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Influence of β-glucan Leiber®Beta-S on selected innate immunity parameters of European eel (*Anguilla anguilla*) in an intensive farming system

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Abstract

Nutritional support plays an important role in promoting high cellular and humoral innate immunity activity and in preventing outbreaks of disease. The effects of β-glucan Leiber®Beta-S dietary supplementation on selected nonspecific immune parameters in juvenile European eel (Anguilla anguilla) in an intensive culture system were studied. The fish were fed commercial pellets containing either 0 (control group) or 200 mg Leiber®Beta-S kg-1 of feed (glucan-fed group). After four and eight weeks of feeding, the levels of the following immunological parameters were measured: phagocyte respiratory burst activity, phagocyte potential killing activity, lymphocyte proliferation stimulated by concanavaline A or lipopolysaccharide, serum lysozyme activity, and total immunoglobulin (Ig) serum levels.

After four and eight weeks of feeding 200 mg Leiber®Beta-S kg feed-1 the levels of all immune parameters were statistically significantly higher ($p < 0.05$) in the glucan-treated group than in the control group.

After eight weeks of feeding the fish 200 mg Leiber®Beta-S kg feed-1 and after an additional eight weeks in ponds, the levels of all immune parameters, excluding lymphocyte proliferation stimulated by concanavaline A, were statistically significantly higher ($p < 0.05$) in the glucan-fed group than in the control group. These data suggest that feeding juvenile eel Leiber®Beta-S for four and eight weeks might improve innate immunity.

Key words: *β-glucan, innate immunity, eel, immunostimulant.*

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Introduction

The European eel (*Anguilla anguilla*) is a catadromous species with a complicated life cycle [1], and its importance as a cultured species has increased significantly in recent years. Eel farming is based on wild catches of glass eels that are used for further rearing. Although EU Council Regulation EC No 1100/2007 established a new plan for sustainable European eel stock exploitation, the global production of this species is still high and according to Food and Agriculture Organisation (FAO) fishery statistics production was nearly 7 tonnes in 2010. Intensive rearing systems for both European and Japanese eel production are being developed systematically in many countries. Unfortunately this could negatively impact fish immune systems by exposing the animals to polyetiological stress, which can increase susceptibility to infectious diseases. Additionally, global aquaculture stock transport is another potential vector for spreading pathogens. Infectious diseases can occur in eel at every stage of life; therefore, it is very important to develop effective disease prevention methods.

Similarly to those in other fish species, the innate immune system of the eel comprises a large number of physical, cellular, and humoral factors that act as the first line of defence against invading organisms [2, 3]. The fish innate immune system probably also plays an important role in activating acquired immune mechanisms [4].

The idea of maintaining optimum fish health and reducing susceptibility to diseases through proper nutrition is well known in the aquaculture industry. In recent decades

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special attention has been paid to immunostimulants, comprising a large group of natural and synthetic compounds capable of activating the immune system. The diet can be supplemented with certain nutrients that provide additional molecules for nonspecific defence mechanisms that also enhance specific immune responses. Because innate immunity is an essential defence against pathogenic microorganisms in fish, which rely more upon nonspecific defence mechanisms than do mammals [3, 5], immunostimulants might play a significant role in controlling outbreaks of disease.

Several promising drugs and natural biological modifiers have been tested *in vitro* and *in vivo* in fish [6, 7]. β-glucans (β(1,3)-D-glucans) are naturally occurring polysaccharides with β(1,3)-D-linkages in the backbone. Common sources of β-glucans include cell walls of bacteria, plants, algae, yeast, and mushrooms [8].

Little is known about the effects of immunostimulants on eel (*Anguilla anguilla*) immune systems; thus, the aim of the present study was to examine the impact of orally administered Leiber®Beta-S on selected nonspecific immune parameters in juvenile eel reared in intensive culture systems.

Material and methods

Leiber®Beta-S (Leiber GmbH, Germany) consists of highly purified 1,3-1,6 β-D-glucan molecules. It is isolated from brewer's yeast (*Saccharomyces cerevisiae*) cell walls. According to information provided by the manufacturer, the yeast is not treated with aggressive enzymes during the production, which protects its structure and allows it to express its full biological activity.

Fish and rearing conditions

Glass eel were obtained from a commercial fish farm in France and transported to the experimental station of the Polish Angling Association in Guzianka, Poland. The fish were held in a recirculation system equipped with mechanical and biological filters. One thousand healthy European eel of an initial mean body weight of 40 g comprised the material for the study. The fish were divided randomly into two groups of 500 each: the control group and the glucan-fed group. The mean initial stocking density was 50 kg m^{-3}. The water parameters were as follows: temperature 22°C (±0.5); oxygen content 8.0 mg l^{-1} (±1.0); pH 7.5 (±0.5).

After eight weeks of feeding, the remaining fish were transported to the "Dgał" Experimental Hatchery of the Inland Fisheries Institute. The control and the glucan-fed groups were reared in separated ponds for eight weeks. Water parameters during the experiment were as follows: temperature 20.8°C (±3.0); oxygen content 5.8 mg l^{-1} (±1.0); pH 7.6 (±0.5).

Feed and feeding

The fish were divided randomly into two groups of 500 each. The control eel group was fed commercial pellets (Europa Eel, Skretting, Netherlands) without Leiber® Beta-S. The experimental group was fed commercial pellets containing Leiber®Beta-S at a dose of 200 mg kg^{-1} of feed, for eight weeks (glucan-fed group). The dose of Leiber®Beta-S was chosen after preliminary studies.

Leiber®Beta-S in known concentration was dissolved in distilled water, sprayed on commercial feed (Europa Eel, Skretting, Netherlands), mixed, and allowed to dry for 24 hours. The feed was then coated with vegetable oil (1% of feed weight). The fish were fed three times daily at 07:00 am, 3:00 pm, and 11:00 pm, and unusual behaviour, morphological changes, and mortality were noted daily. The food coefficient was 1.3.

After being transported to the ponds, the remaining fish were fed commercial pellets (Europa Eel, Skretting, Netherlands) without Leiber®Beta-S. The fish were fed twice daily at 07:00 am and 3:00 pm.

Sample collection

After four and eight weeks of feeding, 10 eel from each group were removed from the tanks and anesthetised with Propiscin [9]. Blood samples were collected from the caudal vein using heparinised syringes. After clotting overnight at 4°C the blood was centrifuged at 5000 × g for 10 minutes to extract serum. The serum was stored at –20°C until testing. The spleen and pronephros were excised aseptically. Individual cells were obtained by fractionating single cell suspensions with Gradisol L (Aqua-Med Poland) or Percoll (Sigma-Aldrich) density centrifugation, according to the manufacturer's protocols.

After an additional eight weeks in the ponds ten fish from each group were sampled under the same conditions.

Immunological assays

The respiratory burst activity (RBA) of the pronephros phagocytes stimulated with phorbol myristate acetate (PMA, Sigma-Aldrich) was measured using a modified Secombes method [10] as described by Siwicki *et al.* [11]. Briefly, aliquots of 100 µl containing 1 × 10^4 cells ml^{-1} in RPMI-1640 medium (Sigma-Aldrich) were added to 96-well micro-titre plates (Sarstedt) and incubated for two hours at RT. After incubation, the non-adherent cells were removed by rinsing with fresh RPMI-1640 medium. The medium was then substituted with 100 µl of RPMI and 100 µl of NBT (nitro blue tetrazolium) solution (Sigma-Aldrich) both with and without additional PMA, to produce final concentrations of 2 mg ml^{-1} NBT and 10 µg ml^{-1} PMA. The plates were incubated for 30 minutes at RT. The medium with NBT was removed and the wells were washed twice with 70% ethanol. The blue formazan produced in cells was solubilised in 120 µl of 2M

KOH and 140 µl DMSO (Dimethyl sulfoxide, Sigma-Aldrich). The optical density of the solution was measured at 620 nm using a microplate reader. The data are expressed as mean values of triplicate determinations.

The potential killing activity (PKA) of the pronephros phagocytes was measured with the Rook technique [12] modified by Siwicki and Anderson [13]. Briefly, aliquots of 100 µl containing 1×10^4 cells ml^{-1} in RPMI-1640 medium were added to 96-well micro-titre plates (Sarstedt) and incubated for two hours at RT. After incubation, the non-adherent cells were removed by rinsing with fresh RPMI-1640 medium. The cells were activated using 100 µl of 0.2% NBT solution in PBS containing live *Aeromonas salmonicida* (1×10^8 ml^{-1}) and incubated for 30 minutes at 22°C. After incubation, the supernatant was removed and the wells were rinsed three times with 70% ethanol. The micro-titre plate was dried by placing it in a warm incubator for 30 minutes, and then 120 µl of 2M KOH and 140 µl of DMSO were added to dissolve the formazan. The optical density of the solution was measured at 620 nm using a microplate reader. All samples were performed in duplicate. The data are expressed as mean values of triplicate determinations.

Lymphocyte proliferation (LP) was determined by MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay according to Mosmann [14] with the modifications described by Siwicki *et al.* [15]. Mitogens concanavaline A (ConA, Sigma) or lipopolysaccharide (LPS, Sigma) was used to stimulate lymphocytes. Isolated lymphocytes were suspended at 5×10^6 cells ml^{-1} in RPMI-1640 medium containing 2 mM L-glutamine, 0.02 mM 2-mercaptoethanol, 1% Hepes buffer, penicillin/streptomycin (100 U/100 µg/ml), and 10% foetal calf serum (FCS). Aliquots of 100 µl cell suspension were distributed in 96-well micro-titre plates (Sarstedt) and then 100 µl of ConA (64 µg ml^{-1}) or LPS (160 µg ml^{-1}) was added to each well. The plates were incubated for 72 hours at RT. After incubation, 50 µl of MTT solution was added to each well and the plates were incubated for 4 hours at 22°C. After the microplates were centrifuged, the media were removed and 100 µl of isopropanol (Sigma-Aldrich) was added to all wells and mixed. The optical density was read on a microreader, at 620 nm. Quadruplicate wells were averaged to obtain mean values. Net optical density (OD) values were obtained by subtracting the mean OD values of negative control cells (without mitogens) from those of stimulated cells.

Serum lysozyme activity was measured using turbidimetric assay, as described by Siwicki and Anderson [13] with further modifications. Briefly, 50 µl of serum samples was placed in test tubes and diluted with 950 µl of 66 mM phosphate buffer pH 6.25. Subsequently, a 2 ml solution of *Micrococcus lysodeikticus* (Sigma-Aldrich) in phosphate buffer (66 mM, pH 6.25) was added and mixed. The final concentration of *M. lysodeikticus* was determined with spectrophotometric methods to give up to 40% T at 450 nm, after one hour of incubation. Absorbance was read at 450 nm, immediately after mixing samples with bacteria and after one hour of incubation. Mean OD values were calculated by averaging duplicate determinations.

Total serum protein levels were measured with the standard biuret reaction, using total protein reagent (Sigma-Aldrich) according to the manufacturer's protocol. Absorbance was read with a spectrophotometer at 540 nm. Duplicate determinations were averaged to calculate mean OD values.

Total serum immunoglobulin (Ig) levels were measured with the spectrophotometric method with further modifications [13]. Briefly, 0.1 ml of serum samples was placed in tubes and 0.1 ml of 12% polyethylene glycol 10 000 kDa (Sigma-Aldrich) in distilled water was added. The samples were mixed well and incubated for two hours at RT. After incubation, the samples were centrifuged for 10 minutes at 10 000 RPM. The supernatant was taken off and the optical density was determined at 540 nm. Mean OD values were calculated by averaging duplicate determinations. Total serum Ig levels were calculating by subtracting supernatant OD values from those of total protein.

Statistical analysis

Mean values and standard deviations from pooled experiments were used for comparisons among groups. Statistical analysis was performed with Statgraphics 2.1 for Windows and Statistica 5.77 software (analysis of variance, comparison of regression lines, Wilcoxon's twin pair analysis). All calculations were determined to be significant at $p < 0.05$.

Results

After four and eight weeks of feeding the fish the diet supplemented with 0.02% Leiber®Beta-S, all immune parameter levels were statistically significantly higher ($p < 0.05$) in the group treated with glucan in comparison to the control group (Table 1).

After eight weeks of feeding the fish the diet supplemented with 0.02% Leiber®Beta-S and rearing them for an additional eight weeks in ponds, the levels of all immune parameters excluding lymphocyte proliferation stimulated by ConA were statistically significantly higher ($p < 0.05$) in the glucan-fed group than in the control group (Table 2).

Discussion

The respiratory burst activity (RBA) and the potential killing activity (PKA) of the pronephros phagocytes were significantly higher ($p < 0.05$) in the group whose feed was supplemented with Leiber®Beta-S, in comparison to the control group, in all the study time periods.

Table 1. Immune parameters in eel after four and eight weeks of feeding with 0.02% Leiber®Beta-S (glucan-fed group) and in the control group (mean ± SD, *statistically significant $p < 0.05$)

Immune parameter	Weeks of fed with Leiber®Beta-S			
	4 weeks		8 weeks	
	control group	glucan-fed group	control group	glucan-fed group
metabolic activity of spleen phagocytes (RBA, OD 620 nm)	0.42 ±0.04	0.50 ±0.03*	0.43 ±0.03	0.59 ±0.04*
potential killing activity of spleen phagocytes (PKA, OD 620 nm)	0.38 ±0.03	0.48 ±0.04*	0.39 ±0.04	0.54 ±0.03*
pronephros lymphocytes ConA (LyP-ConA, OD 620 nm)	0.44 ±0.04	0.56 ±0.05*	0.43 ±0.03	0.59 ±0.05*
pronephros lymphocytes LPS (LyP-LPS, OD 620 nm)	0.30 ±0.03	0.41 ±0.04*	0.32 ±0.04	0.43 ±0.05*
lysozyme activity in serum (mg l^{-1})	9.3 ±0.8	13.5 ±0.7*	9.7 ±0.5	14.0 ±0.8*
Ig level in serum (g l^{-1})	10.5 ±1.0	12.5 ±0.9*	10.2 ±0.9	12.9 ±0.7*

Table 2. Immune parameters in eel after eight weeks of feeding with 0.02% Leiber®Beta-S and after an additional eight weeks in ponds; parameters were measured in both control and glucan-fed group

Immune parameter	Control	Glucan-fed group
metabolic activity of spleen phagocytes (RBA, OD 620 nm)	0.41 ±0.03	0.48 ±0.03*
potential killing activity of spleen phagocytes (PKA, OD 620 nm)	0.40 ±0.04	0.45 ±0.03*
pronephros lymphocytes ConA (LyP-ConA, OD 620 nm)	0.43 ±0.04	0.51 ±0.04
pronephros lymphocytes LPS (LyP-LPS, OD 620 nm)	0.31 ±0.03	0.40 ±0.05*
lysozyme activity in serum (mg l^{-1})	9.6 ±0.7	13.4 ±0.9*
Ig level in serum (g l^{-1})	10.9 ±1.1	12.4 ±0.8*

These results are similar to previous experiments with Asian catfish [16] and snapper [17], in which the fish were fed diets supplemented with 0.1% β-1,3-glucan, and with large yellow croaker [18], in which the fish diet was supplemented with 0.09% β-1,3-glucan. However, in the same experiment with large yellow croaker [18] a higher concentration of β-1,3-glucan (0.18%) did not result in significant differences between the glucan-fed and control groups. These data suggest that low glucan supplementation enhances some innate immunity parameters, while higher glucan concentrations might have no significant impact.

Macrophages and neutrophils, which are phagocytic cells in fish, are essential factors in limiting pathogen growth. Enhanced oxidative respiratory burst and potential killing activity of polymorphonuclear (PMN) and mononuclear (MN) phagocytes indicate their role in defence mechanisms. In addition to a range of degradative antimicrobial enzymes, macrophages and neutrophils can produce agents that are highly toxic to pathogens. Stimulated phagocytes also activate the production of cytokines, which are important signal molecules [19, 20].

Significant changes between the experimental groups after four and eight weeks of glucan feeding were also recorded in the proliferative response of spleen lymphocytes stimulated with LPS, which is a B-cell mitogen, and ConA, which acts as a T-cell mitogen [21]. Eight weeks of giving the fish feed supplemented with Leiber®Beta-S, followed by an additional eight weeks rearing the fish in ponds in this study did not result in significant change in the proliferation of lymphocytes stimulated with ConA.

Lymphocytes T and B are very important cells in non-specific and specific immune responses. Both T and B lymphocytes are responsible for specific pathogen recognition and participate in the regulation of early response mechanisms by producing cytokines and chemokines. Recently, Li *et al.* [22] showed that fish B cells might also have *in vitro* and *in vivo* phagocytic activities. The stimulation of lymphocytes by mitogens noted in the present *in vitro* test is believed to mimic the series of events that occur *in vivo* following stimulation by specific antigens.

The lysozyme activity in the serum was statistically significantly greater in glucan-fed eel than that in the control group in all time periods; this confirms data from previous experiments in which lysozyme activity increased after supplementation with β-glucan in a wide range of fish species [16, 18, 23-25]. Contrary to these studies, Kuntu *et al.* [26] reported no significant influence of dietary β-glucan on lysozyme activity in rainbow trout; however, they did report a significant impact when the fish were

injected with glucan. In addition to damaging Gram-positive bacteria cell walls, fish lysozyme also has antibacterial activity against Gram-negative bacteria in the absence of a complement and it activates phagocytosis [27]. According to Nielsen and Esteve-Gassent [2], lysozyme in eel is an important part of the innate immune system and exhibits the highest activity during the early stages of eel development.

Total Ig serum levels were statistically significantly higher in fish fed Leiber®Beta-S in comparison to the control group. This may suggest that glucan activated the production of immunoglobulins (Ig), which are a very important part of the acquired immune response, but natural antibodies can also be classified as significant innate system factors.

In the current experimental study, the fish exhibited satisfactory feed intake, which was seemingly uninfluenced by the presence of the immunostimulant.

Previous experiments [28] demonstrated that levels of some innate immunity parameters in eel from intensive culture are lower than those measured in wild eel. To conclude, the findings of the present research suggest that Leiber®Beta-S can be used as an immunostimulant to enhance the cellular and humoral innate immune response in eel. Further studies are needed to determine effective dosing and administration times for various fish culture conditions. Experiments are also needed to assess potential side affects that could occur during and after immunostimulation and the effect of high dosages of glucan in fish diets. The use of glucans as adjuvants might also increase the effectiveness of immunostimulants and the potency of vaccines. Dietary supplementation seems to be the best administration route for β-glucans because it allows for fast, large-scale application, which avoids handling-related stress in fish.

The authors declare no conflict of interest.

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