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Isolation and Characterization of Bacteriophages and Experimental Application of Bacteriophage Treatment to Fish in Rainbow Trout Farms in Karkamış Dam Lake

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Therapy

Abstract

This study evaluated the isolation, characterization, and therapeutic efficacy of lytic bacteriophages targeting key bacterial pathogens associated with rainbow trout (*Oncorhynchus mykiss*) aquaculture in Karkamış Dam Lake. *Aeromonas sobria*, *Lactococcus garvieae*, and *Pseudomonas* sp. were isolated from 35 net cages and two hatcheries, and their host-specific phages were designated Aes1, Lac1, and Pah1, respectively. Controlled in vivo challenge assays demonstrated that phage treatments resulted in relative percent survival (RPS) values of 28% for *A. sobria* and *L. garvieae*, and 25% for *Pseudomonas* sp. Phage administration significantly reduced cumulative mortality and delayed time-to-death compared to untreated controls ($P<0.05$). The findings support the applicability of bacteriophage therapy as a targeted biocontrol strategy and a promising alternative to antibiotics in aquaculture systems challenged by antimicrobial resistance. Future research should focus on phage cocktail development and optimization of dosing and delivery protocols to improve therapeutic performance and sustainability.

Introduction

The aquaculture industry has experienced exponential growth in recent years, becoming the fastest-growing food sector worldwide. According to the Food and Agriculture Organization (FAO), global fish production reached approximately 178 million tons in 2022, with aquaculture accounting for nearly 91 million tons. Of this total, around 89% was used for direct human consumption, highlighting the crucial role of aquaculture in meeting global seafood demand. The practice of aquaculture in Turkey originated in the 1960s, when businesses operating in the Bilecik and Akyazı regions introduced imported trout eggs from Europe. Subsequently, state-owned aquaculture operations commenced in Sivas Gökpınar Lake and Konya Konuklar State Production Farm (Memiş et al., 2002). Over the past 15 years, aquaculture production in Turkey has experienced a significant increase, reaching 850 thousand tons (TÜİK, 2022). The field research on bacteriophage, which is the subject of the study, was conducted at Karkamış Dam Lake, where aquaculture production commenced in the 2000s. Currently, rainbow trout (*Oncorhynchus mykiss*) production is carried out with 35 cage facilities and two hatcheries (Kuzucu & Büyükçapar, 2018). *Lactococcus garviae*, *Aeromonas hydrophila*, *Acinetobacter schindler*, *Lactococcus lactis ss lactis*, *Leuconostoc mesenteroides ss cremoris* and *Morexella lacunata* bacteria were isolated from Rainbow Trout Farms in Karkamış Dam Lake (Özcan & Kuzucu, 2019).

The rapid growth of aquaculture has given rise to a number of challenges, including issues related to feed, environmental factors, market dynamics, a qualified labor force, and legislation. Among these challenges, the emergence of fish diseases has emerged as a significant concern, leading to substantial economic losses in the sector (Candan & Karataş, 2010). There are 41 fish medicines licensed in Turkey, including the active ingredients florfenicol, sulfadiazine+trimethoprim, oxytetracycline, amoxicillin, oxolinic acid and enrofloxacin. The use of antibiotics represents a substantial economic input for enterprises. Furthermore, the indiscriminate and unmonitored use of antibiotics by enterprises has resulted in the development of antibiotic resistance among bacteria, necessitating prolonged treatment periods and the presence of antibiotic residues in fish (Akşit, 2016). Excessive and unconscious use of antibiotics has been demonstrated to result in the formation of reservoirs of resistant bacteria, not only in aquaculture but also in environmental ecosystems. This situation poses a significant threat to human health (Abarike, et al., 2024). In fact, 70.8% of bacteria isolated from fish contain at least one resistance gene, and 50% of bacteria are multi-drug resistant and 35% are extensively drug resistant. As a result of the prevalence of MDR bacteria in aquaculture, bacterial infections in fish can be difficult to treat (Ture et al., 2022).

In recent years, the use of bacteriophages (phage therapy) as bioactive substances for the prophylaxis or treatment of bacterial infectious diseases in aquaculture has emerged as a promising approach to address the challenges currently facing the sector (Park et al., 1997).

Bacteriophages represent a subgroup of prokaryotic viruses that invade and replicate within bacterial cells, targeting them specifically. Bacteriophages are simple viruses with either DNA or RNA genomes (O'Flaherty et al., 2009). In recent years, a substantial body of research has been conducted on bacteriophages, resulting in the isolation of numerous new bacteriophages. They represent the largest group of viruses (Ackermann & Prangishvili, 2012). The discovery of bacteriophages is attributed to the British bacteriologist Ernest Hanbury, who observed in 1896 that the water of the Ganges river in India exhibited an antibacterial effect against cholera. Hanbury postulated that this phenomenon could be caused by a living organism. He documented that the waters retained this property even after being filtered through a very fine porcelain filter (Abedon et al., 2011). In 1915, Frederick Twort, a British bacteriologist, published a report on the discovery of an ultra-microscopic agent that caused death in bacterial colonies. a researcher, observed that a filtrate from sewage water samples killed dysentery germs and described this as a "dysentery antagonist germ (D'Herelle, 2007). Bacteriophages are extremely abundant and diverse in natural ecosystems, particularly in marine and freshwater environments. Seawater can contain millions of phages per milliliter, playing a critical role in shaping bacterial population dynamics. In aquaculture, phages have attracted attention as potential biocontrol agents, both in maintaining the natural microbial balance and in suppressing pathogenic bacteria. Bacteriophage diversity varies depending on environmental conditions, host bacterial species, and ecosystem nutrient cycling (Küçükduman et al., 2021).

The objective of this study was to isolate bacterial hosts and their lytic phages, identify and characterize the resulting host bacteria and phages, establish a phage therapy model, and evaluate its therapeutic potential.

Materials and Methods

Collection of Fish Samples

In the context of the study, monthly samples were conducted between April 2021 and October 2022 at aquaculture enterprises operating in Karkamış Dam Lake (Figure 1). A total of 150 fish samples, comprising 5 to 15 fish samples from cages with fish on each sample, were utilized for bacterial isolation.

Isolation Bacteria

Bacterial isolation from clinically affected fish was performed using four culture media, namely Tryptic Soy Agar (TSA), Brain Heart Infusion Agar (BHIA), Nutrient Broth (NB), and Tryptic Soy Broth (TSB). The livers, kidneys, and spleens of the fish samples exhibiting symptoms of clinically infected fish were inoculated on media and incubated. The bacterial colonies on the agar plate were examined and passaged until a single colony was isolated for further analysis. Bacterial were preserved in glycerol for further study.

Bacterial Identification-based Morphological and Biochemical Tests

Colony morphology, Gram staining, and a series of biochemical assays including oxidase, catalase, motility, H_2S production, Methyl Red, Voges–Proskauer, indole, urease formation, and oxidation/fermentation (O/F) tests were performed (Plumb & Bowser, 1983; Austin & Austin 1999).

For the purposes of identification, the bacterial samples removed from the stock were passage into pure culture after revitalization in the appropriate media. First, bacterial isolates were identified using the Biolog system (the Biolog GENIII microplate). This involved incubating the isolates in BHIA and TSA medium at 20°C for two days. The bacterial suspension was prepared in BIOLOG IF-A solution for use as a buffer solution. The bacterial concentration was adjusted to a value between 92 and 98% by turbidimetry in a standard turbidity tube. Bacterial suspensions were inoculated at a density of 100 µl per well in the microplates. The microplates were incubated at 26°C for a period of 4–24 hours (Miller & Rhoden, 1991). Subsequently, the

microplate was analyzed using a reader and the system's database to obtain the Biolog system findings for the bacteria.

Molecular Characterization of *Aeromonas sobria*, *Lactococcus garvieae* and *Pseudomonas* sp

Molecular identification of bacterial isolates was performed using a commercial DNA isolation kit (Vivantis GF-BA-100). The 16S rRNA gene was amplified using universal bacterial primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3'). PCR amplification was conducted with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 48°C for 45 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. PCR products were purified using Exsosap treatment (37°C for 30 minutes, 80°C for 15 minutes) and sequenced using BigDye 3.1 chemistry. Sequence analysis was performed with Bioedit 7.2.5 software and compared to NCBI database using BLAST algorithm for species identification.

Isolation and Characterization of Phages

Water samples were collected from cage sites at three depths and transported aseptically to the laboratory. Samples were incubated at 15°C for 3 h, centrifuged, and the supernatants were enriched in NB at 22°C for 24 h. After chloroform treatment and filtration (0.20 µm), filtrates were stored at 4°C as putative phage lysates (Xu, 2016).

Phages were detected using the double-layer agar method (Kropinski et al., 2009), and plates were incubated at 15°C for 24–48 h to assess plaque formation. Genomic DNA was extracted using phenol–chloroform–isoamyl alcohol (Sambrook & Russell, 2001), and genome profiles were evaluated by restriction digestion (BamHI, EcoRI, SpeI, HindIII, XbaI) (Table 1) following commercial protocols (Pickard, 2009). Genome size estimation was performed using Lambda HindIII markers on agarose gels.

The Examination of the Phage by Field Emission Scanning Electron Microscope

The isolated and purified bacteriophage samples were diluted in a 1:10 ratio with SM buffer and filtered through a 0.20 µm pore diameter injector filter. The filtrates were then visualized by field emission scanning electron microscopy (FE-SEM). Twenty microliters of the samples were deposited onto carbon-coated 300 mesh grids on a parafilm surface and allowed to dry overnight. Subsequently, the grids were washed twice with distilled water and examined by field emission scanning electron microscopy (FE-SEM).

Figure 1. The location of trout farms operating in Karkamış Dam Lake.

Table 1. Restriction enzymes

Restriction enzymes	DNA
EcoRI(Eco32I)	5'…G A T A T C…3' / 3' … C T A T A G… 5'
BamHI	5'…G G A T C C…3' / 3' … C C T A A G… 5'
BcuI(SpeI)	5'…A C T A G T…3' / 3' … T G A T C A…5'
XbaI(Fastdigest)	5'…T C T A G A…3' / 3' … A G A T C T…5'
HindIII	5'…A A G C T T…3' / 3' … T T C G A A…5'

Bacterial Mortality Testing and Safety Testing of Phage Preparations

In Tank 1, 25 fish were injected with *Aeromonas sobria*, in Tank 2, 25 fish were injected with *Lactococcus garvieae*, in Tank 3, 25 fish were injected with *Pseudomonas* sp. In one tank, only 0.8% isotonic solution was injected into 25 fish as a control. The mortality rate was observed in all tanks following the injection.

To ascertain the safety of the identified and visualized phages prior to therapy, a trial was conducted with 25 fish distributed across four tanks. To test the safety of the phage preparations, the *Aeromonas sobria* phage was injected into tank 1, the *Lactococcus garvieae* phage was injected into tank 2, and the *Pseudomonas* sp. phage was injected into tank 3 at a rate of 100 µl 10^8 PFU ml-1 per fish through the peritoneal cavity. Additionally, SM buffer was injected into tank 4 at the same rate as the control.

Experimental Set Up

A controlled phage therapy trial was conducted using rainbow trout fry in standardized tanks, disinfected and prepared per biosecurity protocols, with 80% daily water renewal. Approximately 1,000 fry were sourced from a cage facility in the Surtepe area of Karkamış Dam Lake and transported under aerated, oxygenated conditions.

Phage-bacterium pairs comprised *Aeromonas sobria*, *Lactococcus garvieae*, and *Pseudomonas* sp. with their respective lytic phages, previously isolated, molecularly characterized, and validated via plaque assays. For bacterial challenge, stock cultures were adjusted to OD_{600}= 0.600, centrifuged (4,000 rpm, 10 min), and resuspended in 0.8% isotonic solution; fish received intraperitoneal injections at 1×10^8 CFU mL^{-1}.

After preliminary mortality and safety evaluations, therapy was initiated in five tanks (50 fish each): Tank 1, SM buffer (control); Tank 2, *A. sobria* phage; Tank 3, *L. garvieae* phage; Tank 4, *Pseudomonas* sp. phage; Tank 5, negative control. Phages were administered intraperitoneally 24 h post-challenge, with dosages standardized from preliminary trials to ensure outcome comparability.

Statistical Analysis

Once the sequence results had been organized and sequenced, they were entered into the NCBI GenBank database in Clone Manager 9. This enabled the species identification to be confirmed by comparing the sequences with those of other *Aeromonas sobria*, *Lactococcus garvieae* and *Pseudomonas* species via BLAST in the electronic database.

The results of the phage therapy trial were monitored for a period of seven days. To evaluate cumulative mortality rates, statistical analyses were conducted using IBM SPSS Statistics v25.0 software. All calculations and tests were conducted using log_{10}-transformed values, and differences were deemed statistically significant at $P<0.05$ when compared using appropriate comparison the Wilcoxon tests.

Results

Bacterial Isolation and Identification

Forty purified bacterial isolates were obtained from necropsied fish samples. Classical biochemical tests (Table 2) and the Biolog system (Table 3; Figure 2) identified the isolates as *Aeromonas sobria* DNA group 7, *Lactococcus garvieae*, and *Pseudomonas* sp., which were subsequently confirmed by molecular sequencing and phylogenetic analysis (Figures 3,4,5).

Bacteriophage Isolation and Identification

Bacteriophages were successfully isolated from enriched water samples using host-specific spot assays. Plaque-forming samples (Table 4) were purified by the double-layer agar method (Figure 6), and extracted phage DNA showed intact profiles on agarose gels (Figure 7). Restriction analysis of the *Pseudomonas* sp. phage revealed an estimated genome size of approximately 23.8 kb (Figure 8).

The Examination of the Lytic Phages via FE-SEM

FE-SEM analysis demonstrated distinct phage morphologies. The *L. garvieae* phage exhibited an icosahedral head with a short tail, whereas the *A. sobria* phage displayed a tailless icosahedral structure (Figure 9).

Faj Therapy Application

In bacterial challenge assays, cumulative mortality was highest in infected groups compared with controls. Phage safety tests showed no significant difference from controls ($P>0.05$). Phage therapy applied 24 h post-infection significantly reduced mortality in *A. sobria* and *L. garvieae* groups ($P<0.05$), while no significant effect was observed for *Pseudomonas* sp. In a subsequent trial, immersion with the *Pseudomonas* phage significantly reduced mortality compared with phage-free treatments ($P<0.05$).

Discussion

This study characterized the major bacterial pathogens associated with recurrent mortality in rainbow trout cultured in Karkamış Dam Lake and evaluated the therapeutic efficacy of three lytic bacteriophages (Aes1, Lac1, and Pah1). The identification of *Aeromonas sobria*, *Lactococcus garvieae*, and *Pseudomonas* sp. using classical

Table 2. Morphological and biochemical characteristics of *Aeromonas sobria*, *Lactococcus garvieae* and *Pseudomonas* sp. isolated from Rainbow Trout

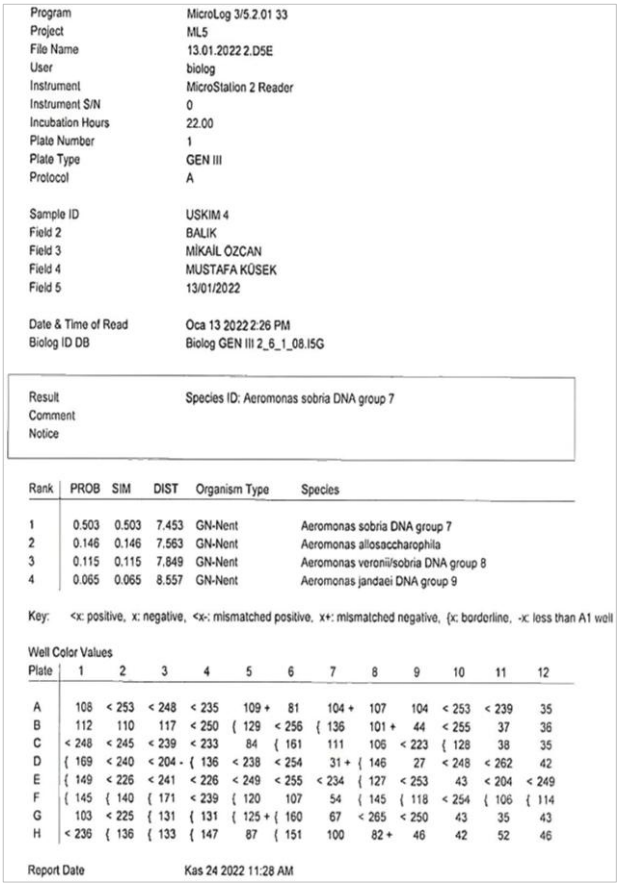
Phenotypic and Biochemical Features	*Aeromonas sobria*	*Lactococcus garvieae*	*Pseudomonas* sp.
Colony color	Cream	White	Pink
Gram Coloring	-	+	-
Shape	Rod	ovoid coccus	Rod
Oxidase	+		+
Catalase	+	-	+
Movement	+	-	+
H_2S	-	-	-
Methyl Red	-	+	-
Voges Proskauer	-	+	-
Indole	+	-	-
Urease Formation	-	-	-
Oxidation / Fermentation	+/+	-/+	+/-
MacConkey Agar	+	-	+
Mueller-Hinton Agar	+	+	+
Reproduction at 0°C	-	-	-
Reproduction at 5°C	+	+	+
Reproduction at 15°C	+	+	+
Reproduction at 20°C	+	+	+
Reproduction at 25°C	+	+	+
Reproduction at 30°C	+	+	+
Reproduction at 37°C	+	+	+
Reproduction in 0.0% NaCl	+	+	+
Reproduction in 0.5% NaCl	+	+	+
Reproduction in 1.0% NaCl	+	+	+
Reproduction in 2.0% NaCl	+	+	+
Reproduction in 6.5% NaCl	-	+	-

Table 3. Other phenotypic characteristics of *Aeromonas sobria*, *Lactococcus garvieae* and *Pseudomonas* sp. isolated from Rainbow Trout (These characteristics were determined by Biolog System Device)

Biochemical criteria	*Aeromonas sobria*	*Lactococcus garvieae*	*Pseudomonas* sp.
Negative Control	-	-	-
Dextrin	+	+	-
D-Maltose	+	+	-
DTrehalose	+	+	-
DCellobiose	+/-	+	-
Gentiobiose	+/-	+	-
Sucrose	+	Weak +	-
D-Turanose	+/-	+	-
Stachyose	+/-	Weak +	-
Positive Control	+	+	+
pH 6	+	+	+
pH 5	+/-	-	-
D-Raffinose	+/-	+/-	-
α-D-Lactose	+/-	+/-	-
DMelibiose	+/-	+/-	-
β-Methyl-DGlucoside	Weak -	+	-
D-Salicin	+/-	+	-
N-Acetyl-D-Glucosamine	+	+	-
N-Acetyl-β-D-Mannosamine	+/-	+	-
N-Acetyl-D-Galactosamine	Weak +	+	-
N-Acetyl Neuraminic acid.	+/-	-	-
1% NaCl	+	+	+
4% NaCl	+/-	+	-
8% NaCl	+/-	Weak +	-
α-D-Glucose	+/-	+	Weak +
D-Mannose	+	+	-
D-Fructose	+	+	-
DGalactose	+/-	+	-
3-Methyl Glucose	+/-	+	-
D-Fucose	+/-	+/-	-
L-Fucose	+/-	+/-	-

Table 3. Continued

Biochemical criteria	*Aeromonas sobria*	*Lactococcus garvieae*	*Pseudomonas* sp.
L-Rhamnose	+/-	+/-	-
Inosine	+	+	-
1% Sodium Lactate	+	+	+
Fusidic Acid	+/-	-	-
D-Serine	+	-	-
D-Sorbitol	+/-	+	-
D-Mannitol	+	+	-
D-Arabitol	+/-	-	-
Myo Inositol	+/-	+/-	-
Glycerol	+/-	+	-
D-Glucose-6-PO_4	+/-	+/-	-
D-Fructose-6-PO_4	+	+/-	+/-
D-Aspartic Acid	+/-	-	-
D-Serine	+/-	-	-
Troleandomycin	+	-	+
Rifamycin SV	+	+	+
Minocycline	+/-	-	-
Gelatin	+/-	-	-
Glycyl-L-Proline	+/-	+/-	+/-
L-Alanine	+/-	-	+
L-Arginine	+/-	+/-	+
L-Aspartic Acid	+	-	+/-
L-Glutamic Acid	+	-	+
L-Histidine	+/-	-	+/-
L-Pyroglutamic Acid	+/-	-	-
L-Serine	+	-	-
Lincomycin	+	+/-	+
Guanidine HCl	+	+/-	+/-
Niaproof 4	+	-	+
Pectin	+/-	+	-
D-Galacturonic Acid	+/-	+	-
L-Galactonic Acid Lactone	+/-	-	+/-
D-Gluconic Acid	+	+	-
D-Glucuronic Acid	+/-	+/-	-
Glucuronamide	+/-	+/-	+/-
Mucic Acid	-	+/-	-
Quinic Acid	+/-	-	Weak +
DSaccharic Acid	+/-	-	-
Vancomycin	+	-	+
Tetrazolium Violet	+	+/-	+
Tetrazolium Blue	+	+/-	+
p-Hydroxy-Phenylacetic Acid	+/-	-	Weak +
Methyl Pyruvate	+/-	+/-	+
D-Lactic Acid Methyl Ester	-	-	-
L-Lactic Acid	+/-	-	+
Citric Acid	+/-	-	+
α-Keto GlutaricAcid	+/-	-	+
D-Malic Acid	+/-	-	-
L-Malic Acid	+	-	+
Bromo-Succinic Acid	+/-	-	+
Nalidixic Acid	+/-	+	-
Lithium Chloride	+/-	-	-
Potassium Tellurite	+/-	+	+
Tween 40	+/-	+/-	+/-
γ-Amino-Butryric Acid	+/-	-	+
α-Hydroxy-Butyric Acid	+/-	+/-	-
β-Hydroxy-D,LButyricAcid	+/-	-	+
α-Keto-Butyric Acid	+/-	+/-	-
Acetoacetic Acid	+/-	+/-	+/-
Propionic Acid	+/-	-	+/-
Acetic Acid	+/-	+/-	+/-
Formic Acid	+/-	-	Weak -
Aztreonam	+/-	+	+/-
Sodium Butyrate	+/-	+	-
Sodium Bromate	+/-	Weak+	-



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3	0.115	0.115	7.849	GN-Nent	Aeromonas veronii/sobria DNA group 8
4	0.065	0.065	8.557	GN-Nent	Aeromonas jandaei DNA group 9

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

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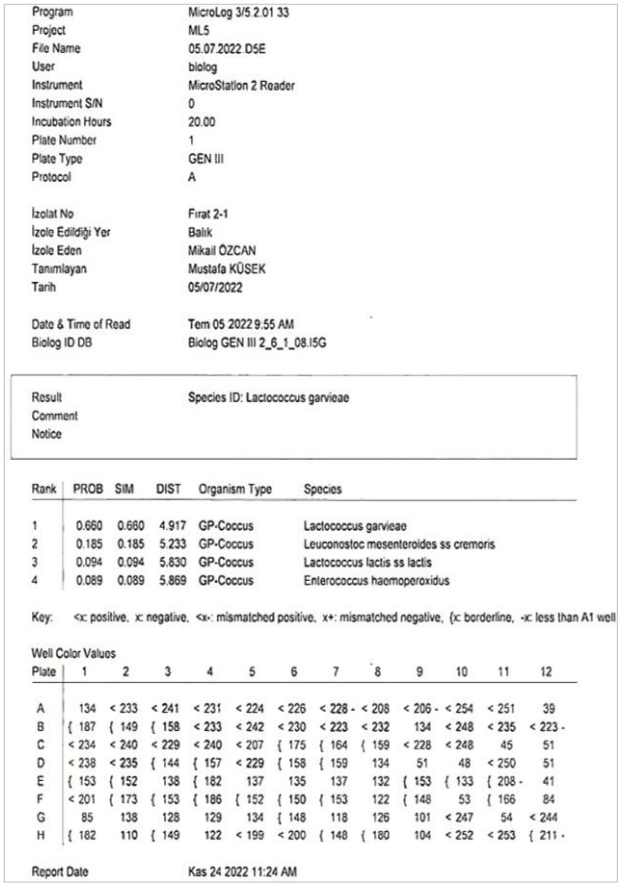

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2	0.185	0.185	5.233	GP-Coccus	Leuconostoc mesenteroides ss cremoris
3	0.094	0.094	5.830	GP-Coccus	Lactococcus lactis ss lactis
4	0.089	0.089	5.869	GP-Coccus	Enterococcus haemoperoxidus

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

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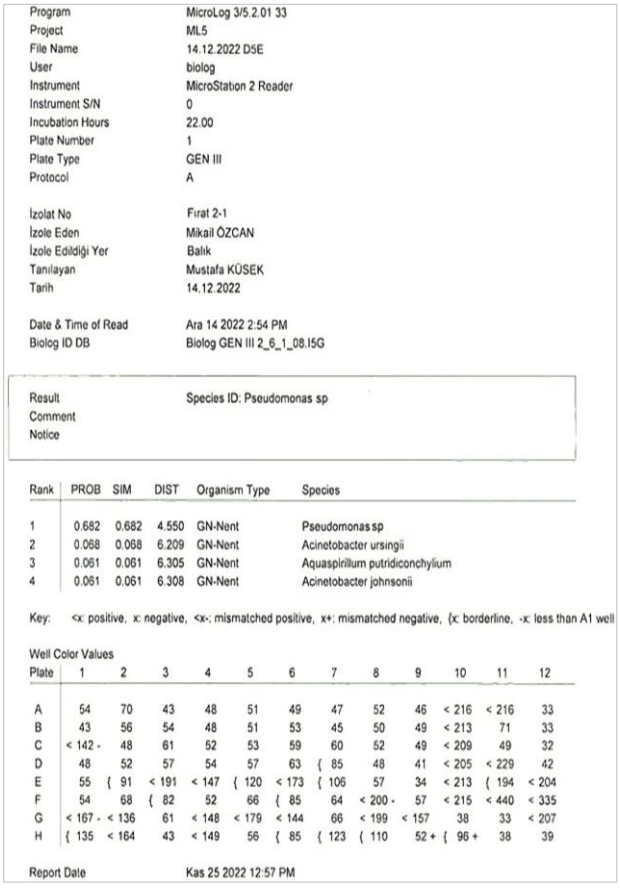

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Tanılayan	Mustafa KÜSEK
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Rank	PROB	SIM	DIST	Organism Type	Species
1	0.682	0.682	4.550	GN-Nent	Pseudomonas sp
2	0.068	0.068	6.209	GN-Nent	Acinetobacter ursingii
3	0.061	0.061	6.305	GN-Nent	Aquaspirillum putridiconchylium
4	0.061	0.061	6.308	GN-Nent	Acinetobacter johnsonii

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	54	70	43	48	51	49	47	52	46	< 216	< 216	33
B	43	56	54	48	51	53	45	50	49	< 213	71	33
C	< 142 -	48	61	52	53	59	60	52	49	< 209	49	32
D	48	52	57	54	57	63	{ 85	48	41	< 205	< 229	42
E	55	{ 91	< 191	< 147	{ 120	< 173	{ 106	57	34	< 213	{ 194	< 204
F	54	68	{ 82	52	66	{ 85	64	< 200 -	57	< 215	< 440	< 335
G	< 167 -	< 136	61	< 148	< 179	< 144	66	< 199	< 157	38	33	< 207
H	{ 135	< 164	43	< 149	56	{ 85	{ 123	{ 110	52 +	{ 96 +	38	39

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Figure 2. Biolog diagnostic system results of *Aeromonas sobria*, *Lactococcus garvieae* and *Pseudomonas sp.* isolated from Rainbow Trout.

biochemical tests, Biolog GEN III profiling, and 16S rRNA sequencing is consistent with previous reports identifying these bacteria as important agents of systemic infections in freshwater aquaculture (Avsever et al., 2012; Duman et al., 2017; Wiklund & Bylund, 1990; Doménech et al., 1993). The difficulty in distinguishing *Aeromonas* species based solely on biochemical traits further supports the combined use of phenotypic and molecular approaches for accurate diagnosis (Janda, 2001).

The metabolic profiles obtained with the Biolog system showed high similarity to reference strains, confirming its reliability for differentiating closely related bacterial taxa, as previously reported (Martínez-Murcia et al., 2007). Clinical signs observed during natural and experimental infections, including exophthalmos, hemorrhages, darkened skin, and petechial bleeding, were in agreement with earlier descriptions of infections caused by *L. garvieae* and *Pseudomonas* spp. in trout (Austin & Austin, 1999; Kusuda & Salati, 1999; Diler et al., 2002).

Electron microscopy revealed that Aes1, Lac1, and Pah1 possess morphologies similar to phages previously isolated from *Aeromonas* and *Lactococcus* species. For

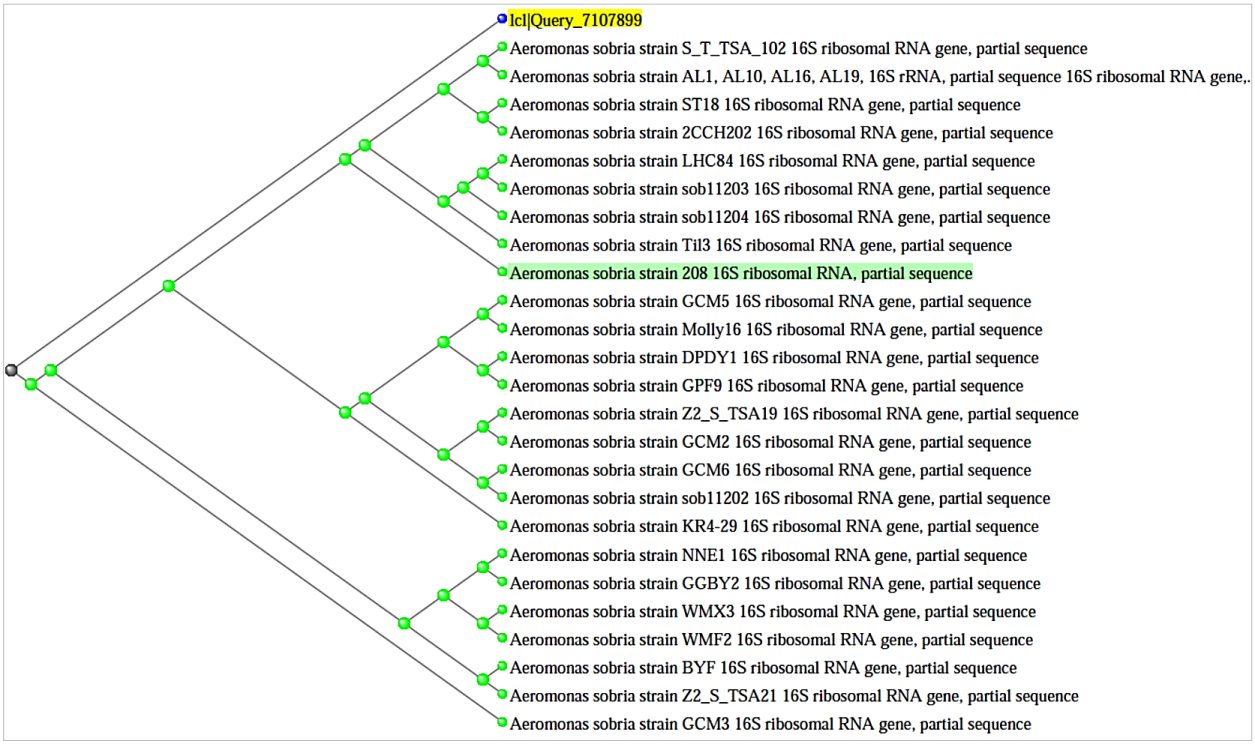

Figure 3. Phylogenetic tree of *Aeromonas sobria*.

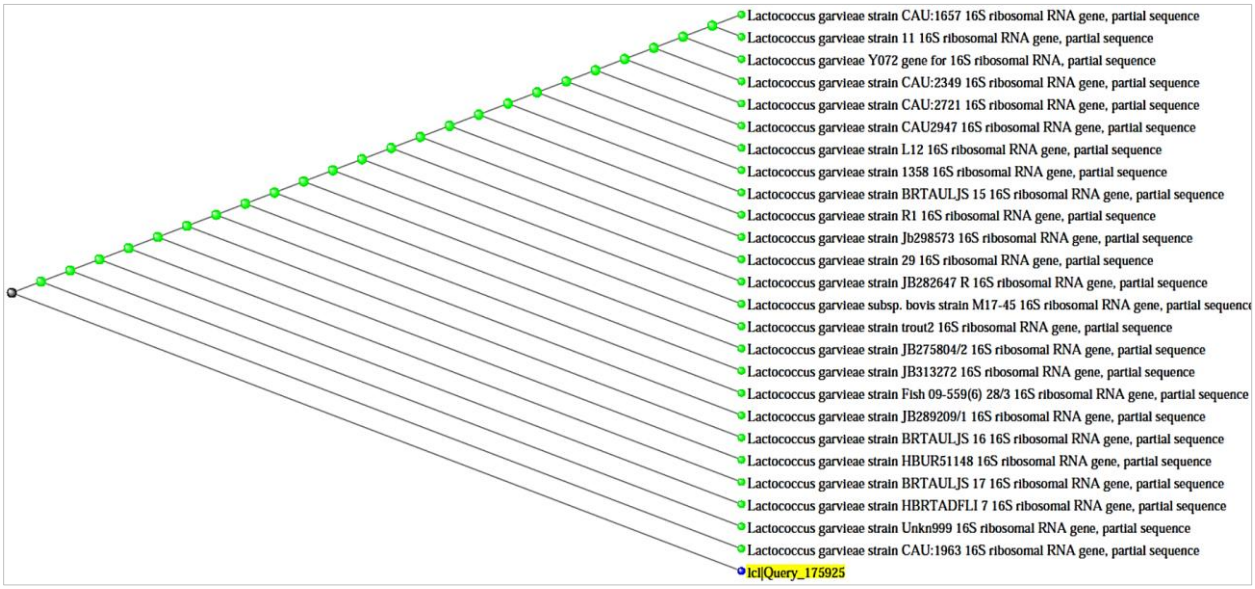

Figure 4. Phylogenetic tree of *Lactococcus garvieae*.

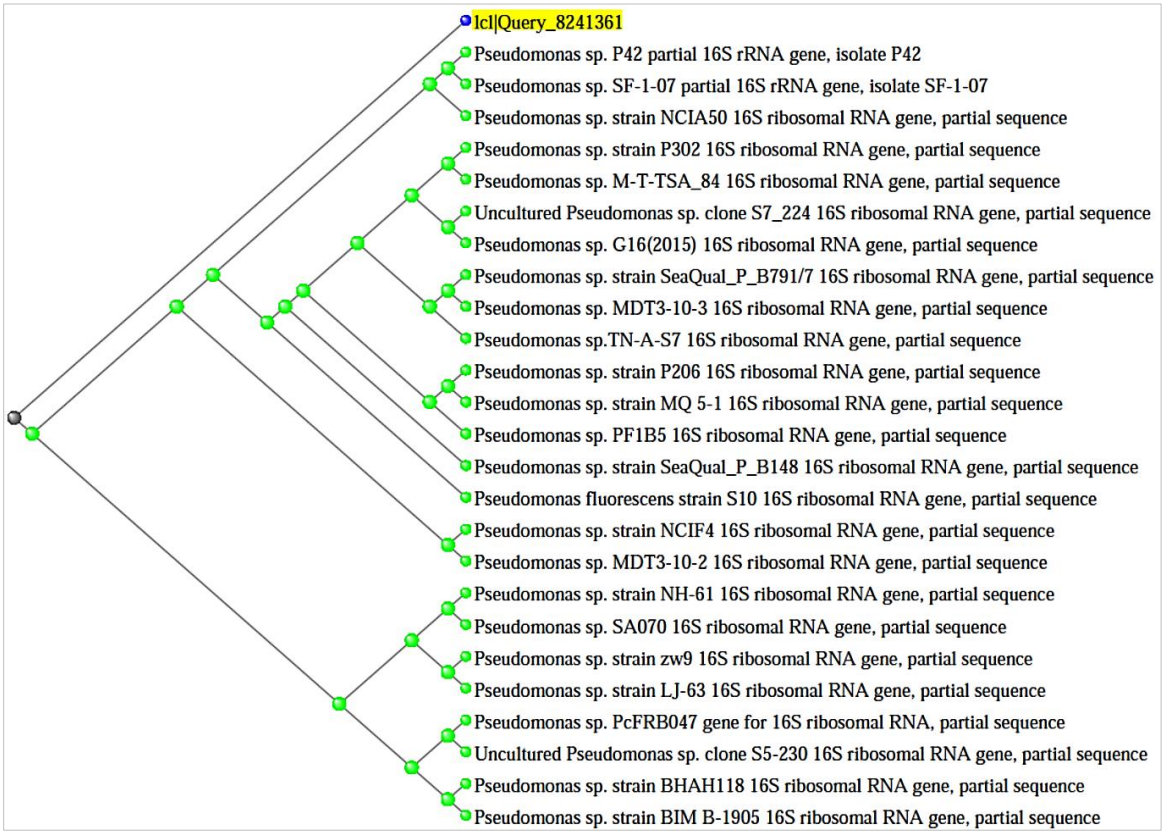

Figure 5. Phylogenetic tree of *Pseudomonas* sp.

Table 4. Bacteriophage spot test (+/-) results

No	Bacteria	Spot test (+/-)	Lab stock no
1.	*Aeromonas sobria*	+	Bakf2
2.	*Aeromonas sobria*	-	Bakf3
3.	*Lactococcus garvieae*	-	Bakf5
4.	*Lactococcus garvieae*	-	Bakf6
5.	*Lactococcus garvieae*	+	Bakf7
6.	*Pseudomonas sp.*	+	Bakf10
7.	*Pseudomonas sp.*	-	Bakf11
8.	*Aeromonas sobria*	-	Bakf16
9.	*Aeromonas sobria*	-	Bakf17
10.	*Lactococcus garvieae*	-	Bakf18
11.	*Lactococcus garvieae*	-	Bakf19
12.	*Lactococcus garvieae*	-	Bakf20
13.	*Lactococcus garvieae*	-	Bakf21
14.	*Pseudomonas sp.*	+	Bakf25
15.	*Aeromonas sobria*	+	Bakf34
16.	*Aeromonas sobria*	+	Bakf35
17.	*Aeromonas sobria*	-	Bakf36
18.	*Aeromonas sobria*	-	Bakf37
19.	*Lactococcus garvieae*	-	Bakf38
20.	*Lactococcus garvieae*	+	Bakf39
21.	*Lactococcus garvieae*	-	Bakf40

example, Aes1 displayed the tailless icosahedral morphology typical of Tectiviridae like phages (Bujak et al., 2022), whereas the Lac1 phage exhibited features consistent with Podoviridae and Siphoviridae reported in Lactococcus phages (Ghasemi et al., 2014). Restriction digestion and whole genome sequencing of Pah1 demonstrated its dsDNA genome and GC content comparable to other *Pseudomonas* phages characterized in aquaculture settings (Chen et al., 2023; Jagdale et al., 2019). These morphological and genomic attributes support the classification of the isolated phages as viable candidates for therapeutic applications.

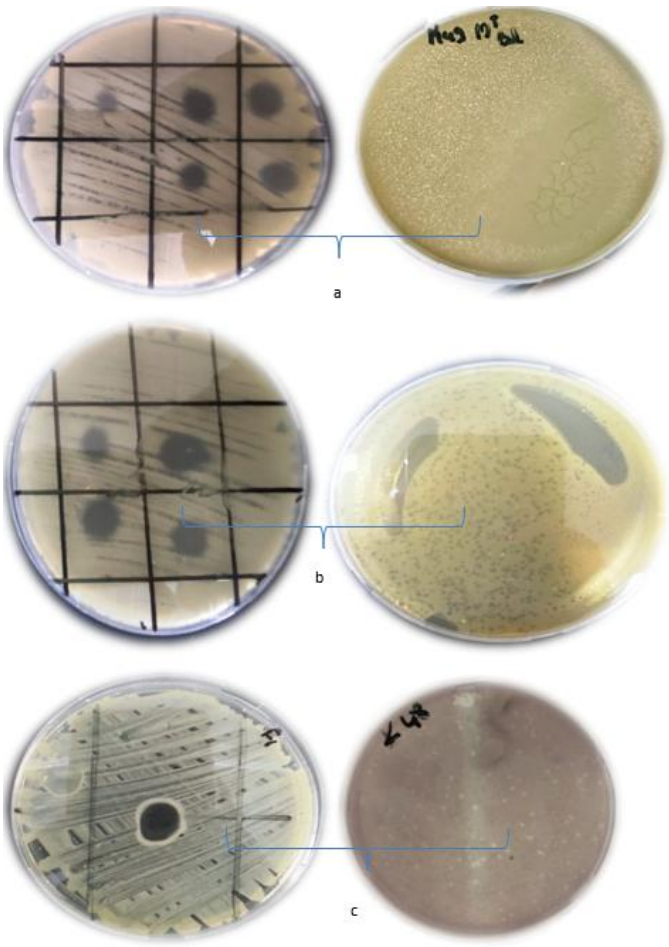

Figure 6. Bacteriophage spot test and double agar assay images (a. *Pseudomonas* sp., b. *A. sobria.* c. *L. garvieae*).

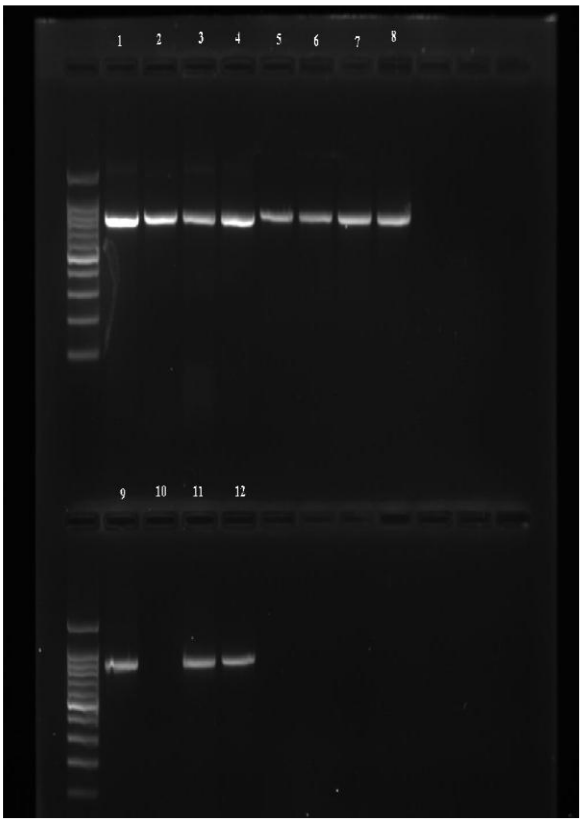

Figure 7. Bacteriophage DNA band images.

Phage safety tests indicated that crude phage preparations caused no adverse clinical responses, consistent with earlier work demonstrating the in vivo safety of bacteriophages in fish, even when endotoxin residues may still be present (Verner-Jeffreys et al., 2007). This justified the development of controlled phage therapy models.

The therapeutic efficacy differed markedly among the three phages. In the Aeromonas model, Aes1 administered 24 hours after infection did not significantly reduce mortality, although treated fish exhibited milder clinical signs. Similar outcome variability has been reported for *Aeromonas* phage therapy, where intramuscular injection models demonstrated strong protection (Kim et al., 2015), yet immersion or intraperitoneal models showed only delayed onset of mortality (Imbeault et al., 2006; Verner-Jeffreys et al., 2007). These findings suggest that timing and route of phage delivery are critical determinants of therapeutic success.

The Lac1 phage displayed partial efficacy, reducing but not preventing mortality following *L. garvieae* challenge. This is consistent with prior studies indicating that lactococcosis, particularly at elevated water temperatures (>16°C), spreads rapidly and may require phage mixtures, repeated dosing, or earlier administration to control infection effectively (Munday et al., 1993; Vendrell et al., 2006). Although earlier research demonstrated successful oral delivery of phage PLgY in yellowtail (Nakai et al., 1999), the different infection dynamics and host–phage interactions observed in trout may explain the lower protective effect observed here.

Among the three phages, Pah1 exhibited the clearest therapeutic potential. Both intraperitoneal and immersion administrations significantly reduced mortality, with immersion proving superior. This finding supports previous reports showing that mucosal exposure enhances phage–pathogen interaction and improves therapeutic efficacy in aquatic species (Park et al., 1997). The consistent outcome across two independent Pah1 trials further strengthens the evidence for its applicability in managing pseudomonad infections, which are often recalcitrant to antibiotic treatment.

Cumulatively, the results emphasize the importance of phage pathogen compatibility, dose optimization, and delivery route. As highlighted in multiple studies (Pereira et al., 2004), host range limitations and rapid bacterial resistance necessitate the development of phage cocktails rather than single phage treatments. The findings of this study support this view: Aes1 and Lac1 alone provided limited therapeutic benefit, whereas Pah1 despite promising efficacy would likely perform even better as part of a multi phage formulation.

Region specific phage banks may be particularly important given the strain level variability of *Aeromonas* and *Pseudomonas* species isolated from Turkish aquaculture (Avsever et al., 2012).

In conclusion, this study demonstrates that Aes1, Lac1, and Pah1 phages are promising biological tools against bacterial pathogens commonly affecting trout in intensive farming systems. While Pah1 showed the strongest therapeutic effect, future work should focus on developing phage cocktails, optimizing dosing schedules, evaluating combined oral–immersion approaches, and testing large scale farm applicability. With further refinement, bacteriophage therapy may become an effective, sustainable, and environmentally sound alternative or complement to antibiotics in modern aquaculture.

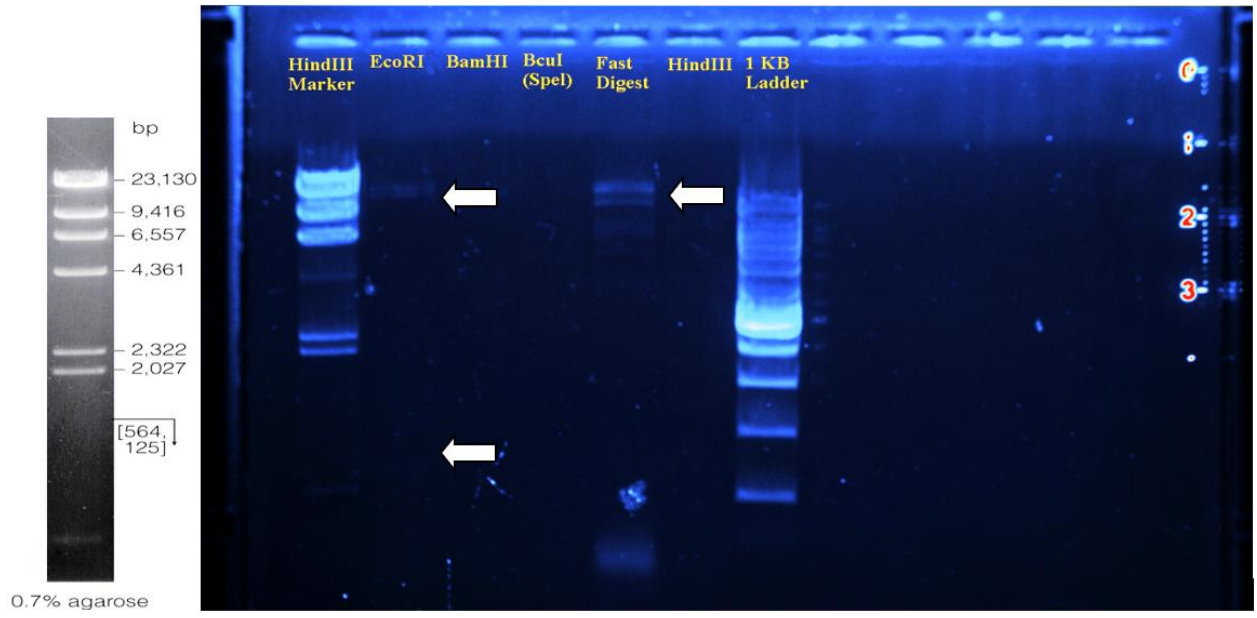

Figure 8. Gel image of *Pseudomonas* sp. phage applied with restriction enzyme.

Conclusions

This study demonstrated the successful isolation and characterization of lytic bacteriophages Aes1, Lac1, and Pah1 targeting *Aeromonas sobria*, *Lactococcus garvieae*, and *Pseudomonas* sp. associated with rainbow trout (*Oncorhynchus mykiss*) aquaculture in Karkamış Dam Lake. In vivo challenge assays confirmed that phage treatments significantly reduced cumulative mortality and delayed time-to-death compared with untreated controls ($P<0.05$), achieving relative percent survival (RPS) values of 25–28%. These results support the applicability of bacteriophage therapy as a targeted biocontrol strategy and a promising alternative to antibiotics in aquaculture systems facing antimicrobial resistance. Further studies focusing on phage cocktail development and optimization of dosing and delivery protocols are warranted to enhance therapeutic performance and sustainability.

Ethical Statement

All animal studies were approved by the Animal Ethics Committee of KSÜZİRHADYEK and Research Institute (Protocol number: 2022/1).

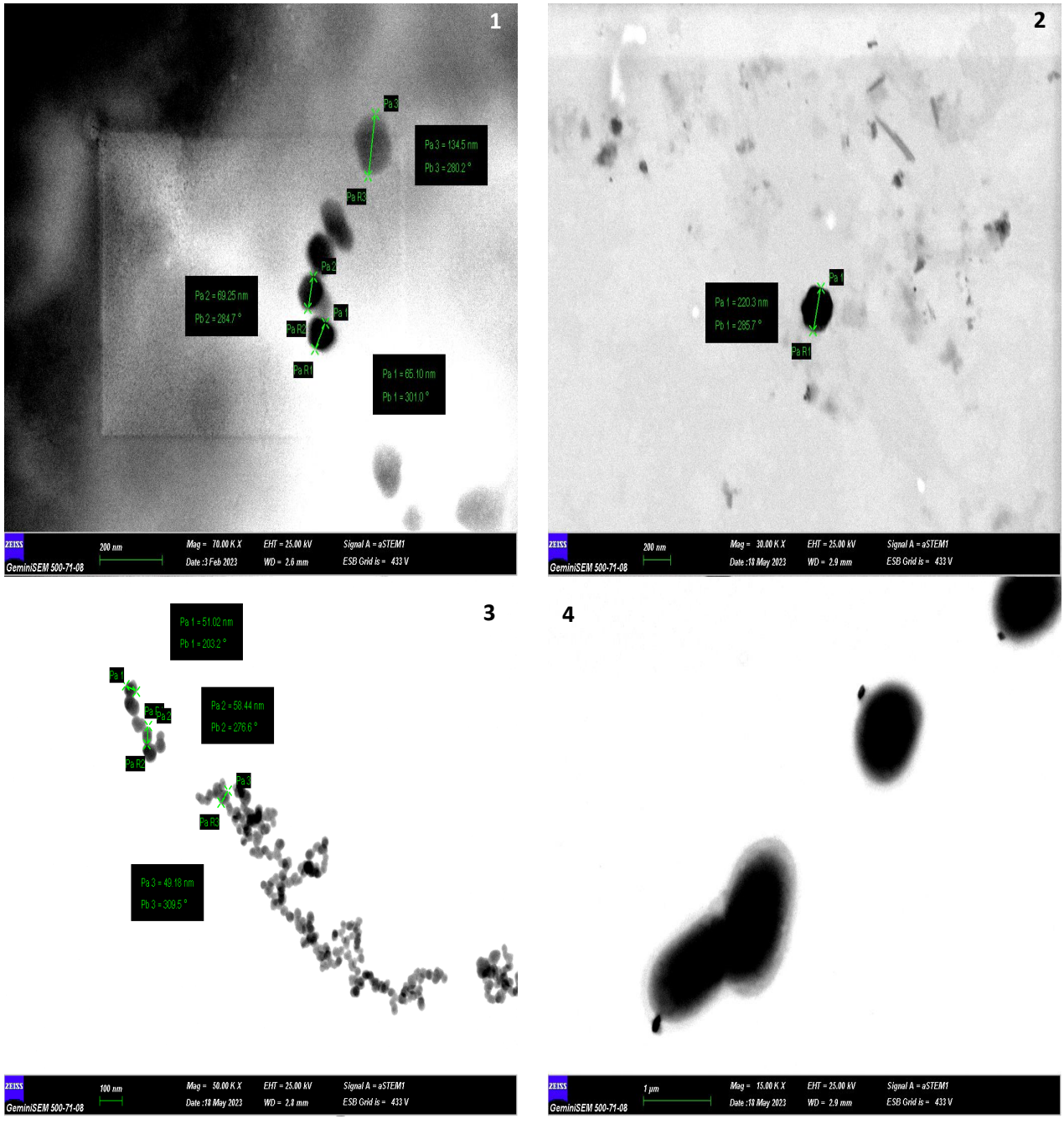

Figure 9. Electron microscope image of phages (1-2: *Pseudomonas* sp. phage, 3: *Aeromonas sobria* phage, 4: *Lactococcus garvieae* phage).

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Author Contribution

Conceived idea and designed experiment: M.Ö.; execution and interpretation of data and preparation of the manuscript and editing: M.Ö. and O.K.; and the authors agreed about the contents of the manuscript and its submission.

Conflict of Interest

The authors have declared that no financial or competing interest exists.

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