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Influence of bacteriophages cocktail on European eel (*Anguilla anguilla*) immunity and survival after experimental challenge

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ABSTRACT

Inland fishery belongs to those branches of animal production that use very large amounts of chemotherapeutics, in particular antibiotics. The accumulation of chemotherapeutic agents in bottom sediments is a direct threat to the aquatic environment and directly affects the condition and health of the fish. Finding a preparation that could be used both prophylactically to increase the resistance of fish and therapeutically in case of infection with pathogenic bacteria, without side effects for fish and aquatic environment could be a great solution to this problem. Our aim was to determine influence of BAFADOR® the new bacteriophage-based preparation on European eel immunity and survival after experimental challenge. Application of BAFADOR® increased total protein level, immunoglobulin level, lysozyme activity and ceruloplasmin level in European eel serum. Potential killing activity and metabolic activity of spleen phagocytes as well as pronephros lymphocyte proliferation of was higher compared to control. The preparation also reduced mortality after experimental infections with the pathogenic bacteria *Aeromonas hydrophila* and *Pseudomonas fluorescens*. Our results showed that preparation BAFADOR® is well tolerated by the fish organism causing stimulation of cellular and humoral immunity parameters and reduces the mortality of the European eel after experimental challenge.

1. Introduction

Aquaculture is one of the fastest growing food-production sectors in the world nowadays. The global demand for aquaculture products is driven by the growing world population, stagnation in the procurement of fishery products and the growing awareness of the positive impact of fish consumption on human health. Currently, aquaculture is a lucrative industry. However, the intensification of this type of production requires breeding at high densities, which promotes the occurrence of diseases, including infectious ones. Along with the increase in restocking, the number of pathogens is also growing, which increases the risk of epidemics. Fish infectious diseases are considered one of the main limiting factors in aquaculture [1]. To protect fish health the hygienic plans of livestock farms were developed as a permanent part of the production procedures. As the basis for their implementation the laboratory diagnostics of pathogenic agents is used, within the so-called ownership supervision. In developed hygienic programs, the most necessary to maintain good fish health and immunity is high-quality fodder, repeated disinfection or periodic metaphylaxis with antibiotics or sulfonamides, vaccination, and eradication of infected populations [2]. However, the usage of chemotherapeutics generates significant risks to public health by promoting the selection, propagation, and persistence of bacterial-resistant strains. Inland fisheries belong to those branches of animal production that use very large quantities of chemotherapeutics, especially antibiotics. Contamination with antibiotics has been detected almost everywhere in the world. Their accumulation in bottom sediments poses an immediate threat to the aquatic environment and directly affects the condition and health of fish. Antibiotics not only destroy the target bacteria but also damage external microorganisms and disrupt the ecological balance of water environment [3]. The alarming phenomenon observed in recent years is the dramatic increase in the bacterial resistance to antibiotics and other chemotherapeutics approved for use in fisheries.

The most commonly diagnosed pathogens that cause problems in controlled fish farming in Poland are infections caused by *Aeromonas spp*. (*A. hydrophila*, *A. sobria*, *A. salmonicida subsp. salmonicida* or atypical *A. salmonicida*) and *Pseudomonas sp*. (*P. fluorescens*) [2,4].

Aeromonas hydrophila is a Gram-negative bacterium. The *Aeromonas* genus is widespread in the environment and causes many different diseases in fish. These include epizootic ulcerative syndrome and fatal

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hemorrhagic septicemia that are manifested by external symptoms like local hemorrhages in the gills and anal area, blisters, dropsy, scale protrusion, abscesses, exophthalmia, fin rot, tail rot and abdominal swelling. It has an adverse effect on all important organs that may lead to high mortality in many species of fish [5]. This bacterium may also appear as a secondary opportunistic pathogen, attacking fish with a compromised immune system or stressed hosts [6]. The therapeutic agent must, therefore, regulate the severe inflammatory immune response while promoting the elimination of the pathogen by the immune system.

Pseudomonas fluorescens is a common Gram-negative bacterial pathogen to a wide range of aquaculture animals including various species of fish. Generally, *P. fluorescens* is associated with fin or tail rot and ulcerative conditions in which the infected area is eroded away [7] but is also an infectious factor of hemorrhagic septicemia which is clinically very similar to motile aeromonad septicemia. *Pseudomonas* infection can lead to the appearance of red skin disease throughout the year. Its development comes especially when the body surface is damaged by improper handling or injury during transport. Also, stress resulting from the inadequate water parameters or overcrowding may trigger outbreaks of disease. Because of the lack of effective control measures, this often leads to high mortality, resulting in significant losses [8].

Due to the high frequency of resistance to antimicrobials among clinical isolates of *P. fluorescens* and *A. hydrophila*, causes serious problems in selection of appropriate antimicrobials Antibiotic resistance occurring in pathogens creates a global health problem that is aggravated by the abuse of antibiotics, horizontal gene transfer and the evolution of bacteria. Epidemiologists warn that it is necessary to develop new antimicrobial therapies. One of the alternatives are bacteriophages (phages), highly specified viruses that kill bacteria.

Bacteriophages use unique mechanism of action, based on the recognition of specific molecules on the target bacterial host surface what fits into the 'novel mode of action' concept desired for all new antibacterial agents [9]. Bacteriophage specificity means a bacteriophage can infect only certain bacteria bearing receptors to which they can bind, which in turn determines the phage's host range. Bacteria that do not have such receptors can't be attacked [10,11]. Phage use as therapeutics of infectious diseases of animals and people refers to the times before antibiotic therapy [12,13]. After the discovery of antibiotics, interest in this type of therapy weakened. The ability of phages to kill bacterial cells is the basis of the idea of using them as therapeutic agents. The lytic phages seem to be the most suitable candidates for therapy due to their effectiveness (rapid multiplying leads to bacterial lysis and the exponential growth of their number) and safety (limited possibility of horizontal gene transfer). There are many reports in various species about the antibacterial activity of phages, but there is little information about their effect on the immune system. Although in recent years there have been reported about the interaction of bacteriophages with the immune system, they mainly concern people [14,15]. There is no data on this subject in the field of aquaculture.

Phage preparations used for phage therapy may have not only direct antibacterial action but also immunomodulating effects mediated by phages themselves as well as by bacterial antigens. Bacterial lipopolisacharides (LPS), also known as endotoxins are the wall component of most of the Gram-negative bacteria. LPS constitutes a very troubling contaminant of crude phage lysates produced in Gram-negative bacteria. Higher animals are very sensitive to endotoxic shock, but fish are stated to be resistant. There are several reports confirming immunomodulatory effect of LPS in different fish species [16,17].

In this study, we evaluated the response of European eel to contact with lipopolysaccharide and bacteriophages contained in the preparation BAFADOR®, through the monitoring of different immunological parameters, as well as checking its prophylactic and therapeutic effectiveness.

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

The experimental material comprised of 90 European eels (*Anguilla anguilla*) used for immunological tests with the 112 g average body mass and 37,7 cm average length, obtained from Department of Ichthyology, Institute of Inland Fisheries in Olsztyn, Poland. A separate group of eels (175) with average body mass 7,7 g and 13,4 cm average length was used for experimental infection to determine the prophylactic and therapeutic effects of the preparation. The fish had not been vaccinated nor exposed to disease and were healthy without any symptoms of infection. All the fish were fed the basal diet during two weeks of acclimatization.

2.2. Bacteriophage cocktail

In the presented study, bacteriophage cocktail called BAFADOR® containing bacteriophages: 3 against *Aeromonas hydrophila* (50AhydR13 PP, 60AhydR15 PP, 25AhydR2PP) and 4 against *Pseudomonas fluorescens* (22PfluR64 PP, 67PfluR64 PP, 71PfluR64 PP, 98PfluR60 PP) were used. For Fodder group preparation was mixed with commercial feed and vacuum sealed with a vacuum pump (AGA Labor) 1 l/1 kg of fodder.

2.3. Experimental design

The fish were kept in a closed water circulating system with a total volume of 2300 L, equipped with temperature sensors and the UV lamp. The circuit consists of basins with a working size of 180 L, a compensatory volume of 200 L and a power volume of 300 L. During the experiment, the temperature, the level of dissolved oxygen and pH have been controlled. The fish were fed a commercial fodder using the automatic band feeders in an amount suitable for body weight and temperature. Physicochemical conditions were maintained at: T 18–20 °C, O_2- 5–8 mg/l, pH 6,5–7,5. This study was conducted in several steps.

2.3.1. Experiment I

After 14 days of acclimation, the animals for immunological tests were randomly divided into three equal groups (n = 30):

Control - not treated with BAFADOR®
Immersion - fish subjected to 1-h bath in BAFADOR® at a concentration 10^5 PFU/mL
Fodder - fish subjected to feeding with BAFADOR® feed in a weight of 2% of body weight

Six fish of each group were sampled on day 1, 7, 14 and 21 of the experiment. Blood samples were collected from a caudal vein for blood serum and stored in −80 °C until analysis. Pronephros and spleen isolated for immunoassays were subjected to testing immediately. The immune cells isolated from the individuals within each group were pooled in pairs before performing the assays and tested in duplicate.

The following parameters were determined: total protein and total-Ig-contents, lysozyme and ceruloplasmin activities, 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. Additionally, the level of cortisol and glucose as stress indicators were examined.

2.3.2. Experiment

After 14 days of acclimation, the fish intended to test the therapeutic effect of BAFADOR® were divided into 5 groups (n = 20):

1t - non-infected and not treated with BAFADOR®, injected with PBS
2t - infected and not treated with BAFADOR®
3t -infected and treated with BAFADOR® in immersion 24 h post-infection
4t - infected and treated with BAFADOR® in immersion48 h post-infection
5t - infected and treated with BAFADOR® in immersion 72 h post-infection

Fish from infected groups (2t-5t) were given a single intraperitoneal injection of a 48 h cultures of *A. hydrophila and P. fluorescens* (0.2 mL/fish at a concentration of 1.5 MF). The fish from the uninfected group received intraperitoneal injection of 0.2 mL PBS (negative control). The fish were observed for 14 days for mortality and post-challenge survival percentage was calculated. The cause of mortality was confirmed by re-isolating the bacteria from the kidney of dead fish using tryptone soya agar (ThermoFisher Scientific, Poland).

2.3.3. Experiment III

After 14 days of acclimation fish intended for prophylactic testing of BAFADOR® were divided into 3 groups (n = 25).

1p - non-infected and not treated with BAFADOR®, injected with PBS
2p - infected and not treated with BAFADOR®
3p - infected and treated with BAFADOR® in a bath 24 h pre-infection

Fish from infected groups (2p and 3p) were given a single intraperitoneal injection of a 48 h culture of *A. hydrophila and P. fluorescens* (0.2 mL/fish at a concentration of 1.5 MF). The fish from the uninfected group (1p) received intraperitoneal injection of 0.2 mL PBS and served as a negative control. Group 2p had no previous contact with bacteriophages and served as a positive control. The animals from the 3p group were subjected to1 hour bathing in a bacteriophage preparation 24 h before the experimental infection. Mortality was tabulated and post-challenge survival percentage was calculated in each group. The presence of pathogens was confirmed by isolation from the kidney of dead or moribund fish using tryptone soya agar (ThermoFisher Scientific, Poland).

2.4. Evaluation of biochemical parameters

2.4.1. Cortisol level

Serum cortisol levels were determined using the enzyme immunoassay (Nova Tec ImmunodiagnosticaGmbh, Germany).

2.4.2. Glucose level

Glucose levels were determined with a set of reagents for enzymatic, colorimetric determination of this parameter in serum (PTH hydrex, Poland).

2.4.3. Protein level

Analysis of total protein levels in serum was based on the Lowry micro method (Sigma, Diagnostic Kits). Total serum protein (TSP) was measured with the standard biuret reaction. 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 total serum immunoglobulin level (T-Ig) was measured using the spectrophotometric method adapted for fish species by Siwicki & Anderson [18]. The level of extinction in the collected supernatant was determined at 540 nm. Mean OD values were calculated by averaging duplicate determinations. Total serum Ig levels were calculating by subtracting supernatant OD values from those of total protein.

2.5.2. Lysozyme activity

The lysozyme activity in plasma was measured by turbidimetric assay [18]. The assay is based upon the lysis of the lysozyme-sensitive 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

Ceruloplasmin activity in the serum was determined according to Siwicki and Anderson with further modifications [18]. Optical density was read immediately at 540 nm. To calculate mean OD values, triplicate determinations were averaged.

2.5.4. Isolation of leukocytes

The spleen and pronephros of each fish were removed aseptically and pressed through a 60 μm nylon mesh. Single cell suspensions were obtained for isolating individual cells using Gradisol L (Aqua-Medica, Łódź, Poland) gradients, as described by Siwicki and Cossarini-Dunier [19]. Cells were suspended in RPMI-1640 medium containing 10% fetal 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. Respiratory burst activity

The respiratory burst activity (RBA) of the spleen phagocytes stimulated with oxygen burst activator -phorbol myristate acetate (PMA, Sigma-Aldrich) was measured using a modified Secombes method [20] as described in Siwicki et al. [21]. Briefly, aliquots of 100 μL containing 1×10^4 cells mL^{-1} in RPMI-1640 medium (Sigma-Aldrich) were added to 96-well microtiter plates (Sarstedt) and incubated for 2 h at 24 °C. After incubation, the non-adherent cells were removed by rinsing with fresh RPMI-1640 medium. The medium was then substituted with 100 μL of RPMI and 100 μL of NBT (nitro blue tetrazolium) solution (Sigma-Aldrich) both with and without additional PMA. The plates were incubated for 30 min at 24 °C. The medium with NBT was removed and the wells were washed twice with ethanol. The blue formazan produced in cells was solubilized in 120 μL of 2 M KOH and 140 μL DMSO (Dimethyl sulfoxide, Sigma-Aldrich). The optical density of the solution was measured colorimetrically in a Sunrise absorbance reader (Tecan, Austria) at 620 nm. The data are expressed as mean values of duplicate determinations.

2.5.6. Potential killing activity

The potential killing activity (PKA) of the spleen phagocytes was measured with the Rook technique [22] modified by Siwicki and Anderson [18]. Briefly, aliquots of 100 μL containing 1×10^4 cells mL^{-1} in RPMI-1640 medium were added to 96-well microtiter plates (Sarstedt) and incubated for 2 h at 24 °C. After incubation, the non-adherent cells were removed by rinsing with fresh RPMI-1640 medium. The cells were activated using 100 μL of 0.2% NBT solution in PBS containing live *A. hydrophila* (1×10^8 $cells^{mL-1}$) and incubated for 30 min at 24 °C. After incubation, the supernatant was removed and the wells were rinsed two times with ethanol, and then 120 μL of 2 M KOH and 140 μL

of DMSO were added to dissolve the formazan. The amount of extracted reduced NBT was measured colorimetrically in a Sunrise absorbance reader (Tecan, Austria) at 620 nm. The data are expressed as mean values of duplicate determinations.

2.5.7. Proliferative response of pronephros lymphocytes

Pronephros lymphocyte proliferation was determined by MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay according to Mosmann [23] with the modifications described by Siwicki et al. [24]. Mitogens concanavaline A (ConA, Sigma) or lipopolysaccharide (LPS, Sigma) were used to stimulate lymphocytes. Isolated lymphocytes were suspended at 5×10^6 cells mL^{-1} in RPMI-1640 medium containing 2 mM L-glutamine, 0.02 mM 2-mercaptoethanol, 1% Hepes buffer, penicillin/streptomycin (100 U/100 μg mL^{-1}), and 10% fetal calf serum (FCS). Aliquots of 90 μL cell suspension were distributed in 96-well microtiter plates (Sarstedt) and then 10 μL of ConA (64 μg mL^{-1}) or LPS (160 μg mL^{-1}) was added to each well. The plates were incubated for 72 h at 24 °C. After incubation, 25 μL of MTT solution was added to each well and the plates were incubated for 4 h at 22 °C. After the microplates were centrifuged, the media were removed and 100 μL of DMSO (Sigma-Aldrich) was added to all wells and mixed. The optical density was read on a microreader at 570 nm. Duplicate determinations were averaged to obtain mean values.

3. 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 $P < 0.05$.

4. Results

In these studies conducted to evaluate the immune system's efficiency, best indicators were selected to allow the assessment of natural processes that are important for the defense against harmful factors.

4.1. Experiment I

Comparisons of the innate cellular defense mechanism in European eel are presented in Table 1. The analysis of the results showed that metabolic activity (RBA) of spleen phagocytes was increased in both groups for the whole monitoring time with a better response after immersion. Potential killing activity (PKA) of spleen phagocytes of European eel was higher throughout the duration of the experiment compared to control with also higher stimulation of the immersion group. Pronephros lymphocyte proliferation (MTT) stimulated by mitogens ConA or LPS was elevated for 2 weeks time in both experimental groups.

The only elevated cortisol level occurred in immersion group 24 h after bacteriophage preparation contact. After one week it was comparable to the control group (Fig. 1). There were no statistically significant changes in the glucose level in any group during the experiment (Fig. 2).

Total protein level in European eel serum was slightly increased for seven days in bath group, unlike fodder group where no change occurred (Fig. 3).

The total globulin level progressively increased in all experimental groups for two weeks compared with the control (Fig. 4).

Lysozyme activity in European eel serum in immersion group was elevated already after 24 h and increased for 14 days, then began to slowly descend. In the group where the cocktail was administered with fodder, the situation was similar, except that the increase in the activity of lysozyme occurred later and was weaker (Fig. 5).

Ceruloplasmin level was slightly elevated for two weeks in Immersion group subjected to a 1 h bath in the solution of bacteriophage preparation (Fig. 6). There was no change in this parameter in BAFADOR®-coated fodder group compared with the control.

4.2. Experiment II

Application of the bacteriophage preparation by immersion 24 h after the experimental infection with *A. hydrophila* and *P. fluorescens* allowed reducing mortality of fish by 40%. The delay of therapy for another 24 and 48 h reduced mortality only by 25% and 15%, respectively (Fig. 7).

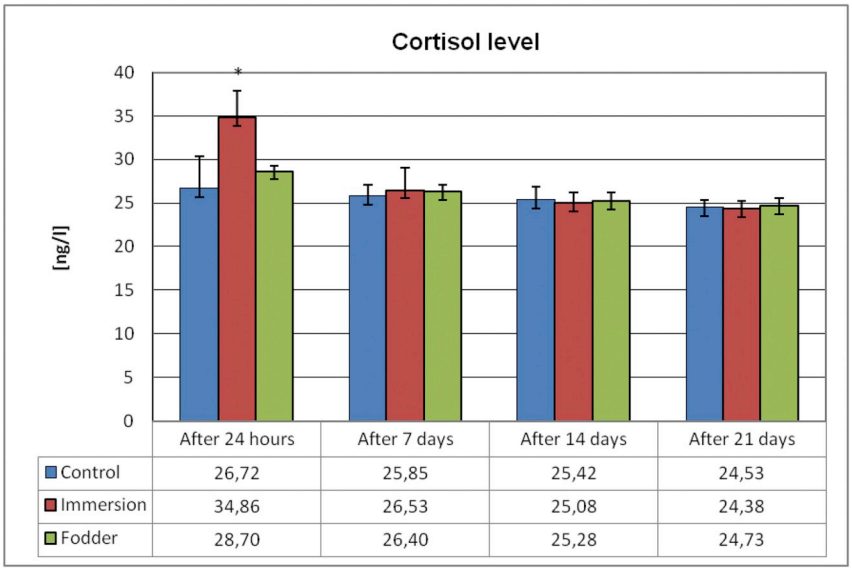
4.3. Experiment III

The cumulative survival percentage of European eel after prophylactic use of bacteriophage preparation 24 h before challenge with *A. hydrophila* and *P. fluorescens* is presented in Fig. 8. Fish treated with

Table 1
The effect of BAFADOR® on metabolic and potential killing activity of spleen phagocytes and on pronephros lymphocyte proliferation stimulated by ConA or LPS of European eel ($P < 0.05$).

Parameter	Group	Experimental day			
		1	7	14	21
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
RBA	C	0.335 ± 0.04	0.326 ± 0.03	0.258 ± 0.03	0.285 ± 0.06
	I	0.789 ± 0.11*	0.521 ± 0.07*	0.426 ± 0.09*	0.309 ± 0.05
	F	0.521 ± 0.08*	0.480 ± 0.05*	0.368 ± 0.04*	0.304 ± 0.04
PKA	C	0.255 ± 0.04	0.224 ± 0.02	0.220 ± 0.02	0.242 ± 0.02
	I	0.595 ± 0.12*	0.583 ± 0.03*	0.307 ± 0.07*	0.263 ± 0.03*
	F	0.358 ± 0.04*	0.373 ± 0.01*	0.296 ± 0.03*	0.272 ± 0.04*
MTT- ConA	C	0.258 ± 0.05	0.247 ± 0.03	0.260 ± 0.04	0.254 ± 0.04
	I	0.343 ± 0.02*	0.452 ± 0.06*	0.557 ± 0.05*	0.264 ± 0.05
	F	0.376 ± 0.03*	0.456 ± 0.06*	0.421 ± 0.08*	0.288 ± 0.06
MTT- LPS	C	0.239 ± 0.01	0.244 ± 0.02	0.261 ± 0.02	0.242 ± 0.06
	I	0.329 ± 0.06*	0.389 ± 0.06*	0.470 ± 0.06*	0.270 ± 0.06
	F	0.294 ± 0.01*	0.379 ± 0.02*	0.431 ± 0.06*	0.209 ± 0.06

* $P < 0.05$.



	After 24 hours	After 7 days	After 14 days	After 21 days
Control	26,72	25,85	25,42	24,53
Immersion	34,86	26,53	25,08	24,38
Fodder	28,70	26,40	25,28	24,73

Fig. 1. The effect of BAFADOR® on cortisol level in European eel serum ($P < 0.05$).

	After 24 hours	After 7 days	After 14 days	After 21 days
Control	63,40	63,18	62,78	62,42
Immersion	65,90	64,15	63,38	62,20
Fodder	65,13	64,12	63,37	63,05

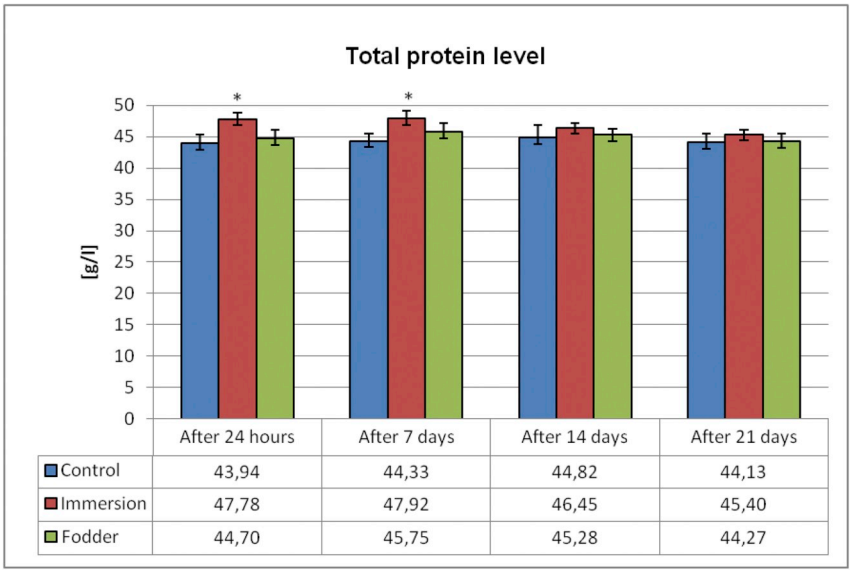
Fig. 2. The effect of BAFADOR® on glucose level in European eel serum ($P < 0.05$).

BAFADOR® before infection showed the same survival rate as non-infected fish.

5. Discussion

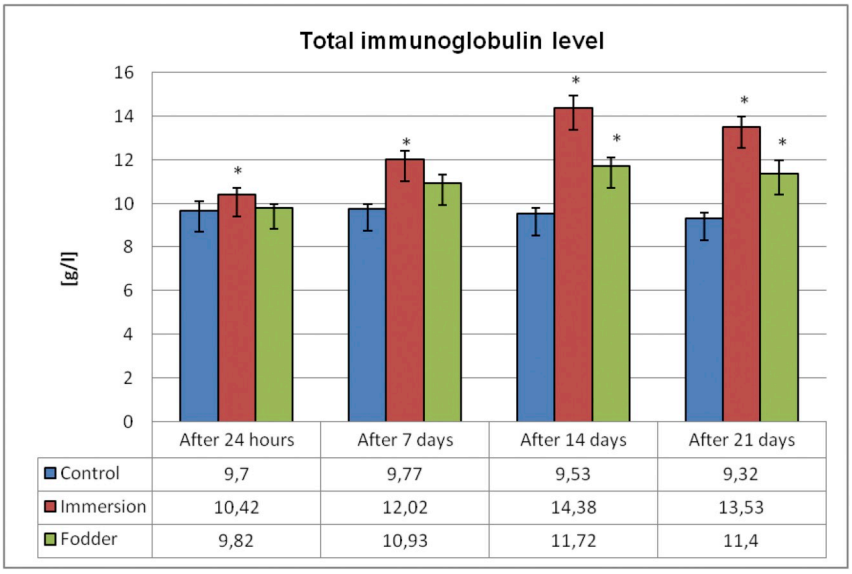
Phage preparations used for phage therapy may have not only direct antibacterial action but also immunomodulating effects mediated by phages themselves as well as by bacterial antigens. Although the effect of LPS on the immune system of fish has been widely described in the literature [25–27], there are no results regarding *A. hydrophila* or *P. fluorescens* LPS influence on European eel. What is more, we for the first time described the influence of bacteriophages cocktail on European eel immune system showing its high effectiveness in combating pathogenic bacteria.

One of the most important fish defense mechanisms is phagocytosis. Some cells such as monocytes, macrophages, and neutrophils are known as professional phagocytes. The respiratory burst activity of phagocytes has been used frequently as an indicator of nonspecific immunity in fish [28–30]. Salati et al. [31] reported an enhanced phagocytic activity of blood leucocytes when treating Japanese eel with LPS from *Edwardsiella tarda*. The analyses of our results showed that the phagocytic ability (RBA) and potential killing activity (PKA) of spleen phagocytes were significantly higher ($P < 0.05$) in European eel both in the immersion and fodder group, compared to control fish (Table 1). The similar pattern was observed in the proliferative response of pronephros lymphocytes (Table 1) stimulated by lipopolysaccharide, a B-cell mitogen, and concanavalin A, a T-cell mitogen [32]. We observed better stimulation after BAFADOR® used in immersion, what might be caused by uneven and insufficient feed intake in the fodder group what can't be prevented during rearing. The lower stimulation of the immune system may also have been affected by a smaller amount of BAFADOR® available in feed than during an hour's bath, which may indicate an excellent way of administering bacteriophages cocktail by this route. Oral administration of drugs tested in experimental animals is a convenient and popular method that mimics the oral ingestion of drugs by humans. BAFADOR® is intended for administration by immersion so the other method of administration was only experimental and requires further research. Such route would be an excellent opportunity to



	After 24 hours	After 7 days	After 14 days	After 21 days
Control	43,94	44,33	44,82	44,13
Immersion	47,78	47,92	46,45	45,40
Fodder	44,70	45,75	45,28	44,27

Fig. 3. The effect of BAFADOR® on total protein level in European eel serum ($P < 0.05$).



	After 24 hours	After 7 days	After 14 days	After 21 days
Control	9,7	9,77	9,53	9,32
Immersion	10,42	12,02	14,38	13,53
Fodder	9,82	10,93	11,72	11,4

Fig. 4. The effect of BAFADOR® on immunoglobulin level in European eel serum ($P < 0.05$).

prevent and treat fish without introducing stress in additional manipulations.

The response to stress in fish is stimulation of the hypothalamus. It results in the activation of the neuroendocrine system and a cascade of metabolic and physiological changes [33]. This allows to increase the tolerance to adverse situations or changes in the environment. Some serum compounds are used for evaluation of the health status and stress condition of the fishes. Because stress has been found to increase cortisol and glucose serum levels, these parameters often serve as stress indicators [34]. Experiments investigating the response of fish to acute stress have shown that the increase in cortisol levels is rapid but quite quickly returns to a normal level [35]. An increase of cortisol caused by bacterial lipopolysaccharide given in injection was reported in yellow perch but not in pallid sturgeon [26], that indicates, that different species of fish have a different level of sensitivity to LPS. Our results showed a significant increase in cortisol levels within the first 24 h after contact with BAFADOR® in the immersion group (Fig. 1). The level of cortisol could be increased by the lipopolysaccharide contained in BAFADOR®. However, as this effect was not found in the fodder group, it can be concluded that increase in cortisol level was caused by the handling stress and not the preparation itself. Cortisol activates glycogenolysis and gluconeogenesis processes in fish. Glucose concentration was widely used, in a variety of fish species, as a stress monitoring parameter. It is believed, however, that it is a less precise indicator than cortisol [36,37]. Some authors showed only a slight increase in glucose [35], while others did not find any changes during and after stress [38,39]. It was also noted that this parameter could be in decline [40]. Our results showed a very slight increase in the level of glucose in the first day in experimental groups, but these changes were not significant (Fig. 2).

Immunoglobulins play an important role in reducing the spread of infectious agents, killing microbes and other potential pathogens. Hang et al. [45] showed that the ability to raise the level of immunoglobulins may depend on the amount of LPS. Their results indicated that a low dose of LPS increased the level of immunoglobulin while high dose did not induce differences in level of this parameter in comparison with the control in striped catfish. Nayak et al. [17] observed no significant difference in total protein and globulin content in LPS of *E. coli* injected

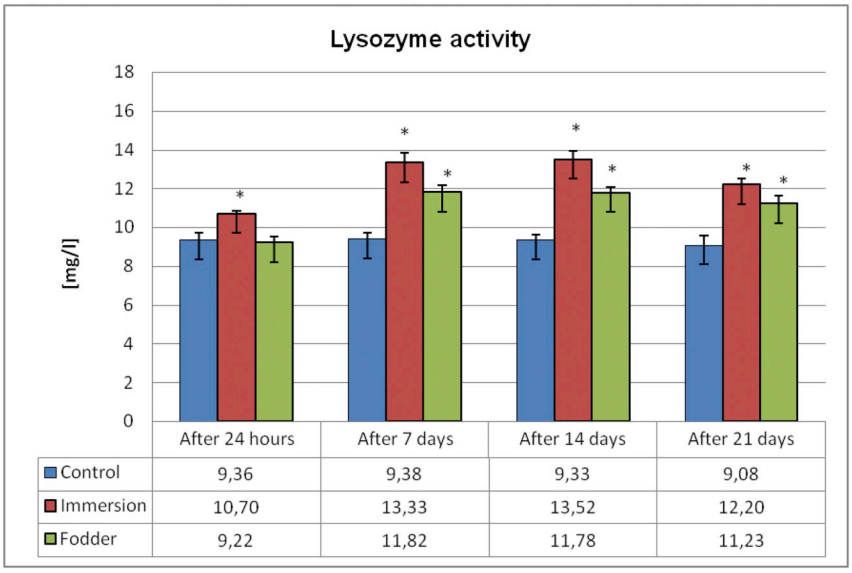


Fig. 5. The effect of BAFADOR® on lysozyme activity in European eel serum ($P < 0.05$).

catfish. In our studies, the level of immunoglobulins in the serum was increased during the whole experiments in the immersion group and had an increasing tendency, reaching a maximum of 14.38 g/l after 14 days (Fig. 4). In the group where BAFADOR® was administered with fodder the level of immunoglobulin also increased throughout the duration of the experiment to a statistically significant value after 14 days but reaching lower values. These results are different from the results of serum total protein level (Fig. 3), where the increase occurred only in the immersion group reaching a maximum 47.92 g/l after 7 days. This may be due to the formation of anti-bacteriophage antibodies and a change of the fractions proportion in the serum. However, this requires further research. A single administration of BAFADOR® with feed does not have an effect on total protein level compared to the control.

Lysozyme in eel is an important part of the innate immune system and exhibits the highest activity during the early stages of eel development [41]. In addition to damaging gram-positive bacteria cell walls, fish lysozyme has antibacterial activity against gram-negative bacteria in the absence of a compliment as it activates phagocytosis. Lysozyme activity increases after supplementation with a wide range of immunostimulants in various fish species [42]. Different data shows a different impact of LPS on lysozyme level in fish. It was noted it can either increase or decrease this parameter [16]. Our results indicate stimulation of lysozyme production after contact with BAFADOR® both in the form of immersion and feed (Fig. 5). It is suggested that the lysozyme of fish serum comes from leukocytes and its activity increases with the number of leukocytes and antibody titers [43]. The present study confirms previous experiments with different immunostimulants [30,44] and indicates that BAFADOR® increases the level of lysozyme activity in European eel.

Animals undergoing external or internal challenge to their state of health mount a vigorous response including the acute phase response to limit the harmful effects of the stimulus. Ceruloplasmin is an acute phase protein found to be activated by the host immune system during stress conditions. It plays a similar role to interferon and transferring in mammals. It inhibits the growth of bacteria by depriving them of access to copper ions. It also has oxidative protection properties, participates in blood coagulation and fibrinolysis processes and protects the body

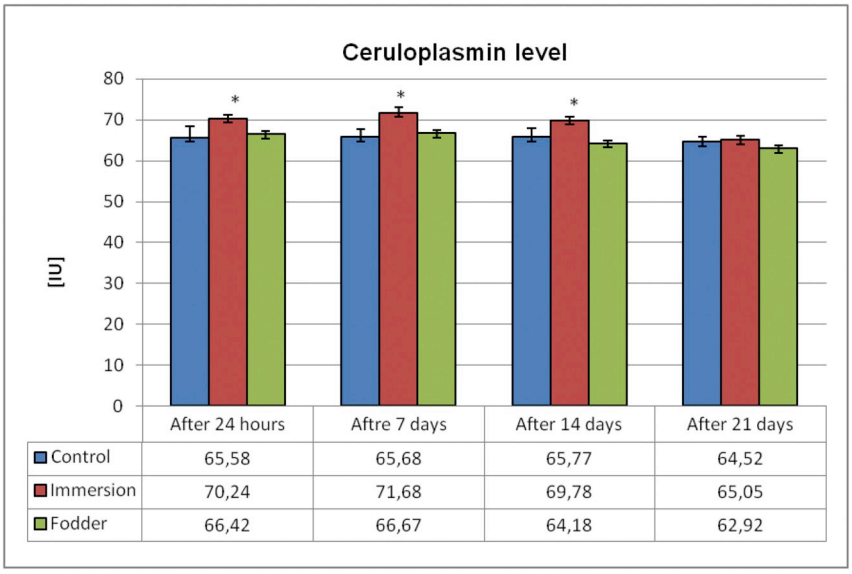


Fig. 6. The effect of BAFADOR® on ceruloplasmin level in European eel serum ($P < 0.05$).

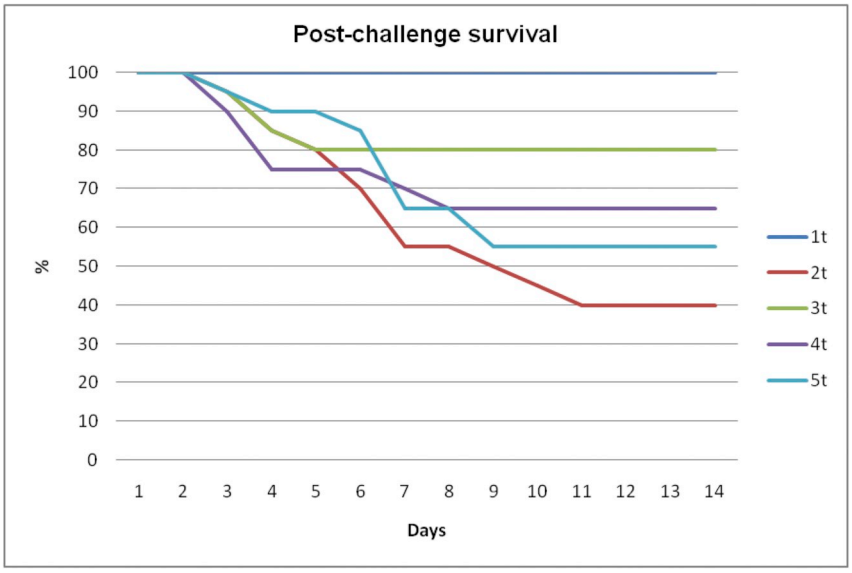


Fig. 7. In vivo effect of BAFADOR® application after experimental infection.

from iron loss. Sahoo et al. [46] showed a significant up-regulation of expression of ceruloplasmin after *A. hydrophila* infection during the up to 15 days post-challenge in the survivors. This coincides with the time of increase in ceruloplasmin levels in our studies in BAFADOR® immersion group (Fig. 6). Considering no increase in other stress indicators in our research, this should not be related to stress. It is most likely triggered by bacteriophage cocktail stimulation. There was no increase in this parameter in the feed group what could have been due to the smaller amount of BAFADOR® in fodder.

A. hydrophila and *P. fluorescens* are two most frequent bacterial pathogens in Poland, of both eel [47] and other fish species [4,48]. In the present study, the highest cumulative percentage of survival was registered in group 3t where BAFADOR® was given in the shortest time from experimental infection with a mixture of bacteria, that is 24 h after infection (Fig. 7). It was 40% higher compared to the infected control 14 days after infection. The delay in therapy resulted in a decrease in survival to 65% in the 4t group and 55% in the 5t group. These results agree with the results of Grochoła et al. [49] who found lower sensitivity of carp after intraperitoneal stimulation to *A. hydrophila* infection.

Previous studies [31] of Japanese eel immunized with crude LPS showed, 15% of relative percent survival, protection to the strong experimental challenge. Nya and Austin [50] examined the use of orally administered bacterial LPS for the prevention of infection by *A. hydrophila* in rainbow trout reaching 89% survival rate. Nayak et al. [17] showed 60% survival of carp after LPS and experimental infection of *P. fluorescens*. Our results showed a definitely higher level of survival as high as uninfected control (Fig. 8), which indicates good protection of bacteriophage preparation. In addition, we received survival rate not differing from the control (98%) despite the one-time use of the preparation, which indicates that bacteriophages in the preparation increase the effectiveness of prophylactic against *A. hydrophila* and *P. fluorescens*.

Due to its specificity, the treatment with bacteriophages has a narrow antibacterial spectrum, limited to one or in the case of a cocktail to several species of bacteria. The use of the preparation is therefore indicated in the case of frequently recurrent infections with the same pathogens. Our results indicate the possibility of using BAFADOR® not only as a therapeutic but also for prophylactic purposes and increasing

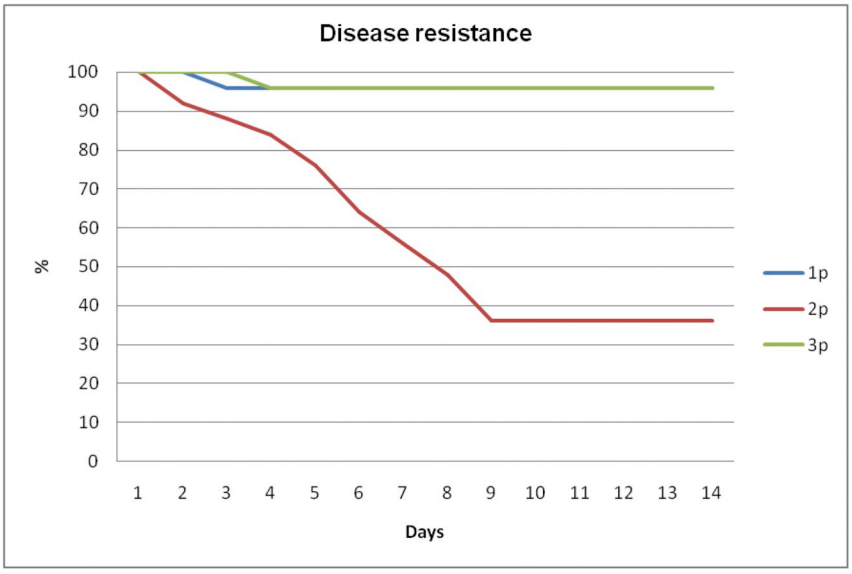


Fig. 8. In vivo effect of BAFADOR® application before experimental infection.

non-specific immunity in European eel.

6. Conclusions

BAFADOR®, the new bacteriophage-based preparation dedicated to fight fish bacterial pathogens, has the potential to stimulate the non-specific immune response in fish which can improve resistance to bacterial infection. Its one-time application in immersion shows better immunological responses in European eel than the application in the diet. Additionally, it fulfills its role as a therapeutic preparation limiting the European eel's death with a mixed infection of *A. hydrophila* and *P. fluorescens* so can be treated as a new strategy for better health management.

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