et al., 2015), so they can be expected to be eliminated from the environment faster than their wild-type relatives. In fact, colonies of phage-resistant *A. salmonicida* were smaller than colonies formed by the non-phage added control and were visible only after two days of incubation. In the non-phage added control, bacterial colonies were visible after 24 hour of incubation. These results suggest that the remaining bacterial mutants (forming small size colonies and showing slow growth) maintained their viability in the presence of phages but their phenotypes were affected. Moreover, after three-five streak-plating steps on solid medium, these *A. salmonicida* cells regained sensitivity to AS-A phage, indicating that the viruses can still infect these bacteria. Similar results have already been observed in other studies using different phages and bacteria (Vieira et al., 2012). These results suggest that the emergence of phage-resistant mutants should not be a major problem to the application of phages to control bacterial infections in aquaculture.

4.3. Safety of the AS-A phage to control *A. salmonicida* infections in aquaculture.

The major challenge of bacterial inactivation by phages is the selection of safe phages, which must not induce lysogenic conversion, carry toxin genes, perform generalized transduction, neither cause a significant impact on the structure of the natural bacterial community of the aquaculture water and fish bacteriome.

The experiments of lysogenic induction showed that the remaining viable bacteria harbored no prophages. These bacteria that remained viable grew slowly and their colonies were smaller than those observed in the treatment with no phages.

As the addition of the phage lysate to the fish juveniles without bacterial challenge did not decrease juvenile fish survival (mortality in this group was similar to that of fish

non-challenged and non-treated fish control, 0% and 3%, respectively), it is not likely that AS-A may carry toxin genes. While phage AS-A produced no observable effects in *Solea* juveniles (morphological changes or mortality), further evaluation trials must still be performed (e.g., whole genome sequencing) to identify the presence of any genes encoding toxins and/or antibiotic resistance. The phage lysate could also potentially contain residual bacterial antigens or endotoxins (Sambrook et al., 1989). In this study, juveniles experimentally inoculated only with the phage preparation (phage lysate) did not evidence any negative effects on their health. Consequently, this phage seems to be safe to be used in phage therapy.

The addition of phage AS-A to aquaculture water did not cause significant impact on the natural structure of the bacterial community. However, the bacterial community of the intestinal tract of juvenile *S. senegalensis* was moderately affected by the addition of the AS-A phage. Those differences were significantly lower when the phage was added in the presence of the host bacteria.

In the *in vivo* assays performed, the phage AS-A controlled the growth of *A. salmonicida* but did not affect other bacteria already present in the juvenile fish, thus confirming that natural bacterial communities were not significantly affected. These findings indicate that survival of AS-A in aquaculture water allows the use of phage therapy to control *A. salmonicida* infections, as phages can inactivate the pathogenic bacterium in the water without significantly affecting the beneficial bacterial community present in the system. Although phage AS-A infect the two *Aeromonas* species tested in the present work, it would be important to investigate if this phage was also able to infect other strains of *A. salmonicida*, namely those isolated from marine environments.

5. Conclusions

The results of the present work suggest that phage therapy using the AS-A phage can be an interesting alternative to fight furunculosis in aquaculture through the direct administration of phages to the culture water. This practice appears to promote a limited regrowth of resistant cells and has no significant impact on natural bacterial communities present in aquaculture water. Nonetheless, further studies are still needed, namely those performed *in situ* under natural aquaculture conditions, where environmental conditions may vary considerably at different time scales. These future trials will play a key role on the evaluation of the true potential of AS-A phage therapy to control bacterial diseases under environmental/seasonal variation.

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