Received: 22 March 2019 | Accepted: 25 April 2019

DOI: 10.1111/jfd.13026

ORIGINAL ARTICLE

Journal of Fish Diseases WILEY

Bacteriophage-based cocktail modulates selected immunological parameters and post-challenge survival of rainbow trout (*Oncorhynchus mykiss*)

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Funding information
European Regional Development Fund, Grant/Award Number: POIG.01.04.00–10-098/12

Abstract

Recently, a rapid increase in the resistance of pathogenic bacteria to antibiotics and chemotherapeutics admitted for use in aquaculture has been observed. This happens especially often in intensive breeding. The use of drugs in closed circuits is problematic because it can damage biological filters. Therefore, in recent years, there has been a growing interest in natural methods of combating pathogens. These include bacteriophages. The aim of the study was to determine the safety of the new BAFADOR® bacteriophage-based preparation, its effect on selected immunological parameters and the effectiveness of prophylactic and therapeutic use after experimental infections with pathogenic bacteria *Aeromonas hydrophila* and *Pseudomonas fluorescens*. The use of BAFADOR® increased the activity of lysozyme, total protein level and immunoglobulin level. The level of ceruloplasmin in the rainbow trout serum remained unchanged regardless of the route of administration of the preparation. Potential killing activity and metabolic activity of spleen phagocytes and proliferation of pronephros lymphocytes were higher compared to the control group. Both therapeutic and prophylactic application of the preparation after mixed experimental infection of *A. hydrophila* and *P. fluorescens* limited the mortality of rainbow trout.

KEYWORDS
Aeromonas hydrophila, innate immunity, prophylaxis, *Pseudomonas fluorescens*, therapy

1 | INTRODUCTION

The basis of this study was an investigation of immunity modulation against infection with *Aeromonas hydrophila (A. hydrophila)* and *Pseudomonas fluorescens (P. fluorescens)* following the administration of novel bacteriophage preparation in immersion and in a feed to rainbow trout, as well as checking its prophylactic and therapeutic effectiveness.

During recent years in Polish-controlled fish farming, one of the most commonly diagnosed pathogens causing problems is infections caused by *Aeromonas* spp. (*A. hydrophila*, *A. sobria*, *A. salmonicida* subsp. *Salmonicida* or atypical *A. salmonicida*) and *Pseudomonas* sp. (*P. fluorescens*) (Bernad et al.,2016 ; Terech-Majewska, 2016). Aeromonads and Pseudomonads are characteristically bacteria from an aquatic environment. The motile *Aeromonas* genus includes bacteria considered not only as an important disease pathogen of fish and other ectothermic species but also as an aetiological factor responsible for various infections in humans (Janda & Abbott, 2010). In salmonids, *A. hydrophila* has been isolated from fish with the clinical signs of bacterial fatal haemorrhagic septicaemia and dermal lesions (Zepeda-Velázquez, Vega-Sánchez, Salgado-Miranda, & Soriano-Vargas, 2015). It is also responsible for local haemorrhages in the gills and anal area, fin rot, tail rot, dropsy, scale protrusion, abscesses blisters, exophthalmia and abdominal swelling in different fish

species (Sahu et al., 2011). This bacterium may also appear as a secondary opportunistic pathogen, attacking fish with a compromised immune system or stressed host. Motile *Aeromonas* septicaemia displays chronic characteristics that persist for weeks, during which the mortality rate increases gradually and the cumulative mortality can be high (Zhang, Moreira, Shoemaker, Newton, & Xu, 2016). Among the Pseudomonads, *P. fluorescens* is a dominant component of the freshwater ecosystem and its infection is widespread in aquaculture. It has been reported to cause disease in a wide range of fish species (Saharia & Prasad, 2001). It is associated with fin or tail rot in which the infected area is eroded away, haemorrhage at the anal region and, internally, petechia of each organ. Ascites fluid can accumulate in the peritoneal cavity, as it is a typical generalized bacterial sepsis usually associated with stress or improper health management. It has been reported that mortality can reach up to 90% of the infected population (Austin & Austin, 2016). Some of the recent studies indicate that the increase of the above bacterial species in recirculating aquaculture systems may be related to feeding dose and total fish biomass and increasing water pollution (Gołaś et al., 2019). Currently, many breeders increase the density of fish, so it is essential to prevent the development of the above-mentioned bacteria.

Over the last decades, the problem of selecting therapeutic agents has become more and more serious due to the high frequency of antimicrobial resistance among clinical isolates, among others *P. fluorescens* and *A. hydrophila* (Ginovyan, Hovsepyan, Sargsyan, Grigoryan, & Thrchunyan, 2017; Gutierrez & Barros, 1998; Sørum, 2008; Trivedi, Patil, Shettigar, Gangwar, & Jana, 2015). Maintaining fish health is one of the most important aims of aquaculture. In modern fish farms, large fish populations are kept which is associated with significant economic losses if the contagious disease is not controlled quickly and effectively. Prevention of fish diseases, therefore, is crucial and can be achieved by various strategies, including most often a combination of different methods such as optimal feed and fish density, as well as strengthening the immune system.

As the excessive use of antibiotics and chemotherapeutics for treatment and prevention in intensive aquaculture has been widely criticized for its negative impact, it is important to look for eco-friendly alternatives to antibiotics that can keep fish healthy. Bacteriophages can be an excellent alternative therapy in aquaculture. Phages are commonly found in nature and are considered as a factor limiting the occurrence of bacteria in the environment. The first applications of bacteriophages as a therapy in aquaculture were described in 1981 by Wu, Lin, Jan, Hsu, and Chang (1981) and a year later by Wu and Chao (1982). They checked the effectiveness of this type of therapy against *Edwardsiella tarda* and *A. hydrophila*. Since then, interest in phage therapy in different aquatic species has gained a lot of interest, among others to control columnaris disease, bacterial cold water disease, edwardsiellosis, enteric septicaemia of catfish, vibriosis, furunculosis, *Pseudomonas plecoglossicida* infection, *Pseudomonas aeruginosa* infection, streptococcosis, lactococcosis (Choudhury, Tharabenahalli Nagaraju, Gita, Paria, & Parhi, 2017), *A. hydrophila* and *P. fluorescens* infection (Schulz, Robak, Dastych, & Siwicki, 2019) and others. More and more often, it is mentioned that phages also directly affect immunity, but most of the work concerns mammalian organisms, in particular humans (Van Belleghem, Dąbrowska, Vaneechoutte, Barr, & Bollyky, 2019; Dąbrowska, 2018; Górski et al., 2017, 2012). There is little data confirming impact of phages on fish immune system.

Non-specific defence of fish can be upregulated by administration of various natural or synthetic immunostimulants. Bacterial lipopolysaccharides (LPS) are responsible for the lethal effects and clinical signs of diseases in humans and animals even at low doses. Lower vertebrates, such as fish, are often resistant to endotoxic shock, which allows considering the use of LPS as an immunostimulant which has been confirmed by many authors (Swain, Nayak, Nanda, & Dash, 2008).

This study has sought to extend earlier work (Schulz et al., 2019) by studying similar parameters in another fish species.

2 | MATERIAL AND METHODS

The experiments were carried out in conformity with Animal Protection Law and the recommendations of the Animal Ethics Committee of the University of Warmia and Mazury in Olsztyn. During the experiment, animals were kept in Faculty premises with the observance of adequate experimental conditions.

2.1 | Fish

Ninety rainbow trout (*Oncorhynchus mykiss*) with an average body weight of 110.0 g and an average length of 20.8 cm were used to study immunological parameters. In order to determine the prophylactic and therapeutic capability of the bacteriophage preparation, an additional group of 175 rainbow trout with similar body weight and average length was used. The total length was measured from the front end of the body to the end of the longest radius of the caudal fin. The measurement was taken along a straight line. Individual body weight was measured using an electronic scale RADWAG WPT 6 CF.

Before starting the experiment, the fish showed no signs of a disease and were not vaccinated or exposed to a disease. All fish were fed a commercial diet according to the manufacturer's instructions during the 14 days of acclimatization.

2.2 | Bacteriophage cocktail

In the presented study, the bacteriophage cocktail called BAFADOR® was used. It contained seven bacteriophages: three against *Aeromonas hydrophila* (50AhydR13PP, 60AhydR15PP and 25AhydR2PP) and four against *Pseudomonas fluorescens* (22PfluR64PP, 67PfluR64PP, 71PfluR64PP and 98PfluR60PP).

The animals from the immersion group were subjected to hour bathing in a bacteriophage preparation in concentration 10^5 PFU/ml for an hour. For the fodder group, preparation was mixed with

commercial feed and vacuum-sealed with a vacuum pump (AGA Labor) 1 L/1 kg of fodder and fed on day 0 of the experiment.

2.3 | Experimental project

The rainbow trout has been placed in a closed water recirculating system with a total volume of 2,300 L. The system was equipped with temperature sensors and a UV lamp. The object is composed of a working volume of 180 L basins and a power volume of 300 L. During the experiment temperature, dissolved oxygen level and pH were controlled. The physicochemical conditions were maintained at T 18–20°C, O_2 5–8 mg/L, pH 6.5–7.5. This study was carried out in several stages.

2.3.1 | Immunological tests

Acclimation of animals for immunological tests lasted 14 days. After this period, fish were randomly divided into three equal groups (n = 30):

Control—not treated with BAFADOR®

Immersion—fish subjected to 1-hr bath in BAFADOR® at a concentration of 10^5 PFU/ml

Fodder—fish subjected to single feeding with BAFADOR® in a weight of 2% of body weight.

The material was collected from six randomly selected fish from each group after 1, 7, 14 and 21 days of the experiment. The spleen and pronephros collected for immunoassays were subjected to immediate examination. Immune cells isolated from individuals in each group were paired prior to testing and tested in duplicate. Blood serum was obtained from tail vein and stored at −80°C until analysis.

The following parameters were determined: the proliferative response of pronephros lymphocytes after stimulation with lipopolysaccharide (LPS) or concanavalin A (ConA), as well as metabolic activity and potential killing activity of spleen phagocytes, total protein and total Ig contents, lysozyme and ceruloplasmin activities.

2.3.2 | Therapeutic tests

Acclimation of animals for therapeutic tests lasted 14 days. After this period, fish intended to test the therapeutic effect of BAFADOR® were randomly divided into five groups (n = 20):

1t—negative control: no *A. hydrophila* and *P. fluorescens* infection and not treated with BAFADOR® (injection of PBS),

2t—positive control: placebo, *A. hydrophila* and *P. fluorescens* infection and not treated with BAFADOR®,

3t—test group: BAFADOR® administered in a bath 24 hr after *A. hydrophila* and *P. fluorescens* infection,

4t—test group: BAFADOR® administered in a bath 48 hr after *A. hydrophila* and *P. fluorescens* infection,

5t—test group: BAFADOR® administered in a bath 72 hr after *A. hydrophila* and *P. fluorescens* infection.

Rainbow trout from the uninfected group (1t) received a single intraperitoneal injection of 0.2 ml PBS (negative control). The 2t–5t groups were infected by a single injection of a 48-hr culture of *A. hydrophila* and *P. fluorescens* (0.2 ml/fish at a concentration of 1.5 MF). Then, the fish from 3t to 5t underwent a bath in BAFADOR® at different time intervals. Fish were observed for 14 days to observe disease signs, report mortality and calculate the percentage of survival after challenge. The cause of mortality was confirmed by re-isolating the bacteria from the kidney of dead fish using tryptone soya agar (ThermoFisher Scientific).

2.3.3 | Prophylactic tests

Acclimation of animals for prophylactic tests lasted 14 days. After this period, fish intended for prophylactic testing of BAFADOR® were randomly divided into three groups (n = 25).

1p—negative control: no *A. hydrophila* and *P. fluorescens* infection and not treated with BAFADOR® (injection of PBS),

2p—positive control: placebo, *A. hydrophila* and *P. fluorescens* infection and not treated with BAFADOR®,

3p—test group: BAFADOR® administered in a bath 24 hr before *A. hydrophila* and *P. fluorescens* infection.

Rainbow trout from the uninfected group (1p) received a single intraperitoneal injection of 0.2 ml PBS (negative control). The animals from the 3p group were subjected to 1-hr bathing in a bacteriophage preparation 24 hr before the experimental infection. The 2p and 3p groups were infected by a single injection of a 48-hr culture of *A. hydrophila* and *P. fluorescens* (0.2 ml/fish at a concentration of 1.5 MF). After injection, fish were observed for 14 days to observe disease signs, report mortality and calculate the percentage of survival after challenge. The cause of mortality was confirmed by re-isolating the bacteria from the kidney of dead fish using tryptone soya agar (ThermoFisher Scientific).

2.4 | Evaluation of biochemical parameters

2.4.1 | Protein level

The analysis of total protein levels in serum was based on the Lowry micromethod (Sigma, Diagnostic Kits). Total serum protein (TSP) was measured based on the biuret reaction principles. The total protein reagent (Sigma-Aldrich) was used according to the manufacturer's protocol. The absorbance was read with a spectrophotometer at 540 nm. Double determinations were averaged to calculate average OD values.

2.5 | Evaluation of immunity parameters

2.5.1 | Total Ig level

The spectrophotometric method adapted for fish species by Siwicki and Anderson (1993) was used to determine the total serum immunoglobulin level (T-Ig). The collected supernatant was subjected to

an extinction level test at 540 nm. Mean OD values were calculated by averaging duplicate determinations. Total serum Ig levels were calculated by subtracting supernatant OD values from those of total protein.

2.5.2 | Lysozyme activity

The turbidimetric assay was carried out according to Siwicki and Anderson (1993) to determine serum lysozyme activity. The assay is based upon the ability of lysozyme to lyse the Gram-positive bacterium *Micrococcus lysodeikticus* (Sigma), which is obtained freeze-dried. A solution of *Micrococcus lysodeikticus* in sodium phosphate buffer was mixed with plasma and incubated at 25°C. The absorbance was measured before and after 15 min of incubation in sterile plastic tubes at 450 nm. The standard was hen egg white lysozyme (Sigma). Mean OD values were calculated.

2.5.3 | Ceruloplasmin activity

For the determination of ceruloplasmin activity in the serum, the method described by Siwicki and Anderson (1993) with further modifications was used. Optical density was read immediately at 540 nm. To calculate mean OD values, triplicate determinations were averaged.

2.5.4 | Isolation of leucocytes

Leucocytes for the tests were isolated from the fish spleen and head kidney. The spleen and pronephros of each fish were removed aseptically and pressed through a 60-µm nylon mesh. The cell suspensions were placed on density gradient Gradisol L (Aqua-Medica) in order to isolate lymphocytes and then centrifuged at 400 *g* for 45 min at 4°C. The interface cells were suspended in RPMI-1640 medium containing 10% foetal calf serum (FCS, Sigma-Aldrich) and 1% antibiotic–antimycotic solution (Sigma-Aldrich), then dispensed into 96-well plates, and cultured/incubated at 24°C and used for the following assays.

2.5.5 | RBA, PKA, MTT

The respiratory burst activity of the spleen phagocytes stimulated with oxygen burst activator–phorbol myristate acetate (PMA, Sigma-Aldrich), and the potential killing activity of the spleen phagocytes and the mitogenic response of pronephros lymphocyte were made in accordance with the methodology described in Schulz et al. (2019).

2.6 | Statistical analysis

Mean values and standard deviations from pooled experiments were used for comparisons among groups. Data are reported as means ± *SE*. Student's *t* test was used to determine the significant difference in immunological parameters between the groups. All calculations were determined to be significant at $p < 0.05$.

3 | RESULTS

3.1 | Immunological tests

In order to evaluate the effectiveness of the immune system, the best indicators were chosen which are important for defending against harmful factors.

Comparisons of the innate cellular defence mechanism in rainbow trout are shown in Table 1. The analysis of the results showed that metabolic activity (RBA) of spleen phagocytes was increased in both groups for 2 weeks time. Potential killing activity (PKA) of spleen phagocytes of rainbow trout was higher for 14 days in the immersion group. In the fodder group, stimulation was weaker and lasted only for 7 days. Pronephros lymphocyte proliferation (MTT)

TABLE 1 Metabolic and potential killing activity of spleen phagocytes and on pronephros lymphocyte proliferation stimulated by ConA or LPS of rainbow trout after BAFADOR® administration (*$p < 0.05$)

		Experimental day			
		1	7	14	21
Parameter	Group	Mean ± *SD*	Mean ± *SD*	Mean ± *SD*	Mean ± *SD*
RBA	C	0.284 ± 0.03	0.290 ± 0.05	0.293 ± 0.08	0.288 ± 0.03
	I	0.410 ± 0.05*	0.348 ± 0.04*	0.363 ± 0.08*	0.314 ± 0.03
	F	0.319 ± 0.04*	0.352 ± 0.06*	0.313 ± 0.03*	0.301 ± 0.06
PKA	C	0.384 ± 0.02	0.272 ± 0.02	0.326 ± 0.07	0.297 ± 0.01
	I	0.526 ± 0.07*	0.371 ± 0.02*	0.366 ± 0.06*	0.321 ± 0.02
	F	0.399 ± 0.06*	0.335 ± 0.02*	0.309 ± 0.05	0.292 ± 0.04
MTT-ConA	C	0.238 ± 0.03	0.319 ± 0.09	0.246 ± 0.02	0.258 ± 0.04
	I	0.375 ± 0.08*	0.368 ± 0.04*	0.349 ± 0.03*	0.279 ± 0.03
	F	0.431 ± 0.06*	0.434 ± 0.06*	0.277 ± 0.05	0.264 ± 0.04
MTT-LPS	C	0.254 ± 0.04	0.239 ± 0.03	0.267 ± 0.03	0.255 ± 0.03
	I	0.394 ± 0.04*	0.289 ± 0.02*	0.287 ± 0.03	0.246 ± 0.06
	F	0.285 ± 0.04*	0.275 ± 0.06*	0.278 ± 0.04	0.256 ± 0.04

stimulated by LPS was elevated for 7 days time in both experimental groups. MTT stimulated by ConA in the immersion group was lower but lasted longer, that is a week longer than in the fodder group.

Lysozyme activity in rainbow trout serum in the fodder group was elevated after 24 hr and increased for 7 days, and then began to slowly decrease. In the group where bacteriophage cocktail was administered in immersion, the situation was similar, except that the increase in this parameter occurred later but was stronger (Figure 1).

The total protein level in rainbow trout serum decreased in the first day of the experiment in the immersion group. A statistically significant increase in this parameter was noticed after 2 weeks in both experimental groups, of which the fodder group remained elevated also after 3 weeks (Figure 2).

The total immunoglobulin level was increased in all experimental groups during the experiment compared to the control group (Figure 3).

Ceruloplasmin level did not show any changes during this experiment compared with any experimental group (Figure 4).

3.2 | Therapeutic tests

Application of BAFADOR® by immersion 24 hr after experimental infection with *A. hydrophila* and *P. fluorescens* reduced the mortality by 25%. Delay of therapy by another day caused a decrease in survival for another 5%. With the therapy applied 72 hr after the infection, the survival rate decreased to 55% which means only 10% survival difference from the non-treated group (Figure 5).

3.3 | Prophylactic tests

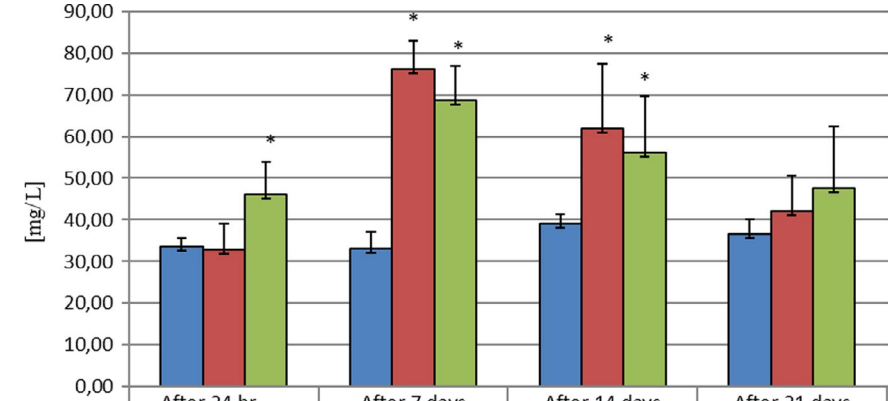
The cumulative survival percentage of rainbow trout after prophylactic use of BAFADOR® 24 hr before the experimental infection with *A. hydrophila* and *P. fluorescens* is shown in Figure 6. Fish that had contact with bacteriophage preparation prior to infection showed a 36% higher survival rate than animals that BAFADOR® was not administered at any time.

4 | DISCUSSION

Fish are the main source of animal protein in many countries, leading to the rapid development of aquaculture. Stress and diseases that accompany intensification led to increased demand for treatment with the help of antibiotics and chemicals. The main use of bacteriophages is the treatment of antibiotic-resistant bacterial infections because their natural hosts are bacterial cells. A strong argument for the safety of phage therapy is the high specificity of the antibacterial activity of phages. Phages kill only bacteria from certain strains or subspecies. Therefore, unlike antibiotics, they are less likely to disturb the balance of bacterial microflora. However, there have already been studies showing the possibility of bacteriophage interactions with some eukaryotic cell populations (Duerkop & Hooper, 2013). These are mainly cells of the immune system associated with both innate and adaptive immunity, including antibody production, T- and B-cell proliferation, phagocytosis and respiratory phagocytic cells, as well as cytokine production (Olszowska-Zaremba, Borysowski, Dabrowska, & Górski, 2012). However, these tests mostly concern human organisms (Van Belleghem et al., 2019; Dąbrowska, 2018; Górski et al., 2017, 2012) and the information on the interactions of phages with fish cells and how phages contribute to health and disease is limited.

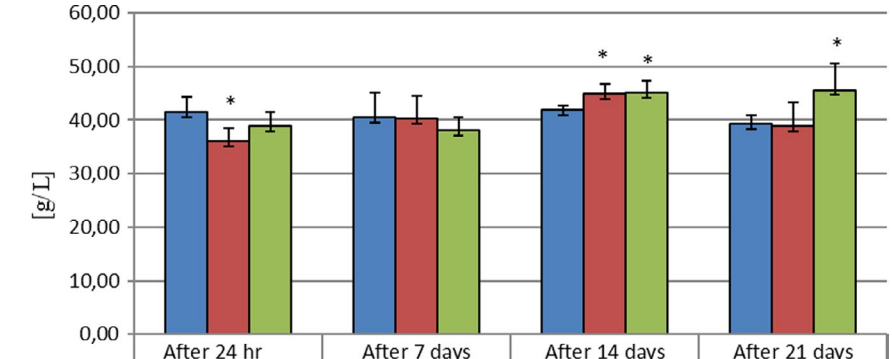
Although there are many reports on the effect of LPS on rainbow trout, there are no data on the effect of the combination of two types of LPS originating from *A. hydrophila* and *P. fluorescens*. What's more, for the first time we described the effect of bacteriophage cocktail on the rainbow trout immune system, demonstrating its high effectiveness in combating pathogenic bacteria.

Many authors chose to determine non-specific immunity in fish by determining the respiratory burst activity and potential killing activity of phagocytes (Bulfon, Galeotti, & Volpatti, 2018; Lundén, Lilius, & Bylund, 2002; Terech-Majewska et al., 2016; Terech-Majewska, Schulz, & Siwicki, 2015). Nya and Austin (2010) reported an enhanced phagocytic activity of head kidney macrophages by feeding rainbow trout with *A. hydrophila* LPS. The analyses of our



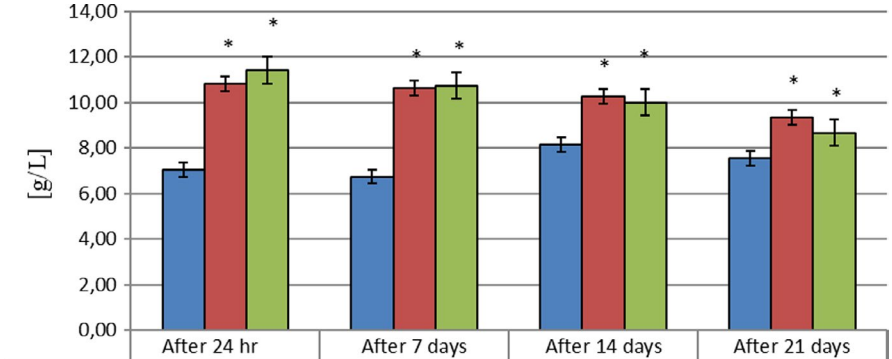
	After 24 hr	After 7 days	After 14 days	After 21 days
Control	33,47	33,07	39,08	36,57
Immersion	32,78	76,13	61,93	42,17
Fodder	46,04	68,63	56,23	47,63

FIGURE 1 Lysozyme activity in rainbow trout serum after BAFADOR® administration (*$p < 0.05$)



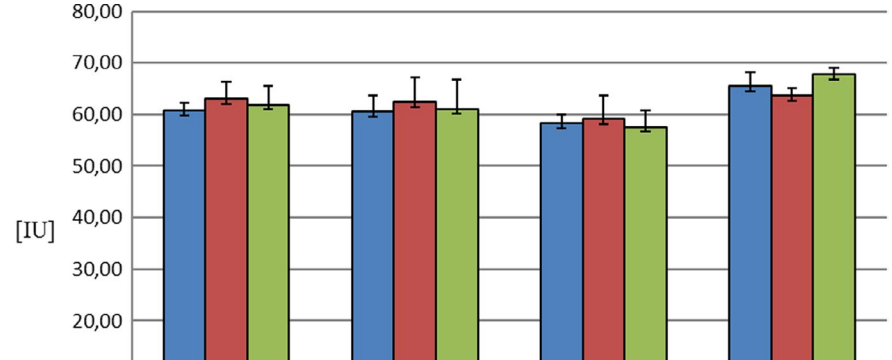
	After 24 hr	After 7 days	After 14 days	After 21 days
Control	41,45	40,43	41,93	39,30
Immersion	36,02	40,20	44,88	38,92
Fodder	38,88	38,12	45,00	45,60

FIGURE 2 Total protein level in rainbow trout serum after BAFADOR® administration (*$p < 0.05$)



	After 24 hr	After 7 days	After 14 days	After 21 days
Control	7,03	6,73	8,15	7,53
Immersion	10,80	10,63	10,25	9,33
Fodder	11,40	10,73	10,00	8,67

FIGURE 3 Immunoglobulin level in rainbow trout serum after BAFADOR® administration (*$p < 0.05$)



	After 24 hr	After 7 days	After 14 days	After 21 days
Control	60,77	60,48	58,35	65,57
Immersion	63,00	62,33	59,12	63,70
Fodder	61,90	61,08	57,58	67,70

FIGURE 4 Ceruloplasmin level in rainbow trout serum after BAFADOR® administration (*$p < 0.05$)

results showed that the phagocytic ability (RBA) was significantly higher in rainbow trout both in the immersion and in the fodder groups for 2 weeks, compared to control fish. The potential killing activity (PKA) of spleen phagocytes was elevated in both experimental groups, but in the immersion group, the stimulation lasted longer (Table 1). Similar stimulation was obtained in the proliferative response of pronephros lymphocytes stimulated by concanavalin A, but lipopolysaccharide stimulated this parameter for a shorter

period of time (Table 1). Other authors also pointed to the role of LPS as a strong immunostimulant and mitogen of B lymphocytes (Nakanishi & Iwama, 1996) and in particular as a stimulant of fish leucocytes (Clem, Sizemore, Ellsaesser, & Miller, 1985). We observed a longer stimulation after using BAFADOR® in immersion in the case of PKA and ConA stimulated MTT, which may be caused by uneven and insufficient feed intake in the feed group. Such a result could also be affected by the smaller amount of BAFADOR® available in the feed than during the 1-hr bath. In the case of a single administration of bacteriophage-based preparation in European eel (Schulz et al., 2019), the stimulation lasted for a longer period reaching even 3 weeks of time. BAFADOR® is intended for administration by immersion, so the method of oral administration is only experimental and requires further investigation. Both administration routes are an excellent way to prevent and treat fish without introducing stress in additional manipulations.

The serum lysozyme level or activity is an important index of innate immunity of fish (Nya & Austin, 2010). Various authors indicate that it plays multiple roles in fish immunity (Kolman, Kolman, Siwicki, Szczepkowski, & Szczepkowska, 2000). It is well documented that fish lysozyme possesses lytic activity against bacteria hydrolysing peptidoglycans in cell walls. Depending on the species of fish, mode of administration and the dose of endotoxin, the effect on lysozyme activity may vary. It was noted that it may increase or decrease this parameter (Belleghem et al., 2019). Our results indicate stimulation of lysozyme activity in rainbow trout after contact with BAFADOR® in the form of both immersion and feed (Figure 1). In the fodder group, lysozyme activity was raised after 24 hr, while in the group in which BAFADOR® was given in immersion, the change in activity was noticed until after 7 days. This may have been due to the stress induced by manipulations necessary to carry out the bath. Möck and Peters (1990) showed that lysozyme activity in rainbow trout could be dependent on stress factors. They assume that changes in lysozyme activity reflect the sensitivity and modulation of the defence system. Less stressful situations can both increase or decrease lysozyme activity. In the case of European eel (Schulz et al., 2019), the situation was reversed. After 24 hr from BAFADOR® contact, the immersion group reacted, but not the fodder group, and lysozyme activity was raised for a longer time than in the case of rainbow trout. This is most likely related to species differences.

Serum proteins can be separated into four basic fractions including albumin, α-, β- and γ-globulins. Proteins are involved in almost all of the reactions occurring in the organism. One of the most important functions of the total proteins is a defence against infection (the accumulation of antibodies reacting to an antigen of bacterial or viral origin) (Řehulka, Minařík, Adamec, & Řehulková, 2005). In our studies, the level of immunoglobulin in rainbow trout serum was increased during the whole experiment in both experimental groups, reaching a maximum value at day 1 (Figure 3). In the case of European eel, the total immunoglobulin level progressively increased in all experimental groups for 2 weeks compared with the control (Schulz et al., 2019). The results of the serum total protein level (Figure 2) reached the highest values in both experimental

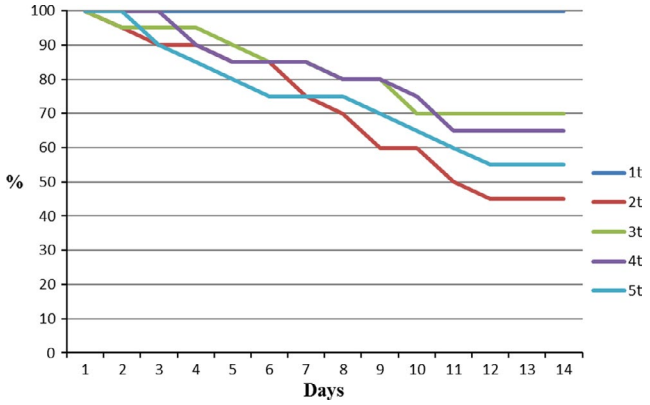


FIGURE 5 The effect of BAFADOR® application on the survival rate of experimentally infected Rainbow trout

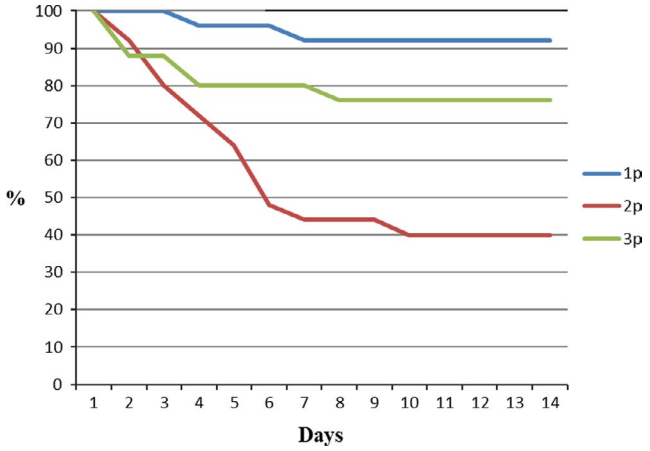


FIGURE 6 The prophylactic effect of BAFADOR® application on the survival rate of experimentally infected rainbow trout

groups after 14 days. This may be due to the formation of antibacteriophage antibodies. Total protein level in European eel serum after a single application of BAFADOR® was slightly increased for seven days in the immersion group, unlike the fodder group where no change occurred (Schulz et al., 2019). The reason may be that different species of fish react differently to bacterial lipopolysaccharide (Kolman et al., 2000). Some authors have pointed to the role of LPS as increasing the serum total protein, albumin and globulin (Wiegertjes, Stet, Parmentier, & Muiswinkel, 1996); however, others (Nayak, Swain, Nanda, Mohapatra, & Behera, 2011) did not observe a significant difference in the total protein and globulin content after LPS administration.

Ceruloplasmin is an acute-phase protein synthesized in the liver and is found to be activated by the host immune system during stress conditions. The role of ceruloplasmin is similar to that of interferon and transferrin. It inhibits bacterial development by depriving it of essential nutrients, that is copper ions (Alexander, 1985). It is also involved in oxidative protection (Kushner & Mackiewicz, 1993). Analyses of ceruloplasmin level in European eel were slightly elevated for 2 weeks in fish which were subjected

to bath in the solution of bacteriophage-based preparation. There was no change in this parameter in BAFADOR®-coated fodder group compared with the control (Schulz et al., 2019). In the case of our research on rainbow trout, the level of ceruloplasmin was not increased in any of the groups. It may be related to species differences and different sensitivity to both bacteriophages and LPS contained in the preparation.

In 2016, the most frequently isolated bacterium from fish with a clinical form of the disease in Poland was *P. fluorescens* (28.2% of the total number). It was isolated mainly from rainbow trout in the first year of rearing. The second in sequence bacterium isolated from diseased fish was *A. hydrophila complex* (26% of the total number), also mainly from rainbow trout in the initial period of rearing (Bernad, Terech-Majewska, Pajak, Schulz, & Siwicki, 2017). In the previous year (Bernad, Terech-Majewska, Pajdak, Schulz, & Siwicki, 2016), studies showed 32% of infections caused by *P. fluorescens*, and 25.4% of *A. hydrophila complex* infections also mainly in salmonids. These bacteria often cause mixed infections. The biggest losses are caused by the sudden development of the disease in young fish, so it is very important to have the possibility of introducing quick therapy. In this study, the highest cumulative survival rate was recorded in the 3t group, where BAFADOR® was administered in the shortest possible time from experimental infection with a mixture of bacteria, that is after 24 hr (Figure 5). It was 25% higher compared to the 2t group after 14 days from infection. Each subsequent delay in administration resulted in a decrease in survival by another 5%. In the case of the therapeutic use of BAFADOR® in the European eel (Schulz et al., 2019), a 40% higher survival rate than the infected control was obtained, and delaying therapy for every next 24 hr resulted in a 10% decrease in survival.

Immunostimulants are commonly used in fish culture. For this purpose, bacterial LPS have also been used many times. Nya and Austin (2010) examined the use of orally administered bacterial LPS for the prevention of infection by *A. hydrophila* in rainbow trout reaching 89% survival rate. In previous studies of European eel (Schulz et al., 2019), the authors received survival rate not differing from the control (98%) after the one-time use of the BAFADOR®, which indicates that bacteriophage-based preparation showed prophylactic activity against *A. hydrophila* and *P. fluorescens* infections. The results of these studies have shown that it is possible to get a better survival of rainbow trout after prophylactic use of BAFADOR® 24 hr before the experimental infection with *A. hydrophila* and *P. fluorescens*. Fish that had contact with bacteriophage-based preparation prior to infection showed a 76% survival rate. It has a lower survival rate than in European eel experiment in the same conditions, but it is still a result showing that prophylactic use of BAFADOR® allows to significantly reduce economic losses in breeding after the occurrence of infection.

Fish live surrounded by microorganisms; therefore, constant and successful discrimination between commensal and pathogenic bacteria is important for proper development. This is a very important problem also in breeding. Due to the very high specificity, the therapy and prophylaxis carried out with the help of bacteriophages are characterized by a high degree of safety. The results of our research indicate the possibility of using bacteriophage preparations as immunostimulants.

5 | CONCLUSIONS

BAFADOR® can stimulate a non-specific immune system in fish that is responsible for combating bacterial infections. BAFADOR®, the new bacteriophage-based preparation dedicated for fighting with fish bacterial pathogens, fulfils its role as a prophylactic and therapeutic preparation limiting the rainbow trout death caused by a mixed infection with *Aeromonas hydrophila* and *Pseudomonas fluorescens*. It should be taken into account as an alternative for antibiotics to maintain fish health.

ACKNOWLEDGEMENT

Research was funded by Project POIG.01.04.00-10-098/12.

CONFLICT OF INTEREST

The authors state that there are no conflicts of interests to declare regarding the presented work.

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How to cite this article: Schulz P, Pajdak-Czaus J, Robak S, Dastych J, Siwicki AK. Bacteriophage-based cocktail modulates selected immunological parameters and post-challenge survival of rainbow trout (*Oncorhynchus mykiss*). *J Fish Dis*. 2019;00:1–10. https://doi.org/10.1111/jfd.13026