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Influence of Dietary Administration of β-hydroxy-β-methylbutyrate (HMB) on the Innate Immunity and Resistance against Bacterial Infections in Pikeperch (*Sander lucioperca*)

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Abstract: The present study examined the influence of leucine metabolite β-hydroxy-β-methylbutyrate (HMB) on the nonspecific cellular and humoral defence mechanisms and protection against *Yersinia ruckeri* and *Aeromonas salmonicida* in juvenile pikeperch (*Sander lucioperca*). The fish was fed with a commercial trout pellets, containing either 0 (control group) or 50 mg HMB kg^{-1} body weight day^{-1} (HMB fed group). After four weeks feeding, 20 pikeperch from each group were anaesthetised and blood and pronephros samples were taken. The levels of the following immunological parameters were measured: respiratory burst activity of phagocytes, potential killing activity of phagocytes, lymphocytes proliferation stimulated by concanavaline A or lipopolisaccharide, lysozyme activity in serum, ceruloplasmin activity in serum, total protein in serum and total immunoglobulin (Ig) level in serum. A disease challenge test using *Yersinia ruckeri* or *Aeromonas salmonicida* was conducted after 4 weeks of feeding. The levels of all immunological parameters excluding ceruloplasmin activity and total protein in serum were statistically significantly higher ($P < 0.05$) in the HMB treated group compared to the control group. Feeding with HMB has led to a significant decrease in the mortality after the challenge trial with *Y. ruckeri* (30% lower mortality than in the control group) and with the *A. salmonicida* (40% lower mortality than in the control group). The current study strongly suggests that feeding pikeperch with HMB in dose 50 mg kg^{-1} body weight day^{-1} may improve the innate immunity and decrease the mortality rates after experimental infection with pathogenic bacteria *Y. ruckeri* and *A. salmonicida*.

Key words: HMB, pikeperch, immunostimulant, fish, *Yersinia ruckeri*, *Aeromonas salmonicida.*

1. Introduction

The immune system of fish in a commercial fish farm is negatively affected by many agents, including stress caused by a variety of factors and chemotherapeutic treatments. Thus the influence of these factors can increase susceptibility of fish to infectious diseases. Interactions between nutrition, defence mechanisms and protection against diseases are widely known although these relations are far more complex than originally thought. Certain nutrients can be supplemented in the diet and act as an immunomodulators by providing building blocks of nonspecific cellular and humoral defence mechanisms. Because fish depend more strongly on nonspecific defence mechanisms than mammals [1], immunostimulants can play a significant role in protection fish against infectious diseases.

HMB is a metabolite of essential amino acid leucine, produced through oxidation of the ketoacid of leucine (α-ketoisocaproate, KIC) in cytosol by the enzyme α-ketoisocaproate dioxygenase [2]. In recent years a

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number of studies have examined the influence of HMB dietary supplementation on immune parameters and mortality in various fish species [3-5] and other farmed animals [6-9]. In rainbow trout, pikeperch and tench, HMB activated cell-mediated immunity [10-13]. This study continues the investigation of the effects of HMB feeding on the nonspecific defence mechanisms and protection against furunculosis and yersiniosis in pikeperch (*Sander lucioperca*) in intensive system of culture.

2. Materials and Methods

2.1 Fish and Rearing Conditions

The fish were reared at the Department of Aquaculture, Inland Fisheries Institute in Olsztyn (Poland). The juvenile pikeperch (n = 200) were maintained in circular fibreglass tanks, 200 L each. The tanks were supplied with biological and mechanical filters and were exposed to 24 h illumination at 30-80 lux. Water temperature was maintained at about 22 °C.

2.2 Feed and Feeding

Fish were fed for 18 h day^{-1} with a commercial trout feed (ALLER AQUA Poland) using automatic band feeders. The fish, divided into two groups, received either 0 (control group, n = 100) or 50 mg HMB kg^{-1} body weight day^{-1} (HMB fed group, n = 100) for 4 weeks. HMB was obtained as a calcium β-hydroxy-β-methylbutyrate (Metabolic Technologies, USA). Calcium salt of HMB in known concentration was dissolved in distilled water, sprayed on commercial feed, mixed and allowed to dry 24 hours before proceeding to the next step. After this step the feed was coated with oil (1% of feed weight). The fish were observed daily for unusual behaviour, morphological changes and mortality.

2.3 Sample Collection

After 4 weeks 20 fish from each group were anaesthetised in Propiscin (Inland Fisheries Institute, Poland) and blood samples were taken into heparinized syringes. The pronephros was removed and single cell suspension was obtained for isolating individual cells by density gradient centrifugation with *Gradisol* L. (Aqua-Med Poland) or Percoll (Sigma-Aldrich).

2.4 Immunological Assays

Respiratory burst activity (RBA) of the pronephros phagocytes stimulated with phorbol myristate acetate (PMA, Sigma-Aldrich) was measured using modifcation of the Secombes method [14] and presented in Siwicki et al. [15]. Briefly, aliquots of 100 µL containing 1×10^4 cells mL^{-1} in RPMI-1640 medium were added to 96-well micro-titer plates (Sarstedt) and incubated for 2 h at RT. After incubation, the non-adherent cells were removed by washing with fresh RPMI-1640 medium. The medium was then substituted by 100 µL of RPMI and 100 µL of NBT (nitroblue tetrazolium) solution (Sigma-Aldrich) both with, and without addition of PMA, to give final concentrations of 2 mg mL^{-1} NBT and 10 µg mL^{-1} PMA. The plates were incubated for 30 min at RT. The medium with NBT was removed and wells were washed twice with 70% ethanol. The blue formazan produced in cells was solubilized in 120 µL of 2 M KOH and 140 µL DMSO (Dimethyl sulfoxide, Sigma-Aldrich). The optical density of the solution was measured at 620 nm using a microplate reader. Data are expressed as mean values of triplicate determinations.

Potential killing ativity (PKA) of the pronephrotic phagocytes was measured by Rook et al. [16] technique with modifications described by Siwicki and Anderson [17]. Briefly, aliquots of 100 µL containing 1×10^4 cells mL^{-1} in RPMI-1640 medium were added to 96-well micro-titer plates (Sarstedt) and incubated for 2 h at RT. After incubation, the non-adherent cells were removed by washing with fresh RPMI-1640 medium. The cells were activated using 100 µL of 0.2% NBT solution in PBS containing living *Aeromonas salmonicida* (1×10^8 mL^{-1}) and incubated for 30 min at 22 °C. After incubation, the supernatant was removed and the wells were rinsed thrice with 70% ethanol. The microtiter plate was dried by placing it in a warm

incubator for 30 min, and then 120 µL of 2 M 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. Data are expressed as mean values of triplicate determinations.

Lymphocytes proliferation (LP) was determined by the MTT (3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) colorimetric assay according to Mosmann [18] with modification described by Siwicki et al. [19]. Mitogens concanavaline A (ConA, Sigma) or lipopolisaccharide (LPS, Sigma) were used for stimulation of 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% fetal calf serum (FCS). Aliquots of 100 µL cell suspension were distributed in 96-well micro-titer plates (Sarstedt). 100 µL of ConA (64 µg mL^{-1}) or LPS (160 µg mL^{-1}) was then added to each well. The plates were incubated for 72 h at RT. After incubation, 50 µL of MTT solution was added to each well and the plates were incubated for 4 h at 22 °C. After centrifugation of the microplates, the media were removed and 100 µL of isopropanol (Sigma-Aldrich) were added to all wells and mixed. The optical density was read on a microreader, at 620 nm. To obtain mean values, the quadruplicate wells were averaged. Net optical density (OD) values were obtained by subtracting the mean OD of negative control cells (without mitogens) from OD of stimulated cells.

The lysozyme activity in serum was measured using turbidimetric assay as described in Siwicki and Anderson [17] with further modifications. Briefly, 50 µL of serum samples were put into test tubes and diluted with 950 µL of 66 mM phosphate buffer pH 6.25. Next, 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* has been determined by spectrophotometric methods to give up to 40% T at 450 nm, after 1 h incubation. The absorbance was read at 450 nm, immediately after mixing samples with bacteria and after 1 h of incubation. To calculate mean OD values, duplicate determinations were averaged.

Ceruloplasmin activity in serum was determined according to Siwicki and Anderson [17] with further modification. Aliquots of 100 µL 0.2% p-phenylenediamine (PPD) solution in acetate buffer (pH 5.2) and 10 µL serum samples were added to 96-well micro-titer plates (Sarstedt) and mixed. The PPD solution in acetate buffer was freshly prepared and brought to 37 °C before mixing with serum. The plates were incubated for 15 min at 37 °C. After incubation 140 µL of 0.02% sodium azide solution was added to each well. The optical density was read immediately at 540 nm. To calculate mean OD values, triplicate determinations were averaged.

Total protein level in serum was measured by standard biuret reaction, using total protein reagent (Sigma-Aldrich) according to the manufacturer's protocol. The absorbance was read using spectrophotometer at 540 nm. To calculate mean OD values, duplicate determinations were averaged.

Total immunoglobulin (Ig) level in serum was measured by spectrophotometric method [17] with further modifications. Briefly, 0.1 mL of serum samples were put 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 2 h at RT. After incubation samples were centrifuged 10 min/10,000 rpm. The supernatant was taken off and optical density was determined at 540 nm. To calculate mean OD values, duplicate determinations were averaged. To calculate the total serum Ig levels, supernatant OD values were subtracted from total protein OD values.

2.5 Challenge Test

A disease challenge test with *Yersinia ruckeri* or *Aeromonas salmonicida* was conducted after 4 weeks of feeding. 20 fish from each group were given a single

intraperitoneal injection of a 48 h growth *Y. ruckeri* (0.2 mL; 1×10^6 colony forming units (CFU) mL^{-1}) suspended in growth medium. 20 fishes from each group were given a single intraperitoneal injection of a 48 h growth *A. salmonicida* (0.2 mL, 1×10^7 CFU mL^{-1}) suspended in growth medium. Any mortality was recorded and swabs from tissues were taken to confirm the presence of pathogens.

2.6 Statistical Analysis

The mean values and standard deviations from pooled experiments were used for comparison between the groups. Statistical analysis was performed using Statgraphics 2.1 for Windows and Statistica 5.77 software (analysis of variance, comparison of regression lines, Wilcoxon's twin pair analysis). For all calculations $P < 0.05$ was assumed as significant.

3. Results and Discussion

The results of this study showed that oral administration of HMB (50 mg kg^{-1} body weight day^{-1}) during 4 weeks statistically stimulated the non-specific cellular (Table 1) and humoral (Table 2) defence mechanisms. The respiratory burst activity (RBA) and the potential killing ativity (PKA) of the pronephros phagocytes were statistically significantly higher ($P < 0.05$) in the HMB treated group compared to the control group. The results showed that HMB at dose 50 mg kg^{-1} increased the metabolic activity and intracellular killing activity of macrophages, very important immunocompetence cells in the innate immunity in fish. The oxigen-dependent killing mechanisms are very effective to intracellular destruction of pathogens in fish. The higher respiratory burst activity and potential killing activity of polymorphonuclear (PMN) and mononuclear (MN) phagocytes result in effective defence mechanisms against bacterial infections. The similar pattern was observed in the proliferative response of pronephros lymphocytes stimulated by ConA or LPS. The lymphocyte proliferation was statistically significantly higher ($P < 0.05$) in pikeperch that were fed with HMB, comparing to the control group. The lymphocytes T and B are very important cells in nonspecific and specific immune response and both T and B lymphocytes acquire specific receptors for antigen, which commit them to a single antigenic specificity for the rest of their life-span. Antigen-induced lymphocyte proliferation normally occurs outside the blood and can be visualized *in vitro* by cultivating lymphocytes with specific antigens. In our experimental study the authors observed higher lymphocyte activity after mitogens stimulation in fish fed with HMB. Mitogen stimulation of lymphocytes in our *in vitro* method is believed to mimic the series of events, which occur *in vivo* following their stimulation, by specific antigens. The results suggested that HMB may activate the lymphocytes, which provide protection from invading microorganisms.

Antibody molecules that protect the fish are produced by plasma cells, which are mature B lymphocytes, inducing humoral immunity. A distinctive feature of fish IgM antibody is that the majority is in characteristic tetrameric form and production is temperature-dependent. In our study the statistically significant ($P < 0.05$), the greater total Ig level in serum was observed in fish fed with HMB, compared to the control group. This result suggested that HMB activated the production of immunoglobulins (Ig), very important part of non-specific and specific immune response.

The results of this study showed that HMB activated nonspecific humoral-mediated immunity. Because fish have fewer complex specific immune capabilities than higher vertebrates to delay and prevent the entrance of violating microorganisms, the nonspecific mechanisms play very important role. These nonspecific humural defences respond relatively quickly, even at low temperatures. Fish tissues and body fluids contain naturally occurring non-immunoglobulin proteins that react with a diverse set of pathogens and constitute an important part of innate immunity in fish. They consist of microbial growth inhibitors like ceruloplasmin or c-reactive protein.

Table 1 The levels of nonspecific cellular defence parameters in pikeperch after 4 weeks feeding with 50 mg HMB kg^{-1} body weight day^{-1} (HMB-fed group) and in control group (mean ± SD, *statistically significant $P < 0.05$).

Immunological parameter	Control group (n = 20)	HMB-fed group (n = 20)
Respiratory burst activity of phagocytes (OD 620 nm)	0.45 ± 0.04	0.59 ± 0.05*
Potential killing activity of phagocytes (OD 620 nm)	0.41 ± 0.04	0.62 ± 0.04*
Lymphocytes proliferation stimulated by ConA (OD 620 nm)	0.49 ± 0.05	0.57 ± 0.05*
Lymphocytes proliferation stimulated by LPS (OD 620 nm)	0.34 ± 0.04	0.51 ± 0.05*

Table 2 The levels of nonspecific humoral defence parameters in pikeperch after 4 weeks feeding with 50 mg HMB kg^{-1} body weight day^{-1} (HMB-fed group) and in control group (mean ± SD, *statistically significant $P < 0.05$).

Immunological parameter	Control group (n = 20)	HMB-fed group (n = 20)
Lysozyme activity in serum (mg L^{-1})	45.5 ± 5.8	57.5 ± 3.9*
Ceruloplasmin activity in serum (IU)	22.8 ± 7.5	21.5 ± 2.5
Total protein in serum (g L^{-1})	64.5 ± 4.0	67.7 ± 3.5
Total Ig in serum (g L^{-1})	18.0 ± 1.4	21.5 ± 2.0*

Fish also have a variety of relatively specific lytic molecules that cause microbial cell lysis. Lysozyme has a wide range of antibacterial and antifungal activity and has also been reported to have antiviral and antiparasitic properties. Lysozyme is widely distributed in fish tissues and has been detected in serum, mucus and internal organs and is produced by phagocytes. In our study, the lysozyme activity in serum was statistically significantly greater ($P < 0.05$) in the HMB-fed fish than in the control group. There were no statistically significant differences in the levels of ceruloplasmin activity and total protein levels in serum between HMB-fed pikeperch and control-fed pikeperch.

Feeding fish with HMB has led to a significant decrease in the mortality after the challenge trial with *Y. ruckeri*, where 30% lower mortality was observed than in the control group. The similar finding was observed after challenge test with the *A. salmonicida* where 40% lower mortality was in HMB-fed group compared to the control. Results of challenge tests correspond with higher levels of nonspecific cell-mediated and humoral-mediated immunity parameters in fish fed with HMB, compared to the HMB-free group of fish.

The current study indicates that feeding pikeperch with HMB in dose 50 mg kg^{-1} body weight day^{-1} in intensive system of culture can improve the innate immunity and decrease the mortality rates after experimental infection with pathogenic bacteria *Y. ruckeri* and *A. salmonicida*. This study corresponds with previous research of Siwicki et al. [10-13] showing that HMB dietary supplementation can enhance the nonspecific immune parameters and protection against pathogens in various fish species. In contrary, Kunntu et al. [5] showed that feeding juvenile rainbow trout with HMB had no effect on survival after challenge with *Flavobacterium columnare*. As the authors suggested the specific character of columnaris diseases may cause it. Although in the same study HMB oral administration increased the complement activity in plasma and reactive oxygen species (ROS) production in whole blood. Whittington et al. [3] showed that dietary supplementation with different doses of HMB did not influence the survival of vaccinated Nile tilapia followed challenge with *Streptococcus iniae*. The study of Li and Gatlin [4] revealed that feeding hybrid striped bass with HMB had no significant effect on mortality rate after challenge with *S. iniae.* However, the neutrophil oxidative burst significantly increased in groups fed with HMB. The discordances in research results can be explained by various factors including differences between fish species in response to pathogens or different mode of leucine metabolism [4, 20].

4. Conclusions

Treatment of bacterial fish diseases with chemotherapeutics is not always effective and can cause adverse effects in fish and be harmful for

environment. Thus, prevention methods, including diet supplemented with potential immunostimulants should be considered. The findings of the present research suggest that β-hydroxy-β-methylbutyrate may be used as an immunostimulant to enhance the cellular and humoral immune response in fish. Although further studies are needed to determine the effective dosing and feeding protocol for certain fish species.

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