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Impact of brewer's yeast extract and levamisole in diets with vegetable oils on the growth, chemical composition, and immunological and biochemical blood parameters of pikeperch (*Sander lucioperca*)

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ABSTRACT: The impact of applying NuPro® (40 g/kg feed) and levamisole (300 mg/kg feed) in European pikeperch (*Sander lucioperca*) diets with vegetable oils on growth, immunological, hematological, and blood chemical parameters was assayed. The fish were fed the feed containing sunflower and linseed oils (48 and 21 g/kg feed) (group VO) or this feed with levamisole (group VOL) or NuPro® (group VON). The fourth group was fed the feed with fish oil added in the amount of 69 g/kg feed (group FO). The pikeperch were reared for 56 days. Feeding the fish feed supplemented with levamisole or NuPro® increased the immunological responses of the phagocytes and lymphocytes, lysozyme activity, and total gamma-globulin levels. The feed supplemented with NuPro® resulted in decreased aspartate and alanine aminotransferase and alkaline phosphatase activity. Vegetable oils in the feed were linked to increased contents of linoleic acid in fish bodies. The quantity of eicosapentaenoic and docosahexaenoic acids was significantly the highest in group FO. The immunological index obtained in group VON indicated that the diet supplemented with brewer's yeast extract (NuPro®) was the most advantageous when feeding juvenile pikeperch feeds with vegetable oils.

Keywords: lipids; fatty acids; immunomodulators; disease resistance

INTRODUCTION

The recent expansion of aquaculture has led to growing interest in controlling fish diseases with the aim of preventing or treating them. The intensifying production and the associated stress the fish experience have been confirmed to impact resistance negatively and to cause disease (Li and Gatlin 2006). Replacing fish oil (FO) with vegetable oils (VO) results in increased amounts of polyunsaturated fatty acids with C18 (PUFA) and decreased eicosapentaenoic (EPA, 20:5 n-3) and docosahexaenoic acids (DHA, 20:6 n-3) content in feed, which can affect several aspects of the immune response (Thompson et al. 1996). High levels of PUFA and deficits of EPA and DHA reduced the responses of phagocytes, the killing ability of macrophages, and elevated mortality after infection with pathogenic bacteria in turbot (*Scophthalmus maximus*), rainbow trout (*Onco-*

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rhynchus mykiss), and channel catfish (*Ictalurus punctatus*) (Blazer 1992; Kiron et al. 1995). Low contents of n-3 PUFA, EPA, and DHA in feed with VO causes increased activities of ceruloplasmine (Cp), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) in pikeperch (*Sander lucioperca*) (Kowalska et al. 2010 a,b). Very high quantities of linoleic acid (LA, 18:2 n-6), which dominates in some VO, suppress lysozyme activity in grouper (*Epinephelus malabaricus*) (Lin and Shiau 2007) and lower immunity functions in juvenile pikeperch (Kowalska et al. 2012). However, diets supplemented with synthetic or natural substances that activate cellular and humoral immunity can improve fish resistance and rearing parameters (Gopalakannan and Arul 2006; Li et al. 2006a, b; Oliva-Teles 2012).

Among the synthetic immunomodulators, levamisole (2,3,5,6-tetrahydro-6-phenylimidazo(2,1-b) thiazole), which has been thoroughly studied, is highly effective in increasing the nonspecific immune response of fish (Mulero et al. 1998). Feeds supplemented with levamisole enhance phagocytosis and natural killing cell activity in gilthead seabream (*Sparus aurata*) (Mulero et al. 1998) and lysozyme activity in common carp (*Cyprinus carpio*) (Gopalakannan and Arul 2006) and whitespotted clarias (*Clarias fuscus*) (Li et al. 2006a), and reduce mortality in fish infected with pathogenic bacteria (Gopalakannan and Arul 2006; Li et al. 2006a). Moreover, dietary supplementation with levamisole enhances the growth of common carp juveniles (Gopalakannan and Arul 2006). The stimulatory effect of levamisole on the immune system depends on the dosage. The administration of 250 mg levamisole/kg feed to common carp and striped bass (*Morone* sp.) or 300 mg levamisole/kg feed to whitespotted clarias is optimal for stimulating fish growth, nonspecific defense mechanisms, and specific immune responses against pathogenic bacteria (Li et al. 2006a, b; Maqsood et al. 2009).

Natural substances in yeast cells, such as oligosaccharides and glucans, are biologically active and stimulate fish immune systems (Oliva-Teles 2012). An extract derived from *Saccharomyces cerevisiae* cells that are rich in nucleotides is available as the commercial product NuPro® (Alltech Inc., USA). Feeding fish diets with 40 g NuPro® per kg/feed increases lysozyme activity, total immunoglobulin levels, phagocyte and macrophage activity, and lymphocyte responses in juvenile pikeperch (Jarmolowicz et al. 2012, 2013). Although the brewer's yeast extract does not impact pikeperch growth (Jarmolowicz et al. 2012, 2013), it is a source of components which could positively affect lipid metabolism, and, consequently, digestibility through the assimilation and utilization of nutrients (Li and Gatlin 2006). This can be important when feeding fish feeds with vegetable oils.

Administering immunomodulators with feed is convenient, but success rates have varied. Many factors can determine the effectiveness of dietary immunostimulation regimes. Since VO can impair fish immune systems, in this study we investigated the effect of 0.3 g levamisole/kg feed and 40 g NuPro®/kg feed on growth performance, body composition, and blood parameters in juvenile pikeperch fed feed with VO.

MATERIAL AND METHODS

Animals and rearing conditions. Pikeperch with mean initial body weights of 73.4–74.7 g (6-month-old) were stocked into 12 tanks with volumes of 0.2 m^3 in a recirculating aquaculture system (RAS). The mean initial stocking density was 12.9 kg/m^3 (35 animals/tank). The water temperature and oxygen content, total ammonia nitrogen (TAN = NH_3–N + NH_4^+–N), and water pH at the rearing tank outflow were as follows: 21.8 ± 0.4°C; 6.6 ± 0.6 mg O_2/l; 0.05 ± 0.02 mg TAN/l; 7.8–8.0. Water flow in the tanks was 4 l/min. The photoperiod applied was permanent 24 h light, while the light intensity measured at the surface of the rearing tanks was 40–50 lux.

Diets and feeding animals. The experimental diets were prepared with Aller Safir base feed (Aller-Aqua, Golub-Dobrzyn, Poland) and fish oil (FO) from a commercial feed production line (Aller-Aqua) and vegetable oils (VO) – sunflower oil (SFO) (ZT Kruszwica, Kruszwica, Poland) and linseed oil (LO) (Oleofarm, Pietrzykowice, Poland). Extracts of selected strains of brewer's yeast, *Saccharomyces cerevisiae* (NuPro®; Alltech Inc., Nicholasville, USA) or the synthetic compound Levamisole (Sigma-Aldrich, St. Louis, USA) were the immunomodulators. The base feed contained 540 g crude protein/kg feed, 71 g crude fat/kg feed, and 80 g crude ash/kg feed (based on dry matter; granule size 3.0 mm in diameter). The main source of fat in the base feed was fish meal, while that of protein were fish and soy meals. The base feed

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was supplemented with immunomodulators and oils using a vacuum pump (AGA Labor, Lublin, Poland) (Jarmolowicz et al. 2012). Four groups of fish were fed the base feed supplemented with FO in quantities of 69 g/kg feed (group FO) or a mixture of the two vegetable oils at 48 g LO and 21 g SFO/kg feed (group VO) or a mixture of these two vegetable oils with the addition of levamisole (0.3 g/kg feed) (group VOL) or NuPro® (40 g/kg feed) (group VON). Levamisole and NuPro® were incorporated separately before the addition of oils. The quantities of immunomodulators were mixed with distilled water and added to feed and then sealed using the vacuum pump. The total fat content of the experimental diets ranged from 119 to 130 g/kg feed (Table 1). The contents of protein, fat, and nitrogen free extract (NFE) in the diets tested were within the ranges recommended for juvenile pikeperch (Kowalska et al. 2011). The four dietary treatment groups of fish, each in three

Table 1. Proximate (g/kg of dry weight) and selected fatty acid (FA) composition (g FA/kg of total FA) of experimental diets

Specification	Experimental diets			
	FO	VO	VOL	VON
Components				
Crude protein	504.2	504.3	460.9	478.5
Crude fat	128.3	130.9	119.6	118.6
NFE	267.1	262.7	291.2	303.7
Crude ash	76.9	78.2	72.9	73.3
Gross energy (MJ/kg feed)	20.7	20.8	19.3	19.6
Fatty acids				
ΣSFA[a]	232.6	184.7	175.9	186.4
ΣMUFA[b]	432.5	316.7	314.4	336.1
18:2 n-6	115.2	372.3	386.4	353.5
18:3 n-3	31.2	31.1	26.5	27.9
20:4 n-6	5.4	3.0	3.1	3.0
20:5 n-3	64.3	37.5	38.3	39.1
22:5 n-3	14.9	6.8	7.4	7.1
22:6 n-3	72.9	36.0	35. 9	34.4
ΣPUFA[c]	334.2	498.3	509.2	477.7
Σn-9[d]	292.3	246.2	247.8	264.9
Σn-6[e]	123.2	375.9	390.1	357.0
Σn-3[f]	191.8	114.4	110.9	111.3
Σn-3 HUFA[g]	159.1	83.4	84.8	83.6
Σn-3/**Σ**n-6	1.6	0.3	0.3	0.3

FO = feed with fish oil (69 g/kg feed), VO = feed with sunflower and linseed oils (48 and 21 g/kg feed), VOL = feed with sunflower and linseed oils (48 and 21 g/kg feed) and levamisol (0.3 g/kg feed), VON = feed with sunflower and linseed oils (48 and 21 g/kg feed) and NuPro® (40 g/kg feed), NFE = nitrogen free extract calculated as 1000 – (protein + lipid + ash + fibre) g/kg feed

[a]Σ saturated FA: 14:00, 15:00, 16:00, 18:00, 20:00, 22:00

[b]Σ monoenes: 14:1, 16:1, 17:1, 18:1 *cis*9, 18:1 *cis*11, 20:1 n-9, 20:1 n-7, 22:1 n-11, 22:1 n-9

[c]Σ polyenes: 16:2, 16:4, 18:2 n-6, 18:3 n-3, 18:3 n-4, 18:4, 20:2, 20:3 n-6, 20:4 n-6, 20:3 n-3, 20:4 n-3, 20:5 n-3, 21:5, 22:5 n-6, 22:5 n-3, 22:6 n-3

[d]Σ n-9: 18:1 *cis*9, 20:1 n-9, 22:1 n-9

[e]Σ n-6: 18:2 n-6, 20:3 n-6, 20:4 n-6, 22:5 n-6

[f]Σ n-3: 18:3 n-3, 20:3 n-3, 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3

[g]Σ n-3 HUFA: 20:3 n-3, 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3

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replicates (n = 3), were reared for eight weeks (56 days) and fed continuously for 19 h/day with an automatic band feeder 4305 FIAP (FIAP GmbH, Ursensollen, Germany). The daily feed ration, set at weekly intervals, was 0.9% of the fish biomass.

Calculation. The stock biomass in the tanks was determined at weekly intervals during the experiment (± 1 g). On the first and last days of the experiment, the fish were weighed (W ± 0.01 g) and measured (standard body length, BL ± 0.1 cm). During measurements the fish were anesthetized with a solution of etomidate at 1.0 ml/l (Propiscin; Inland Fisheries Institute in Olsztyn, Olsztyn, Poland). At the end of the experiment 5 individuals were taken from each replicate (tank) and their body weight, viscera (± 0.01 g), and livers (± 0.001 g) were weighed to determine the values of the viscerosomatic (VSI) and hepatosomatic (HSI) indexes. The fish used for these tests were anesthetized in a overdose solution of etomidate (4.0 ml/l) and then decapitated. The data collected were used to calculate the following parameters:

Daily growth rate (DGR, g/day) = $(W_f - W_i)/T$

Condition factor (K) = $100 \times (W/BL^3)$

Feed conversion ratio (FCR) = TFI/(FB – IB)

Viscerosomatic index (VSI, %) = $100 \times (VW/W)$

Hepatosomatic index (HIS, %) = $100 \times (LW/W)$

where:

W_f = final body weight (g)
W_i = initial body weight (g)
T = time of rearing (days)
BL = fish standard body length (cm)
FB = final absolute fish weight (g)
IB = initial absolute fish weight (g)
TFI = total actual feed intake (g)
VW = viscera weight (g)
LW = liver weight (g)

Non-specific cellular and humoral-mediated immunity assays. At the end of experiment, blood (from the caudal veins), pronephros, and spleens from 15 individuals from each of the experimental groups were collected for immunological study (n = 15; 5 fish/tank).

Assay procedures for separating plasma and cells. The spleen and pronephros were removed aseptically and single cell suspensions were obtained by teasing the tissues in medium through steel mesh and isolating the individual cells using either a Gradisol (Polfa, Lodz, Poland) or Percoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden) gradient. The leukocytes from the spleen or pronephros were washed three times in phosphate buffered saline (PBS) and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) supplemented with 10% Fetal Calf Serum (FCS; Gibco-BRL, Berlin, Germany) at a stock concentration of 2×10^6 cells/ml of medium. Cell viability was assessed with supravital staining with 0.1% w/v trypan blue (1 : 1 mixture of cell suspension and trypan blue solution). 200 cells were counted, and only samples containing at least 90% viable cells were used in the experiments. The plasma was isolated after the blood centrifugation at 400 g for 15 min.

Hematological assays. The blood drawn from the caudal veins was used previously for hematological study. After withdrawal, two microhematocrit capillary tubes were filled directly from the Vacutainer tubes for hematocrit examinations according to the method presented in Siwicki et al. (2003a). The erythrocyte and total leukocyte counts (RBC and WBC, respectively) were determined using the methods for pikeperch described in Siwicki et al. (2003a). The hemoglobin concentration was measured using the cyanmethemoglobin method (Alpha Diagnostics, Warsaw, Poland).

Biochemical assays. The biochemical examinations of the blood plasma were performed photometrically using a Hach-Lange spectrophotometer DR 3900 (Hach-Lange GmbH, Düsseldorf, Germany) and study kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alcaline phosphatase (ALP) activities (Alpha Diagnostics, Warsaw, Poland). The bilirubin, cholesterol, and glucose levels in plasma were also measured spectrophotometrically (Hach-Lange DR 3900) with kits (Biolabo SA, Maizy, France).

Cell-mediated immunity assays. The phagocytic ability of spleen macrophages was determined by measuring intracellular respiratory burst after stimulation with phormol myristate acetate (PMA; Sigma-Aldrich) as described in Siwicki and Robak (2011). The isolated cells were resuspended in a RPMI-1640 medium (Sigma-Aldrich). The amount of extracted, reduced nitro blue tetrazolium (NBT; Sigma-Aldrich) was measured colorimetrically at 620 nm in a plate microreader MRX 3 Dynatech (Dynex Technologies, Billigshurst, UK). All samples were tested in triplicate, and the mean values served

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as the results. The potential bactericidal activity of phagocyting cells was determined in isolated spleen macrophages stimulated with killed microorganisms according to the method presented for fish in Siwicki and Robak (2011). This was done by mixing 100 µl of spleen macrophages with 100 µl of 0.2% NBT in phosphate buffer at pH 7.2 and 10 µl of killed *Aeromonas salmonicida* (containing 1×10^6 bacteria/ml) was added. The amount of extracted, reduced NBT was measured at 620 nm in the plate microreader MRX 3 Dynatech. All samples were tested in triplicate, and the mean values served as the results.

The proliferative response of the pronephros lymphocytes stimulated with mitogen concanavaline A (ConA; Sigma-Aldrich) or lipopolysaccharide (LPS; Sigma-Aldrich) were determined with 3-(4,5-dimethyl thiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) assay described in Siwicki et al. (2003b). MTT was dissolved in PBS at a concentration of 5 mg/ml and filtered, then 100 µl of pronephros lymphocytes in RPMI 1640 containing 10% FCS, 2mM L-glutamine, 0.02mM 2-mercaptoethanol, 1% hepes buffer, penicillin/streptomycin (100 U/100 µg/ml) were mixed with 100 µl of RPMI 1640 containing mitogens ConA (5 µg/ml) or LPS (20 µg/ml) on Costar® 96-well culture plates (Corning Inc., Corning, USA). After 72 h of incubation at 22°C without 5% carbon dioxide atmosphere, 50 µl of MTT solution were added to each well, and the plates were incubated at 22°C for 4 h. After incubation, the plates were centrifuged (1400 *g*, 15°C, 5 min), the supernatants were removed, and 100 µl of DMSO (Sigma-Aldrich) were added to each well, and incubated for 15 min at room temperature. After incubation, the solubilized reduced MTT was measured colorimetrically at 620 nm in the plate microreader MRX 3 Dynatech. All samples were tested in triplicate, and the mean values served as the results.

The lysozyme activity in the plasma was measured with turbidimetric assay presented for pikeperch in Siwicki et al. (2003b). The standard used was hen egg white lysozyme (Sigma-Aldrich) and a *Micrococcus lysodeikticus* suspension in phosphate buffer. The ceruloplasmine activity in the plasma was determined according to Siwicki et al. (2006). The total protein and gamma-globulin (Ig) levels in the plasma were measured with the spectrophotometric Lowry micro-method (Sigma-Aldrich diagnostic kit) developed for pikeperch by Siwicki et al. (2003b).

Biochemical analysis of proximate and fatty acid composition. The proximate composition (contents of water, total protein, crude fat, and crude ash in wet weight) was determined in samples of whole fish (5 animals from each tank). The fish obtained from the same tank were combined and analyzed together (n = 3, for each dietary treatment). The chemical composition of the experimental feeds was also determined. The protein content was determined with the Kjeldahl method. The fat content was determined with the Soxhlet method (AOAC 1975). The gross energy content of the diets was measured with bomb calorimetry. On the final day of the experiment, whole fish bodies were analyzed to determine the contents of particular fatty acids (g/kg of total fatty acid (tFA)). Qualitative and quantitative analyses of fatty acids were conducted according to methods described in Folch et al. (1957). The fatty acids were methylated using a mixture of chloroform, anhydrous methanol, and sulfuric acid (100 : 100 : 1) (Peisker 1964). Chromatographic separation was performed with an Agilent Technologies 6890N gas chromatograph with a flame ionization detector (FID) (Agilent Technologies Inc., Santa Clara, USA). The detector signal was registered with a Philips device on a scale of 1 mV at a tape speed of 10 mm/min. The fatty acids were identified with standards from Supelco (Bellefonte, USA).

Statistical analysis. Parameter measurements and calculations were analyzed statistically with the GraphPad Prism program (Version 5.04, 2010). The means were compared with single factor analysis of variance (ANOVA). When statistically significant differences were confirmed among dietary treatments ($P \leq 0.05$), further statistical analysis was performed with Tukey's test. For all calculations $P < 0.05$ was assumed as significant. All values expressed as percentages were transformed with arcsin before statistical processing.

RESULTS

Growth and somatic index parameters. The vegetable oils and immunostimulators fed to the juvenile pikeperch had no impact on growth or condition coefficient K (Table 2). The mean final body weights and the final condition factor values of the pikeperch ranged from 108 to 116 g and from 0.7 to 0.8 in groups VON and VOL, respectively. No significant differences were noted among the FCR, VSI, or HSI coefficients (Table 2).

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Table 2. Growth, condition factor, and feed conversion ratio of juvenile pikeperch fed experimental diets for 56 days (mean ± SD; n = 3)

Specification	Dietary treatments			
	FO	VO	VOL	VON
Initial body weight (g)	73.6 ± 2.2	74.5 ± 0.3	74.7 ± 1.9	73.4 ± 1.1
Final body weight (g)	113.4 ± 10.5	114.1 ± 4.6	116.1 ± 3.1	108.7 ± 3.3
Daily growth rate (g/day)	0.72 ± 0.16	0.72 ± 0.08	0.75 ± 0.07	0.64 ± 0.07
Initial condition factor K	0.77 ± 0.01	0.79 ± 0.01	0.78 ± 0.01	0.79 ± 0.02
Final condition factor K	0.80 ± 0.04	0.80 ± 0.01	0.82 ± 0.01	0.80 ± 0.02
Feed conversion ratio	1.10 ± 0.26	1.07 ± 0.12	1.10 ± 0.17	1.23 ± 0.12
Viscerosomatic index (%)	7.89 ± 0.65	8.05 ± 0.45	8.57 ± 0.27	8.08 ± 1.13
Hepatosomatic index (%)	1.02 ± 0.15	1.05 ± 0.02	1.04 ± 0.02	1.13 ± 0.06

FO = feed with fish oil (69 g/kg feed), VO = feed with sunflower and linseed oils (48 and 21 g/kg feed), VOL = feed with sunflower and linseed oils (48 and 21 g/kg feed) and levamisol (0.3 g/kg feed), VON = feed with sunflower and linseed oils (48 and 21 g/kg feed) and NuPro® (40 g/kg feed)
no statistically significant differences were noted among the dietary treatments ($P > 0.05$)

Hematology, blood chemistry, and immunology. Feeding the pikeperch the experimental feeds containing immunostimulators resulted in significant increases in hemoglobin and leukocyte levels (Table 3). Supplementing feed with NuPro® (group VON) significantly lowered the values of blood biochemical parameters (Table 3). Lower cell-mediated immunity was noted in the pikeperch fed feeds without immunomodulator supplementation (groups FO and VO). Moreover, the serum lysozyme activity and gamma-globulin level were significantly the lowest in these groups (Table 4).

Chemical composition of pikeperch bodies. No significant differences were noted in the quantity of water, protein, fat, or ash in whole fish bodies from the groups tested (Table 5). Significantly the highest quantities of MUFA and the lowest quantities of PUFA were noted in the bodies of fish from the FO group in comparison to those from the other dietary treatments (Table 5). The amount of n-3

Table 3. Hematological and biochemical blood parameters in pikeperch fed experimental diets for 56 days (mean ± SD; n = 15)

	Dietary treatments			
	FO	VO	VOL	VON
Hematological parameters				
Erythrocytes RBC (1×10^6/l)	1.05 ± 0.15	1.10 ± 0.12	1.25 ± 0.20	1.20 ± 0.10
Hematocrit Ht (%)	36.0 ± 4.0	34.0 ± 3.0	35.0 ± 3.0	33.0 ± 4.0
Hemoglobin Hb (g/l)	61.5^{a} ± 3.5	64.0^{ab} ± 4.5	67.5^{b} ± 4.0	66.5^{b} ± 2.5
Leukocyte WBC (U/l × 10^3)	26.0^{a} ± 1.5	28.0^{ab} ± 2.5	29.5^{b} ± 2.0	30.5^{b} ± 2.5
Biochemical parameters				
Aspartate aminotransferase AST (IU)	12.5^{b} ± 1.5	12.0^{ab} ± 1.4	11.5^{ab} ± 1.5	10.6^{a} ± 1.2
Alanine aminotransferase ALT (IU)	6.5^{b} ± 0.7	5.9^{ab} ± 0.8	5.7^{ab} ± 0.9	5.3^{a} ± 0.7
Alcaline phosphatase ALP (IU)	3.0^{b} ± 0.5	2.5^{ab} ± 0.6	2.4^{ab} ± 0.7	2.1^{a} ± 0.4
Bilirubin (mmol/l)	5.1^{b} ± 0.6	4.7^{ab} ± 0.5	4.6^{ab} ± 0.6	4.3^{a} ± 0.4
Cholesterol (mmol/l)	2.9^{b} ± 0.4	2.6^{ab} ± 0.5	2.5^{ab} ± 0.4	2.0^{a} ± 0.3

FO = feed with fish oil (69 g/kg feed), VO = feed with sunflower and linseed oils (48 and 21 g/kg feed), VOL = feed with sunflower and linseed oils (48 and 21 g/kg feed) and levamisol (0.3 g/kg feed), VON = feed with sunflower and linseed oils (48 and 21 g/kg feed) and NuPro® (40 g/kg feed)
^{a,b}data in the same row with different superscript letters are significantly different ($P < 0.05$)

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Table 4. Cell- and humoral-mediated immunity in pikeperch fed experimental diets for 56 days (mean ± SD; n = 15)

	Dietary treatments			
	FO	VO	VOL	VON
Cellular immunity				
Respiratory burst activity of spleen phagocytes (RBA; OD 620 nm)	$0.46^{a} \pm 0.05$	$0.45^{a} \pm 0.04$	$0.56^{b} \pm 0.05$	$0.53^{b} \pm 0.05$
Potential killing activity of spleen phagocytes (PKA; OD 620 nm)	$0.35^{a} \pm 0.05$	$0.34^{a} \pm 0.04$	$0.47^{b} \pm 0.05$	$0.42^{b} \pm 0.03$
Lymphocyte proliferation stimulated by ConA (LP-ConA; OD 620 nm)	$0.49^{a} \pm 0.06$	$0.47^{a} \pm 0.05$	$0.59^{b} \pm 0.04$	$0.56^{b} \pm 0.05$
Lymphocyte proliferation stimulated by LPS (LP-LPS OD 620 nm)	$0.35^{a} \pm 0.05$	$0.37^{a} \pm 0.05$	$0.46^{b} \pm 0.05$	$0.43^{b} \pm 0.04$
Humoral immunity				
Lysozyme activity in serum (mg/l)	$63.4^{a} \pm 3.5$	$61.5^{a} \pm 2.5$	$69.7^{b} \pm 3.5$	$67.5^{b} \pm 3.6$
Ceruloplasmin activity in serum (IU)	51.2 ± 5.5	54.3 ± 5.7	52.6 ± 3.3	52.5 ± 5.5
Total protein level in serum (g/l)	43.3 ± 3.6	42.5 ± 3.5	42.1 ± 3.5	43.0 ± 2.5
Total gamma-globulin level in serum (g/l)	$3.8^{a} \pm 0.7$	$3.4^{a} \pm 0.6$	$5.1^{b} \pm 0.5$	$4.7^{b} \pm 0.6$

FO = feed with fish oil (69 g/kg feed), VO = feed with sunflower and linseed oils (48 and 21 g/kg feed), VOL = feed with sunflower and linseed oils (48 and 21 g/kg feed) and levamisol (0.3 g/kg feed), VON = feed with sunflower and linseed oils (48 and 21 g/kg feed) and NuPro® (40 g/kg feed)

[a,b]data in the same row with different superscript letters are significantly different ($P < 0.05$)

highly unsaturated fatty acids (HUFA) ranged from 174.9 g/kg tFA in group VOL to 225.3 g/kg tFA in group FO, and this difference was statistically significant. The whole bodies of fish fed the feed with vegetable oils were characterized by higher content of total n-6 compared to group FO. A similar amount of ALA was observed in fish bodies among the experimental groups. The contents of LA, EPA, and DHA were significantly different in the bodies of fish from groups VO, VOL, and VON compared to group FO (Table 5). In group FO, the quantities of LA were more than twice lower. The EPA and DHA contents ranged from 71.3 to 86.5 g/kg tFA and from 83.3 to 113.1 g/kg tFA in groups VOL and FO, respectively. The n-3/n-6 ratio in pikeperch bodies was the highest in group FO (Table 5).

DISCUSSION

Growth performance – VO and immunomodulators. The application of feeds with VO and with or without supplementary immunomodulators had no impact on the growth performance of the fish. When recommended quantities of protein, carbohydrates, and lipids are maintained in pikeperch diets, vegetable oils do not impact the growth of this species (Kowalska et al. 2010a, b; Zakes et al. 2010). Among pikeperch (W = 8; 40; 75 g), NuPro® (20–60 g/kg feed) was not observed to have had a stimulatory effect on body weight and FCR ratios following 8 weeks of rearing (Jarmolowicz et al. 2012, 2013; current study). Similar results were obtained when using levamisole in pikeperch (current study), and also in Indian carp *Labo rohita* (W = 37 g; dosage 125–500 mg/kg feed; 8 weeks of rearing) (Misra et al. 2009). Possibly, the somatic growth achieved by the juvenile pikeperch (W = 73–75 g, current study) fed the experimental diets and reared in RAS was the limit of the potential growth possible under these conditions. The FCR values obtained were also likely the result of the levamisole dose (300 mg/kg feed) administered. It has been shown in juvenile hybrid striped bass (W = 40 g) that levamisole-supplemented feed either enhanced growth and feed intake (100 mg/kg feed) or reduced growth and intake when 100 mg levamisole/kg feed was added for 3 weeks (Li et al. 2006b).

Hematological parameters of blood – diet lipid sources and immunomodulators. The fact that there were no differences in hematological parameters between groups VO and FO was confirmed by the corresponding FA ratios in the pikeperch fed a diet with the VO lipid source (Kowalska et al. 2012).

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Table 5. Proximate (g FA/kg of wet weight) and selected fatty acid (FA) composition (g FA/kg of total FA) of juvenile pikeperch whole body fed experimental diets (mean ± SD; n = 3)

	Dietary treatments			
	FO	VO	VOL	VON
Components				
Water	703.6 ± 9.4	713.2 ± 4.9	703.6 ± 6.1	705.5 ± 3.9
Total protein	178.4 ± 2.7	174.5 ± 4.2	178.3 ± 3.5	176.7 ± 0.6
Crude fat	78.0 ± 9.0	73.6 ± 2.1	78.9 ± 2.2	78.6 ± 4.5
Crude ash	39.2 ± 1.1	38.2 ± 0.9	38.4 ± 1.1	37.8 ± 1.0
Fatty acids				
ΣSFA[a]	240.8 ± 6.5	234.52 ± 10.2	235.7 ± 5.7	230.5 ± 3.3
ΣMUFA[b]	390.3[b] ± 11.1	338.5[a] ± 8.3	354.7[a] ± 1.7	349.7[a] ± 4.4
18:2 n-6	96.6[a] ± 4.0	193.5[b] ± 7.7	194.0[b] ± 5.7	186.8[b] ± 11.1
18:3 n-3	18.7 ± 2.2	18.6 ± 0.5	17.3 ± 5.1	20.8 ± 2.4
20:4 n-6	6.5[b] ± 0.1	5.1[a] ± 0.1	4.9[a] ± 0.1	5.1[a] ± 0.2
20:5 n-3	86.5[b] ± 6.0	76.9[a] ± 1.4	71.3[a] ± 2.2	76.7[a] ± 5.6
22:5 n-3	17.3[b] ± 0.4	15.1[a] ± 0.4	13.9[a] ± 0.5	14.8[a] ± 1.3
22:6 n-3	113.1[b] ± 3.4	91.4[a] ± 3.4	83.3[a] ± 2.7	89.6[a] ± 7.9
ΣPUFA[c]	368.3[a] ± 4.6	426.4[c] ± 3.4	409.1[b] ± 4.2	419.3[c] ± 2.9
Σn-9[d]	236.3 ± 28.0	224.3 ± 5.9	233.3 ± 5.6	226.4 ± 9.9
Σn-6[e]	108.2[a] ± 3.5	204.5[b] ± 6.9	204.1[b] ± 5.4	197.4[b] ± 10.7
Σn-3[f]	243.2[b] ± 7.3	207.6[a] ± 5.1	191.1[a] ± 1.3	207.4[a] ± 12.6
Σn-3 HUFA[g]	225.3[b] ± 10.0	190.5[ab] ± 5.5	174.9[a] ± 5.4	187.9[ab] ± 15.2
Σn-3/**Σ**n-6	2.2[b] ± 0.1	1.0[a] ± 0.1	0.9[a] ± 0.1	1.1[a] ± 0.1

FO = feed with fish oil (69 g/kg feed), VO = feed with sunflower and linseed oils (48 and 21 g/kg feed), VOL = feed with sunflower and linseed oils (48 and 21 g/kg feed) and levamisol (0.3 g/kg feed), VON = feed with sunflower and linseed oils (48 and 21 g/kg feed) and NuPro® (40 g/kg feed)

Σ saturated FA: 14:00, 15:00, 16:00, 18:00, 20:00, 22:00

[b]Σ monoenes: 14:1, 16:1, 17:1, 18:1 *cis*9, 18:1 *cis*11, 20:1 n-9, 20:1 n-7, 22:1 n-11, 22:1 n-9

[c]Σ polyenes: 16:2, 16:4, 18:2 n-6, 18:3 n-3, 18:3 n-4, 18:4, 20:2, 20:3 n-6, 20:4 n-6, 20:3 n-3, 20:4 n-3, 20:5 n-3, 21:5, 22:5 n-6, 22:5 n-3, 22:6 n-3

[d]Σ n-9: 18:1 *cis*9, 20:1 n-9, 22:1 n-9

[e]Σ n-6: 18:2 n-6, 20:3 n-6, 20:4 n-6, 22:5 n-6

[f]Σ n-3: 18:3 n-3, 20:3 n-3, 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3

[g]Σ n-3 HUFA: 20:3 n-3, 20:4 n-3, 20:5 n-3, 22:5 n-3, 22:6 n-3

[a–c]data in the same row with different superscript letters are significantly different ($P < 0.05$)

Similarly to carp (Siwicki et al. 1998), levamisole-supplemented feed (dose range of 100–300 mg/kg feed) significantly increased the WBC count and Hb values in the blood of the currently studied fish. For the first time in the studies of pikeperch, the impact of brewer's yeast *S. cerevisiae*, which is rich in nucleotides, was confirmed to increase the hematological parameters of Hb and WBC. Supplementation with thermally extracted baker's yeast has been demonstrated to have similar effects in rainbow trout fed the feed supplemented with nucleotides (0.1 and 0.2%) (Tahmasebi-Kohyani et al. 2011). Active iron absorption by nucleotides could possibly result in increased Hb in fish blood (Tahmasebi-Kohyani et al. 2011). In turn, the high number of leukocytes, which induce and participate in defense mechanisms against pathogens (Schreck and Moyle 1990), have a positive impact on the blood immunological parameters, as was demonstrated by the current study of pikeperch (groups VON and VOL).

Biochemical parameters of blood – body composition and immunomodulators. Dietary PUFA can be linked to body composition and blood biochemical parameter values. The fish bodies from groups VO, VOL, and VON were characterized

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by the contents of n-6 PUFA within LA that were twice as high, and also by significantly lower levels of EPA and DHA as compared to those in group FO. In our earlier study (Kowalska et al. 2010a, b, 2012), increased values of ALT, AST, and bilirubin resulted from VO in the fish feed, and these were also linked to dietary EPA and DHA deficiency, while PUFA from the n-3 and n-6 series, i.e., ALA and LA, were noted in excessive quantities. In the present study, the diet with FO contained twice the amount of n-3 PUFA, while that of VO had a 3-fold higher amount of n-6 PUFA than the other diets. This experiment indicates that even though FO is a source n-3 PUFA, it can adversely affect the value of blood biochemical parameters. In groups FO, VO, and VOL, the values of AST, ALT, ALP, bilirubin, and cholesterol were similar. In group VON, the values of these parameters were significantly the lowest compared to group FO. The high levels of PUFA in fish diets are particularly susceptible to oxidation, and various products of lipid oxidation can react with proteins, vitamins, and other dietary components and limit their nutritional value. Yeast extracts are important sources of amino acids, nucleotides, and vitamins, and since no negative effects on the biochemical parameters in fish blood have been observed, yeast extracts (group VON) could be used as alternative sources of some dietary components.

Cellular parameters of blood – n-3/n6 fatty acid and immunomodulators. Dietary lipids and fatty acids can affect immunocomponent cells in fish. Essential fatty acid deficiencies can impair the killing ability of macrophages or the proliferation of lymphocytes in rainbow trout (Kiron et al. 1995), channel catfish (Blazer 1992), and pikeperch (Kowalska et al. 2012). Conversely, high levels of n-3 fatty acids (HUFA or PUFA) had immunosuppressive effects on immune response in Atlantic salmon (*Salmo salar*) and rainbow trout (Kiron et al. 1995), and pikeperch (Kowalska et al. 2012). This can be linked to decreased synthesis of immune system components, which was noted in Atlantic salmon and channel catfish fed diets with high contents of n-3 PUFA (Lin and Shiau 2007). The use of VO with n3/n6 ratios ranging from 0.7 to 1.3 in fish diets do not disturb bioconversion or do not cause excesses or deficiencies of fatty acids, and, consequently, do not impair cell immune response in fish (Kowalska et al. 2012). The n3/n6 ratio in the feeds fed to groups VO and FO were 0.3 and 1.6, respectively, and the activity of phagocytes and lymphocyte proliferation in pikeperch was either similar to or lower than the values reported in an earlier study (Kowalska et al. 2012). The addition of levamisole and NuPro® (groups VOL, VON) to the pikeperch diet increased cells activity in immune response. Since doses of levamisole that are either too high or administered too long have an immunosuppresant effect (Maqsood et al. 2009; Misra et al. 2009), it can be concluded that the dose of 300 mg/l administered for 8 weeks to pikeperch is effective in improving fish immunity. NuPro® in doses of 40 g/kg feed stimulated cellular immunity robustly in pikeperch (Jarmolowicz et al. 2012, 2013; current study). Brewer's yeast as a source of nucleotides in the pikeperch diet proved to be an effective immunomodulator. The feed supplemented with NuPro® and levamisole can increase fish resistance to pathogens and can be used in the prevention of fish diseases.

Humoral parameters of blood – PUFA and immunomodulators. Lipid amount and quality of diets can cause some immunity disorders in fish. Lin and Shiau (2007) showed that fatty acid oxidation products suppress lysozyme activity. High quantities of LA or ALA in pikeperch diets reduce lysozyme activity probably because of the oxidation process potentiation (Kowalska et al. 2012). In groups FO and VO, the lysozyme activity was similar to and more than twice as high (62–63 mg/l) than the values reported by Kowalska et al. (2012) (21–30 mg/l) and could have been the result of the appropriate quantity of PUFA in the feed. The lysozyme activity of pikeperch fed commercial trout feed was nearly 50 mg/l (Siwicki et al. 2006), but it increased significantly (by 68–70 mg/l) in groups VOL and VON, which were fed feed supplemented with levamisole or NuPro®. Levamisole enhances lysozyme activity in turbot, cyprynidae, and salmonidae (Maqsood et al. 2009; Misra et al. 2009). Although the results of the present experiment indicated high lysozyme activity in fish fed VO based feed, this fat source may cause reduced activity and immunological resistance. The addition of immunostimulators to fish diets could limit this effect; moreover, as has been demonstrated in the studies of NuPro® and levamisole in pikeperch diets (groups VON and VOL), it could potentially increase immunity in fish reared in RAS.

The analysis of blood parameters, including Cp, could be used as a marker in assessing fish health.

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Increased Cp activity is linked to anomalies in the regulation of its secretion, changes in liver structure, and disturbances in lipid metabolism (Kowalska et al. 2010a, b). Dietary administration of NuPro® (20–60 g/kg feed) has no effect on the histological structure of the liver or intestine or on the Cp activity of pikeperch (W = 35–38 g) (Jarmolowicz et al. 2013). Misra et al. (2009) reported that levamisole did not disturb any physiological functions, including metabolism, as indicated by glucose levels. The value of Cp in dietary treatments indicated that the oils (FO and VO) and supplements (levamisole and NuPro®) tested did not impair hepatocyte function, which confirms the effectiveness and the safety of the immunomodulators tested in pikeperch feeding.

CONCLUSION

In summary, well-balanced fatty acid content in diets with VO had no impact on the cellular or humoral immunity of pikeperch, but feeding pikeperch feeds supplemented with levamisole (300 mg/kg feed) and NuPro® (40 g/kg feed) for 8 weeks significantly increased their immunological response. However, because of the content of C18 PUFA in the VO and oxidation processes, the addition of yeast extracts (NuPro®) to the feed can be a valuable source of essential nutrients potentially used in defense to oxidation. The best blood parameter values were recorded in group VON.

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