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Effects of dietary probiotics supplementation on several biomarkers in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The aim of the study was to assess the effects of dietary supplementation with two potential probiotics, *Bacillus subtilis* and *Bacilis cereus toyo*, on selected biomarkers, not directly related to nutrition, in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792). A nine week-long diet with the probiotics was found to affect hepatic EROD and GPx; muscular AChE, BChE, POx and LP; and branchial Na^+/K^+-ATPase, as compared to an equal diet without probiotics supplementation. No effects on hepatic GST, SOD, CAT, POx, LP, LPO, PY; muscular GPx, LPO; brain AChE, BChE; and gross morphometric indices were found.

Key words: probiotics, diet, nutrition, rainbow trout, biomarkers, gross indices.

Introduction

Various factors affect biomarkers in fish, both pollution-related and environmental. Changes in marine organisms at the biochemical level have been extensively studied as biomarkers, mainly in connection with exposure to a variety of pollutants (Van der Oost et al., 2003), and effects recognised at the biochemical level are generally used as 'early warning' signals for assessing the influence of stress-related factors on organisms. However, some of the well established biomarkers are also affected by environmental factors, e.g. temperature, salinity, oxygen concentration (Kopecka & Pempkowiak, 2008), nutrition, dietary supplements (Fernandez-Diaz et al., 2006), and hydrostatic pressure (Kopecka-Pilarczyk & Coimbra, 2010a, 2010b).

To quote the most widely used definition (Fuller, 1989), "Probiotics are a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance." The role of dietary nutrients or additives on the functions of the immune system in fish has been investigated since the 1980s. Research conducted during the recent years has provided a comprehensive understanding of the role of probiotics in fish health, including aspects related to dosing and feeding, disease resistance, modulation of the immune system, body composition, growth performance, reduced malformations and improved gut morphology and microbial balance (Avella et al., 2010; Merrifield et al., 2010a, 2010b; Nayak, 2010). Moreover, the effects of probiotics (bacterial strain *Lactobacillus rhamnosus* IMC 501) on stress response biomarkers: glucocorticoid receptor (GR mRNA) and 70-kDa heat shock protein (hsp70) gene expression, have been documented in larvae of clownfish *Amphiprion ocellaris* (Cuvier, 1830) (Avella et al., 2010).

Some research on the impact of vitamins on biomarkers has already been conducted (Fernandez-Diaz et al., 2006). Moreover, effects of L-carnitine on fish immune system (Dias et al., 2011) or dietary yeast supplementation on growth performance (Ozório et al., 2012) or antioxidant enzyme activities and gene expression (Tovar-Ramirez et al., 2010) have also been analyzed. Research conducted throughout the recent years provides comprehensive understanding of the role of probiotics in fish health (Merrifield et al., 2010a, 2010b; Nayak, 2010), growth performance or mechanisms responsible for probiotic enhancement in fish development (Avella et al., 2010).

Detailed evaluation of the influence of nutrition-related factors on the biomarkers typically measured in relation to pollution is lacking in the literature. Therefore, some effects caused by changes in diet can be mistakenly attributed to pollution or other stress factors, thus affecting the results of

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biomonitoring based on biomarkers. This state prompts the urgent need to conduct a comprehensive analysis of the overall biochemical reaction of fish in response to nutritional factors.

The aim of the present work was to assess the effects of dietary supplementation with two potential probiotics, *Bacillus subtilis* and *Bacilis cereus toyoi* supplied in equal proportions, on selected biomarkers in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792). In particular, this work is expected to contribute to the understanding of the role of probiotics as antioxidants when included in the diet. To the best knowledge of mine, such assessment has been done for the first time.

Materials and Methods

Design of the experiments

Five hundred juvenile rainbow trouts (15.6 ± 0.5 g), were distributed into 18 tanks (6 tanks/diet) and hand-fed twice a day, 6 days per week, until visual satiation. Fish were reared at 17ºC in a fresh water recirculating system, with the photoperiod of 12 h light : 12 h dark, at a stocking density of 7 kg/m^3. The fish were distributed for the analysis of biomarkers (this paper) and for growth performance assessment and gut morphology analysis (Ramos et al., 2012)

The probiotic (4.2×10^9 cfu g^{-1} dry powder) was incorporated into a basal diet (a practical trout fishmeal containing 48% crude protein and 12.5% crude lipid) in two concentrations: 0.03% (PRO1) and 0.06% (PRO2), and tested against a control diet (without the probiotic). All diets were formulated and manufactured by CIIMAR, by pelleting at <60ºC at 2 mm pellet size.

At 9 weeks of supplementation, 15 fish (5 fish per treatment: control, PRO1 and PRO2) were netted for biomarker analyses and then anaesthetised by immersion in benzocaine. The length (L) and the total weight of each fish (W_T) were taken, and thereafter the fish were killed by decapitation. The tissues (liver, muscles, brain and gills) were immediately removed from each fish, weighed (including the weight of the entire liver W_H), placed in 1.5 mL Eppendorf tubes, and frozen in liquid nitrogen. All samples were stored at −80 °C until the analyses.

Analyses

The following biomarkers were measured in the liver samples: 7-ethoxyresorufin-O-deethylase (EROD), glutathione-S-transferase (GST), catalase (CAT), superoxidase dismutase (SOD), glutathione peroxidase (GPx), lipid peroxidation (LP - measured in two ways: using the TBA – thiobarbituric acid, and using 1-methyl-2-phenylindole, the latter further referred to as LPO), protein oxidase (POx), and also protein yield (PY) and total protein contents; in the muscle samples: GPx, POx, LP, LPO, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), protein contents; in the brain samples: AChE, BChE, protein contents; and in the gills samples: sodium/potassium ATPase (Na^+/K^+-ATPase) and protein contents. Moreover, the following gross morphometric indices were calculated for each individual fish:

- condition factor (CF) = (W_T / L^3) x100;
- hepatosomatic index (HSI) = (W_H / W_T) x100,

where W_T = total wet weight [g], L = total length [cm], W_H = liver weight [g]. As far as the author is aware, none of these biomarkers has ever been analysed in relation to probiotics supplementation in fish.

All the biomarkers and morphometric indices were measured by the author of this paper, closely following the methods are described by Kopecka-Pilarczyk (2013), and the interested reader is referred to that paper for details.

Statistics

Results of the measurements (n = 5 for each group) expressed as $\bar{x}$ (mean) ± S.D. (standard deviation of the mean of 3 replicate measurements for each sample) are gathered in Table 1, where asterisks indicate statistically significant differences ($p < 0.05$) of the results with respect to control for each study assessed by Student's *t*-test for independent groups. For comparison among all the groups (control, PRO1, PRO2), one-way parametric ANOVA was used together with Bonfferroni correction whenever the variances were homogeneous, or the Kruskal-Wallis non-parametric rank test was applied if the variances were heterogeneous. All statistical calculations were carried out using the StatSoft Statistica® 5.0 software package (www.statsoft.com) and SyStat SigmaPlot® 11 for Microsoft Windows® (www.sigmaplot.com).

Results

Among the analysed biomarkers, statistically significant differences between the treatment groups and control (as indicated in Table 1) were only found in hepatic EROD, GPx and muscular BChE in case of PRO1, and in muscular AChE, POx, LP and branchial Na^+/K^+-ATPase in case of PRO2.

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Table 1. *The level of biomarkers in the liver (EROD, SOD, GST, CAT, GPx, POx, LP, LPO, PY, protein), in muscles (AChE, BChE, GPx, POx, LP, LPO, protein), in brain (AChE, BChE, protein), in gills (Na^+/K^+-ATPase, protein), and gross indices (CF, HSI) in Oncorhynchus mykiss, expressed as the mean ± S.D. Statistically significant differences from the control found by t-test are indicated as: * - $p<0.05$, ** - $p<0.01$.*

Endpoint	Control	PRO 1	PRO 2
CF [%]	1.11 ± 0.07	1.13 ± 0.03	1.10 ± 0.05
HSI [%]	1.04 ± 0.06	0.93 ± 0.21	0.97 ± 0.06
LIVER			
EROD [pmol min^{-1} mg $prot^{-1}$]	2696.5 ± 336.2	1679.1 ± 278.0 **	2820.5 ± 593.4
GST [nmol min^{-1} mg $prot^{-1}$]	107.70 ± 15.02	95.69 ± 16.15	115.80 ± 15.75
SOD [U min^{-1} mg $prot^{-1}$]	28.07 ± 6.85	23.04 ± 4.54	29.30 ± 4.86
CAT [µmol min^{-1} mg $prot^{-1}$]	95.24 ± 1.64	94.43 ± 1.45	93.55 ± 4.18
GPx [nmol min^{-1} mg $prot^{-1}$]	8.83 ± 1.11	6.51 ± 0.88 *	7.94 ± 1.76
POx [nmol mg $prot^{-1}$]	0.75 ± 0.42	0.78 ± 0.23	1.17 ± 0.53
LP [nmol MDA g^{-1} w.w.]	77.64 ± 12.73	85.57 ± 10.90	78.23 ± 14.55
LPO [nmol MDA g^{-1} w.w.]	25.52 ± 4.24	30.38 ± 6.34	30.33 ± 5.60
PY [mg g^{-1} w.w.]	80.21 ± 4.86	87.74 ± 8.00	86.51 ± 4.78
protein [mg ml^{-1}]	16.04 ± 0.97	17.55 ± 1.60	17.30 ± 0.96
MUSCLES			
AChE [nmol min^{-1} mg $prot^{-1}$]	131.81 ± 29.70	143.06 ± 25.98	180. 01 ± 21.66 *
BChE [nmol min^{-1} mg $prot^{-1}$]	5.22 ± 2.14	9.91 ± 3.31 *	8.38 ± 3.87
GPx [nmol min^{-1} mg $prot^{-1}$]	3.91 ± 1.15	4.35 ± 0.68	3.79 ± 0.75
POx [nmol mg $prot^{-1}$]	0.58 ± 0.17	0.86 ± 0.18 *	1.08 ± 0.25 **
LP [nmol MDA g^{-1} w.w.]	11.96 ± 1.40	16.13 ± 5.76	22.48 ± 6.60 *
LPO [nmol MDA g^{-1} w.w.]	12.28 ± 3.65	10.70 ± 2.91	13.40 ± 1.10
protein [mg ml^{-1}]	6.81 ± 1.31	7.32 ± 0.43	7.03 ± 0.67
BRAIN			
AChE [nmol min^{-1} mg $prot^{-1}$]	55.32 ± 10.15	68.56 ± 10.07	69.72 ± 11.61
BChE [nmol min^{-1} mg $prot^{-1}$]	2.68 ± 0.47	3.04 ± 0.40	2.95 ± 0.29
protein [mg ml^{-1}]	6.40 ± 0.50	6.18 ± 0.77	6.00 ± 0.40
GILLS			
Na^+/K^+-ATPase [µmol ADP mg $prot^{-1}$ h^{-1}]	1.17 ± 0.43	0.76 ± 0.26	0.54 ± 0.03 *
protein [mg ml^{-1}]	15.67 ± 2.39	16.54 ± 2.41	15.72 ± 3.19

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Moreover, statistically significant differences between all the tested groups (control, PRO1, PRO2) were found by one-way ANOVA in hepatic EROD (control-PRO1, $p<0.05$; PRO1-PRO2, $p<0.05$); muscular AChE (control-PRO2, $p<0.05$), POx (control-PRO2, $p<0.01$), and LP (control-PRO2, $p<0.05$). The differences in the other measured biomarkers were statistically insignificant ($p>0.05$).

Discussion

It is known that nutrition can affect the activity of detoxification enzymes in fish (Bucheli & Fent, 1995, Di Giulio et al., 1995). Much of this effect may be due to the differences in the presence of natural inducing agents or inhibitors in the diet (Di Giulio et al., 1995). In particular, it has been stated that nutritional composition and value of diet, e.g. vitamins, protein, lipid, carbohydrate levels, trace elements, may affect EROD activity, and only adequate nutrition allows microsomal enzyme systems to function properly (Whyte et al., 2000). Therefore, it is natural to expect that EROD might also be affected by probiotics as one of exogenic factors different from xenobiotics (Bucheli & Fent, 1995; Whyte et al., 2000). Indeed, a statistically significant decrease in EROD activity was observed at the lower probiotics concentration (PRO1). Moreover, hepatic GPx, which is known to play an especially important role in protecting membranes from damage due to lipid peroxidation, shows a statistically significant decrease at the PRO1 diet. It seems that lower activity might be attributed to the decrease in production of reactive oxygen species (ROS), and there is no detectable oxidative damage caused by the presence of the probiotics in the diet. Failure of the antioxidant system may result in LP and induction of carbonyl content (POx). Data obtained in this study show a small increase in hepatic POx at the higher probiotics concentration (at PRO2), and slightly higher levels of hepatic LP (at PRO1) and LPO (at both PRO1 and PRO2), although all these differences in hepatic POx, LP and LPO not statistically significant.

The response in the muscular biomarkers to the presence of the probiotics in the diet was stronger than that of the hepatic ones (see Table 1). Statistically significant increase was encountered in neurotransmitters (AChE in the case of PRO2 and BChE in the case of PRO1), muscular POx (both treatments), and LP (for PRO2). From these results, it is clear that muscles turned out to be more effective in response to probiotics. Also activity of brain neurotransmitters (AChE and BChE) is slightly higher in the case of the probiotics-enhanced diet than for the control fish, although these differences are not statistically significant. These results may be due to an increased rate of fish metabolism and a better condition of the nervous system in fish, which are both indicators of positive effects of the probiotics on the fish. Moreover, the fact that probiotics can actually induce the activity of the neurotransmitters must be taken into account in field studies, especially that inhibition of both AChE and BChE activity is a commonly applied indicator of biota exposure to the organophosphorus and carbamate compounds used as active agents in many pesticides (Mayer et al., 1992).

In general, the observed changes in the hepatic, muscular and brain biomarkers seem to indicate a probable hypothesis that the probiotics improve health status of fish. This hypothesis, however, must be verified by more studies with probiotics.

An intriguing fact is that the activity of branchial Na^+/K^+-ATPase decreased when probiotics diet was used, although the decrease was not statistically significant at the weaker dose of the probiotics. Na^+/K^+-ATPase plays a pivotal role in the osmoregulation of estuarine animals, and its activity is primarily affected by differences in salinity (Wilson et al., 2007). It is also known that some pollutants can affect the activity of Na^+/K^+-ATPase in gills (Mayer et al., 1992). However, there have been no studies conducted on any effects of nutrition on branchial Na^+/K^+-ATPase activity. The encountered decrease in the activity of this biomarker caused by the probiotics supplementation is the first result of this kind, and definitely requires further attention.

As far as the gross morphometric indices are concerned, CF is typically used to assess general condition of fish, and HSI may be used to detect a possible liver disease. Since both indices were essentially at the same level in control and both probiotic treatment groups, apparently the general condition of liver was not affected by the 9 weeks of probiotics supplementation.

Conclusion

The results of the research described in this article show that probiotics diet supplementation in *O. mykiss* was reflected in changes in some of the comprehensive battery of biomarkers typically measured in relation to pollution. Further comprehensive research on this topic is necessary to

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establish a repeatable pattern of changes in biomarkers caused by probiotics, also depending on the quantity and on the period of time of supplementation, and to provide a more in-depth explanation of the reasons of these changes.

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