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RESEARCH ARTICLE

Do immunomodulatory substances facilitate recovery from stress caused by feed changes in juvenile brown trout (*Samo trutta* m. *fario* L.)?

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Abstract. The aim of the study was to determine the influence of feeds with or without β-glucan-based immunomodulatory supplements (groups BF and UB or B and U, respectively; feeding period 14 and 28 days) on the welfare of brown trout. The diets tested did not influence the rearing indices. The type of feed and the feeding period were confirmed to have a significant influence on the white blood cell (WBC) count. WBC counts were lower than those in the initial sample particularly on day 14 of the experiment. Red blood cell (RBC) counts and hematocrit (HCT) were determined by feeding period, and the values of these parameters decreased the longer the tested feeds were applied. These changes were accompanied by increases in the values of mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Feed type determined levels of chloride ion (Cl^-) and ammonia (NH_3) and alkaline phosphatase (ALP) activity and feeding period affected sodium (Na^+) and albumin (ALB) concentrations, while aspartate aminotransferase (AST) activity depended on both factors. Changes in leukograms were noted in all the groups, inter alia, significant increases in the share of lymphocytes, and after changing diets, decreases in the percentage of neutrophils, myeloperoxidase activity (A_{MPO}) in phagocytes and cidal ability (CA). After day 28, A_{MPO} and CA values increased and were significantly higher in groups BF and UB.

Keywords: conservative aquaculture, fish, β-glucan, welfare

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Introduction

Aquaculture fish production focuses on producing food, stocking material for open waters, and ornamental fishes (Huntingford et al. 2012). The prevailing principle in intensive aquaculture is quantity over quality, which means effecting maximum fish growth

and maximum production (Trushenski et al. 2010). Increasingly, however, with the production of stocking material it is recommended to develop rearing procedures that take into consideration appropriately preparing the material for release into open waters, for example, increasing immunity, changing foraging behavior, and shaping anti-predator behavior (Bergqvist and Gunnarsson 2011, Saikkonen et al. 2011, Näslund and Johnsson 2016). Brown trout (*Salmo trutta* m. *fario* L.) is one of the important angling species that is mainly produced for stocking purposes (Linløkken 1995, Linløkken et al. 2019). This species is of great importance in the stocking of the rivers of the Baltic Sea catchment area, including Polish open waters (Bernaś and Wąs-Barcz 2020). Brown trout stocking material is produced at farms that focus on the production of rainbow trout (*Oncorhynchus mykiss* (Walbaum)) using procedures that are used for this species (S. Dobosz, personal communication).

The effectiveness of stocking with material reared under aquaculture conditions depends on many factors including anthropogenic, environmental, and individual (Brown and Day 2002). Of the latter, good fish immune system function is of vital importance (Senger et al. 2012). When released into new environments, these fishes come into contact with a completely new range of potentially pathogenic microbes. Additionally, the variability of environmental conditions in open waters means that the microflora and microfauna species composition is unpredictable, which calls into question the validity of applying vaccines to increase fish immunity. Consequently, the level of innate immunity of fishes that are introduced or released into open waters must be seen as an extremely important factor that determines the effectiveness of stocking (Martin and Król 2017).

The innate defenses of a body include the first line of physical barriers, phagocytes (neutrophils, monocytes, tissue macrophages), and thrombocytes and a range of innate immune proteins including those of the complement system, interferon, and others (Smith et al. 2019). The mechanisms of innate immunity can act immediately after contact with pathogens and are often sufficient to eliminate them, and, moreover, they enable the mechanisms of adaptive immunity to react faster. Thus, it is extraordinarily important that the immune system functions effectively (Smith et al. 2019). Cortisol is one of the key hormones influencing this system; it has anti-inflammatory properties and inhibits immune responses, which can affect the results of rearing, especially in juvenile stages of fishes, and the effectiveness of stocking fishes into open waters. Cortisol also influences the functioning of other systems and organs (e.g., cardiovascular, respiratory, urinary, digestive), the release of neurotransmitters and hormones (e.g., gonadotropins and thyroid hormones), and metabolism. What is significant is that this hormone plays an important role in the stress response by interacting with the sympathetic system and enhancing the action of other stress hormones, such as adrenaline and noradrenaline (Ellis et al. 2012).

Studies of stress reactions and the immune system conducted in vitro and/or in vivo contribute to the body of knowledge about fishes, but they also present perspectives and create effective methods to ensure the welfare and health of fishes that can be applied in practice (Van Muiswinkel 2008, Ellis et al. 2012, Van Muiswinkel and Nakao 2014). One of the strategies developed to protect fish health is to feed them functional feeds enriched with immunomodulatory substances (Dawood et al. 2018). Immunomodulators are characterized by the lack of toxic effects on fishes, humans, and the natural environment, and they are available in a wide range of both natural and synthetic products (Sahoo and Mukherjee 2002, Misra et al. 2007, Ringø et al. 2012, Assefa and Abunna 2018). The most well-documented are studies of the immunomodulatory properties of polysaccharide fiber, i.e., glucan (such as 1,3/1,6 β-glucan obtained from fungi, primarily *Saccharomyces cerevisiae*). The positive effects of this have been proven in many fish species (Ringø et al. 2012, Meena et al. 2013, Terech-Majewska 2016), which is why many commercial feeds containing this component have begun to appear on the market. Examples of these feeds include EFICO Enviro FOCUS Plus® from BioMar A/S (Aarhus, Denmark) for salmonids that is enriched

with β-glucan and vitamins C and E (Minářová et al. 2021). Advantageous effects have also been obtained with Bioimmuno (IFI Olsztyn, Poland), which, in addition to 1,3/1,6 β-glucan, contains the synthetic immunostimulator methisoprinol (Kazuń and Siwicki 2013, Terech-Majewska 2016). This product is used as a supplement in feeds for carp (*Cyprinus carpio* L.), rainbow trout, wels catfish (*Silurus glanis* L.), and European eel (*Anguilla anguilla* (L.)). Feeding fishes feeds supplemented with Bioimmuno increased the phagocytic and metabolic activity of phagocytes and the proliferation capacity of T and B lymphocytes. The fishes reacted faster and better to experimental infection and more readily eliminated pathogens from their bodies (Terech-Majewska 2016). Products such as these can also have advantageous influences on fish growth, feed utilization efficiency, the digestive system microbiota, hematological parameters, and resistance to stress and diseases (e.g. Hisano et al. 2007, Meena et al. 2013, Dawood et al. 2018). Data from the literature indicate that providing feeds containing immunomodulatory substances for two to four weeks prior to exposure to stress conditions or factors such as changes in environmental conditions, periods of increased risk of disease, sorting, or transport can strengthen the immunity, condition, and natural defense mechanisms of fishes (Terech-Majewska 2016, https://www.biomar.com/en/denmark/product-and-species/trout/functional-feeds).

The aim of this study was to determine the influence of feeding brown trout feeds supplemented with immunomodulatory substances on the welfare of this species. The values of rearing indicators and hematological, biochemical, and immunological parameters were analyzed.

Material and Methods

Fish and rearing conditions

A total of 480 fish (12 tanks × 40 fish in each) with a mean initial body length (SL) of 17.3 ± 1.0 cm and a mean body weight (BW) of 75.95 ± 14.61 g were used in the study. The fish were stocked into tanks with volumes of 1.14 m^3 (1.95 × 1.95 × 0.3 m; L × W × H) that were part of a open system with a continuous supply of fresh water from an intake on the Radunia River (Kashubian Lake District, northern Poland). Throughout the four weeks of the experiment, the fish were held under optimal conditions recommended for rearing individuals of this species (S. Dobosz, unpublished materials). Measurements of water temperature (± 0.1°C) and oxygen concentration (± 0.01 mg O_2 l^{-1}) at the rearing tank inflows and outflows were taken daily. The water parameters of total ammonia nitrogen (TAN = NH_4^+-N + NH_3-N) (± 0.01 mg TAN l^{-1}), nitrates (± 0.01 mg NO_2-N l^{-1}), and pH (± 0.01) were measured at the tank outflows every seven days. The mean water temperature during the experiment was 12.0 ± 0.1°C. Oxygen concentrations at the rearing tank outflows did not decrease below 7.43 mg l^{-1} (83.9% saturation). The oxygen levels at the tank inflows was within a range of 90–98% saturation. TAN and NO_2-N concentrations at the tank outflows did not exceed 0.032 mg TAN l^{-1} or 0.008 mg NO_2-N l^{-1}, respectively. The water pH range was 6.81–7.04.

Diets and feeding

During the experiment, the fish were fed four feeds, two of which were functional and two of which corresponded to standard feed without immunomodulating supplements. The commercial feeds with a granulate size of 3 mm from BioMar A/S (Aarhus, Denmark) used were: functional – EFICO Enviro FOCUS Plus® supplemented with 1,3/1,6 β-glucan and vitamins C and E (feed/group BF); standard – BioMar EFICO Enviro (feed/group B). The other two feeds (UB and U) were prepared in the laboratory of Department of Ichthyology and Aquaculture, University of Warmia and Mazury in Olsztyn. All dry ingredients were ground in a hammer mill, sieved through a 0.8 mm screen, and mixed thoroughly. The mixed ingredients (100 g kg^{-1} of moisture was added before extrusion cooking) were extruded through a single screw extruder (METALCHEM, Poland) with a 2.0

Table 1
Proximate composition and the components of the diets tested

Specification	Tested diets			
	B	BF	U	UB
Proximate composition (% of wet weight)				
Crude protein	44.37	42.84	48.68	46.29
Crude fat	22.38	21.33	18.74	19.98
Nitrogen free extract (NFE)[1]	22.64	23.89	16.18	17.47
Crude ash	5.21	6.06	8.34	8.03
Water	5.40	5.88	8.06	8.23
Gross energy (MJ kg^{-1} feed)[2]	23.22	22.66	21.74	21.87
Components composition (g 100 g^{-1})				
Fishmeal[a]	Producer data: fish meal, poultry meal,		30.0	30.0
Poultry meal[b]	soya concentrate, blood meal, fish oil,		18.0	18.0
Soya concentrate[c]	rapeseed oil, horse beans, wheat,		5.0	5.0
Blood meal[d]	hydrolysed feather protein meal, sunflower		5.0	5.0
Wheat flour[e]	cake, guar protein, wheat gluten,		10.0	10.0
Yeast[f]	monosodium phosphate, monocalcium		5.0	5.0
Fish oil[g]	phosphate, yeast, FOCUS Plus (diet BF)		18.0	18.0
Rapeseed oil[h]			6.0	4.0
Bioimmuno[i]			0.0	2.0
Premix[jk]			3.0	3.0

B: BioMar EFICO Enviro; BF: EFICO Enviro FOCUS Plus®; U: experimental diet; UB: experimental diet + 20 g kg^{-1} Bioimmuno; [1]NFE: nitrogen free extract (NFE = 100 – (crude protein + crude lipid + crude ash + water)); [2]gross energy calculated from the chemical composition using the following energy conversion factors: 24 kJ g^{-1} proteins, 39 kJ g^{-1} lipids, and 17 kJ g^{-1} NFE (Jobling 1994); [a]FF SKAGEN, Denmark; [b]SONAC, Poland; [c]HP 300 HAMELT, Denmark; [df]SONAC, Poland; [e]CASTELLO, Poland; [f]ARTEX, Poland; [g]AGROFISH, Poland; [h]ZT Kruszwica S.A., Poland; [i]IFI Olsztyn, Poland; [j]DOLFOS, Poland; [k]composition of the premix (in dry matter): vitamin A - 70,000 IU kg^{-1}; vitamin D – 200,000 IU kg^{-1}; vitamin E – 17,500 IU kg^{-1}; vitamin K – 867 mg kg^{-1}; vitamin C – 28,500 mg kg^{-1}; vitamin B1 – 1,067 mg kg^{-1}; vitamin B2 – 2,000 mg kg^{-1}; vitamin B5 – 5,334 mg kg^{-1}; vitamin B6 – 1,334 mg kg^{-1}; vitamin B12 – 400 mg kg^{-1}; biotin – 200 mg kg^{-1}; niacin – 12,000 mg kg^{-1}; folic acid – 800 mg kg^{-1}; inositol – 20,000 mg kg^{-1}; choline chloride -120,000 mg kg^{-1}; betaine – 75,000 mg kg^{-1}; $FeSO_4 \times H_2O$ – 4,334 mg kg^{-1}; KJ – 734 mg kg^{-1}; $CuSO_4 \times 5H_2O$ – 267 mg kg^{-1}; MnO – 734 mg kg^{-1}; $ZnSO_4 \times H_2O$ – 1,250 mg kg^{-1}; ZnO – 750 mg kg^{-1}; Na_2SeO_4 – 34 mg kg^{-1}

mm die using a conventional low-moisture extrusion cooking process (Liu et al. 2021). The extrusion parameters were controlled during feed production as follows: temperature in conditioner outlet – 90°C; temperature in the second segment – 110°C; temperature at the endplate – 120°C; pressure at the endplate 15 bar; screw revolution speed – 85 rpm; cutter revolution speed – 70 rpm. The oil was added to the feed after extrusion. Bioimmuno (1,3/1,6 β-glucan – 96 g 100 g^{-1}, Biolex®, Leiber, Germany, and methisoprinol – 4 g 100 g^{-1}, Polfa, Grodzisk Mazowiecki, Poland) was added at a ratio of 20 g kg^{-1} feed (Kazuń and Siwicki 2013) during oil coating to UB experimental feed. Methisoprinol is a synthetic compound formulated with p-acetamidobenzoate salt of N-N-dimethylamino-2-propanol and inosine in a 3:1 molar ratio (Kazuń and Siwicki 2013). The proximate composition of the feed used in the diets tested was analyzed according to AOAC (2007) procedures: crude protein was determined with the Kjeldahl method (Kjel-FossAutomatic 16210 analyzer; AISN Foss Electric, Hillerød, Denmark); crude fart with the Soxhlet method (extraction with ethyl ether for 12 h); crude ash by incinerating a dried sample at a temperature of 550°C for 12 h (furnace by Linn High Therm GmbH; Eschenfelden, Germany). The gross energy of the feed was calculated based on the chemical composition using the following energy conversion factors: 39 kJ g^{-1} fat; 24 kJ g^{-1} protein; 17 kJ g^{-1} carbohydrate (Jobling 1994). The

level of nitrogen free extract (NFE) was calculated based on the following difference: (100 – (water + crude fat + crude protein + crude ash)) (Table 1). Fish were fed manually to satiation every four hours (08:00, 12:00, 16:00) according to D-journal Freshwater Farm® software (From and Rasmussen 1984).

Experimental procedures

Four experimental fish groups were created each in three replicates. Diet type was the factor that was different (groups B and BF and groups U and UB). Additionally, before beginning the feeding trial, an initial fish sample (IFS) was collected. On the initial day of the experiment (d0) and after d14 and d28 of the experiment, the fish were measured for body length (SL, ± 0.1 cm), caudal length (CL, ± 0.1 cm), and body weight (BW, ± 0.01 g). Based on these data, the values of the following parameters were calculated: daily growth rate: DGR (g d^{-1}) = (BW_2 – BW_1) × t^{-1}; specific growth rate: SGR (% d^{-1}) = 100 × (lnBW_2 – lnBW_1) × t^{-1}; Fulton's condition factor: F = 100 × BW × SL^{-3}; feed conversion ratio: FCR = TFS × (FB – IB)$^{-1}$, where: BW_1 – initial fish body weight (g), BW_2 – final fish body weight (g), t – rearing time (days), SL – fish body length (cm), FB – final stock biomass (g), IB – initial stock biomass (g), TFS – total feed supply (g). Fish mortality was also monitored daily.

Blood samples were drawn every two weeks (d0, d14, d28) to determine the hematological and biochemical parameters and innate immunity indices. Each time, blood was drawn from seven specimens chosen at random (d14, d28) from each experimental group (2–3 fish from each tank/replicate). The number in the initial sample (d0, IFS) was also seven specimens. Prior to drawing blood, the fish were anesthetized in an aqueous solution of tricaine methanesulfonate (MS-222) (Sigma-Aldrich Co., Missouri, USA) at a concentration of 100 mg l^{-1}. Blood was drawn from the caudal vein with heparinized syringes (Smiths Medical, Minnesota, USA).

Complete blood count was analyzed in a calibrated BC-2800 VET semi-automated hematology analyzer (Mindray, Shenzhen, China). The analyzer readings were calibrated to the blood of several fish species, including brown trout, based on traditional methods for fish blood hematological determinations (Dacie and Lewis 2001). The analyzer was calibrated by Stamar (Dąbrowa Górnicza, Poland). After the plasma was centrifuged (4,000 rpm, 3 min; Fresco 17, Thermo Scientific, Massachusetts, USA), biochemical analyses were performed in a BS120 automated biochemistry analyzer (Mindray, Shenzhen, China). The following hematological parameters were analyzed: white blood cell (WBC) and red blood cell (RBC) counts, hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT). The following biochemical blood plasma parameters were determined: glucose (GLU), total protein (TP), albumin (ALB), globulins (GLB), C-reactive protein (CRP), total bilirubin (BIL-T), ammonia (NH_3), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP). The levels of sodium (Na^+) and chloride (Cl^-) ions were also determined.

Cortisol (CORT) was determined with an ELISA enzyme-linked immunosorbent assay test (Cayman Chemical Company, Michigan, USA). The hormone was extracted from the plasma with ethyl ether according to the method in Hermelink et al. (2011). The analyses were conducted in 96-well plates into which the following were placed: EIA test buffer; cortisol standards; the samples tested (each in two replicates). The optimal dilution of the samples analyzed was determined earlier based on a series of tests. After incubation and rinsing the wells, the samples were read at a wavelength of 412 nm. According to manufacturer instructions (Cayman Chemical Company, Michigan, USA), the cortisol levels in given samples were calculated using the standard curve.

The level of innate immunity in brown trout was determined by analyzing leukograms and phagocyte

ability and assessing the activity of myeloperoxidase (MPO), a specific enzyme present in the granulocytes and monocyte granules (Das and Sahoo 2014). Blood was drawn from seven specimens from each group and smears were made, which were subjected to multistage cytochemical staining according to the manufacturer's procedures (Sigma-Aldrich Peroxidase [Myeloperoxidase] test, Procedure No. 391). The key stage of this procedure was incubating smears in diaminobenzidine (DAB) and hydrogen peroxide. MPO catalyzes DAB oxidation and turns it brown, and subsequent staining stages change the color to a gray-black depending on MPO activity. The stained smears were analyzed under a light microscope at 900× magnification. Depending on color intensity, the dimensions, and the extent of the granules inside the macrophages, four degrees of MPO activity (A_{MPO}) were distinguished ranging from a lack of activity (0 degree) to high activity (3rd degree). To activate MPO, before smear preparation, blood samples were incubated with a suspension of zymosan particles (Sigma-Aldrich). For each fish, 200 µl aliquots of blood were placed into two wells of a microplate (NUN96ft, Thermo Fisher Scientific) and 30 µl of zymosan solution was added to each well. After 25 min incubation in a chamber (MaxQ 4450, Thermo Scientific) at 24°C with continuous stirring, two blood smears were made for each well.

The relative shares of the different types of leukocytes: small lymphocytes (S lymphocytes), large lymphocytes (L lymphocytes), total lymphocytes, monocytes, immature neutrophils with non-segmented nuclei (JN neutrophils), mature neutrophils with segmented nuclei (neutrophils MN), total neutrophils, eosinophils, were determined for each fish based on the analysis of at least 200 leukocytes. The following measures of phagocytic activity were determined: the percentage of phagocytes (PhC); the percentage of active phagocytes (APhC); the phagocytic index (PhI); cidal ability (CA). The relative number of phagocytes and active phagocytes were determined with the following equations (Nikoskelainen, et al. 2004):

PhC (%) = 100 × [(neutrophil count + monocyte count) × leukocyte count^{-1}],

APhC (%) = 100 × (count of phagocytes with zymosan particles inside × total phagocyte count^{-1}).

The total phagocyte counts (PhC_c), the total active phagocyte counts ($APhC_c$), and the phagocytic index (PhI) were calculated with the following formulas (Santulli-Marott et al. 2015):

$$PhC_c\ (10^3\ \mu l^{-1}) = PhC\ (\%) \times WBC;$$

$$APhC_c\ (10^3\ \mu l^{-1}) = APhC\ (\%) \times PhC_c;$$

$$PhI = ZP \times APhC_c^{-1};$$

where: WBC – white blood cell count, ZP – count of zymosan particles inside phagocytes.

Cidal ability (CA) was calculated as follows:
$CA = A_{MPO} \times APhC_c$.

Data analysis

The normality of the data distribution was tested with the Shapiro-Wilk test, and the homogeneity of variance with Levene's test. When these assumptions were met, the differences between the means were analyzed with ANOVA and Tukey's post hoc test (TT). Since the ANOVA assumptions were not met for thrombocyte counts, total bilirubin, or AST, the Kruskal–Wallis ANOVA (KWA) test and Dunn's test (DT) were used. The results were analyzed with Statistica 13.1 (Statsoft, USA) at a significance level of $P \leq 0.05$. Outliers were removed with the Grubbs-Beck test. Growth data and hematological and biochemical index data were analyzed with two-way ANOVA.

Results

No fish mortality was noted during the experiment. The feeds tested did not influence the rearing parameters (CL, SL, BW, DGR, SGR), condition, or feed conversion factors (Table 2).

The type of feed and the feeding period had a significant influence on WBC counts ($P \leq 0.05$; Table 3). WBC values were lower in the IFS, while the greatest differences were noted after a two-week

Table 2
Rearing indices of brown trout fed two different feeds with or without functional supplementation on subsequent days of rearing

	Fish groups			
Specification	B	BF	U	UB
CL (cm)				
d0	18.70 (± 0.10)	18.73 (± 0.08)	18.69 (± 0.11)	18.72 (± 0.14)
d28	19.71 (± 0.14)	19.81 (± 0.41)	19.67 (± 0.23)	19.81 (± 0.19)
SL (cm)				
d0	17.22 (± 0.11)	17.30 (± 0.13)	17.20 (± 0.15)	17.27 (± 0.14)
d28	18.23 (± 0.21)	18.31 (± 0.37)	18.18 (± 0.18)	18.32 (± 0.26)
BW (g)				
d0	75.76 (± 1.01)	75.91 (± 2.08)	75.70 (± 1.55)	75.88 (± 1.07)
d28	92.75 (± 4.73)	94.77 (± 7.64)	95.07 (± 4.56)	95.85 (± 2.14)
F				
d0	1.45 (± 0.01)	1.46 (± 0.00)	1.44 (± 0.01)	1.46 (± 0.00)
d28	1.51 (± 0.08)	1.52 (± 0.03)	1.56 (± 0.04)	1.54 (± 0.03)
DGR (g d^{-1})				
d0-14	1.01 (± 0.32)	1.06 (± 0.21)	1.04 (± 0.20)	1.01 (± 0.08)
d15-28	1.30 (± 0.11)	1.39 (± 0.28)	1.48 (± 0.11)	1.41 (± 0.19)
SGR (% d^{-1})				
d0-14	1.00 (± 0.32)	1.06 (± 0.21)	1.04 (± 0.19)	1.01 (± 0.08)
d15-28	1.29 (± 0.11)	1.38 (± 0.27)	1.47 (± 0.11)	1.40 (± 0.19)
FCR				
d0-14	0.97 (± 0.25)	0.89 (± 0.17)	0.97 (± 0.19)	0.98 (± 0.08)
d15-28	1.20 (± 0.19)	1.09 (± 0.23)	1.05 (± 0.12)	1.12 (± 0.15)
Survival (%)				
d0-14	100	100	100	100
d15-28	100	100	100	100

B: BioMar EFICO Enviro; BF: EFICO Enviro FOCUS Plus®; U: experimental diet; UB: experimental diet + 20 g kg^{-1} Bioimmuno; d0: initial day of the experiment; d14: day 14 of the experiment; d28: day 28 of the experiment; CL: fork length; SL: standard length; BW: body weight; F: condition factor; DGR: daily growth rate; SGR: specific growth rate; FCR: feed conversion ratio. Mean values (± SD: standard deviation); n = 3. Details in Table 1 and in the Materials and methods section. No statistically significant differences were noted among groups ($P > 0.05$).

period of feeding the fish the feeds tested, especially in groups B and BF, which were fed commercial feeds. The feeding period was significant for the parameters of RBC, HCT, MCH, and MCHC. RBC and HCT values decreased the longer the feeding period was, while MCH and MCHC values increased ($P \leq 0.05$; Table 3). HGB values were determined by the type of diet applied (Table 3). After d14 of the experiment, HGB values were lower than those in the initial sample (d0), while the greatest differences in comparison with the IFS were confirmed in the fish fed commercial feeds (groups B and BF). Two weeks later, (d28) the HGB values in all groups were higher than those noted on d14. The highest increases in HGB values were observed in group BF, while the lowest were in groups B and UB (Table 3).

The diets tested determined sodium (Na^+) and chloride (Cl^-) ion concentrations in the blood plasma. Feeding period influenced Na^+ concentrations, while feed type did so with Cl^- ($P \leq 0.05$; Table 4). After d14 of the experiment, concentrations of Na^+ were lower than those in the IFS, while on d28 the level of

Table 3
Hematological parameters of brown trout fed feeds with or without functional supplementation on the initial day of the experiment (d0) and after 14 and 28 days of rearing (d14 and d28, respectively)

		Time of feeding (d14)				Time of feeding (d28)				Factor effect significancen (F statistic and P value)		Model fit goodness	
		feeding groups				feeding groups							
Parameter	IFS (d0)	B	BF	U	UB	B	BF	U	UB	feed	feeding time	R^2	F statistic and P value
WBC	281.20	246.30	243.43	264.20	255.37	256.90	265.07	270.70	258.04	F = 4.54	F = 9.7	0.425	F = 9.9
	(9.87)	(6.41)	(15.12)	(9.18)	(9.15)	(6.16)	(4.70)	(24.73)	(8.91)	P = 0.006	P = 0.003		P < 0.001
RBC	0.82	0.62	0.59	0.71	0.6	0.52	0.59	0.57	0.59	F = 2.3	F = 13.8	0.454	F = 11.0
	(0.14)	(0.05)	(0.10)	(0.07)	(0.06)	(0.07)	(0.11)	(0.07)	(0.06)	P = 0.08	P = 0.0005		P < 0.001
Hb	83.71	69.71	67.83	77.00	71.29	71.43	76.71	79.71	72.14	F = 3.9	F = 3.7	0.335	F = 7.0
	(6.40)	(3.25)	(8.33)	(4.65)	(5.38)	(2.82)	(6.42)	(12.72)	(3.39)	P = 0.013	P = 0.058		P < 0.001
HCT	16.88)	13.16	12.52	14.97	14.51	10.99	12.41	11.96	12.64	F = 2.6	F = 16.0	0.450	F = 10.8
	(2.35)	(1.00)	(2.02)	(1.53)	(1.33)	(1.52)	(2.27)	(1.29)	(1.20)	P = 0.061	P = 0.0002		P < 0.001
MCV	207.15	212.24	215.17	212.76	211.56	212.89	211.64	211.34	214.19	F = 0.20	F = 0.1	0.077	F = 2.0
	(6.80)	(2.34)	(4.57)	(2.94)	(3.71)	(3.07)	(4.21)	(5.33)	(3.47)	P = 0.892	P = 0.756		P > 0.05
MCH	106.37	112.27	118.17	109.66	103.79	140.13	135.23	141.20	122.61	F = 1.7	F = 22.9	0.314	F = 6.5
	(17.75)	(6.73)	(21.92)	(9.47)	(7.23)	(21.01)	(33.70)	(21.95)	(12.94)	P = 0.186	P = 0.00001		P < 0.001
MCHC	513.67	531.14	550.00	517.71	492.14	660.57	640.29	671.71	574.43	F = 1.8	F = 24.0	0.316	F = 6.5
	(72.30)	(30.34)	(92.63)	(50.01)	(28.79)	(97.06)	(158.08)	(114.90)	(59.29)	P = 0.165	P = 0.00001		P < 0.001
PLT	59.33	44.86	35.67	53.14	43.14	38.57	41.14	37.29	34.71	F = 0.85	F = 2.7	0.015	F = 1.2
	(11.57)	(23.53)	(5.09)	(20.82)	(13.97)	(3.95)	(4.91)	(10.63)	(6.42)	P = 0.473	P = 0.105		P > 0.05

IFS: initial fish sample, Aller Bronze; B: BioMar EFICO Enviro; BF: EFICO Enviro FOCUS Plus®; U: experimental diet; UB: experimental diet + 20 g kg^{-1} Bioimmuno; WBC: white blood cells ($\times 10^3$ μl^{-1}); RBC: red blood cells ($\times 10^6$ μl^{-1}); HGB: hemoglobin (g l^{-1}); HCT: hematocrit (%); MCV: mean corpuscular volume (fl); MCH: mean corpuscular hemoglobin (pg); MCHC: mean corpuscular hemoglobin concentration (g l^{-1}); PLT: platelet count ($\times 10^3$ μl^{-1}). Mean values (± SD: standard deviation); n = 7). Results of two-factor ANOVA main effects analysis of hematological parameters of brown trout fed experimental feeds; P values lower than 0.05 indicate the significant effect of the factor.

Table 4
Biochemical parameters of blood plasma in brown trout fed feeds with or without functional supplementation on the initial day of the experiment (d0) and after 14 and 28 days of rearing (d14 and d28, respectively)

		Time of feeding (d14)				Time of feeding (d28)				Factor effect significance		Model fit goodness	
		feeding groups				feeding groups				(F statistic and P value)			
Parameter	IFS (d0)	B	BF	U	UB	B	BF	U	UB	feed	feeding time	R^2	F statistic and P value
Na+	154.54	153.83	150.16	154.01	152.85	155.60	159.51	158.24	155.28	F = 0.60	F = 16.02	0.194	F = 3.89
	(3.71)	(4.83)	(3.90)	(3.16)	(2.60)	(4,67)	(4.23)	(3.04)	(5.05)	P = 0.617	P = 0.0001		P < 0.01
Cl^-	275.16	285.61	283.16	308.93	291.13	287.06	278.19	296.31	303.47	F = 3.34	F = 0.03	0.138	F = 2.92
	(15.56)	(18.81)	(14.19)	(35.09)	(9.48)	(26.41)	(21.31)	(13.31)	(18.66)	P = 0.025	P = 0.863		P < 0.05
GLU	96.00)	93.57	82.43	84.43	75.71	91.43	82.14	78.71	85.71	F = 1.48	F = 0.010	0.081	F = 2.06
	(17.80)	(19.16)	(18.88)	(23.21)	(8.16)	(12.66)	(11.89)	(14.59)	(22.57)	P = 0.230	P = 0.919		P > 0.05
TP	3.66	3.63	3.51	3.71	3.59	3.50	3.69	4.08	3.95	F =2.08	F = 3.37	0.062	F = 1.80
	(0.63)	(0.29)	(0.43)	(0.23)	(0.41)	(0.30)	(0.30)	(0.48)	(0.38)	P = 0.112	P = 0.072		P = 0.129
ALB	1.87	1.96	1.97	2.18	2.07	2.05	2.15	2.09	2.23	F = 2.76	F = 4.27	0.220	F = 4.38
	(0.12)	(0.11)	(0.14)	(0.22)	(0.24)	(0.15)	(0.12)	(0.10)	(0.11)	P = 0.050	P = 0.043		P < 0.01
GLB	1.79	1.68	1.54	1.54	1.52	1.45	1.55	1.72	1.99	F = 0.851	F = 1.03	0.006	F = 0.93
	(0.67)	(0.31)	(0.48)	(0.21)	(0.27)	(0.28)	(0.33)	(0.47)	(0.32)	P = 0.472	P = 0.313		P = 0.470
CRP	0.80	0.54	0.99	0.46	0.47	0.24	0.57	0.39	0.43	F = 1.60	F = 2.11	0.063	F = 1.81
	(0.88)	(0.79)	(0.77)	(0.30)	(0.48)	(0.10)	(0.29)	(0.46)	(0.19)	P = 0.200	P = 0.152		P > 0.10
NH_3	345.01	286.24	250.16	331.16	191.21	373.44	325.50	169.54	215.51	F = 4.59	F = 0.06	0.183	F = 3.70
	(79.21)	(83.11)	(45.81)	(98.77)	(27.25)	(122.43)	(129.09)	(42.10)	(41.50)	P = 0.006	P = 0.803		P < 0.01
BIL-T	0.33	0.15	0.17	0.16	0.13	0.18	0.19	0.06	0.06	F = 1.45	F = 0.81	0.132	F = 2.83
	(0.27)	(0.08)	(0.10)	(0.09)	(0.10)	(0.15)	(0.14)	(0.02)	(0.02)	P = 0.238	P = 0.373		P < 0.05
ALT	4.29	6.14	3.86	10.57	4.86	4.57	3.86	3.29	6.86	F = 1.45	F = 2.62	0.029	F = 1.36
	(4.96)	(1.46)	(2.19)	(5.06)	(3.02)	(4.69)	(3.80)	(2.93)	(3.13)	P = 0.237	P = 0.111		P > 0.10
AST	519.14	387.86	296.43	586.86	360.00	301.71	359.57	397.29	83.14	F = 5.52	F = 6.67	0.281	F = 5.69
	(234.73)	(45.75)	(97.38)	(388.52)	(83.32)	(64.03)	(72.54)	(89.32)	(138.61)	P = 0.002	P = 0.012		P < 0.001
ALP	123.29	147.14	115.71	182.29	181.57	122.29	128.14	205.57	231.29	F = 24.61	F = 3.18	0.553	F = 15.87
	(34.36)	(30.89)	(17.84)	(24.62)	(14.81)	(24.68)	(17.44)	(35.18)	(49.87)	P < 0.0001	P = 0.080		P < 0.0001

IFS: initial fish sample, Aller Bronze; B: BioMar EFICO Enviro; BF: EFICO Enviro FOCUS Plus®; U: experimental diet; UB: experimental diet + 20 g kg^{-1} Bioimmuno; Na^+: sodium (mmol l^{-1}); Cl^-: chloride (mmol l^{-1}); GLU: glucose (mg dl^{-1}); TP: total protein (g dl^{-1}); ALB: albumin (g dl^{-1}); GLB: globulin (g dl^{-1}); CRP: C-reactive protein (g ml^{-1}); NH_3: ammonia (ěg dl^{-1}); BIL-T: total bilirubin (mg dl^{-1}); ALT: alanine aminotransferase (U l^{-1}); AST: aspartate aminotransferase (U l^{-1}); ALP: alkaline phosphatase (U l^{-1}). Mean values (± SD: standard deviation); n = 7.. Results of two-factor ANOVA main effects analysis of biochemical parameters of brown trout fed experimental feeds; P values lower than 0.05 indicate the significant effect of the factor.

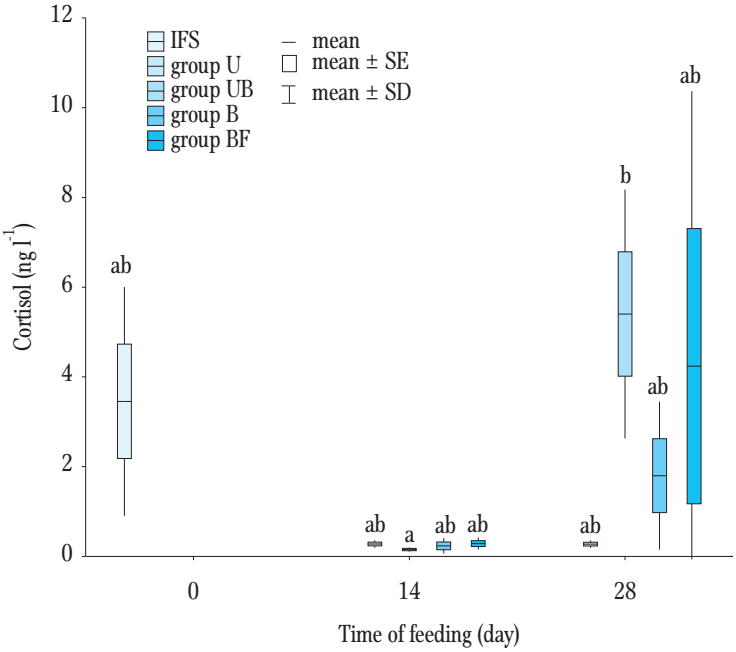

Figure 1. Level of cortisol in brown trout brown trout fed feeds with or without functional supplementation on the initial day of the experiment (d0) and after 14 and 28 days of rearing (d14 and d28, respectively). IFS: initial fish sample, Aller Bronze; B: BioMar EFICO Enviro; BF: EFICO Enviro FOCUS Plus®; U: experimental diet; UB: experimental diet + 20 g kg^{-1} Bioimmuno. Means marked with different letter indexes are significantly different (Kruskal-Wallis ANOVA and Dunn's test $P \leq 0.05$; n = 7).

this cation was significantly higher in comparison to that in the IFS. Increased values of chloride ions compared to the IFS were noted on both d14 and d28. The greatest increases in Cl^- concentrations were noted in groups U and UB (Table 4).

No significant differences among groups were noted in cortisol or GLU values, which are two important indicators of stress (Figure 1, Table 4). It should be mentioned that after two weeks of feeding the feeds tested, clearly lower mean cortisol concentrations were noted in comparison with those in the IFS, while over the subsequent 14 days, cortisol concentrations increased. Simultaneously, differences in the levels of this hormone among individuals were large (Figure 1). Of the nine other biochemical parameters, significant changes were noted in the values of ALB, NH_3, ALP, and AST (Table 4). ALB increased along with the feeding period of the feeds tested. The highest ALB values were noted on d28 in groups BF and UB that were fed feeds supplemented with immunomodulatory substances. In turn, NH_3 and ALP levels were determined by feed type; after d14 of the experiment, NH_3 concentrations in all groups were lower than those in C, and the largest decrease in NH_3 was confirmed in the fish from groups BF and UB. On d28, the mean NH_3 concentrations in groups B and BF fed commercial feeds were at a similar level to those in the IFS, while in groups U and UB they were significantly lower (Table 4). Substantially higher ALP activity was confirmed in groups U and UB in comparison to that in groups C, B, and BF. The activity of the second enzyme, AST, depended on both the feed type and feeding period. The highest AST activity was noted on d14 in the brown trout from group U, which was 67.72 U l^{-1} higher than that in the IFS and as much as 199.00–290.43 U l^{-1} higher than in the other groups. Over the subsequent two weeks, increases in AST activity were noted in group BF, but further decreases were noted in the both groups fed feeds without immunomodulator supplementation and in group UB (Table 4).

Feeding fish the feeds tested did not have a significant influence on the shares of monocytes or eosinophils; however, no eosinophils were detected in any of the fish sampled for testing on d28

Table 5
Relative share (%) of different types of leukocytes (leukogram) of brown trout fed feeds with or without functional supplementation on the initial day of the experiment (d0) and after 14 and 28 days of rearing (d14 and d28, respectively)

		Time of feeding (d14)				Time of feeding (d28)			
		feeding groups				feeding groups			
Parameter	IFS (d0)	B	BF	U	UB	B	BF	U	UB
Lymphocytes S	77.1 ± 22.8^{acd}	94.8 ± 1.5^{abe}	94.9 ± 2.5^{bce}	94.5 ± 2.1^{ad}	95.0 ± 3.2^{acd}	92.7 ± 2.4^{abcd}	88.0 ± 2.1^{ae}	93.5 ± 2.8^{d}	88.9 ± 3.1^{bce}
Lymphocytes L	13.3 ± 9.0^{a}	2.4 ± 1.5^{c}	2.2 ± 1.0^{bc}	3.0 ± 1.6b^{c}	2.7 ± 2.0b^{c}	4.4 ± 1.8^{abc}	5.5 ± 2.1^{ab}	4.1 ± 2.4^{ab}	5.1 ± 2.1^{abc}
Σ Lymphocytes	90.4 ± 16.4^{a}	97.2 ± 0.7^{bd}	97.1 ± 1.6^{b}	97.5 ± 1.7^{bc}	97.7 ± 1.4^{bc}	97.1 ± 2.1^{ad}	93.5 ± 2.4^{ad}	97.6 ± 1.0^{a}	94.0 ± 1.4^{cd}
Monocytes	0.6 ± 0.9^{a}	0.3 ± 0.2^{a}	0.2 ± 0.2^{a}	0.3 ± 0.4^{a}	0.6 ± 0.3^{a}	0.4 ± 0.4^{a}	0.3 ± 0.4^{a}	0.3 ± 0.2^{a}	0.2 ± 0.3^{a}
Neutrophils JN	2.5 ± 2.9^{a}	1.2 ± 0.4^{a}	1.6 ± 1.0^{a}	1.0 ± 0.7^{a}	0.6 ± 0.6^{a}	0.8 ± 0.4^{a}	2.1 ± 1.0^{a}	0.7 ± 0.4^{a}	1.7 ± 0.5^{a}
Neutrophils MN	6.3 ± 12.4^{ab}	1.1 ± 0.9^{a}	1.1 ± 0.9^{a}	1.0 ± 1.0^{a}	1.0 ± 0.8^{a}	1.7 ± 1.7^{ab}	4.1 ± 1.7^{bc}	1.4 ± 0.8^{ac}	4.1 ± 1.0^{bc}
Σ Neutrophils	8.8 ± 15.2^{ab}	2.3 ± 0.7^{ab}	2.6 ± 1.6^{ab}	2.1 ± 1.4^{ab}	1.6 ± 1.3^{a}	2.5 ± 1.9^{ab}	6.2 ± 2.3^{b}	2.1 ± 0.9^{ab}	5.8 ± 1.3^{b}
Eosinophils	0.2 ± 0.4^{a}	0.2 ± 0.3^{a}	0.1 ± 0.2^{a}	0.1 ± 0.2^{a}	0.1 ± 0.2^{a}	0^{a}	0^{a}	0^{a}	0^{a}

IFS: initial fish sample, Aller Bronze; B: BioMar EFICO Enviro; BF: EFICO Enviro FOCUS Plus®; U: experimental diet; UB: experimental diet + 20 g kg^{-1} Bioimmuno; lymphocytes S: small lymphocytes; lymphocytes L: large lymphocytes; Σ lymphocytes: total lymphocytes; neutrophils JN: neutrophils with non-segmented nuclei; neutrophils MN: neutrophils with segmented nuclei; Σ neutrophils: total neutrophils. Mean values (± SD: standard deviation); n = 7. The significance of differences were tested with Kruskal–Wallis ANOVA and Dunn's test after conversion to absolute values. Values in rows marked with different letter indexes are significantly different at $P \leq 0.05$.

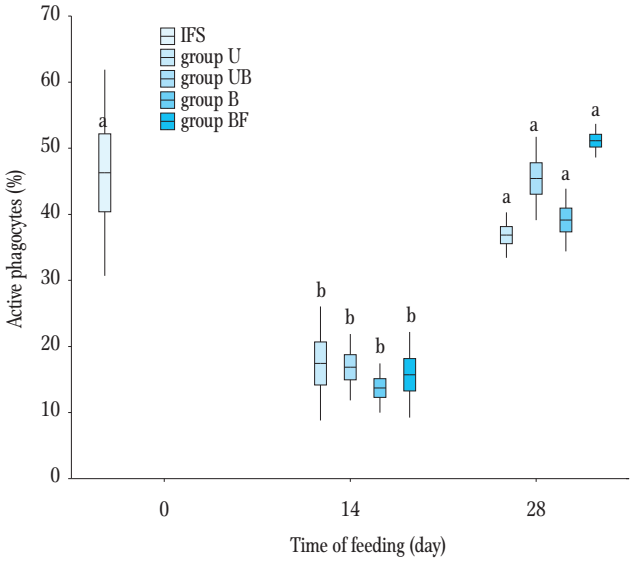

Figure 2. Percentage of phagocytes engulfing zymosan particles in the blood of brown trout brown trout fed feeds with or without functional supplementation on the initial day of the experiment (d0) and after 14 and 28 days of rearing (d14 and d28, respectively). IFS: initial fish sample, Aller Bronze; B: BioMar EFICO Enviro; BF: EFICO Enviro FOCUS Plus®; U: experimental diet; UB: experimental diet + 20 g kg^{-1} Bioimmuno. Values marked with different letter indexes are significantly different at $P \leq 0.01$ (n = 7).

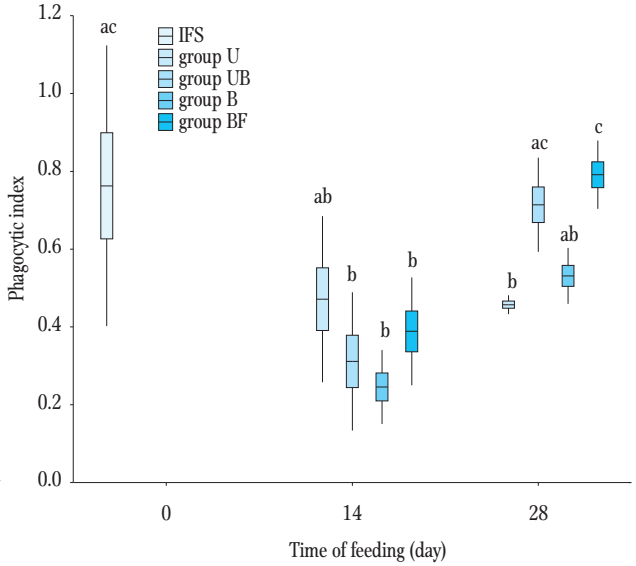

Figure 3. Phagocytic index (PhI) of brown trout fed feeds with or without functional supplementation on the initial day of the experiment (d0) and after 14 and 28 days of rearing (d14 and d28, respectively). IFS: initial fish samlpe, Aller Bronze; B: BioMar EFICO Enviro; BF: EFICO Enviro FOCUS Plus®; U: experimental diet; UB: experimental diet + 20 g kg^{-1} Bioimmuno. Values marked with different letter indexes are significantly different at $P \leq 0.05$ (n = 7).

(Table 5). Feeding the fish experimental feeds had a statistically significant influence on the percentage of L lymphocytes in the peripheral blood ($P \leq 0.05$). A distinct decrease in the shares of these lymphocytes was noted in all fish groups from 13.3% in the IFS to 2.2–3.0% in the experimental groups on d14 and 4.1–5.5% on d28 of the feeding periods. A significant increase in the share of total lymphocytes was also noted in all groups in comparison to the IFS (Table 5), while the share of neutrophils decreased, especially that of MN neutrophils with segmented nuclei.

The percentage of phagocytes actively engulfing zymosan particles (APhC) measured after d14 of the experiment was significantly lower in all groups than that in the initial sample ($P \leq 0.01$), while on d28 it was similar to that in the IFS (Figure 2). Similar trends were noted in phagocytic activity measured with the number of engulfed zymosan particles (PhI), myeloperoxidase activity in phagocytes (A_{MPO}), and phagocyte cidal ability (CA) (Figures 3, 4, 5). Compared to the PhI value in the IFS, significant differences were noted in these values in groups B, BF, and UB on d14 and also in group U on d28 ($P \leq 0.05$; Figure 3). Differences in A_{MPO} values were noted among all groups on d14 and also on d28 in groups BF and UB, which were fed feed supplemented with immunomodulatory substances ($P \leq 0.01$; Figure 4). Significant changes were also confirmed in the values of the cidal ability index. After two weeks of the experiment, the CA index was approximately 20-fold lower in all groups than that in groups C. Over the subsequent two weeks, the value of this index increased to a level that was not significantly different from the initial value in the IFS, and the highest CA values were noted in groups UB and BF, which were fed feeds supplemented with immunomodulators ($P \leq 0.05$; Figure 5).

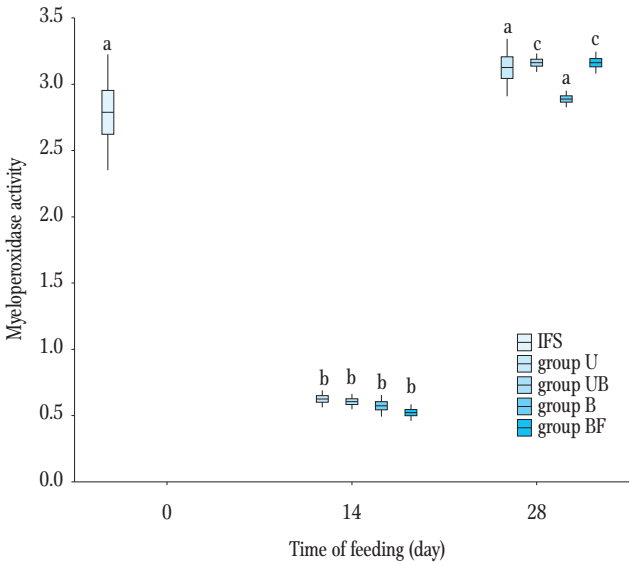

Figure 4. Myeloperoxidase activity (A_{MPO}) in phagocytic cells of brown trout fed feeds with or without functional supplementation on the initial day of the experiment (d0) and after 14 and 28 days of rearing (d14 and d28, respectively). IFS: initial fish sample, Aller Bronze; B: BioMar EFICO Enviro; BF: EFICO Enviro FOCUS Plus®; U: experimental diet; UB: experimental diet + 20 g kg^{-1} Bioimmuno. Values marked with different letter indexes are significantly different at $P \leq 0.01$ (n = 7).

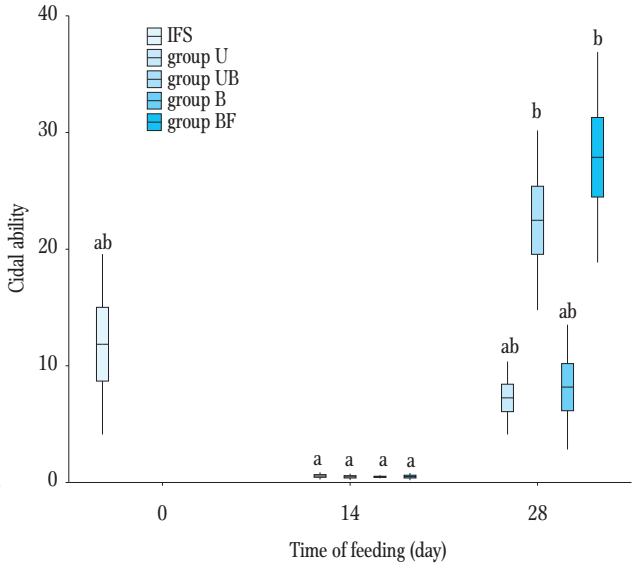

Figure 5. Cidal ability (CA) of brown trout brown trout fed feeds with or without functional supplementation on the initial day of the experiment (d0) and after 14 and 28 days of rearing (d14 and d28, respectively). IFS: initial fish sample, Aller Bronze; B: BioMar EFICO Enviro; BF: EFICO Enviro FOCUS Plus®; U: experimental diet; UB: experimental diet + 20 g kg^{-1} Bioimmuno. Values marked with different letter indexes are significantly different at $P \leq 0.05$ (n = 7).

Discussion

β-glucan is widely recognized as one of the most important active substances produced by fungi that has many health benefits. It mainly stimulates macrophages enhancing their phagocytic properties and increasing the production of free radicals. It stimulates the complement system and the secretion of interferon. It also affects other cells in the immune system including B lymphocytes and dendritic and natural killer (NK) cells (Vetvicka et al. 2019), while also increasing immunity to fungal, viral, and bacterial infections (Vetvicka et al. 2002, 2019). Human clinical trials indicated that glucan can lower cholesterol and GLU blood concentrations, affect intestinal peristalsis, and reduce appetite and food intake (Chen and Raymond 2008).

In the current study, feeding brown trout feeds supplemented with 1,3/1,6 β-glucan did not influence survival, rearing parameters (CL, SL, BW, DGR, SGR), condition, or feed conversion factors. Similarly glucan at a dose of 0.5–1.5% for a feeding period of 62 days did not influence the growth or survival of juvenile tropical gar (*Atractosteus tropicus* Gill) (Nieves-Rodríguez et al. 2018). Similar observations were also reported for rohu (*Labeo rohita* (Hamilton)) (Tayyab et al. 2019), which was fed feed supplemented with 0.1% glucan for a feeding period of 120 days. Tayyab et al. (2019) reported that, in addition to a lack of differences in growth among groups of this fish species, the diet had no influence on fish body composition, total serum proteins, or hematological parameters. In turn, Caspian trout (*Salmo trutta caspius* Kessler) fed feed supplemented with prebiotics and probiotics (β-glucan, 3 mg g^{-1}; mannan oligosaccharide, 4 mg g^{-1}; and *Lactobacillus plantarum*, 10^8CFU mg^{-1} diet), either individually or in combination, were noted to have reduced feed intake and FCR and increased weight gain (WG), protein efficiency ratio, and final body weight (Jami et al. 2019). β-glucan was also confirmed to have a positive influence on rearing indices in studies conducted on rainbow

trout (supplementation levels of 0.05%, 0.1%, and 0.2%; feeding period of 42 days), and higher dietary β-glucan levels improved SGR, WG, and feed efficiency (Ji et al. 2017). Additionally, survival rates of fish fed feeds with β-glucan increased significantly compared with the control group after *Aeromonas salmonicida* infection. Studies of kutum (*Rutilus frisii kutum* (Kamensky)) showed improved growth performance, body protein levels, and survival rates after applying glucan at doses of 0.5 and 1.0% for a 60-day feeding period (Rufchaie and Hoseinifar 2014). However, higher doses of the supplement of 1.5 and 2.0% did not influence rearing indices. This indicates that fish species react differently to β-glucan and also to the dose and the period during which it is applied.

While the application of products containing β-glucan usually have a positive influence on fish hematological parameters, they are not always identical in all species. Increased WBC counts in fish groups fed feed supplemented with β-glucan are confirmed in, among others, common carp (supplementation of 1%, feeding period 60 days. Gopalakannan and Arul 2010), rohu (0.25 g kg^{-1}, 42 days; Misra et al. 2006), and striped catfish (*Pangasianodon hypophthalmus* (Sauvage)) (1.0 g kg^{-1}, 30 days; Nguyen et al. 2016). In turn, significant increases in WBC counts were noted in channel catfish (*Ictalurus punctatus* (Rafinesque)) after just two weeks of β-glucan application (dose 0.05, 0.1, 0.5%; Sánchez-Martínez et al. 2017). The increase in WBC counts in this species was accompanied by gradual decreases in RBC and HCT the longer the feeding period was with the diet supplemented with glucan (Sánchez-Martínez et al. 2017). Similar observations were made of red snapper (*Lutjanus guttatus* (Steindachner)) after using 0.05% glucan (Del Rio-Zaragosa et al. 2011); however, Nguyen et al. (2016) report that in juvenile striped catfish feed supplemented with β-glucan at a dose of 1 g kg^{-1} for 30 days caused increases in all of the basic blood parameters of WBC, RBC, HCT, and HGB. Interestingly, lower (0.5 g kg^{-1}) and higher (1.5, 2.0, and 2.5 g kg^{-1}) doses did not cause significant changes in these indicators ((Nguyen et al. 2016). The study results cited above indicate that there are significant differences among fish species in their reactions to β-glucan, both with regard to the dose of the supplement and the period for which it is applied. This thesis is also confirmed by the results of studies of rainbow trout (Minářová et al. 2021) in which two feeds were applied, as in the current study; these were the standard EFICO Enviro and the functional EFICO Enviro FOCUS Plus® (supplemented with β-glucan and vitamins C and E). After 28 days of feeding rainbow trout with these feeds, no significant differences among groups were confirmed in the values of WBC, RBC, HGB, HCT, MCH, MCV, or MCHC (Minářová et al. 2021).

In the current study conducted on juvenile brown trout, no differences were noted only in the values of MCV or PLT. However, the values of WBC, RBC, HGB, HCT, MCH, and MCHC were significantly determined by the type and/or the feeding period of the given feeds. Changes in diet caused significant decreases in the values of WBC and HGB in all fish groups. The values of these parameters after d28 of the experiment were lower than those in the control group, while the lowest WBC and HGB values were noted on d14, especially in groups B and BF that were fed commercial feeds. What is significant is that the lower WBC and HGB values were confirmed in the fish groups fed feeds supplemented with β-glucan. Decreases in RBC and HCT values were noted the longer the experimental feeds were applied. The opposite tendency was noted for MCH and MCHC values. Increases in the values of these parameters with a simultaneous decrease in the values of RBC, HCT, and also HGB (until d14) could be explained by the fish refusing to eat following the change in diet (Ahmed et al. 2020, Witeska et al. 2021). However, in the current study, the results of daily monitoring of feed consumption and the values of the rearing parameters obtained indicated that these changes stemmed mainly from the modification of the diet. This could also suggest hemolytic anemia caused by shortened red blood cell survival and also decreased production or release of erythrocytes into the peripheral blood. Increased MCH and MCHC values could also be symptomatic of respiratory or metabolic acidosis. In this case,

erythrocyte swelling and disruption is observed because of changes in blood pH (Gomułka et al. 2008), and these changes are usually accompanied by increases in AST activity. However, significant changes in MCV values were not noted, and AST activity was positively correlated with erythrocyte numbers ($R^2 = 0.32$, $P < 0.01$).

Important information about the health status, growth potential, and vitality of animals is not only provided by the absolute WBC value but also by the leukogram, which is the relative share of different types of leukocytes (Davis et al. 2008, Sopinka et al. 2016). Often the responses of organisms to given environmental factors are manifested more quickly in changes in leukocyte profiles than in WBC counts (Fazio 2019). In studies conducted on sea trout (*Salmo trutta* L.) fed feed supplemented with β-glucan (1 or 3 g kg^{-1} feed), Revina et al. (2019) analyzed WBC counts, leukograms, and HCT values. In blood samples drawn in January, these researchers noted significant increases in the percentage of neutrophils and eosinophils, while also noting changes in the WBC and HCT values. An increased share of neutrophils was observed in fish fed feed supplemented with 3 g glucan kg^{-1}; however, providing sea trout feed with a lower glucan content (1 g kg^{-1}) caused an increase in the percentage of eosinophils.

In the current experiment, brown trout was characterized by a high potential for adaptive immunity response, which was reflected in the high number of lymphocytes in all the groups studied. Increased shares of lymphocytes to over 90% in all groups after two weeks of feeding experimental feeds (d14) was determined primarily by the increased number of small lymphocytes (S), which indicated the body's readiness for a humoral response to any potential infection (Witeska et al. 2021). The increased share of total blood lymphocytes was also caused by a decrease in the neutrophils in the peripheral blood, particularly of MN neutrophils, the share of which decreased in this period more than 4.5-fold. Similar changes, although not statistically significant, were noted in rainbow trout fed EFICO Enviro FOCUS Plus® (Minářová et al. 2021), while a more than a five-fold decrease in neutrophils was noted in Nile tilapia fed feed supplemented with β-glucan (Sado et al. 2016). The general consensus is that MN neutrophils are mature cells that are fully ready for defense responses (Clauss et al. 2008). Since these cells are capable of migrating to other tissues (Havixbeck and Barreda 2015, Lerman and Kim 2015), one could presume that the change in diet stimulated the immune system and shifted the MN neutrophils to the digestive tract without, however, causing intestinal tissue inflammation as indicated by the lack of change in CRP values. In effect, the share of JN and MN neutrophils in the peripheral blood evened out (in the IFS the proportion was approximately 1.0:2.5). This can be explained by the significant decrease in PhC and the decrease in myeloperoxidase activity in phagocytes in the peripheral blood of brown trout after two weeks of being fed experimental feeds. The changes above were very well reflected in changes in the values of the cidal ability index.

These findings are also in agreement with the recent discovery of neutrophil extracellular traps (NETs) and their role in fighting microbial infection. NETs are web-like extracellular structures released by neutrophils following microbial activation. The formation of NETs allows neutrophils to trap microbes and prevent further dissemination in place of inflammation (Brinkmann et al. 2004). According to Vong et al. (2014) there is emerging evidence suggesting that uncontrolled or excessive NET formation, and the associated liberation of cell-free DNA and degradative proteases, damages surrounding cells and contributes to disease pathophysiology. Vong et al. (2014) discovered that probiotic *Lactobacillus rhamnosus* inhibits NET formation, decreases reactive oxygen species (ROS), and phagocytic capacity of neutrophils in mice thus protecting against cell cytotoxicity especially in the alimentary tract. We believe that the changes observed in brown trout reflected a similar process, specifically the activation of neutrophils by β-glucan, neutrophil migration to gut tissue, and the apparent decrease of ROS production and phagocytic capacity in the bloodstream. However, this hypothesis

requires confirmation in future studies, particularly because the link between these processes and cortisol depletion is unclear. For example, Montoya et al. (2017) suggests that β-glucan induced cortisol increases that improved the innate immune response in matrinxă (*Brycon amazonicus* (Spix & Agassis)), while Marinho de Mello et al. (2019) found that β-glucan had a protective effect by avoiding higher levels of the hormone and improving resistance against bacterial infection in pacu (*Piaractus mesopotamicus* (Holmberg)).

After four weeks of the experiment, a return to levels close to (or significantly higher in myeloperoxidase activity in UB and BF) than those of the initial sample was observed in all measures of phagocytic activity. These changes are best reflected by CA values, the highest levels of which were noted in groups UB and BF at 27.9 and 22.5 respectively. These results indicate that both Bioimmuno (group UB) and BioMar EFICO Enviro FOCUS Plus® (group BF), when applied for a minimum of four weeks, stimulated increases in the levels of innate immunity in brown trout. No differences in oxygen burst, immunoglobulin, or protein levels were noted in rainbow trout fed EFICO Enviro FOCUS Plus® for 28 days, and complement activity was significantly lower in comparison to that in the control groups (Minářová et al. 2021); however, after experimental infection with *A. salmonicida*, the survival of the fish in this group was higher.

Cortisol levels in the blood of individual brown trout specimens varied, but in most specimens is ranged from 0.111 to 3.928 ng ml^{-1}. Only in a few specimens were higher values noted within a range of 7.043–13.345 ng ml^{-1}. In all the groups tested, cortisol was at the baseline level (mean value of less than 8 ng ml^{-1}) (Bry 1982, Pottinger and Moran 1993). Since Tukey's post hoc test did not reveal any significant differences but ANOVA did ($P < 0.05$), Kruskal–Wallis ANOVA and Dunn's test were performed. The only significant difference was found between fish fed EFICO Enviro FOCUS Plus® after two- and four-week feeding periods. However, there was an obvious decreasing trend in cortisol levels after two weeks of feeding the fish the experimental feeds. This effect was not dependent on feed type but was caused by changing the feed itself. This phenomenon is difficult to explain and requires further research.

Concentrations of electrolytes (Na^+ and Cl^-) and GLU can be used as stress indicators since the cortisol released during stress responses influences the levels of these substances in the blood (Evans et al. 2003, Barandica and Tort 2008). In the current study, the level of Cl^- was determined by the type of diet, while that of Na^+ by the feeding period. Elevated Cl^- values were noted on both d14 and d28, and the highest increase in the concentrations of these ions occurred in the groups of fish fed the experimental feeds with and without immunomodulator supplementation. Changes in diets caused decreases in the concentrations of Na^+ in all groups. After four weeks of the experiment, the concentrations of this electrolyte were higher than the those in the IFS. These phenomena are linked with changes in gill diffusion capacity resulting in changes in plasma osmolality and ion concentrations. These types of body reactions depend on many internal and external factors, which is why it is difficult to interpret these results unambiguously (Sopinka et al. 2016). Vetvicka et al. (2013) emphasized that glucan lowers blood GLU levels and reduces stress. In the present study, GLU concentrations in all groups were slightly higher than those in the IFS, but the differences were statistically insignificant. The diets applied also did not influence levels of TP, GLB, or CRP. Sánchez-Martínez et al. (2017) reported similar observations in their study on channel catfish fed for one to five weeks with feeds supplemented with different doses of glucan, and regardless of the amount of supplementation or the feeding period, they noted no significant changes in TP or GLB concentrations in the blood of the fish studied. Similar observations were reported for red snapper and sea bass (*Dicentrarchus labrax* (L.) (Sitjá-Bobadilla and Pérez-Sánchez 1999, Bagni et al. 2005, Del Rio-Zaragosa et al. 2011). In turn, Pionner et al. (2013) demonstrated in carp two-fold increases in CRP concentrations and 35-fold increases in complement activity after two weeks of

applying feed with glucan (MacroGard®); it must be noted here that the supplement dose was 6 mg kg^{-1} of fish body weight.

In brown trout significant changes were determined in ALB and NH_3 levels and ALP and AST activities. ALB concentrations increased with the length of the feeding period with the feeds tested especially in groups BF and UB that were fed feed supplemented with immunomodulatory substances. In turn, the NH_3 level was determined by the type of feed applied. After d14 of the experiment, NH_3 concentrations in all groups were lower than in the IFS, and the largest decrease in NH_3 was confirmed in the fish from groups BF and UB that were fed feed supplemented with immunomodulatory substances. On d28 the mean NH_3 concentrations increased, while in groups B and BF, which were fed commercial feeds, the levels were close to those in the IFS, but in groups U and UB levels were still lower. Increased ALB and TP concentrations might suggest improvement in innate immunity or stronger nonspecific reactions in fish (Tavares-Dias and Moraes 2007). However, changes in the levels of NH_3 could be explained by changes in diet and metabolic rate.

The brown trout from groups U and UB exhibited much higher ALP activity in comparison to that in groups C, B, and BF. Significantly, on d28 the activity of this enzyme was higher in the groups fed feed supplemented with glucan. Increased ALP activity in the blood plasma is usually linked with disruptions in the functions of the liver, bile ducts, or the gallbladder. However, an activity of this enzyme of 7–8% in the blood plasma reflects the level of isoforms secreted in the intestines (Lallès 2019). According to Lallès (2019), intestinal ALP plays an important role in developing immune tolerance to microbes during gut colonization. It is also pivotal in keeping under control gut inflammation that is caused by a number of microbial and endogenous factors (e.g., lipopolysaccharide – LPS; adenosine triphosphate – ATP). In contrast, a tissue nonspecific isoform of ALP from the colon is reported to be of neutrophil origin and increases in its activity often reflects colonic tissue inflammation (Lallès 2014). Ji et al. (2019) indicated that feeds supplemented with β-glucan help to control intestinal inflammation in rainbow trout. This hypothesis of the extrahepatic origin of increased ALP activity in the blood plasma appears to be confirmed by ALT and AST activities, which are considered to be enzyme indicators of parenchymal tissue damage. ALT activity, which is considered to be more "liver specific", did not exhibit significant changes in any of the experimental groups. Further, ALT levels below 20 U l^{-1} were noted in all the groups, and this most likely excluded any parenchymal tissue damage. AST activity, however, depended on both the type of feed and the feeding period during which it was applied. On d14 the highest AST activity, which was higher than that in the initial sample, was noted in brown trout from group U. The values of this parameter in the other groups was lower by 199.00–290.43 U l^{-1}, while the lowest AST activity was noted in the fish that had been fed feed supplemented with glucan. In the subsequent two weeks, increased AST activity was noted in group BF, while further decreases in it were noted in both groups fed feeds without immunomodulators and in group UB. Although the changes in AST activity in brown trout cannot be interpreted unambiguously, one can postulate that they did not occur as a result of parenchymal tissue damage. It is likely that the elevated levels of this enzyme, which were noted in both the initial and experimental groups, could have resulted from providing formulated feed that damages the epithelial tissue of the intestines or other tissues in the body. Elevated AST levels exceeding 40 U l^{-1} are often reported in salmonids fed formulated feeds. The cause of the tissue damage, mainly to intestinal tissue, are the non-nutritional components derived from plants; for example, feeds containing soy meal are rich in saponins that cause enteritis (Krogdahl et al. 2015). Explaining this phenomenon requires further study.

Conclusions

The current study demonstrated that changes in diet, regardless of diet type, cause negative changes in the values of hematological, biochemical, and

immunological parameters in brown trout juveniles. Inter alia, they lead to decreasing numbers of phagocytes in the peripheral blood, which can significantly influence immune capacity against new pathogens. These changes, which are linked with adapting to new feeds, were mainly observed in the first 14 days after diet changes. Following this period and thanks to compensatory processes, homeostasis is gradually restored. Consequently, changing diets two weeks before exposing brown trout to stressful conditions or stress factors, such as stocking, is not recommended. It must be underscored that only after d28 of the experiment did the range of hematological, biochemical, and immunological parameters increase to levels that did not differ from initial ones or that were statistically significantly higher, such those of A_{MPO} and CA. Therefore, the conclusion that can be drawn is that feeding brown trout feeds supplemented with immunomodulatory products has no clear advantageous influence on the welfare of this species. It is recommended, however, to apply these types of feeds at least four weeks prior to transporting the fish or releasing them into open waters.

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