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Effects of long-term administration of various dietary prebiotic supplements on the growth, immune cell activity and digestive tract histology of juvenile vimba *(Vimba vimba)*

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Abstract

Introduction: The experiment was set to determine the effects of long-term (55-day) use of three commercial prebiotics including *Saccharomyces cerevisiae*–derived β-glucans and one including inulin on juvenile vimba (*Vimba vimba*) reared intensively under controlled conditions. **Material and Methods:** Six-month-old fish were fed commercial feed (Control group, n = 90), or the same feed supplemented with 0.02% Leiber Beta-S (BS group, n = 90), 0.20% Biolex MB40 (MB group, n = 90), 0.30% CeFi (CE group, n = 90) or 1.00% inulin Orafti GR (IN group, n = 90) for 55 days. **Results:** In the BS group, the final growth parameters were significantly lower than in the Control group, while the feed conversion ratio was significantly higher. No significant differences were found between any other group and the Control group in the respective parameters. The respiratory burst activity of the head-kidney phagocytes was significantly lower in all fish groups fed the prebiotic-supplemented diets compared to the Control group. The proliferative response of the head-kidney lymphocytes stimulated by concanavalin A was lower in the BS group than in the Control group, while in other groups this response was not affected. No significant differences were found in histopathological analyses of the digestive tract, liver or pancreas. **Conclusion:** The long-term supplementation of fish diets with prebiotics can negatively influence the growth, feed conversion, nonspecific cellular resistance and proliferative activity of the T lymphocytes of vimba juveniles.

Keywords: innate immunity, aquaculture, β-glucan.

Introduction

Populations of many fish species living in the wild are subject to strong fishing pressure. As a result, they are now maintained largely by human intervention, mostly restocking. Thus, the demand for stock of an increasing number of fish species grows constantly. This also applies to numerous populations of vimba (*Vimba vimba*), which is a species of Cypriniformes and was once commercially valuable. The success of stocking depends on the availability of the stocking material and its biological quality, as well as other factors such as the stocking procedure and the environmental conditions in target water bodies (*e.g.* the abundance of food, number of predators, quality of water and carrying capacity).

Fish production for restocking purposes increasingly takes place in recirculating aquaculture systems (RAS), which, in addition to having many advantages, present certain shortcomings. Economic considerations obligate fish farmers to maintain high fish densities and provide a diet composed of commercial dry feeds, which often leads to negative consequences for the growth and disease resistance of fish. Having no contact with pathogens present in the natural environment, fish reared

in RAS do not develop effective immune system mechanisms; therefore, they are unable to defend themselves against diseases and succumb to bacteria occurring in natural water bodies (34).

The latter problem has encouraged the search for natural substances that could strengthen the resistance of fish and minimise the multiplication of microorganisms. For this purpose, various feed additives such as prebiotics, probiotics, synbiotics and vitamin mixes are used in contemporary aquaculture practice.

Prebiotics are indigestible nutrients that demonstrate a positive effect on the host organism by selectively activating the growth and/or activity of bacteria dwelling in the fish digestive tract. Prebiotics are not hydrolysed or absorbed in the proximal section of the intestine and instead reach the distal part of the gut in an unchanged form, where they undergo fermentation by intestinal bacteria. Lactic acid and short-chain fatty acids (SCFAs), products of this fermentation, stimulate the activity of immune system cells and release proinflammatory mediators (interleukins), regulate the pH of the intestinal contents and facilitate the absorption of elements such as calcium, iron and magnesium. Short-chain fatty acids are the main nutrients for enterocytes; therefore, they contribute to the multiplication of cells lining the intestinal villi, to more rapid healing of possible injuries or wounds in the gut, and to increasing the number of capillaries in the intestinal mucosa. As a result, SCFAs expand the area for nutrient absorption, leading to a higher growth rate for the animals.

The prebiotics that have been used as feed supplements in aquaculture comprise a variety of substances, mainly polysaccharides. Some are β-glucans or fructans such as inulin, and others are oligosaccharides such as fructo-oligosaccharides, mannan-oligosaccharides (MOS), galacto-oligosaccharides, xylo-oligosaccharides, arabinoxylo-oligosaccharides and isomalto-oligosaccharides.

It has been demonstrated that polysaccharides activate innate mechanisms of the immune system in two ways, *i.e.* by having a direct influence on the immune system, where they bind to specific receptors, causing a cascade of the cellular and humoral immunological response; and by activating the beneficial intestinal flora, which inhibits the multiplication of pathogenic bacteria (11). Thus far, however, the mechanism through which immunostimulants enhance the growth rate of fish has not been elucidated thoroughly. Beta-glucans in the form of solid particles are neither absorbed through the digestive tract nor digested by the digestive enzymes of fish and are therefore not a source of nutrients for these animals.

Inulin is a well-known prebiotic widely administered to animals and used by humans, although it has less often been used in aquaculture thus far, and the assessments of its effect on the growth parameters of fish found in the literature are contradictory. Enhanced growth has been induced by an inulin supplement in rainbow trout (*Oncorhynchus mykiss*), Siberian sturgeon (*Acipenser baerii*) and Nile tilapia (*Oreochromis niloticus*) (15, 26). However, a dietary supplement of inulin can also reduce fish growth, as was found in beluga (*Huso huso*) (29).

Obtained from the cell walls of the yeast *Saccharomyces cerevisiae*, β-glucans are a group of polysaccharides that have been most thoroughly investigated. They are also structural components of the cell walls of fungi (*Sclerotium glucanicum*, *Grifola frondosa*, *Lentinula edodes*, *Schizophyllum commune*, *Coriolus versicolor* and *Pleurotus ostreatus*), some bacteria (*Alcaligenes faecalis*), algae (*Laminaria digitata*), and even cereals. Glucans are a heterogeneous group of polymers of glucose with different structures and molecular weights, which demonstrate a significant effect on the immune response. Glucans with higher molecular weights activate the innate immune response, while those with low molecular weights do not produce such an effect. However, the glucan extraction and purification methods applied, as well as the source of glucans, can have varied effects on the organism which ingests the glucan. In fish, the effects derive from the presence of appropriate receptors on the immunocompetent cells (28). Studies have demonstrated that neutrophils, macrophages, monocytes and dendritic cells of fish are capable of recognising β-glucans (21).

It is feasible to apply β-glucans alone or to mix them with other immunostimulants to fortify the immune response of fish. Examples of immune stimulants that have been used together with β-glucans are mannan-oligosaccharides and glucomannanoprotein complexes originating from the cell walls of *S. cerevisiae* (11). However, studies on the effectiveness of β-glucans applied together with MOS on the immune system of fish are scarce. When given to rainbow trout and Nile tilapia, the following effects have been observed: enhancement of the blood leucocyte count, higher total protein concentration, elevated bactericidal activity, improved activity of lysozyme and of the alternative complement activity, slightly lower mortality and significantly more rapid growth (1, 31). However, results reported by other authors suggest that supplementation with this mixture of prebiotics does not always produce a positive effect on the growth rate or feed conversion ratio (13, 22).

Supplementation with polysaccharides is an increasingly important routine prophylactic measure. Nonetheless, there are few articles dealing with the adverse effects caused by the prolonged application of immunostimulants, and the results obtained thus far have frequently been contradictory. To gain a more in-depth insight into this problem, an attempt was undertaken to determine the effects of three commercial fish prebiotics containing β-glucans and those of one containing inulin. The preparations were given to juvenile vimba fish for 55 days and observations were made of how they affected the feed conversion ratio, growth rate, immune response and morphology of the digestive system.

Material and Methods

Experimental fish. Juvenile vimba used in the experiment were the pooled progeny of four females and five males. They were obtained from an experimental hatchery at the National Inland Fisheries Research Institute in Żabieniec, Poland. Experimental fish were raised from the beginning of their lives under controlled conditions preparing them for the experiment. At the beginning of the experiment, the fish were 6 months old and their mean (± standard deviation – SD) body weight (BW) and total length (TL) were 0.47 ± 0.10 g and 4.30 ± 2.90 cm, respectively.

Experimental conditions. Prior to being introduced into the experimental aquaria, the fish were checked for the absence of body deformities and graded according to their BW to unify their initial size distribution in all experimental groups according to the method described by Myszkowski *et al.* (25). The fish were stocked in fifteen 20-L flow-through glass aquaria and were divided into five groups, *i.e.* the Control group and four experimental groups, all with 30 fish in each aquarium and three replicate aquaria.

The aquaria were continuously supplied with filtered, heated and aerated water from an RAS flowing at approximately 0.25 L min^{-1}. The water temperature and dissolved oxygen concentration in the water were measured twice a day. The mean water temperature in different experimental groups ranged from 25.08 to 25.15°C. The water in the aquaria was continuously aerated with airstones. The oxygen concentration in water was maintained above 59% saturation, and the mean values ranged from 77.7 to 80.9%. Other water quality parameters were monitored weekly in one aquarium per group. The concentrations of total ammonia and nitrates were 0.103–0.688 mg L^{-1} and 0.080–0.101 mg L^{-1}, respectively; the conductivity was 407–426 µS cm^{-1}, and the pH was 7.87–8.03. The aquaria were illuminated from 08:00 to 21:00 by LED light at approximately 450 lx at the water's surface.

Experimental design. In the Control group, fish were fed dry Aller Futura EX GR 0.5–1.0 mm commercial starter feed (Aller Aqua, Christiansfeld, Denmark). According to the producer, the proximate composition of the feed was 60.0% crude protein, 12.6% ash, 15.0% total lipids, 5.7% nitrogen-free extract, 0.7% fibre, 1.4% phosphorus, and 2.5% calcium and it contained 21.2 MJ kg^{-1} of gross energy. Four different prebiotic preparations were used in the experiment (Table 1). The prebiotic-supplemented diets were prepared according to Kazuń *et al.* (16). The dosage of the prebiotic preparations in groups BS, MB and CE was based on the producer's recommendations. In the IN group, the supplement dose was based on the method for setting this dose described by Cerezuela *et al.* (9). All fish feeds were stored in a refrigerator. Feed was given manually in equal portions five times a day every 3 h between 08:00 and 20:00. The daily food ration per aquarium was initially 0.56 g and 3.97% of the fish biomass. However, it was increased on day 15 to 0.90 g and on day 29 to 1.45 g to adjust to fish biomass increase during the experiment. On these days all the fish were counted and weighed under mild anaesthesia after a 12-h overnight starvation period and the feeding period was 11:00 to 20:00 instead of 08:00 to 20:00. The experiment lasted 55 days.

Sample collection for immunological assays. At the end of the experiment, all fish were anaesthetised, and their individual BW and TL were measured. All 30 fish from each experimental aquarium were euthanised by immersion with an overdose (150 mg L^{-1}) of unbuffered tricaine methanesulphonate solution (MS-222, Sigma-Aldrich, St. Louis, MO, USA), after which the liver, spleen, head kidney and intestinal tract samples were taken.

Isolation of vimba immune cells. Vimba head kidneys were pooled within the groups (the organs from 15 individuals kept in the same aquarium per pool, giving two pools per aquarium, and all three aquaria in a group being sampled: n = 6). Head kidney immune cells were isolated using Histopaque 1077 (Sigma-Aldrich) density gradient centrifugation, suspended at a concentration of 1×10^6 cells mL^{-1} in RPMI-1640 medium supplemented with 10% foetal calf serum and 1% antibiotic–antimycotic solution (both reagents from Sigma-Aldrich), and cultured/incubated as described in a previous publication (16). Isolated cells were then used for assays of respiratory burst activity (RBA), potential killing activity (PKA) and proliferative response of lymphocytes. Samples obtained from each pool were tested in duplicate.

Respiratory burst activity and potential killing activity tests. The intracellular respiratory burst and potential killing activities of phagocytes were determined as previously described by Kazuń *et al.* (18).

Table 1. Prebiotic supplements used in the experiment

Group	Prebiotic name	Producer	Brief characteristics	Dosage
BS	Leiber Beta-S	Leiber GmbH, Germany	High-purity 1.3-1.6-beta-D-glucan molecules from the cell wall of *Saccharomyces cerevisiae*	0.02%
MB	Biolex MB40	Leiber GmbH, Germany	The cell walls of *S. cerevisiae*	0.20%
CE	CeFi pro	Leiber GmbH, Germany	The cell contents and the cell walls of *S. cerevisiae*	0.30%
IN	Inulin Orafti GR	BENEO-Orafti SA, Belgium	93.4% inulin and 6.6% glucose+fructose+sucrose in dry matter	1.00%

In short, the adherent immune cells were incubated in a fresh medium containing 0.1% nitroblue tetrazolium (NBT; Sigma-Aldrich) and 1 μg mL^{-1} phorbol myristate acetate (PMA; Sigma-Aldrich) or *Aeromonas hydrophila* (1×10^8 cells mL^{-1}) for 60 min at 22°C. Once the supernatant was removed, the cells were fixed with absolute ethanol and the reduced NBT was extracted using potassium hydroxide (KOH; Chempur, Piekary Śląskie, Poland) and dimethyl sulphoxide (DMSO; POCh/Avantor, Gliwice, Poland). The optical density (OD) of the samples was measured colourimetrically at 620 nm. The results were expressed as a stimulation index (SI), which was calculated by dividing the mean OD of PMA (RBA test) or bacteria-stimulated cells (PKA test) by the OD of the unstimulated control cells.

Proliferative response of lymphocytes – MTT reduction assay. The mitogenic response of vimba lymphocytes was determined using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colourimetric assay described by Kazuń *et al.* (18). The head kidney immune cells were cultured in the presence of mitogens: concanavalin A (ConA) as a T-cell mitogen or lipopolysaccharide from *Escherichia coli* (LPS) as a B-cell mitogen (both mitogens purchased from Sigma-Aldrich and used at concentrations of 10 μg mL^{-1}) for 72 h at 22°C. Unstimulated control cells were maintained in a medium without mitogens. Following incubation, 10 μL of MTT; (Sigma-Aldrich) solution at 10 mg mL^{-1} concentration was added to each well, and the plate was incubated for 3 h. After the supernatant was removed, the reduced MTT was dissolved in DMSO, and the optical density was measured at a wavelength of 570 nm, with 640 nm as a reference wavelength. The results were expressed as a stimulation index, which was calculated by dividing the mean OD of the mitogen-stimulated cells by the OD of the unstimulated control cells.

Histological analysis. Histological samples were fixed in Bouin solution for 24 h and submitted to the standard paraffin procedure. Sections of the digestive tract from the proximal to the distal part of the liver and pancreas were stained with the haematoxylin and eosin (HE) and Alcian blue/periodic acid Schiff (AB/PAS) (pH 2.5). The following intestine measurements were taken with DLT-CamViewer software (Delta Optical, Mińsk Mazowiecki, Poland): fold height, enterocyte height and fold width. All measurements were conducted in two individuals of each triplicate (six per group) for 10 to 30 measurements per parameter. The hepatocyte area and the hepatonucleus area were measured manually in the liver using QuPath (v. 0.3.0) software (8).

Fish growth and feed utilisation calculations. The condition factor (K) of the fish was calculated as $BW\ (TL^3 \times 10^5)^{-1}$. The relative growth rate (RGR) was determined with the formula $(e^g - 1) \times 100$, where $g = (\ln BW_f - \ln BW_i) \times d^{-1}$, with BW_f and BW_i being the final and initial mean body weights of the fish, respectively, and d being the duration of the experiment in days. It was also necessary to know the increment in total length (ITL), and this was obtained from $(TL_f - TL_i) \times d^{-1}$, where TL_f and TL_i were the final and initial mean total lengths of the fish, respectively. The final production indicator required was the feed conversion ratio (FCR), which was derived by dividing the feed intake by the biomass gain of the fish.

Statistical analysis. After validation of normality with the Shapiro–Wilk test and homogeneity of variances with Brown–Forsythe's test, all data were subjected to a one-way analysis of variance followed by a two-sided Dunnett's *post-hoc* test. The difference between the means was tested at the 5% probability level. All statistical analyses were carried out using the GraphPadPrism 7 software package (GraphPad Software, San Diego, CA, USA).

Results

Fish growth and feed utilisation. No mortality occurred during the experiment. No significant ($P < 0.05$) differences were found in fish final BW, TL, K, RGR, increment in total length (ITL) or feed conversion ratio (FCR) between the Control group and any of the experimental groups MB, CE or IN (Table 2). However, in the experimental group BS, the final mean values for BW, TL, RGR and ITL were lower and FCR was higher compared to the Control group.

Evaluation of nonspecific cellular immunity. Results of the RBA of the head kidney phagocytes in all groups of fish fed a prebiotic supplemented diet were significantly lower than in the Control group (Fig. 1A). The PKA of the head kidney phagocytes was similar in all experimental groups (Fig. 1B).

Proliferative response of lymphocytes – MTT reduction assay. In the BS group, the proliferative response of the head kidney lymphocytes stimulated by ConA was significantly lower than that in the Control group (Fig. 1C), while differences regarding all other groups were not significant. Neither were differences found in the proliferative response of the head kidney lymphocytes stimulated by LPS (Fig. 1D).

Histological evaluation. No significant histopathological changes were found in any of the analysed fish groups. The histological structure of the digestive tracts of individuals from each group varied only insignificantly comparing sections like with like, except for the height of enterocytes in the proximal part of the intestines, which was significantly higher in the CE group than in the Control group (Table 3). In all posterior sections, PAS-positive absorption vacuoles were present in the intestinal epithelium in the supranuclear region (Fig. 2).

No histopathological changes were found in the liver or pancreas of the studied fish. The structure of the liver was similar in all the groups, without melanomacrophage centres, steatosis or inflammation symptoms. The pancreas of all individuals was composed of acinar cells with a well-differentiated polar structure and visible zymogen granules but without inflammatory features, infiltrative cells or necrosis and only moderate adipose tissue hyperplasia.

Table 2. Comparison of the final results for fish size, growth rate and efficiency of feed utilisation for vimba (*Vimba vimba*) fed different experimental prebiotic-supplemented diets for 55 days (Dunnett's test, n = 3)

Parameter	Experimental group								
	Control	BS		MB		CE		IN	
	mean ± SD	mean ± SD	P	mean ± SD	P	mean ± SD	P	mean ± SD	P
BW (g)	3.11 ± 0.09	2.74 ± 0.28	0.025	3.04 ± 0.06	0.930	3.00 ± 0.02	0.708	2.96 ± 0.06	0.543
TL (mm)	74.5 ± 0.80	71.1 ± 2.50	0.025	74.2 ± 0.70	0.989	73.3 ± 0.80	0.598	73.4 ± 0.50	0.698
K	0.75 ± 0.00	0.76 ± 0.00	0.526	0.74 ± 0.01	0.877	0.76 ± 0.02	0.499	0.74 ± 0.01	0.999
RGR (%/d)	3.47 ± 0.06	3.23 ± 0.20	0.036	3.43 ± 0.04	0.960	3.41 ± 0.01	0.808	3.39 ± 0.04	0.665
ITL (mm/d)	0.57 ± 0.01	0.51 ± 0.04	0.020	0.56 ± 0.01	0.924	0.55 ± 0.02	0.509	0.54 ± 0.01	0.285
FCR	0.82 ± 0.03	0.97 ± 0.13	0.047	0.84 ± 0.02	0.977	0.86 ± 0.01	0.871	0.87 ± 0.02	0.753

BS – 0.02% Leiber Beta-S–supplemented; MB – 0.20% Leiber Biolex MB40–supplemented; CE – 0.30% Leiber CeFi pro–supplemented; IN – 1.00% BENEO-Orafti Inulin Orafti GR–supplemented; SD – standard deviation; BW – body weight; TL – total length; K – Condition factor; RGR – Relative growth rate; ITL – Increment in total length; FCR – Feed conversion ratio

Table 3. Comparison of histomorphometric analysis of the anterior and posterior intestines and livers in vimba fed different prebiotic-supplemented experimental diets for 55 days (Dunnett's test, n = 6)

Parameter	Experimental group								
	Control	BS		MB		CE		IN	
	mean ± SD	mean ± SD	P	mean ± SD	P	mean ± SD	P	mean ± SD	P
Anterior intestine fold height (µm)	398 ± 40	368 ± 28	0.708	396 ± 8	0.999	399 ± 36	0.990	360 ± 15	0.580
Anterior enterocyte height (µm)	38.2 ± 3.6	42.3 ± 2.5	0.232	41.2 ± 3.9	0.368	44.9 ± 4.3	0.025	34.2 ± 0.6	0.557
Anterior intestine width (µm)	94.2 ± 6.2	100.6 ± 4.8	0.471	90.0 ± 6.4	1.000	104.8 ± 11.9	0.134	90.2 ± 10.3	0.993
Posterior intestine fold height (µm)	330 ± 40	284 ± 32	0.103	335 ± 72	0.168	322 ± 37	0.706	316 ± 59	0.155
Posterior enterocyte height (µm)	41.4 ± 2.8	54.0 ± 22.7	0.130	41.4 ± 9.9	0.920	47.1 ± 6.3	0.983	40.2 ± 6.7	0.894
Posterior intestine width (µm)	96.5 ± 9.6	92.5 ± 12.8	0.992	100.9 ± 14.6	0.466	103.9 ± 11.5	0.807	94.7 ± 6.3	0.967
Hepatocyte area (μm^2)	120 ± 10	133 ± 21	0.257	122 ± 10	0.986	109 ± 8	0.434	118 ± 10	0.995
Hepatonucleus area (μm^2)	20.5 ± 1.1	21.9 ± 1.9	0.300	20.3 ± 1.7	1.000	19.5 ± 1.0	0.601	20.1 ± 1.6	0.983
Hepatonuclear index (%)	17.8 ± 1.2	17.4 ± 1.7	0.969	17.3 ± 1.6	0.932	18.6 ± 1.8	0.791	17.8 ± 1.2	1.000

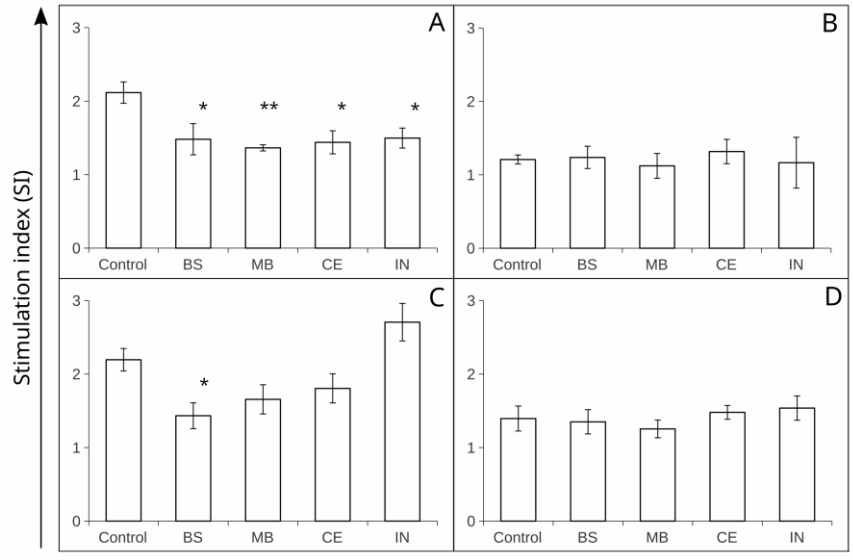

Fig. 1. Prebiotic effects after 55 days' administration on the activity of vimba head-kidney immune cells: respiratory burst activity (A), potential killing activity (B), proliferation of T cells stimulated by concanavalin A (C) and proliferation of B cells stimulated by liposaccharide from *E. coli* (D). BS – 0.02% Leiber Beta-S–supplemented; MB – 0.20% Leiber Biolex MB40–supplemented; CE – 0.30% Leiber CeFi pro–supplemented; IN – 1.00% BENEO-Orafti Inulin Orafti GR–supplemented. All data are expressed as means ± standard deviation (SD); n = 6. Asterisks refer to statistically significant differences between Control and treatment groups: * $P < 0.05$, ** $P < 0.01$

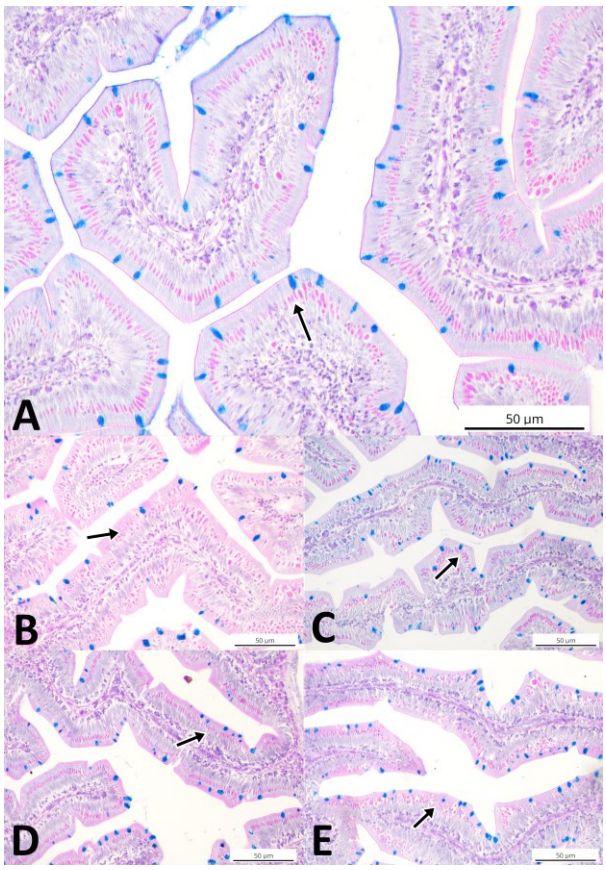

Fig. 2. Histological structure of vimba posterior intestine in Control (A), BS (B), MB (C), CE (D) and IN (E) groups with PAS-positive, magenta stained absorptive vacuoles in the supranuclear cytoplasm of mucosa (arrow) and intense blue acidic mucous cells. BS – 0.02% Leiber Beta-S–supplemented; MB – 0.20% Leiber MB40–supplemented; CE – 0.30% Leiber CeFi pro–supplemented; IN – 1.00% BENEO-Orafti Inulin Orafti GR–supplemented

Discussion

Prebiotics are often used in aquaculture, where they are frequently added to feed. There are many reports in the literature describing the immunostimulatory properties of prebiotics demonstrated on various fish species. However, most of the studies conducted thus far have not studied the effects of long-term supplementation of polysaccharides but have examined the outcomes of short-term administration (14–30 days), when a greater intensity of respiratory burst and phagocytosis, increased lysozyme and complement levels, improved bactericidal activity, and stronger resistance to numerous pathogenic bacteria of several cultured fish have been observed (30).

A supplement of β-glucans in feed given to Nile tilapia also activated the production of cytokines (increasing levels of tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-10 and IL-12 in the serum), and their immersion administration to rainbow trout increased the expression of genes of proinflammatory cytokines (IL-1β, TNF-α and IL-6) (11). Studies concerning the evaluation of the immunostimulatory effect of polysaccharides have been carried out on many fish species, such as Nile tilapia, common carp (*Cyprinus carpio*), grouper (*Epinephelus coioides*), sea bass (*Dicentrarchus labrax*), roho labeo (*Labeo rohita*), Philippine catfish (*Clarias batrachus*), rainbow trout and Persian sturgeon (*Acipenser persicus*) (6, 7, 20, 24, 28). Also short-term use of the β-1,3/1,6 glucan (Leiber Beta-S) tested in the current experiment resulted in increase of some parameters of the non-specific immune system (16, 17, 32).

However, the results of studies on prolonged administration of immunostimulants are often contradictory (2, 14, 20). Our experiment suggests that the provision of all tested prebiotics to vimba for 55 days weakened innate cellular immunity. This is contradictory to the results of the study by Koch *et al.* (19), who concluded that regardless of how long β-glucan had been administered, Nile tilapia fed this prebiotic presented significantly higher values of the immunity parameters, including the RBA, which is an indicator of the organism's ability to defend itself against pathogens. Increased RBA was also noted by Gopalakannan and Arul (14) and Lin *et al.* (20) in common carp following the supplementation of the diet with β-glucan for 60 and 65 days, respectively. Furthermore, Rodrigues *et al.* (30) reported that the negative effect of β-glucans on the immune system of fish could only be observed when high doses of these compounds (>0.1%) were added to the diet. In the experiment carried out by Ai *et al.* (2), dietary supplementation with a 0.18% addition of β-glucan caused a significant decrease in the percentage of phagocytosis and RBA in a large yellow croaker (*Larimichthys crocea*). These researchers demonstrated that the dose of glucan was the key parameter to be considered when supplementing the fish's diet for a longer period, because excessively high doses of glucan could lead to the exhaustion of the immune cells and cause immunosuppression. Yoshida *et al.* (36) showed that the number of NBT-positive cells in North African catfish (*Clarias gariepinus*) increased after oral administration of glucan (Macrogard) and oligosaccharide (Vetregard) for 30 days, but these effects were not observed after 45 days. Additionally, a study conducted on roho labeo demonstrated that phagocytic indices increased only until day 42 of the application of β-glucan-supplemented diets, after which their values decreased (24). In another study, the long-term oral application of peptidoglycans weakened the immune response of rainbow trout infected with *Vibrio anguillarum* (23). These authors suggested the occurrence of negative feedback leading to immunosuppression after excessively long supplementation.

The decrease in the RBA value observed in our study following dietary supplementation with inulin coincides with the observations reported by Cerezuela *et al.* (9), who noted the same effect in gilthead seabream (*Sparus aurata*) after one week of supplementation of the fish diet with inulin. However, further studies conducted by the same research team demonstrated higher values of the RBA in the same fish species fed a diet with added

inulin for two weeks (10). The effectiveness of nonspecific cellular mechanisms is an important indicator of the resistance status of fish and is used to evaluate their ability to defend against pathogens. Lower RBA values may indicate a greater vulnerability of fish to bacterial infections.

The research results indicate that immunostimulants can also affect the activity of lymphocytes, which are involved in the acquired immune response. In earlier studies on the use of immunostimulants, we observed an increase in the proliferation of lymphocytes stimulated with mitogens in tench (*Tinca tinca*), European eel (*Anguilla anguilla*) and common carp (17, 18, 32), but no effect on these cells in roach (16). Verlhac *et al.* (33) observed a positive impact of β-glucan administered for two weeks in the diet of rainbow trout on the proliferative response of lymphocytes stimulated with ConA. Misra *et al.* (24), who tested a 56-day period of diet supplementation given to roho labeo with different doses of β-glucan, also noted a higher production rate of lymphokines, which was a consequence of the concanavalin-induced proliferation of lymphocytes. In the current experiment, however, we noted that 55-day administration of β-glucan in a diet fed to vimba caused a considerable decline in the proliferation of T lymphocytes.

Some studies prove that a diet that strengthens the immune system and improves resistance to disease can influence the growth rate of fish. Beta-glucan, β-(1,3) (1,6) glucan, β-(1,3) (1,6) D-glucan and crude polysaccharides isolated from *S. cerevisiae*, *C. versicolor*, *P. ostreatus* and *Ganoderma lucidum* are the dietary fungal polysaccharides that have been the most thoroughly studied in experiments on the Cypriniformes. These natural polysaccharides can improve growth performance, although this effect has not always been observed. There are numerous papers suggesting that the effect of prebiotics depends on many factors, such as their dose, duration and frequency of administration, as well as the species of fish (6, 20, 24, 28). Some results also indicate that other factors to be considered when planning the supplementation of fish diets are the type of glucans and the structure and method of their extraction (3, 31). Pilarski *et al.* (28) found that glucans produced using different technologies differed in their biological activity. In our study, the long-term administration of the Leiber Beta-S preparation containing highly purified molecules of β-glucans caused both immunosuppression and an adverse effect on the growth parameters of fish. The data found in relevant references suggest that an excessive dose of β-glucans and/or their prolonged supplementation can lead to a non-reactive physiological status and, consequently, a poor immune response (12). Lin *et al.* (20) observed that a local inflammatory response caused by β-glucans in the intestines can result in smaller body weight gains in fish. However, the influence of long-term administration of prebiotics has not been clarified precisely and further studies are needed to determine the factors that contribute to slower fish growth.

Studies dealing with the impact of the prolonged use of prebiotics on the morphology of the digestive tracts of fish are scarce since the main interest is focused on their influence on the immune system. As with the other aspects analysed in this work, reports on the effects of prebiotics on digestive tract morphology are often contradictory. Some experiments suggested that the application of prebiotics has a positive effect on intestinal morphology, manifested in increased height of intestinal folds, in Nile tilapia (31) and rainbow trout (35). This conclusion is partly confirmed by our findings in vimba. The vacuolisation of the mucosa observed in the posterior region was a result of normal absorption in this section of the digestive tract (27). However, an experiment conducted on red drum (*Sciaenops ocellatus*) showed that diet supplementation with inulin had a negative effect on the height of intestinal folds, both in anterior and posterior sections, compared to other prebiotics (5). In barramundi (*Lates calcarifer*), supplementation with inulin at various doses increased the absorption surface area and glycogen content in the liver during 60-day administration (4), an effect that was not observed in our experiment. These research findings suggest that factors other than supplements could have a significant impact on these different outcomes.

There are many commercial polysaccharides used in aquaculture as immunomodulators with the intention of enhancing immunity and improving the growth parameters of fish. The results of our experiment show that besides the doses of supplements and duration of feed supplementation, there must also be consideration of the type of supplements, the way the applied substances were produced and the degree of their purification when designing a protocol for administration of immunostimulants. An inadequate supplementation strategy can lead to excessive stimulation and exhaustion of immune cells, which will result in immunosuppression of the immune system and a lower growth rate in fish. The research results provide evidence that marketed products differ in activity. Attention should also be given to the fact that no other studies have been performed on the fish species analysed in our experiment, and species-specific differences may play a more important role than might be expected. Immunostimulants have great potential in aquaculture; however, further studies are necessary to explain whether long-term supplementation of feeds with prebiotics raises the risk of unwanted effects, such as decreased growth rates or immune parameters in fish.

In summary, the results of our experiment demonstrated that prolonged administration of prebiotics can impair nonspecific cellular immunity, decrease the proliferative activity of T lymphocytes and negatively affect growth parameters of juvenile vimba. However, prebiotics did not appear to have a significant effect on gastrointestinal tract morphology.

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